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Modeling human neurodegenerative disorders in *Drosophila melanogaster*

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Abstract

Drosophila is a well-understood, genetically tractable model organism with approximately 75% of human disease-related genes having functional homologs. In the last two decades, Drosophila has contributed significantly to the fields of life sciences and drug discovery, owing to its powerful and unparalleled genetic tools that have provided intricate understanding of cellular and molecular pathways conserved in humans. Drosophila is suitable for elucidating disease-related complexities and can manifest responses to potential treatments. The genetic systems have revealed factors involved in pathological pathways, leading to the identification of novel druggable targets. In this review, we summarize Drosophila as a high-throughput model for recapitulating various aspects of major human neurodegenerative diseases, such as Parkinson's disease, Alzheimer's disease, and Huntington's disease, to identify active compounds and analyze their pharmacological properties by performing different assays. We also illustrate representative studies and findings executed in fruit fly models to explore their potential in therapeutic discovery, validation, and repositioning of effective drugs to manage and ameliorate these diseases, as well as their subsequent translation and validation in higher animal models.

Key words : *Drosophila,* Neurodegenerative diseases, Parkinson's disease, Alzheimer's disease, Huntington's disease, Drug discovery.

D*rosophila* has been cardinal in the fields of genetics and developmental biology. *Drosophila* resembles mammalian species in some aspects such as biological, physiological, neurological and biochemical. Many genes and molecular processes in fly are found to be functionally conserved in other higher organisms including humans making it a powerful model system for research. Moreover, about 75% of human disease-related genes have functional homologs in the fruit fly and thus proved to be highly versatile in modeling human diseases[31]. *Drosophila* was the first major complex organism to have its genome elucidated and

annotated. The genome contains approximately 16,000 genes and more than 60% of protein encoding genes have orthologous equivalent in humans[17]. Comparative genomic studies of fly and human genome have revealed that fundamental biological pathways are conserved between them[40]. In *Drosophila*, about 700 human disease-related genes have homologues providing a great tool to understand gene function and its molecular mechanism[30]. Genetic homology between *Drosophila melanogaster* and *Homo sapiens* make it a potential model for studying human diseases and putative pharmacological interventions. Genetic screens in *Drosophila* have led to the discovery of important signaling pathways, including *Notch*, *wingless*, and *hedgehog* which are critical for developmental studies in vertebrates as well as to understand the origins of human disease[6].

Humans are currently facing a serious challenge of tackling various new age diseases. With the rising economic development, new lifestyle related diseases have emerged which were very insignificant in number few decades ago. Depression, neurological disorders, cardio vascular diseases, cancers and diabetes are among the most notorious ones[20]. Due to ethical concerns, human studies are less and limited in their designs. Several in vitro and in vivo approaches are utilized to study various aspects of these complex diseases so that therapeutic targets could be identified. *In vitro* models suffer from their less relevancy to whole systems. Despite of being quick, they provide only preliminary evidence. Therefore, *in vivo* animal model become highly desired. Various invertebrate and vertebrate organisms have been utilized in life sciences to model human diseases such as *Caenorhabditis elegans*, yeast, *Xenopus*, *Drosophila*, zebra fish and mouse. Model organisms help to progress our knowledge of development, cellular functions, human disease mechanisms. The findings in one organism can be applied to other organisms to generalize them[48]. A comparison of merits and demerits of each animal model is beyond the scope of this review, hence, only *Drosophila melanogaster* is discussed below as a disease model.

Drosophila melanogaster as an emerging disease model :

Drosophila offer several advantages over mammalian models, including short life cycle, high fecundity, low maintenance cost, and rapid study turnaround[31,39]. Their genetic malleability and the ease of detecting gene products makes them powerful models for molecular and genetic analysis, fulfilling the 3R criteria: Replacement, Refinement and Reduction of animal utilization in research[44]. *Drosophila* models human diseases, aiding identification of therapeutic targets or biomarkers and the screening of potential treatment substances. They are utilized in neurodegeneration, Parkinson's disease, Alzheimer's disease, sleep disorders, seizure disorders, cognitive disorders, cardiovascular diseases and more[31,48]. They help in understanding disease pathways due to substantial sequence and functional conservation with human disease-related genes[32].

Powerful genetic tools available include UAS-GAL4 binary expression system and "RNA interference" technique for ectopic gene expression of human-disease genes in

Drosophila[30,37]. Specific GAL4 driver lines help in tissue-specific gene expression resulting in specific phenotypes. Transposable elements, typically P-elements, are inserted at known genome sites[31,38,48]. Drosophila studies follow three main approaches: (1) forward genetics, (2) reverse genetics and (3) diagnostic strategy to discover disease-causing genes[38,42]. Forward genetics use random mutations to screen for phenotypes, while reverse genetics generates mutations in fly homologues of human genes to observe in vivo phenotypes. Gene knockouts or knockdowns in flies are achieved through (1) transposon-mediated mutagenesis, (2) CRISPR/Cas9 system, or (3) removal of existing transposable elements and gene silencing through RNA interference (RNAi).

Fly Models of neurodegenerative diseases:

Drosophila melanogaster (*D. melanogaster*) is extensively used to model various human neurodegenerative diseases[8,40]. The fly brain contains approximately 90,000 neurons, million times less than the human brain but with a similar complexity in central nerve system (CNS) similar to higher organisms consisting of neurons and glial cells, and is protected by a blood-brain barrier. It is similar in organization, but less intricate than the mammalian brain and have a similar neurotransmitter profile to mammals (GABA, Glutamate, Acetylcholine)[30,45,28,34]. Moreover, fly and mammals share several neuromodulatory peptides and have some common biogenic amines like serotonin and dopamine. Similar to mammals, flies contain same families of calcium and potassium channels which regulate membrane potential and also have sodium channels for action potential propagation. Both fly and mammals share common protein architecture at synapses making them beneficial for neuronal function study and modelling of diseases. Thus, understanding of fly nervous system may be pertinent to other species also[45]. *Drosophila* offers advantage of completely removing neurons without killing animals. Like mammalian spinal cord, flies also have a segmented nerve cord (FLYBRAIN neuron database). *Drosophila* research has provided comprehensive knowledge of various aspects of neuroscience such as neural development, synaptic transmission, axon guidance, learning and memory and neural disease.

Parkinson's disease (PD) :

Parkinson's disease, the second most common age-related neurodegenerative disorder, is due to degradation of dopaminergic neurons in the brain[5,9,31]. Dopamine, vital for muscle activity control, is transported by these neurons to the basal ganglia, and in fruit flies, plays roles in olfaction, locomotion, memory, learning, and sleep[17]. Loss of these neurons causes motor symptoms like muscle rigidity, resting tremor, and bradykinesia, alongside non-motor symptoms like fatigue, mood alterations, and sleep disturbances[9,31,38]. Intracytoplasmic Lewy bodies, aggregates of a neuronal protein called α-synuclein, signify Parkinson's, with other pathological mechanisms being neuroinflammation, mitochondrial dysfunction, impaired autophagy, oxidative stress, etc[9,13].

Majority of Parkinson's cases are sporadic, with a minority being familial, linked to specific gene mutations. Familial forms are due to SNCA and LRRK2 (dominant) or

PINK1, Parkin, and DJ-1 (recessive). Many genes, including α-synuclein, Parkin, LRRK2, PINK1, and DJ-1, are implicated in monogenic Parkinson's forms[17,24,31]. Although Drosophila lack an SNCA homolog, LRRK2's fly homolog, Lrrk, and proteins PINK1, Parkin, and DJ-1 influence mitochondrial homeostasis. Flies lacking parkin exhibit lifespan reduction, motor defects, and DA neuron degeneration. Drosophila has two DJ-1 orthologs, DJ-1β and DJ-1α, implicated in antioxidant responses[17,25]. Research suggests chronic exposure to agro toxins, along with age, sex, genetic profile, and diet, may be linked to idiopathic Parkinson's cases. Toxins like paraquat, MPTP, 6-OHDA, and rotenone are identified as risk factors[24].

Modeling Parkinson's disease in Drosophila:

Fly exhibit PD-like phenotypes which are characterized by degeneration in DA neurons, locomotion defects, protein aggregation, mitochondrial dysfunction and oxidative stress[19]. Researchers have modelled PD in vertebrate and invertebrate animal models to reproduce pathological features of the disease to understand the pathophysiology. Two types of fly models are generated in PD to mimic familial and sporadic forms of disease: the genetic models, which involves mutation in PD-related genes and toxin models, which involves degeneration of dopaminergic neurons by administrating neurotoxin. Although *Drosophila* is an invertebrate model and evolutionarily distant from humans, it offers appreciable genetic manipulation tools and assays to model pathology and have orthologs of many PD genes. PD is associated with motor symptoms which can be studied in fly through climbing and locomotor activities. In *Drosophila*, toxin models are produced by administering a specific neurotoxin leading to dopaminergic neuronal loss and display of certain PD related behavioural and histopathological changes. Many homologs of Parkinson's related genes are found in fruit fly such as parkin, PINK1, PARK2, DJ–1, LRRK2, HtrA2, UCH–L1, Tau and GBA[9,19,50]

Therapeutic studies in Drosophila PD models :

While no definitive cure exists for Parkinson's disease, therapies such as dopamine replacement, using dopamine precursors (levodopa), metabolism inhibitors (carbidopa, entacapone, selegiline), and dopamine agonists (ropinirole, bromocriptine, etc.) manage symptoms[9,13,38]. These therapies can have limitations, such as levodopa leading to dyskinesia and lessening in effectiveness over time. *Drosophila* Parkinson's models have helped discover potential therapeutic compounds. These include those inhibiting α-syn aggregation, such as rifampicin and nortriptyline, and antioxidants like curcumin, tocopherol, and resveratrol, all improving locomotor activity and reducing ROS levels in fly models[35,38]. Methylene blue, another compound, can prevent the damaging effects of a deficient electron transport chain in Parkinson's[38]. Investigations using redox-sensitive probe possessing transgenic flies allow us to study the redox changes in disease and normal conditions. This technique, reliant on redox-sensitive green fluorescent protein constructs, was used to assess the therapeutic potential of natural and synthetic antioxidant compounds in a *Drosophila* Parkinson's model[18].

α-synuclein fly model :

Flies expressing wild-type and mutant forms of the α-synuclein protein show formation of α-synuclein aggregates, specific loss of dopaminergic neurons and a decline in locomotory behaviour. In α-synuclein transgenic flies, these PD-related phenotypes were used to explore the protective effects of exogenous molecules. Glutathione metabolism is important for the maintenance of dopaminergic neurons and α-synuclein toxicity can be supressed by the overexpressing the components involved in glutathione biosynthesis and conjugation pathway. For instance, flies fed with sulphorafane and allyl disulfide showed protection against oxidative stress and α-synuclein toxicity by increasing glutathione abundance. The enzyme methionine sulfoxide function as a catalytic antioxidant and its overexpression has showed to protect dopaminergic neurons from degeneration and also reduce climbing defects induced by wild-type or A30P α-synuclein. In another study the overexpression of an antioxidant enzyme, superoxide dismutase 1 (SOD1) reduced both neuronal degeneration and locomotion impairment by removing superoxide radicals[17].

DJ-1 fly model for Parkinson :

A study involving DJ-1β mutant flies, linked to early-onset Parkinsonism, showed increased oxidative stress indicators and locomotor dysfunction. Long-term treatment with antioxidants vitamin C and α-tocopherol lessened oxidative stress and improved lifespan. Dietary α-tocopherol specifically benefitted the DJ-1β mutant flies by reducing ROS and H_2O_2 levels. Both antioxidants increased catalase activity, vital for combating oxidative stress[17,18]. Another study used these mutant flies in high-throughput screening of Prestwick® chemical library's compounds for disease-modifying Parkinson's therapies. Notably, Zaprinast (ZAP), a phosphodiesterase (PDE) inhibitor, showed remarkable activity by reducing oxidative stress-induced cell death, attenuating apoptosis, and enhancing mitochondrial viability. ZAP's effectiveness suggests the potential for drug repurposing in neurodegenerative disease treatment discovery[34].

Bawani and Anandhi demonstrated the neuroprotective properties of *Nigella Sativa* seed extract, containing pharmacologically active compounds like thymoquinone and carvacrol, on a transgenic *Drosophila* Parkinson's model. N. Sativa, known for its antioxidant, anti-cancer, and anti-inflammatory properties, was analyzed using GC-MS, identifying 51 compounds. Transgenic flies, expressing human α-synuclein in dopaminergic neurons, exhibited locomotor dysfunction and reduced lifespan. *N. sativa* treatment for 21 days delayed loss of climbing ability, indicative of improved motor function, and extended lifespan in a dose-dependent manner. This study affirms the therapeutic potential of *N. sativa*'s antioxidative phytoextracts in modulating neurodegenerative pathways, enhancing motor abilities, cognitive functions, and lifespan in Parkinson's models[7].

Alzheimer's disease (AD) :

In 1906, Alzheimer's disease was described for the first time. The leading cause of dementia is Alzheimer's disease (AD), a progressive neurodegenerative disease[2]. AD is an age-related disease and associated with cognitive and motor decline, disrupted memory,

behavioural alterations, intellectual impairment, reduced life expectancy and language difficulties with no cure till date[4,16,40,41,43]. In AD, nerve cells (neurons) are damaged or destroyed in the brain parts that are involved in basic body functions and cognitive functions[2]. Sporadic AD is found in over 95% of the cases while less than 5% cases are categorized as familial AD. Familial AD (FAD) is inheritable and caused by mutations in the gene encoding APP (amyloid precursor protein), Presenilin 1 (PS1) and Presenilin 2 (PS2)[8,24,40].

NDs are often characterized by a decrease in neurotransmitters such as neuroactive amines and acetylcholine which are associated with brain. Acetylcholine is used by the cholinergic neurons and play a vital role in central and peripheral nervous systems. Therefore, therapeutic interventions made use of cholinesterase inhibitors namely, donepezil and galantamine and monoamine oxidase inhibitors to control these diseases. However, these approaches were found to have harmful effects, therefore, alternative medicinal or dietary interventions are needed for their prevention[29].

Pathogenesis of Alzheimer's disease :

In humans, the neuropathological characteristics of Alzheimer's disease brain include extracellular deposition of misfolded amyloid-β peptide (Aβ40 and principally Aβ42) as senile plaques and intracellular accumulation of hyperphosphorylated forms of microtubule-associated Tau protein as neurofibrillary tangles (NFTs)[2,3,4,23,24].

Neurofibrillary tangle formation is the hallmark of AD and also of other taupathies. Tau stabilizes microtubules by bridging them with cytoskeletal filaments[11,51]. Under natural conditions Tau is soluble and in unfolded form but, under the pathological conditions, hyper-phosphorylation of tau by several kinases decreases its microtubule binding affinity and axon localization and consequently results in the formation of aggregates of tau tangles in the neuronal soma[14]. It is evident from the human post-mortem and animal model studies that Tau-mediated mechanisms contribute to genomic instability in affected neurons[23]. In *Drosophila* studies also, the taupathy mediated factors have been found including abnormal activation of heterochromatic genes at transcriptional level, breaks in DNA double strands and relaxation of global nuclear chromatin[26].

The formation of amyloid plaques is the second hallmark of AD. Aβ is a small peptide composed of 39–42 amino acids and is produced by proteolytic cleavage of its precursor, amyloid precursor protein (APP) transmembrane receptor at the beta and gamma sites. In the first step, APP is cleaved by β-secretase (BACE1) or α-secretase (mainly ADAM10) and then BACE1- and α-secretase-generated C-terminal fragments are cleaved by γ-secretase complexes (Presenilin 1, presenilin 2, nicastrin, aph-1, and pen-2 are subunits) to produce Aβ peptide (pathogenic) and p3 peptide (non-pathogenic) respectively[3,24]. The most prevalent form of Aβ peptide is Aβ42 which is found in plaques and is neurotoxic[12]. Aβ42 oligomers being hydrophobic form insoluble fibres and are predominantly implicated in AD pathology[4,24]. At synapses beta-amyloid plaques are believed to interfere with neuronal communication and thus

promoting cell death whereas the transportation of nutrients and essential molecules inside the neurons is obstructed by tau neurofibrillary tangles[2].

Modeling Alzheimer's disease in Drosophila:

Several Alzheimer's disease (AD) models in *Drosophila* exist, developed by manipulating AD-related genes like APP, Aβ42, tau, and presenilin[4,21,31,36]. Fly homologues of these genes, such as APP-like (APPL) and Kuzbanian (kuz) which cleave APPL, have been identified, with abnormal behavior seen in APPL deficient flies[8]. Fly models also exhibit traits like locomotor defects and memory issues, enabling therapeutic potential assessment of different compounds[38].

Zhang *et al*.[51] investigated the pharmacological activity of Salidroside (Sal), extracted from *Rhodiola rosea* L., in a tau *Drosophila* transgenic model. Sal has anti-oxidative, anti-inflammatory, and anti-apoptotic properties, and treatment improved lifespan, survival rate, and locomotor activity in AD flies. Sal prevented vacuole appearance in fly brains, signaling attenuated neuronal loss, and regulated GSK-3β phosphorylation and tau phosphorylation in the GSK-3β signalling pathway. GSK-3β is a kinase that phosphorylates tau, causing AD pathogenesis, and its pathway is crucial for neuronal survival. Sal's effectiveness suggests it as a potential therapeutic agent for AD[51].

A study evaluated the neuroprotective effects of polyphenolic extract from *Arabidopsis thaliana* in Aβ25-35 peptide treated BV2 cells and human-Aβ expressing *Drosophila* AD models. Polyphenols exert beneficial effects on memory, modulate pro-inflammatory gene expression and suppress microglial activation. They stimulate α-secretase activity, inhibit β- and γ-secretase activity and competitively interact with aromatic residues to prevent amyloid aggregates[27]. Examples of such polyphenols include quercetin, myricetin, curcumin, and trans-resveratrol, among others. Analysis of polyphenols in *Arabidobsis* extract revealed neuroprotective effects in transgenic AD flies expressing human APP (Aβ1–42)[10,27].

Another study examined the role of biotin in mitochondrial pathology and neurodegeneration in a *Drosophila* taupathy model. The gene Btnd, essential for biotin homeostasis, was found to have abnormal expression in tau transgenic flies, leading to biotin deficiency, impairing mitochondrial function and neuronal health, and causing neurodegeneration[26]. Biotin supplementation was found to improve mitochondrial dysfunction and neuronal health in diseased model. TauR406W-expressing flies displayed pathological features of taupathy, including hyperphosphorylation of tau, neurodegeneration, and decreased longevity. Chronic biotin feeding improved locomotor defects and reduced TauR406W-mediated neurotoxicity[26,47].

Modeling Huntington's disease in Drosophila:

Huntington's disease (HD) is a rare, hereditary neurodegenerative disease caused by expansion of a polyglutamine (polyQ) stretch in the huntingtin protein (Htt), leading to motor dysfunction, psychiatric disorders, and cognitive defects. The expanded CAG repeats in the huntingtin gene result in defective HTT protein, causing cellular dysfunctions[1,33].

Agrawal *et al*. leveraged a Drosophila HD model to investigate combinatorial drug regimens. They developed protocols to assess drug combinations at low threshold concentrations, using photoreceptor neuron degeneration as a measure. Combinations of cystamine, congo red, and suberoylanilide hydroxamic acid (SAHA) effectively suppressed HD pathogenesis in flies expressing Httex1p Q93[1].

Chongtham and Agrawal explored the efficacy of curcumin, a polyphenolic compound with neuroprotective effects, in ameliorating HD symptoms in a Drosophila poly Q disease model. Their study revealed curcumin could suppress neuron degeneration and neuronal loss, while improving morphological abnormalities and motor neuronal impairments. Thus, curcumin has potential as a treatment for HD and other poly Q diseases[1,15].

Biological assays used in Drosophila models of ND to test the therapeutic potential of compounds :

A number of assays are used in *Drosophila* to determine neurodegenerative diseases and for the testing of therapeutic potential of compounds.

Developmental assays

Developmental assays are used to determine the *in vivo* effects of drugs on the development of flies. Lifespan analysis is one of the most common developmental assays in adult flies. The model flies can be supplemented with drug-containing diet associated with test compound and the results are compared to the control flies which are fed with drug free normal diet. There are numerous *Drosophila* disease models that exhibit developmental arrest and die before eclosion. There are many *Drosophila* models of human diseases that show developmental arrest and die before eclosion. For instance, in transgenic fly model of Huntington's disease pan-neuronal expression of defective Htt causes pupal lethality and excess growth of synaptic associations. The pupal lethality could be used to determining the therapeutic potential of screening compounds for Huntington's disease[30,38].

Behavioural assays

These assays are commonly used to study *Drosophila* models of neurodegenerative diseases such as Parkinson's disease and Alzheimer's disease which are characterized by increasing locomotor disabilities. *Drosophila* larvae show many coordinated locomotor behaviours such as turning, crawling, rolling and burrowing which become impaired in fly models of neurodegenerative diseases. Most commonly, larval crawling (locomotor) assay is performed in third instar larvae which is performed with or without drug to measure locomotor activities in treated and untreated larvae to identify therapeutics for the respective neurodegenerative disease. *Drosophila* have a natural tendency to move against gravity that is they are negatively geotactic. Therefore, adult diseased fly models are often studied by negative geotaxis assay (climbing/locomotor) for their disease-related motor dysfunction and are help in identifying plant derived components associated with neuroprotective potential. Another assay is rapid interactive negative geotaxis assay (RING) which allows testing of various groups of flies treated with different drugs[30,38].

Biochemical assays

Neurodegenerative diseases are often characterized by neuronal damage and loss in definite areas of nervous system which is contributed by inflammation, oxidative stress or mitochondrial dysfunction that alter the cellular antioxidant system. Most commonly used biochemical assays are glutathione peroxidase (GPx), malonaldehyde (MDA), superoxide dismutase (SOD), catalase (CAT) and glutathione S-transferase (GST). These assays used to identify the antioxidant therapeutic potential of plant extracts[30,38].

Current limitations of Drosophila model :

Drosophila as a model for human diseases and drug discovery have several limitations. A present limitation is the requirement of tedious work in keeping the score of drug effects on different fly models of human diseases. Likewise, as in analytical and imaging tools, at least partial automation is needed in phenotypic scoring to surpass these limitations. The translational usefulness of *Drosophila* in drug discovery and further validation in human diseases is another drawback and a recurrent question that arises is whether or not the compounds identified in screening in fly models would exhibit the similar function in mammals[22,32]. However, a few drugs or compounds identified and already validated in fly models have been investigated in higher organisms such as vertebrate models and found to produce favourable results, thus believed to establish *Drosophila* as a significant translational tool in drug discovery[32]. Despite the similarity that *Drosophila* share with humans in fundamental physiological pathways, concerning to the drug discovery platform the differences in the pharmacodynamics and pharmacokinetics of small molecules may create disparity in drug response profiles between *Drosophila* and mammals due to the evolutionary gap[17,32].

Future directions :

Drosophila has emerged as a powerful *in vivo* screening platform in the primary drug discovery process allowing the assessment of large number of compounds in short duration of time. Over the last two decades, *Drosophila* research has made significant contributions in elucidating the conserved mechanisms of fundamental biological pathways associated with human diseases and in the translational research in pharmaceutical areas[49]. In the drug discovery process *Drosophila* has demonstrated its potency on screening platforms to generate a collection of high-quality compounds from a large collection of hits for future development[46]. Chemical screens are performed using various experimental approaches *in vivo* in *Drosophila* to evaluate the candidate compounds for their therapeutic potential and to eliminate undesirable leads found in biochemical assays. These candidate compounds initially identified in fly models must be established in mammalian models before their translational potential in human patients. Future research using *Drosophila* models of human diseases will undoubtedly lead to development of effective therapeutic approaches and provide promising strategies to alleviate neurological disorders.

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Declarations of interest :

None

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