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REVIEW

Diverse models in Alzheimer's research: exploring alternative approaches beyond traditional rodent frameworks

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Abstract

Alzheimer's disease (AD) is a debilitating neurodegenerative disorder that progressively impairs cognitive function and memory. It is characterized by the buildup of beta-amyloid plaques and neurofibrillary tangles, leading to the progressive destruction of brain cells, especially in the hippocampus and neocortex. Despite extensive research, the precise molecular mechanisms underlying AD remain unclear. Rodent models, that are the mainstay of AD research, offer inherent limitations, thus prompt the search and exploration of alternative models. To address this knowledge gap, scientists have turned to non-mammalian models such as zebrafish, fruit flies, and worms. These organisms provide a valuable platform for studying AD due to their shorter lifespans, ease of genetic manipulation, and lower maintenance costs. Additionally, they facilitate high-throughput screening and real-time imaging, which accelerates the investigation of underlying pathogenesis and discovery of potential drug targets. By exploring the complex pathogenesis of AD using these models, researchers aim to develop innovative therapies to combat this devastating disease.

Keywords: Alzheimer's disease, *Drosophila melanogaster* (fruit flies), *Caenorhabditis elegans* (worms), *Danio rerio* (zebra fish), transgenic models, neurodegeneration

Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder (NDD) characterized by a gradual decline in memory, learning, and cognitive abilities. It is the leading cause of dementia, a condition marked by the deterioration of cognitive function [1]. AD poses a significant and growing public health concern. It currently affects over 6 million Americans aged 65 and older, with projections indicating that this number could more than double by 2060 in the absence of significant medical breakthroughs [2]. Tragically, AD has become a leading cause of death worldwide, with mortality rates escalating dramatically over the past two decades, a trend likely exacerbated by the COVID-19 pandemic [3]. According to data from the GBD study 2021, there was an elevation in AD prevalence by 160.8% from 1990 to 2021 [4].

Despite advancements in symptom management, a definitive cure for AD remains elusive. Discovering effective treatments for AD and other NDDs is a paramount challenge of the 21st century [5, 6] AD is indeed defined by the abnormal accumulation of proteins in the brain, which ultimately leads to neuronal death and tissue degeneration [7]. The underlying causes of AD are complex and multifaceted, involving impaired communication between brain cells, oxidative stress, mitochondrial dysfunction [8], apoptosis [9], inflammation [10], and aberrant brain signaling [11]. Current treatments can only alleviate symptoms and do not halt disease progression [7]. Recent advancements in human genetics have significantly enhanced our comprehension of the genetic underpinnings of NDDs, including Parkinson's disease and AD. However, ethical considerations and technical limitations impede human-based research. Consequently, animal models, primarily rodents, have become indispensable tools for AD research. While these models can recapitulate certain AD characteristics, such as neuronal alterations and behavioral abnormalities, they fall short of fully capturing the disease's complexity [12-14]. Rodent models, despite their contributions, exhibit substantial limitations, including inadequate replication of human AD, extended lifespans, slow disease progression, ethical concerns, and high resource demands. To circumvent these challenges,

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Table 1. Comparison between rodent and non-rodent models for AD research.

Features	Rodent model	Non-rodent model (*C.elegans*, *Drosophila*, and zebrafish)
Complexity of nervous system	Highly complex, with a brain and spinal cord	Relatively simple nervous system
Genetic manipulation	Advanced genetic techniques are required for genetic manipulation	Highly amenable to genetic manipulation
Lifespan	Months to years	Days to week
Cost	Relatively high cost, due to housing, feeding, and experimental procedures	Low cost, due to simple housing and feeding requirements
Ethical considerations	Significant ethical considerations	Minimal or no ethical considerations
Behavior and cognition	Complex	Simple

researchers have turned to non-mammalian models like zebrafish, fruit flies, and nematodes [15-17]. Characterized by shorter lifespans and lower maintenance requirements, these organisms offer advantages for investigating the fundamental mechanisms of AD. Moreover, the development of numerous transgenic strains expressing both human and complementary amyloid-β (Aβ) sequences in non-rodent models has expanded research possibilities, yielding strains that produce either $A\beta_{1-42}$ or $A\beta_{3-42}$ [18-21]. Table 1 highlights the key advantages of these novel models over the traditional rodent model for AD research.

The nematode, *Caenorhabditis elegans* (*C. elegans*), has emerged as a powerful model organism for dissecting the underlying mechanisms of human NDDs, including AD [19, 20, 22]. Its rapid lifespan, microscopic size, and economical maintenance render it highly efficient for research [23]. Notably, *C. elegans* possesses numerous human gene orthologs, including those implicated in AD, as shown in Figure 1. This genetic homology, coupled with well-established neurodegenerative pathway analysis techniques, positions the nematode as an ideal system for exploring the cellular and molecular intricacies of AD [24, 25].

For over a century, *Drosophila melanogaster* (fruit fly), has been a fundamental tool in biological research, significantly advancing our understanding of genes, chromosomes, and genetic inheritance [26]. Beyond its genetic contributions, *Drosophila* has proven to be a valuable model organism for investigating human NDDs at molecular, physiological, and behavioral levels [27, 28]. It's simple yet conserved anatomy and genetic framework make it an ideal system for unraveling complex disease mechanisms [29]. *Drosophila* research has led to groundbreaking discoveries regarding critical AD-related processes and pathways, and numerous models have been established to study amyloidosis and tauopathy [26].

The zebrafish has also emerged as a valuable vertebrate model organism for investigating human NDDs, particularly AD [30, 31]. Its relatively simple yet conserved nervous system facilitates detailed imaging and analysis of neuronal proteins, providing unparalleled insights into

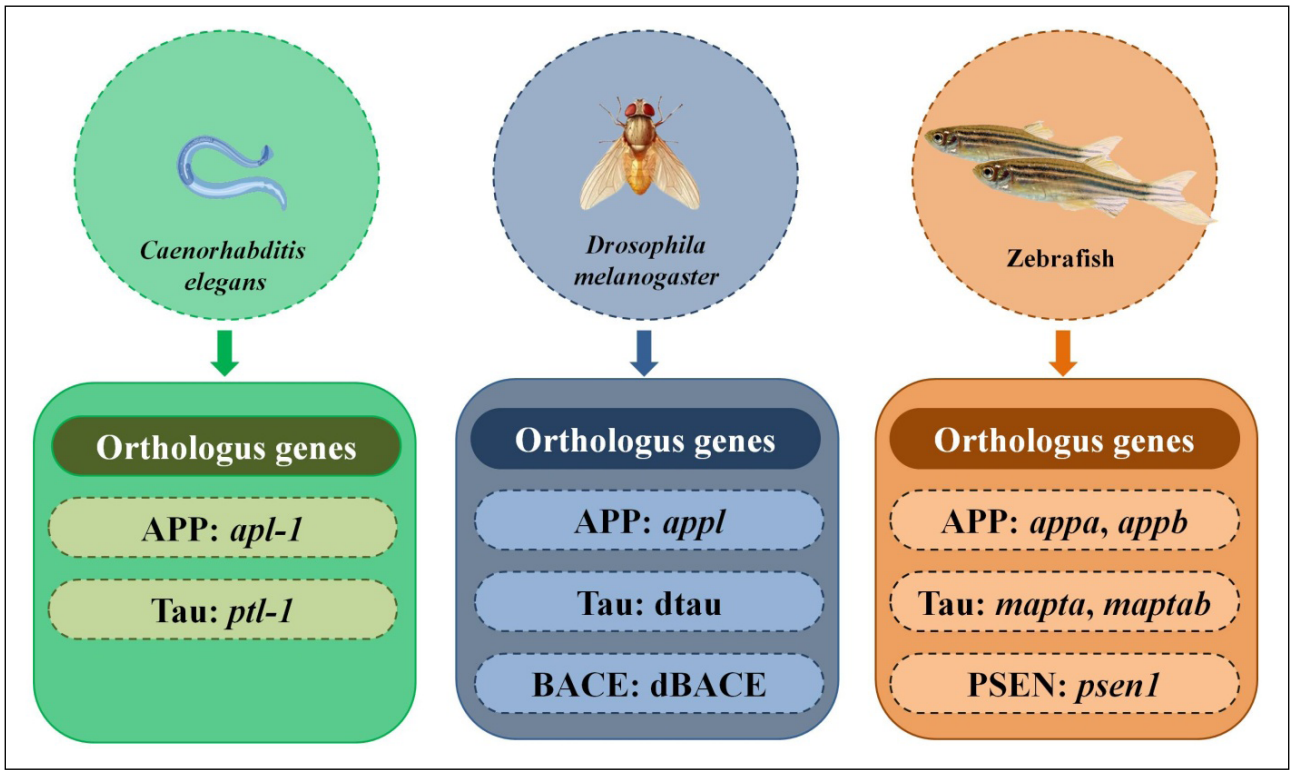

Figure 1. Non-rodent models and their orthologous genes with humans.

neurological processes. Notably, zebrafish exhibit behavioral patterns strikingly similar to humans, suggesting-shared neural mechanisms underlying behavior such as learning, social interactions, and decision-making [28]. Researchers have successfully developed zebrafish models that recapitulate key pathological features of AD, including tauopathy and amyloid-beta accumulation. These models offer a powerful platform for elucidating disease mechanisms and identifying potential therapeutic targets. As a result, the zebrafish is rapidly gaining prominence as a cornerstone in neuropharmacological research [32-34].

This article highlights the increasing significance of non-rodent animal models, particularly zebrafish, fruit fly, and *C. elegans*, in AD research. These models offer distinct advantages over traditional rodent models, such as accelerated development timelines, reduced costs, and enhanced capacity for high-throughput screening, as illustrated in Figure 2. Zebrafish, with their transparent embryos and comprehensively characterized nervous system, are uniquely suited for studying neuronal development and function. *C. elegans*, a genetically tractable nematode, provides a robust toolkit for dissecting complex biological processes. By harnessing the strengths of these models, researchers can expedite the discovery and development of novel therapeutic interventions for AD and other neurodegenerative diseases. These invertebrate systems serve as invaluable platforms for elucidating disease mechanisms and identifying promising drug targets.

Caenorhabditis elegans as a model for AD

C. elegans, a microscopic nematode, has emerged as a valuable model organism for studying AD and other NDDs. They were introduced by Sydney Brenner in 1963, its simple anatomy, transparent body, and short lifespan make them highly amenable to genetic and biochemical studies [35]. The complete sequencing of the *C. elegans* genome revealed striking similarities to the human genome, including shared genes involved in AD pathogenesis, such as APP and tau. This organism offers several advantages over other *in vivo* models. Its relatively small nervous system enables detailed investigation of neuronal processes, while its transparent body facilitates real-time observation of cellular events [36, 37]. Additionally, *C. elegans* is highly amenable to genetic manipulation, allowing for the creation of transgenic lines and high-throughput screening, which have been instrumental in elucidating the functions of AD-related genes and proteins [38, 39].

C. elegans provides a valuable platform for understanding the molecular mechanisms underlying AD. While lacking the Aβ sequence, the nematode possesses a single amyloid precursor protein (APP) homolog, apl-1, which shares structural similarities with the human APP family [40]. Although lacking a clear BACE ortholog, *C. elegans* harbors *ADAM10* and *ADAM17* homologs, enzymes involved in APP processing [41, 42]. A transmembrane motif as well as a small cytosolic region with 71% sequence identity to the mammalian APP is also present. Notably, *apl-1* lacks the Aβ sequence in contrast to APP but is otherwise comparable to *APLP1* and *APLP2* [43, 44]. The sup-17 and adm-4 genes throughout *C. elegans* are present and resemble the human *ADAM10* and *ADAM17* genes, respectively [45]. Bioinformatic investigations have not shown the existence of a BACE ortholog or other β-secretase

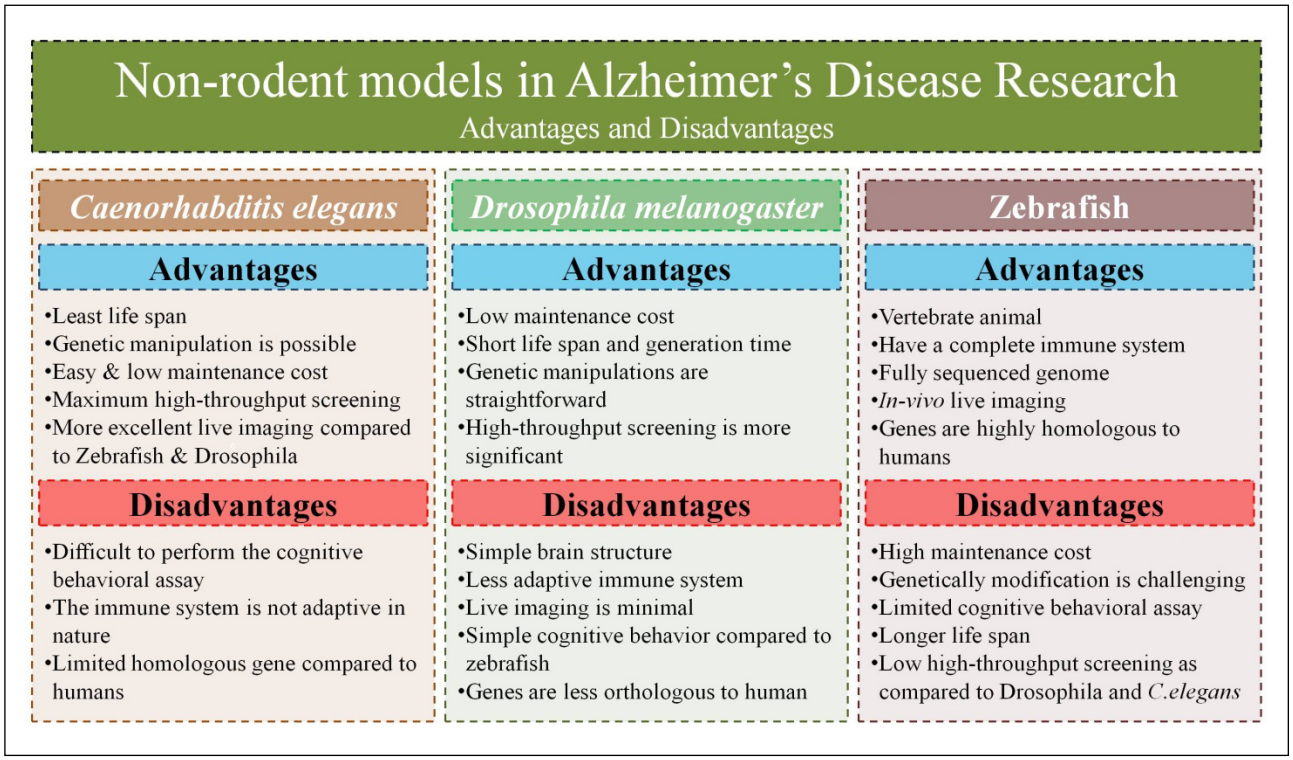

Figure 2. Advantages and disadvantages of various alternatives to rodent models in AD research.

activities that break down mammalian APP into *C. elegans* [46]. Human *PSEN1* and *PSEN2* also known as S182 and E5-1 respectively, were initially characterized as new proteins with numerous transmembrane domains [47]. Presenilins, key components of the γ-secretase complex implicated in AD, are conserved between humans and *C. elegans* [46]. The two *C. elegans* Notch genes, *lin-12* and *glp-1*, are implicated in germline development and various cell fate decisions throughout development and are also included in vulvae cell specifications [48]. The nematode *sel-12* protein is the functional homolog of human presenilins and is essential for Notch signaling, a pathway crucial for development. The ability to rescue loss-of-function *sel-12* mutations with human presenilins underscores the functional conservation of these proteins [49, 50]. Additionally, *C. elegans* possesses additional components of the γ-secretase complex, including *Aph-1*, *Aph-2/NCT*, and *pen-2*, which interact with *sel-12/psen* to facilitate Notch signaling [51]. Expanding on the role of presenilin homologs in *C. elegans*, *spe-4*, *sel-12*, and *hop-1* were also identified as members of the PSEN gene family. Notably, *sel-12* exhibits a higher degree of sequence homology to mammalian PSEN, suggesting a conserved role in regulating APP processing. Overexpression of *apl-1* in *C. elegans* led to increased mortality, which could be rescued by *sel-12* variants, implicating *sel-12* in the control of *apl-1* cleavage. Furthermore, *sel-12* mutations in the nematode have been associated with disruptions in mitochondrial calcium homeostasis, a hallmark of AD pathology [52, 53]. These genetic and biochemical similarities between humans and *C. elegans* make the nematode a powerful model for investigating the molecular basis of AD and for identifying potential therapeutic targets.

Aβ and tau in *C. elegans*

The progressive nature of AD is reflected in animal models such as earthworms expressing mammalian $A\beta_{3-42}$ exhibited age-dependent paralysis, with a more rapid and severe onset observed with $A\beta_{1-42}$ expression [54, 55]. Similarly, *C. elegans* exposed to aggregated Aβ display muscle paralysis and neuronal toxicity. This model system offers a platform for identifying molecules and pathways capable of inhibiting Aβ aggregation or promoting disaggregation [56]. Researchers have demonstrated that *C. elegans* extracts possess the ability to disassemble aggregated human $A\beta_{3-42}$, independent of protease activity, suggesting the involvement of novel protein factors in this process [57]. The *C. elegans* model has proven invaluable in identifying potential therapeutic agents capable of disaggregating Aβ. High-throughput drug screening using this model has accelerated the discovery process [58]. For instance, PBT2 an analog of 8-hydroxyquinoline, demonstrated remarkable efficacy in reversing AD-like symptoms in mice within days. This compound also prevented paralysis in *C. elegans* expressing inducible $A\beta_{1-42}$, highlighting its potential to target Aβ-induced neurotoxicity [59].

C. elegans offers insights into the role of heat shock chaperone proteins in modulating Aβ toxicity. Researchers have identified several *C. elegans* orthologs of human heat shock chaperone proteins that interact directly with $A\beta_{3-42}$ [60]. Notably, B-crystallin and HSP-16 proteins, including HSP-16-48, HSP-16-1, and HSP-16-2, bind to both intracellular $A\beta_{3-42}$ monomers and soluble oligomers, but not fibrillar forms [61]. Interestingly, $A\beta_{3-42}$ transgenic lines exhibit increased *hsp-16-2* transcript levels, suggesting a potential role for this chaperone in response to Aβ stress. However, the precise function of these chaperones in promoting or inhibiting Aβ-induced paralysis remains to be elucidated [62]. In contrast to the HSP-16 family, another study by Fonte *et al*. demonstrated a protective role for the HSP70 chaperone in preventing paralysis induced by Aβ in *C. elegans*. These findings align with human studies reporting increased levels of both HSP70 and B-crystallin in AD brains, suggesting a potential role for these chaperones in binding and mitigating Aβ toxicity [63-65]. The clearance of Aβ from the brain is a also critical process linked to apolipoprotein E (ApoE) and the LRP2/megalin receptor [66]. Yochem and their team have discovered *LRP-1* in the nematode, a protein that shares significant structural and functional similarities with the human protein LRP2/megalin. This homology makes *C. elegans* an ideal organism for exploring the role of LRP-2/megalin-like functions in these pathways [67].

Neurofibrillary tangles (NFTs), primarily composed of hyperphosphorylated tau protein, are also a hallmark of AD. *C. elegans* possesses a single tau ortholog, *ptl-1*, which shares structural similarities with human tau, including multiple tau protein-like repeats. The two main isoforms, *ptl-1a* and *ptl-1b*, exhibit high sequence homology to the C-terminal region of human tau. Like human tau, *C. elegans ptl-1* binds to microtubules and promotes tubulin polymerization *in vitro* [68]. The *C. elegans* protein *ptl-1*, similar to human tau, shows changing distribution patterns throughout its development. Initially present in the head nerve cells and growing outer layer of embryos, *ptl-1*, is later detected in most neurons that respond to light touch [69]. Notably, loss-of-function mutations in *ptl-1* result in embryonic lethality, mirroring the phenotype observed with *ptl-1* overexpression, suggesting a critical role for this protein in early developmental processes [68]. While *ptl-1*/tau mutant animals that survive embryogenesis exhibit seemingly normal microtubule organization at the light microscopic level, they display significant deficits in gentle touch sensitivity compared to wild-type controls. Despite normal growth and development, these mutants exhibit reduced lifespan, suggesting a broader impact of *ptl-1* dysfunction on organismal health and longevity [70].

Transgenic *C.elegans* models for AD

In an effort to unravel the complexities of AD, researchers have developed a diverse range of *C. elegans* strains that carry specific genetic alterations. These engineered worms serve as valuable models to study the underlying mechanisms of NDDs [60]. Treusch and colleagues identified several neurodegenerative modifiers related to the cytoskeleton, including *GRR1*, *YAP 1802*, *SLA1*, *KEM1*, *CRM1*, *INP52*, and *RTS*, which offer potential targets for

therapeutic intervention [71, 72]. A significant milestone in AD research occurred in 1995 with the creation of the CL2006 strain, the first *C. elegans* transgenic model to express human $A\beta_{1-42}$ in body wall muscle. This model has become a crucial platform for testing the therapeutic potential of various herbal and synthetic compounds [71, 72]. Furthermore, Diomede and colleagues tested the effects of silver nanoparticles on transgenic *C. elegans* producing amyloidogenic proteins. These worms, specifically strains CPV10 and CL2120, mimic diseases caused by the buildup of Aβ, a substance linked to many age-related illnesses. The researchers found that silver nanoparticles harm both normal and transgenic *C. elegans* in various ways. To gauge the sensitivity of the worms, they compared their results to those obtained using common mammalian cell lines, D384 brain cells and A549 lung cells [73]. Additionally, he and colleagues evaluated seven newly developed compounds (2-aryl ethenyl quinolines) for their ability to protect nerve cells using the CL2355 strain of *C. elegans* as an AD model. Two of these compounds improved memory problems and reduced the buildup of harmful Aβ clumps in these worms [74]. Nevertheless, a variety of *C. elegans* transgenic models have been generated to study AD, including UA166, CL2355, CL2122, CL2006, and CL4176 [75, 76]. Despite significant advancements in transgenic *C. elegans* models of AD, a critical gap remains. No existing strain displays constitutive, pan-neuronal expression of $A\beta_{1-42}$, nor does it accurately replicate the age-dependent behavioral deterioration that characterizes human AD. This limitation hinders the ability to fully understand the complex mechanisms underlying the disease and develop effective therapeutic strategies. Recently, by utilizing transgenic AD model strain (GRU102), which expresses pathogenic human $A\beta_{1-42}$ exclusively in neurons, Toe and team successfully identified metformin, lithium, and curcumin as promising candidates for AD treatment [77].

In AD, tau proteins undergo changes in structure and function. To study these changes and their effects, Brandt and colleagues created *C. elegans* worms with human tau proteins. These worms were made in two versions: one with normal tau and another with a modified, abnormally structured tau (pseudo-hyperphosphorylated tau). Both types of worms showed similar movement problems. However, only the worms with the abnormal tau developed clumps of tau protein and had problems with motor neuron development [78]. Three different *C. elegans* models—myo-3, snb-1, and unc-54—are commonly used to test how well drugs protect nerve cells from damage caused by Aβ. The unc-54 model, where Aβ builds up inside cells, offers a unique opportunity to identify cellular proteins that interact with Aβ. These proteins might be involved in either causing Aβ-related damage or protecting the cell from it [63].

C.elegans AD models for screening of new therapeutic agents

Several natural and synthetic compounds have shown promise in combating AD when tested on *C. elegans* models [79, 80]. McCormick and colleagues tested azaperone, an antipsychotic drug that blocks dopamine D2 receptors, in a *C. elegans* model of AD. They found that this drug improved the worms' movement and decreased the levels of abnormal tau protein. These results suggest that blocking dopamine D2 receptors might be a promising approach to preventing nerve damage caused by tau protein [81]. Additionally, caffeic acid has shown promising results in combating the harmful effects of Aβ in CL4176 *C. elegans* worms. This compound significantly reduced Aβ toxicity, extended lifespan, improved reproductive issues, and alleviated body paralysis. It also activated protective heat-shock proteins by increasing *hsf-1* and *hsp-16.2* gene activity [82]. In another study, researchers examined the effects of magnolol on Aβ-related diseases using transgenic *C. elegans* worms producing human Aβ. They discovered that magnolol can activate PPAR-gamma, boost ApoE levels, and enhance the ability of microglia cells to clear Aβ, thereby reducing Aβ-induced toxicity and memory impairment [83]. Further the water extract from *Terminalia chebula* reduced Aβ clumps and improved paralysis in AD model worms (CL2006 and CL4176) [84]. Nicotine also lessened paralysis symptoms in CL2120 and CL4176 models by decreasing Aβ buildup and harmful oligomers, although it did not prevent Aβ clumping [85]. *Ginkgo biloba* extract (EGb 761) and its component ginkgolide A improved various AD-related behaviors in *C. elegans* by reducing Aβ clumping and harmful oligomers [86]. Finally, oleuropein, when tested in CL2006 worms, reduced Aβ plaque buildup, Aβ oligomers, and paralysis while extending lifespan [87]. Recently, it was observed that ethyl caffeate treatment significantly delayed the onset of paralysis in the CL4176 *C. elegans* model of AD, following the reduction in the severity of 5-hydroxytryptophan (5-HT) induced paralysis, suggesting a neuroprotective effect. Furthermore, it also activated the nuclear translocation of the transcription factors DAF-16 and SKN-1, leading to increased expression of the stress response genes *sod-3*, *gst-4*, and *hsp-16.2* [88]. Hence, these findings suggest that natural compounds may offer potential therapeutic targets for AD.

Drosophila melanogaster as a model for AD

Drosophila melanogaster has emerged as a cornerstone model organism in biological research, particularly for understanding complex human diseases [27, 28]. With a rich genetic history spanning over a decade, this fruit fly offers a unique combination of scientific, genetic, behavioral, anatomical, and economic advantages. Its genome shares remarkable homology with humans, with nearly two hundred *Drosophila* genes corresponding to human disease-associated genes [29]. The simpler genetic makeup of *Drosophila* compared to vertebrates facilitates gene characterization, while its less complex but structurally similar brain enables the study of NDDs. Furthermore, *Drosophila* exhibits age-related behavioral changes and physiological mechanisms, such as oxidative stress, that

mirror aspects of human NDDs [89, 90]. These combined attributes make *Drosophila* an invaluable tool for investigating the underlying biology of complex disorders and identifying potential therapeutic targets [26]. It has proven to be a valuable model for studying AD due to its genetic simplicity, rapid life cycle, and conserved biological pathways. With a fully sequenced genome of about 30,600 protein-coding genes spread across four chromosomes, *Drosophila* offers exceptional genetic tractability. Its well-characterized nervous system, including the complex eye, allows for detailed phenotypic analysis. The fly exhibits a range of behaviors, from simple reflexes to complex learning and memory, which can be easily quantified [91-93]. This model of AD has been created by overexpressing human AD-related genes like tau and $A\beta_{42}$, resulting in neuronal decline and mimicking key pathological features of AD. Observable phenotypic changes, such as impaired flight, reduced motility, blindness, and early death, provide clear endpoints for assessing disease progression. Additionally, *Drosophila* models have shown memory deficits, a hallmark of AD. This ability to study both cellular and behavioral aspects of neurodegeneration has greatly advanced our understanding of AD pathogenesis [94, 95].

Aβ and tau in *Drosophila* model

Comparative genomics reveals striking similarities between human and *Drosophila* gene structures, providing a strong foundation for using *Drosophila* as an AD model [96]. A significant proportion of human genes associated with AD have homologous counterparts in *Drosophila*. While *Drosophila* possesses a β-secretase-like enzyme, its activity is notably low [97-99]. However, the fly's APP homolog, dAPPl, shares structural similarities with its vertebrate counterpart. Overexpressing the *Drosophila* β-secretase-like enzyme can lead to the cleavage of dAPPl and the generation of peptides resembling human Aβ peptides. Intriguingly, the accumulation of these peptides in *Drosophila* is linked to neurodegeneration and age-related behavioral deficits. These findings underscore the potential of *Drosophila* as a valuable tool for understanding the molecular mechanisms underlying AD pathogenesis [100]. In addition to $A\beta_{42}$ plaques, hyperphosphorylated tau protein, and the resulting NFTs are key pathological features of AD. While the interaction between these two hallmark features is not fully understood, *Drosophila* models expressing $A\beta_{42}$ have been used to explore whether endogenous tau can form fibrillary aggregates [101]. Despite the presence of $A\beta_{42}$, these models did not show hyperphosphorylated tau or NFT formation. On the other hand, overexpressing both wild-type and mutant human tau in *Drosophila* resulted in intracellular lesions and hyperphosphorylation, though classic filamentous structures were not observed. Nevertheless, these tau-expressing flies exhibited neurodegeneration and abnormal tau protein distribution, as shown by immunostaining [27, 102]. Additionally, the development of vacuolar lesions in the *Drosophila* nervous system, along with age-dependent neurodegeneration and early mortality, highlights the model's value for studying tau-related pathologies [103]. To investigate the synergistic effects of tau and $A\beta_{42}$, Folwell and colleagues co-expressed both proteins in *Drosophila*. The combination of these pathological proteins exacerbated tau-induced axonal transport defects, reduced survival, and neuronal dysfunction. This model system provides a valuable platform for studying the early stages of AD pathogenesis and identifying potential genetic modifiers [104]. Utilizing the rough eye phenotype as a screening tool, researchers identified *Drosophila* homologs of GSK-3β as modulators of tau-mediated toxicity. Overexpression of GSK-3β intensified tau-induced eye defects, while its reduction had a protective effect. Mechanistic studies revealed that GSK-3β plays a critical role in promoting pathogenic tau phosphorylation [105].

To elucidate the relationship between tau phosphorylation and toxicity, researchers have generated *Drosophila* models expressing tau variants resistant to specific phosphorylation sites. By mutating serine or threonine residues to alanine, Chatterjee *et al*. created fly lines expressing tau proteins resistant to phosphorylation by kinases such as GSK3β and PAR-1. This approach allowed for a detailed examination of the role of specific phosphorylation sites in tau-mediated toxicity. Interestingly, while the overexpression of GSK3β exacerbated the rough eye phenotype in flies expressing wild-type tau, it had a less pronounced effect on flies expressing the TauS2A mutant, despite increased overall tau phosphorylation. Conversely, the TauS11A mutant, which was resistant to GSK3β phosphorylation, exhibited an exacerbated rough eye phenotype when GSK3β was overexpressed [106]. Furthermore, building on previous research, Iijima-Ando *et al*. generated an additional Tau variant, TauS262A, which is resistant to phosphorylation at serine 26 [107]. Similarly, Steinhilb and team unveiled the critical role of phosphorylation in Tau-mediated neurotoxicity. The creation of phospho-deficient (TauAP) and phospho-mimetic (TauE14) Tau variants provided compelling evidence that altered phosphorylation states can significantly impact disease progression [108]. These findings suggest a complex interplay between tau phosphorylation, specific phosphorylation sites, and toxicity. Moreover, the study revealed no consistent correlation between tau's microtubule binding properties, aggregation, and the rough eye phenotype, indicating that other factors may contribute to tau-mediated toxicity.

Role of metal ion in AD by using the *Drosophila* model

Drosophila models have also been pivotal in exploring the impact of metal ions on AD pathogenesis [109]. Excessive aluminium, cadmium, lead and zinc exposure leads to neurodegenerative symptoms such as shortened lifespan, impaired locomotion, olfactory learning deficits, and brain degeneration [110-114]. The complex relationship between iron and Aβ peptides has been a key area of investigation. Iron has been found to influence $A\beta_{42}$ toxicity, with iron chelation mitigating $A\beta_{42}$-induced damage. Interestingly, while ferritin knockdown protected against $A\beta_{42}$ toxicity, it did not impact Aβ accumulation. Biophysical studies have shown that Fe^{2+} affects Aβ fibril formation,

indicating a complex interaction between iron and Aβ in disease progression [115]. Research has demonstrated that dietary supplementation with copper or zinc exacerbates $A\beta_{42}$-induced neurotoxicity in flies, resulting in a reduced lifespan and locomotors abnormalities [116-119].

Transgenic *Drosophila* model for AD

To investigate endogenous Aβ production and human $A\beta_{42}$-induced neurotoxicity, several transgenic Drosophila models have been developed, expressing variant genes associated with familial AD [120]. Greeve *et al*. created a triple transgenic fly expressing human APP and human β-secretase, incorporating familial AD mutations (N141I, L235P, and E280A). These flies exhibited age-dependent neurodegenerative phenotypes including photoreceptor cell loss, severe axonal degeneration, and premature death [121]. The co-expression of human APP and β-secretase in *Drosophila* facilitated the production of a highly glycosylated form of human APP, leading to the formation of $A\beta_{40}$ and $A\beta_{42}$ plaques in transgenic tissues. This triple transgenic model effectively recapitulates key metabolic processes associated with $A\beta_{42}$ accumulation in humans, highlighting the utility of *Drosophila* for studying AD pathogenesis [122]. To circumvent the complexities of APP processing, researchers have developed *Drosophila* models expressing fully processed Aβ peptides. Finelli *et al*. generated transgenic fly lines expressing human $A\beta_{40}$ and $A\beta_{42}$, enabling the investigation of Aβ accumulation and toxicity in various cell types, including brain cells. Notably, $A\beta_{42}$, but not $A\beta_{40}$, accumulated in the *Drosophila* brain, leading to dose- and age-dependent neurodegeneration, cognitive deficits, locomotor impairments, and reduced lifespan [123]. Furthermore, Crowther *et al*. employed a more direct approach by fusing $A\beta_{40/42}$ peptides to a native *Drosophila* secretory signal sequence. Using the UAS/Gal4 system, these researchers achieved spatiotemporal control over $A\beta_{40/42}$ expression, bypassing the influence of APP processing. This model offers a streamlined approach to studying the toxic effects of Aβ peptides independently of APP-related factors [120]. Although *Drosophila* does not possess a native APOE gene, Haddadi *et al*. developed a groundbreaking transgenic model by introducing human APOE. This innovative approach allows for the investigation of neurodegenerative disease mechanisms and potential therapeutic interventions in a genetically tractable organism [124].

***Drosophila* AD model for screening of new therapeutic agents**

Recent studies have unveiled a complex interplay between epidermal growth factor receptor (EGFR) and $A\beta_{42}$ in *Drosophila* models of AD. Overexpression of EGFR exacerbated $A\beta_{42}$-induced cognitive decline, suggesting a detrimental role for EGFR in AD pathology. Conversely, EGFR inhibitors, gefitinib and erlotinib, commonly used in cancer therapy, effectively ameliorated $A\beta_{42}$-associated memory loss in *Drosophila*. These findings highlight the potential therapeutic benefits of targeting EGFR in AD. Additionally, the study demonstrated the efficacy of memantine, a standard-of-care AD drug, in mitigating $A\beta_{42}$-induced cognitive impairments in flies, further validating the *Drosophila* model for AD research [125]. Several natural compounds have shown promise in combating AD such as Curcumin, a compound derived from turmeric, has shown promise as a potential therapeutic for AD in *Drosophila* models. When administered to flies expressing $A\beta_{42}$, curcumin significantly improved lifespan by up to 75% and enhanced locomotor activity. Mechanistic studies suggest that curcumin promotes the conversion of Aβ oligomers (toxic species) into less harmful amyloid fibrils, thereby reducing neurotoxicity [126]. The therapeutic potential of a novel compound, XJP-1, has been explored using the Aβ-arc transgenic *Drosophila* model of AD. Derived from a substance found in Musa *sapientum L*. peel, XJP-1 demonstrated promising effects in ameliorating AD-related symptoms in flies. Treatment with XJP-1 led to improved locomotor function, reduced amyloid plaque formation, and extended lifespan [127]. Further, acacetin, isolated from *Agastache rugosa* has shown promise by suppressing APP synthesis and Aβ production, potentially through downregulation of APP and BACE-1 mRNA [128]. D737, another compound, extended lifespan and improved locomotor activity in AD flies by inhibiting $A\beta_{42}$ aggregation and reducing oxidative stress [129]. Additionally, Quercetin, a flavonoid, has also demonstrated therapeutic potential in *Drosophila* AD models by mitigating Aβ toxicity. It regulates the expression of cell cycle-related genes, restoring cyclin B levels and ultimately improving lifespan and climbing ability [130]. These findings collectively highlight the potential of natural compounds as promising candidates for AD drug development.

Zebrafish as a model for AD

In the 1970s, George Streisinger at the University of Oregon pioneered the use of zebrafish as a model organism, noting their simpler biology and easier genetic manipulation compared to mice. Over time, zebrafish models have proven crucial in dissecting the complex mechanisms connecting AD features, such as tau and Aβ buildup, to neuronal damage and cognitive decline [131, 132]. While rodent models have also been valuable in AD research, zebrafish have provided unique insights into genes linked to AD that were challenging to study before. Despite significant progress in understanding AD through zebrafish research, the exact molecular processes driving neurodegeneration are still not fully understood [34, 133]. Nonetheless, zebrafish have become a fundamental tool in biomedical research in the past decade, with applications extending beyond NDDs [134]. They allow for precise genetic manipulation at physiologically relevant levels, exceeding the capabilities of rodents. Their relatively large size, accessibility, and transparent embryos make it easy to observe gene function during development. As a result, zebrafish embryos are an invaluable vertebrate model for investigating the cellular and molecular mechanisms associated with AD-related genes [135, 136]. Although many

zebrafish genes are recognized as orthologs of human genes, the complete scope of genetic homology is still being explored.

Zebrafish exhibit a brain structure strikingly similar to that of mammals, including key neurotransmitter systems and a functionally comparable blood-brain barrier. Many genes associated with AD in humans have direct counterparts in zebrafish, such as the "co-orthologs" *appa* and *appb*, which are analogous to the human APP [137-139]. The zebrafish model is particularly suited for genetic manipulation. Techniques such as mRNA injection, genome editing, and transgenesis provide precise control over gene expression, allowing researchers to study both subtle and significant changes in gene function during development [140]. Combined with the transparency of zebrafish embryos, these features offer exceptional opportunities for observing the cellular and molecular impacts of genetic alterations.

Zebrafish exhibit striking similarities to humans in terms of brain structure and function, making them a valuable model for studying neurological diseases. Key proteins involved in AD, such as β-secretase and tau, have identifiable counterparts in zebrafish [141-143]. β-secretase and tau, both species share similar brain regions, neurotransmitter systems, and cellular components [144-146]. Additionally, they have enzymes for production and metabolism, as well as neurotransmitters such as glutamate, serotonin, histamine, GABA, dopamine, and acetylcholine [147, 148]. Astrocytes, microglia, oligodendrocytes, motor neurons, cerebellar Purkinje cells, and myelin present in zebrafish have characteristics that are also similar to those of human cells, demonstrating that similarities exist even at the cellular level [149-151]. Furthermore, zebrafish display comparable behavioral patterns and learning capabilities to humans. Researchers have demonstrated that zebrafish exposed to $A\beta_{1-42}$, a hallmark peptide of AD, develop cognitive impairments and increased tau phosphorylation, mimicking key features of the human disease [133]. Researchers also have looked into non-associative learning throughout zebrafish larvae according to the cognitive and behavioral reactions of AD. Larvae have been subjected to a wide range of different stimuli seven days post fertilization (dpf) and they significantly reduced their startle response [152]. Indeed, another group administered $A\beta_{1-42}$ through injection to zebrafish embryos that were 24 hours post-fertilization (hpf). They noticed significant cognitive impairments in the 5 dpf larvae of the embryos having higher tau hyper-phosphorylation in GSK3 targeted residues [153]. These findings underscore the potential of zebrafish as a model organism for understanding the underlying mechanisms of AD and exploring therapeutic interventions.

Zebrafish have also emerged as valuable models for studying the vascular and neurodegenerative aspects of AD [154]. Research has shown that elevated levels of Aβ in zebrafish can induce abnormal blood vessel growth, while Aβ deficiencies lead to cerebral hemorrhage. Interestingly, the administration of human $A\beta_{1-42}$ can reverse these hemorrhages, suggesting a complex role for Aβ in cerebrovascular development. Additionally, high $A\beta_{1-40}$ exposure is linked to abnormal blood vessel formation and cell death in zebrafish embryos. Beyond vascular changes, zebrafish models have been developed to investigate tauopathy, a hallmark of AD. These models express mutant human tau proteins, leading to cytoskeletal disruptions and the formation of NFTs similar to those found in human AD brains [155-158]. In a groundbreaking study, researchers successfully overexpressed human tau protein in zebrafish by replacing a native zebrafish gene with human tau cDNA. This genetic manipulation resulted in an eightfold increase in tau levels, leading to the accumulation of tau protein within axons, mimicking the formation of NFTs observed in AD [159]. Recent research has expanded the utility of zebrafish as an AD model. By introducing okadaic acid, a protein phosphatase 2A inhibitor, researchers have developed a pharmacological model that mimics AD-like pathologies. This treatment leads to increased tau phosphorylation, similar to that observed in AD patients, and results in cognitive and behavioral impairments [160]. Additionally, the formation of Aβ plaques is enhanced in these zebrafish. Another model involves exposure to aluminum in acidic conditions, which induces AD-like symptoms, including impaired swimming and learning abilities. These studies collectively demonstrate the versatility of zebrafish as a platform for investigating AD pathogenesis and for developing potential therapeutic interventions [161].

Zebrafish model for Aβ toxicity

The human APP has co-orthologs, *appa*, and *appb*, which show extensive and overlapping expression patterns during early zebrafish embryogenesis, starting around mid-gastrulation. While these genes are expressed in various tissues, *appb* is uniquely present throughout the developing spinal cord at 24 hours post-fertilization [162, 163], whereas *appa* is present in ependymal cilia, having critical role in cerebrospinal fluid flow and brain homeostasis [164]. Lee and Cole linked a segment of the *appb* promoter to green fluorescent protein in *Danio rerio*, revealing unexpected *appb* expression in the emerging vasculature, contrary to previous observations. Similarly, *appa* and *APP*-like protein 2, which contain transposon gene trap insertions encoding the red fluorescent protein, result in fusion proteins that accumulate in the vasculature. Despite this, transcripts of these genes were not detected in endothelial cells but were found in neurons, suggesting that the proteins are initially produced in neural cells before accumulating in the vasculature [165]. To explore the function of *appa* and *appb*, translation-blocking morpholinos were used [166]. Inhibiting *appb* led to abnormal cellular movements and reduced body length while suppressing *appa* had no noticeable impact on embryonic development. Notably, injecting human APP mRNA rescued the *appb*-deficient phenotype, although the FAD variant of human APP was less effective. Further research linked *appb* deficiency to impaired axonal outgrowth and synapse formation during brain development [167]. Song and Pimplikar demonstrated that both the extracellular and

intracellular domains of human APP are crucial for its function, as only full-length, not truncated, human APP could correct neural defects. Hence these findings collectively highlight the zebrafish embryo as a valuable model for studying various mutant forms of human APP [168]. Additional research has indicated that the zebrafish *psen1* wild type contributes to the abnormal production of $A\beta_{1-42}$, a process linked to familial FAD mutations [169]. Inhibiting *psen1* translation in zebrafish embryos through the use of morpholinos results in viable embryos but triggers p53-dependent neuronal cell death. Furthermore, studies on *psen1*-deficient zebrafish mutants have unveiled a novel role for *psen1* in regulating the development of histaminergic neurons [170, 171]. These viable mutant fish provide evidence that psen1 is essential for the growth of these specific neurons.

Zebrafish are a valuable model organism for studying the effects of hypoxia on various biological processes. Researchers can induce hypoxic conditions in both adult and embryonic zebrafish using chemical methods, such as administering sodium azide, or by directly reducing the oxygen levels in their aquatic environment [163]. Importantly, hypoxia triggers the upregulation of *appa*, *bace1*, *appb*, *psen2*, and *psen1* genes in the brains of both adult and larval zebrafish, mirroring the response observed in humans. These findings suggest that Aβ production in both humans and zebrafish may serve as a protective mechanism against hypoxic conditions [172].

Transgenic zebrafish model for AD

Transgenic zebrafish, which are engineered to carry exogenous genes, offer a versatile platform for studying gene function, regulation, and overexpression, among other applications. Three main techniques *i.e.* meganuclease injection, transposon insertion, and microinjection or electroporation of linearized DNA, facilitate the creation of transgenic zebrafish embryos [161]. A commonly used method involves microinjecting embryos with a construct containing a transposon and transposase mRNA, leading to the integration of extrachromosomal DNA. This approach has been employed to create transgenic zebrafish expressing human MAPT to explore its function. Notably, zebrafish CNS neurons uniquely exhibit human MAPT expression. Bai *et al*. utilized the enolase 2 gene promoter to achieve high-level MAPT 4R expression in zebrafish neurons, resulting in tau protein accumulations that resemble NFTs [159]. Further developments by Paquet *et al*. used the HuC promoter to drive Gal4:VP16 expression in neurons, followed by the induction of DsRed and mutant TAU-P301L via a bidirectional promoter. These transgenic zebrafish showed biochemical alterations typical of human tauopathies. Additional models expressing Tau-P301L and Tau-A152T mutations were developed using the HuC promoter to closely mimic clinical features. These models display hyperphosphorylation, behavioral deficits, neuronal loss, and protein aggregation, similar to human tauopathies [170]. Lopez *et al*. observed reduced proteasome activity, increased tau phosphorylation, and neurodegeneration in Tau-A152T transgenic zebrafish, with autophagy promoting tau clearance [173]. Similarly, Tau-P301L-Tg zebrafish mainly showed tau hyperphosphorylation without significant oligomerization or NFT formation [174].

Zebrafish AD models for screening of new therapeutic agents

Zebrafish models have become essential for studying AD and evaluating potential treatments. Additionally, research has explored the therapeutic benefits of various compounds in zebrafish AD models. In a research $AlCl_3$-induced zebrafish model linarin significantly reduced AChE activity and improved dyskinesia recovery [175]. Similarly, Necrostatin-1 was tested in an $AlCl_3$-induced AD model, showing the potential to reverse learning and memory deficits, increase acetylcholine levels, and affect the expression of genes associated with necroptosis [47]. Furthermore, Silibinin and naringenin were found to alleviate neuroinflammation, oxidative stress, and neuroapoptosis in a bisphenol $AlCl_3$-induced AD model, leading to improved cognitive function [176]. Another study examined the neuroprotective effects of TDZD-8 in an okadaic acid-induced AD model, showing that it decreased mortality, improved cognitive impairments, restored protein phosphatase 2A activity, and balanced GSK3β phosphorylation [177].

Conclusions

AD is a multifaceted condition influenced by factors such as inflammation, oxidative stress, head injuries, genetics, and diabetes. While mammalian models have been used to study AD, their long lifespans, ethical concerns, and high costs presents significant challenges. To address these issues, researchers have turned to novel model organisms. This review highlights the potential of non-mammalian models, such as zebrafish, fruit flies, and worms, to advance our understanding of AD. These organisms offer advantages over traditional rodent models such as shorter lifespans, ethical permissibility, and lower maintenance costs. With advanced genetic tools, scientists can manipulate genes in these models to investigate their roles in AD development and progression Additionally, these models are crucial for screening environmental factors that might increase AD risk and for validating genetic risk factors identified in human studies. By leveraging these unique features, researchers can investigate the underlying mechanisms of AD, identify novel drug targets, and rapidly screen potential therapeutic compounds. As our knowledge of AD advances, these non-vertebrate models will remain essential for accelerating drug discovery and therapeutic development. While these models have proven to be valuable tools, it is important to recognize their limitations and to integrate them with other approaches, including human clinical studies and advanced imaging techniques. By combining the strengths of diverse model systems, researchers can gain a more comprehensive understanding of AD and ultimately develop effective strate-

gies for its prevention and treatment.

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