CHAPTER 3

"The Greatness of the Smallest Ones": The Most Valuable Attributes of Flies and Worms for the Study of Neurodegeneration

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Abstract: Over the last decades, a large number of experimental models have been developed to explore the mechanisms underlying neurodegenerative disorders. Invertebrate models of neurodegeneration, such as the fruit fly *Drosophila melanogaster* and the nematode *Caenorhabtidis elegans*, have emerged as successful complementary systems to mammalian models, facilitating identification of relevant pathways and novel disease-associated genes. These organisms provide reliable systems for identifying genetic modifiers of neuropathologies and the interesting possibility of screening and testing potential drugs for treatments to prevent and/or alleviate disease symptoms.

This chapter will focus on the main experimental strategies used in *Drosophila melanogaster* and *Caenorhabtidis elegans* to study neurodegeneration. Insights from forward genetic approaches, transgenic models of human neurodegenerative disorders and studies of fly/worm homologs of human disease genes will be presented. The value of using invertebrate models for the study of neurodegeneration will be discussed, highlighting advantages and limitations associated with these studies.

Keywords: Alzheimer's disease, *Caenorhabtidis elegans, Drosophila melanogaster*, drug screening, forward genetics, fruit flies, genetic enhancers, genetic screen, genetic suppressors, Huntington's disease, invertebrate models, neurodegenerative diseases, neurodegeneration, neuronal death, Parkinson's disease, Polyglutamine diseases, reverse genetics, transgenic models, worms.

3.1. INTRODUCTION

Human pedigree analyses and gene linkage studies on families and populations with patterns of inherited neurodegenerative diseases have revealed genetic mutations responsible for disorders such as Alzheimer's disease, Amyotrophic

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lateral sclerosis, Huntington's disease and Parkinson's disease [1]. The identification of such mutations has opened a new field in neurosciences focused on deciphering the function of human disease genes in transgenic animals, with the ultimate aim of discovering the mechanisms of the associated neuropathology.

A large number of experimental disease models based on mutations in homologs of human disease-associated genes or direct expression of human neurodegenerative-associated disease genes have been established in the past years [1-8]. Traditional animal models, such as rodents, seem ideal disease model systems given their close evolutionary relationship with humans [6]. Yet, rodents are expensive and laborious to maintain. In this respect, invertebrate model organisms such as the fruit fly *D. melanogaster* and roundworm *C. elegans* are excellent alternatives for studying mechanisms of neurodegeneration. These simple model systems offer enormous experimental advantages, including short generation times, large number of offspring and low cost of maintaining in the laboratory. In addition, there exist powerful techniques for manipulating gene expression and function that have emerged in worms and flies, including the ability to perform large-scale genetic screens and genome-wide analyses of genetic interactions based on the modification of a given phenotype [9-11]. Finally, compared to vertebrates, *Drosophila* and *C. elegans* have the key benefit of relatively low genetic redundancy, as genes are usually present in one copy. This greatly simplifies genetics studies.

Despite these clear experimental advantages, it could be argued that invertebrate physiology is far too different from human physiology to directly translate findings in flies or worms to humans. Indeed, there are important differences that should be taken into consideration, *e.g.*, invertebrates have a less complex nervous system - with fewer neurons, glia and synapses than the human brain. Additionally, worms and flies lack some important components for many vertebrate pathological phenomena, such as inflammatory processes and neuronal myelination. Nevertheless, flies and worms share many fundamental cellular and molecular pathways with mammals, including those governing gene expression, cell cycle regulation, membrane trafficking, cellular toxicity and cell death. Importantly, the basic structural and functional components of the nervous systems are highly conserved between invertebrates and humans, including

Synaptic Proteins, ion channels and neurotransmitters (*e.g.* dopamine, acetylcholine, glutamate and GABA) [12-15]. This suggests that the fundamental mechanisms underlying neuronal viability and synapse function are evolutionarily conserved across species. Interestingly, various human pathological processes including cancer, ageing, neurodegeneration and infectious diseases also affect flies and worms. In this regard, the annotation of the *C. elegans* and *Drosophila* genomes revealed that more than 70% of the genes associated with human genetic disorders are present in these organisms (http://superfly.ucsd.edu/homophila/; [16-19]). Importantly, expression of several human neurodegenerative disease-associated genes in flies or worms recreates key neuropathological features of the disease including, in some cases, age-dependent neuronal degeneration, vulnerability of specific neuronal types and accumulation of proteins in abnormal aggregates [3, 20-26]. This demonstrates important parallels between these organisms and humans. These simpler model systems have served as platforms for identifying genes and pharmacological compounds that modulate the pathology, thus providing insights into the genetic and molecular basis of neurodegeneration [3, 4, 20-23, 25, 26]. In addition, unbiased genetic screens in invertebrates have uncovered genes not previously suspected to be involved in neuronal maintenance and viability, and interestingly, disrupting the function of some of these genes in more complex organisms also results in neurodegeneration. These findings validate such approaches as a meaningful way to identify conserved genes required to maintain nervous system integrity [24, 27, 28].

It is thus clear that *Drosophila* and *C. elegans* represent valuable model systems to study basic mechanisms governing neuronal dysfunction and death associated with human diseases.

3.2. *DROSOPHILA MELANOGASTER:* BASICS OF A POWERFUL GENETIC MODEL SYSTEM

Over a century of intensive research using *Drosophila melanogaster* to study complex biological processes has generated a vast knowledge about its genetics, anatomy and development. This small insect (of about 3 mm in length) lives near unripe and rotten fruit in nature (Fig. **1A**). Fruit flies develop through embryo, larval, and pupal stages followed by metamorphosis into the adult fly. They have

a short life cycle, of 10 days at 25 °C, and adults live for ~2–3 months, with the potential of producing hundreds of offspring. This is in contrast to rodent models, where only a few offspring are produced every 3 to 4 months. Although the fly nervous system (Fig. **1B**) has ~1 million-fold fewer neurons than the human brain, it is still capable of producing many complex behaviors, including mating behavior, intra-specific aggression, and learning and memory [29]. The adult fly brain contains around 200,000 neurons, including neurons involved in sensory perception, integration and motor output.

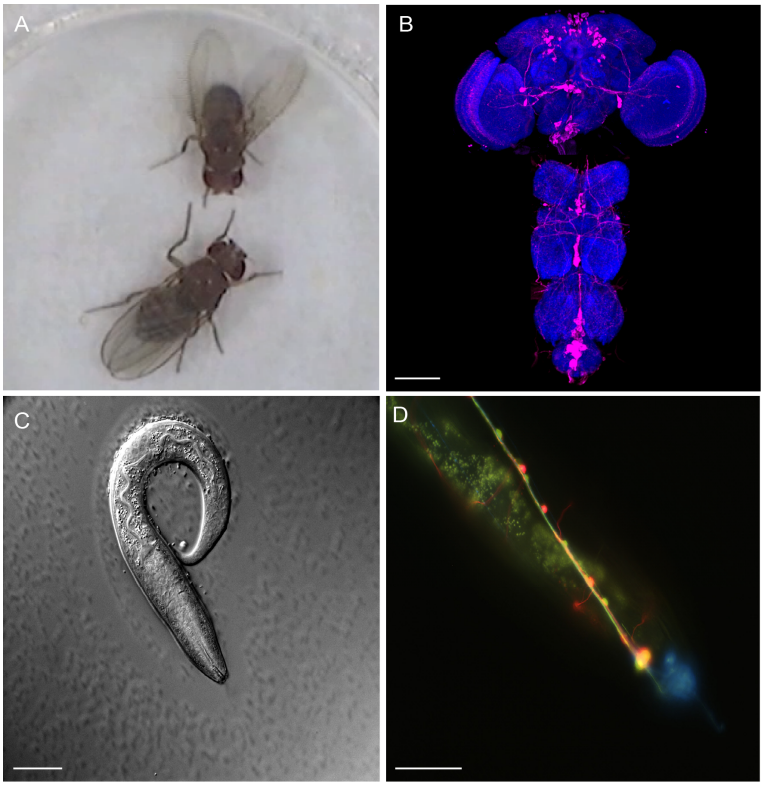

Figure 1: Fruit flies and worms: their anatomy and nervous system. (A) Image of an adult male and female *Drosophila melanogaster* performing the courtship ritual (top and bottom, respectively). (B) The *Drosophila* nervous system. The adult brain (top) and ventral nerve cord (bottom) are labeled with anti-nc82, a synaptic marker (shown in magenta). A sex-specific neuronal circuitry is visualised with anti-GFP antibody (shown in green). Scale bar: 100 µm. (C) *Caenorhabtidis elegans* (L1) larvae, soon after hatching. Scale bar: ~50 µm. (D) The *C. elegans* nervous system. Three types of motor neurons are shown in the ventral nerve cord of a triple transgenic animal: B-type motor neuron (DB; shown in green), A-type motor neuron (DA; shown in yellow) and D-type motor neuron (DD; shown in red). Scale bar: 50 µm. Images in A and B were taken by Carolina Rezával, University of Oxford. Pictures in C and D were kindly donated by M Gravato-Nobre, University of Oxford.

Sophisticated genetic techniques, such as random transposon tagging, site-specific transgenesis and recombination-mediated genetic engineering allow efficient manipulation of gene expression and function. There exist online databases containing valuable information regarding different aspects of *Drosophila* biology, including genes, mutations, phenotypes and available stocks (Flybase: http://flybase.org/), as well as nervous system anatomy (Flybrain: http://flybrain.neurobio.arizona.edu/; flymind) and development (the Interactive Fly: http://www.sdbonline.org/fly/aimain/1adult.htm).

3.3. *C. ELEGANS*: BASICS OF A CELLULARLY DEFINED MODEL SYSTEM

C. elegans is a free-living nematode of only ~1 mm in length that lives in temperate soil environments (Fig. **1C**). These roundworms have a rapid generation cycle (~3 days), short lifespan (~3 weeks) and two sexes: hermaphrodites (comprising most of the population) and males (comprising approximately 0.1% of the total population). After hatching, they undergo four larval stages (L1–L4) to become an adult. Under non-favorable environmental conditions, such as starvation or stress, *C. elegans* can enter an alternative third larval stage: the "dauer state". They can persist as stress-resistant dauer larvae for weeks or even months. When suitable environmental conditions are resumed, animals re-enter the life cycle at the fourth larval stage. An adult hermaphrodite produces about 300 self-fertilized eggs over a period of 3 days, and more than 1,000 eggs after male insemination.

Worms are straightforward to cultivate and propagate in the laboratory, as thousands of them can be reared on small agar-filled Petri plates or liquid media seeded with bacteria (*e.g. Escherichia coli*). In addition, worm strains can be frozen in glycerol, allowing for long-term storage. Bioinformatics and functional genomic databases providing valuable information of *C. elegans* are available on line [11], such as WormBase (http://www.wormbase.org) and WormAtlas (http://www.wormatlas.org).

One of the most remarkable features of worms is their transparent body, allowing for the visualization of all cells at all stages of development. Indeed, the complete cell lineage of *C. elegans* has been precisely described. The adult consists of 959 somatic cells, 302 of which are neurons that include chemosensory, mechanosensory, and

thermosensory types [11, 30] (Fig. **1D**). In contrast to flies, worms lack a centralized brain. Interestingly, the position of each neuron, fate and synaptic connections has been characterized in great detail [30, 31], simplifying the study of neurodegenerative phenotypes.

3.4. GENETIC APPROACHES TO STUDY NEURODEGENERATION IN FLIES AND WORMS

There are four interconnected and complementary approaches based on "reverse" and "forward" genetics to study neurodegeneration in flies and worms (Fig. **2**):

i. Transgenic models of human diseases

ii. Loss-of-function of fly or worm homologs of human disease-associated genes

iii. Screen of novel genes involved in neurodegeneration

iv. Identification of modifiers of neurodegenerative phenotypes

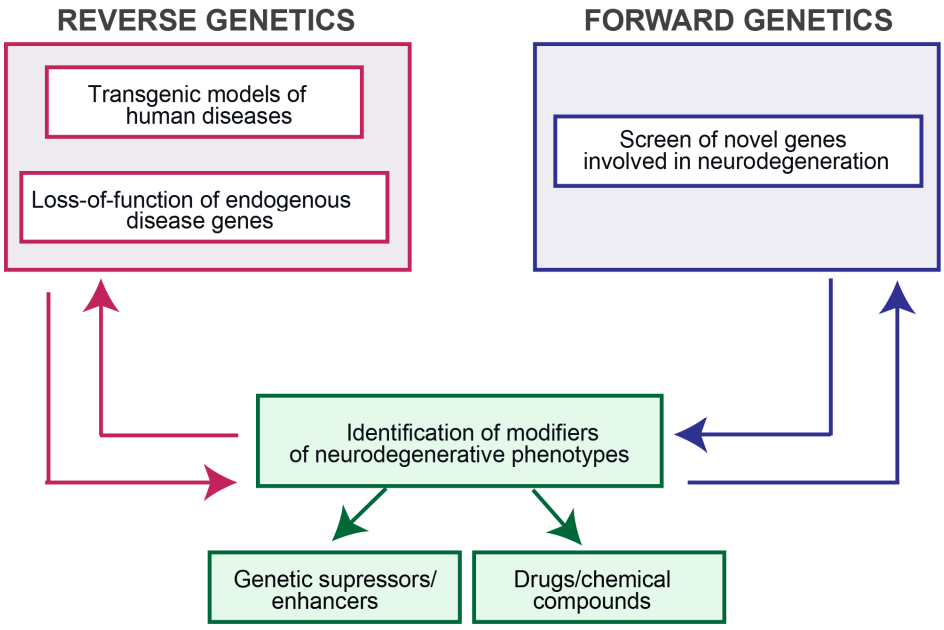

Figure 2: Using invertebrates to study neurodegeneration. The main experimental strategies employed in *D. melanogaster* and *C. elegans* to study neurodegeneration. These approaches are interconnected: a neurodegenerative phenotype caused by either (1) expression of a human disease

gene, (2) inactivation of a given disease gene ortholog or (3) mutation of a novel fly/worm neurodegenerative gene can be used as a platform to identify genetic enhancers and suppressors, or chemical compounds, that modify the original neuropathology. Genetic and chemical modifiers identified in forward genetic approaches can be studied in reverse genetic models and *vice versa.*

i) Transgenic Models of Human Diseases

"Reverse" genetics (from causal gene to phenotype) consists of evaluating the function of a candidate gene in a given biological process. Once the function of the gene has been altered, the effect on the physiology and/or behavior of the organism can be subsequently analyzed. Following such approach, wild type or mutant human genes previously linked to neurodegenerative disorders can be expressed in flies or worms. A successful transgenic model should recapitulate behavioral and pathological features of the human neurodegenerative disorder, allowing researchers to study the fundamental pathways influenced by pathological genes. By overexpressing human disease-associated genes in a specific subset of cells or tissues in both flies and worms, lethal effects resulting from broad mis-expression can be circumvented. In *Drosophila*, the GAL4/UAS binary system [32] provides a very efficient method for expressing genes in a tissue and time-dependent manner. In one parental strain, promoter regions for a particular gene drive expression of the yeast transcription factor GAL4 in defined tissues or cellular types. The second fly strain bears a transgene under the control of the upstream activation sequence (UAS) that is recognized by GAL4. The resulting progeny will express the gene of interest only in those tissues or cells expressing the GAL4 protein (Fig. **3**). In most fly disease models a human pathogenic transgene is fused to UAS (*UAS-transgene*) and expressed in a specific pattern. There are several collections of transgenic "GAL4 drivers" specific for different tissues or cell-types available to direct the expression of the gene of interest (http://flybase.org/). The eye-specific promoter GMR (Glass Multimer Reporter) has been extensively used to express pathological transgenes in the developing compound eye. This regular structure, composed of highly organized eye units known as ommatidia, allows the identification of neurodegenerative phenotypes using a standard light microscope or scanning electron microscopy to detect altered ommatidial numbers or arrangement that typically lead to a "rough" eye phenotype and loss of photoreceptor neurons (Fig. **4 A-E**). Use of GMR-based constructs has been particularly useful for genetic

enhancer/suppressor screens [33-35]. Neurodegeneration in the brain can be detected by the appearance of vacuoles (holes) (Fig. **4F,G**). Specific transgenes can be broadly expressed in the brain (*e.g. via* the pan-neuronal driver *elav-GAL4)* or, alternatively, in specific subsets of neurons (*via* neuron type-specific drivers, such as those for dopaminergic or serotonergic neurons (*TH-GAL4* or *TRH-GAL4,* respectively). Thus, it is possible to investigate cell-type-specific death associated with the expression of pathological genes. Different modifications of the GAL4/UAS system have been developed to further refine tissue specificity as well as temporal expression [36, 37]. By employing these techniques, it is possible to manipulate biological processes in the adult brain without affecting nervous system development. It should be noted, however, that high levels of GAL4 protein can trigger neuronal death *per se*, thus an excess of GAL4 might enhance neuronal defects observed in neurodegeneration models [38].

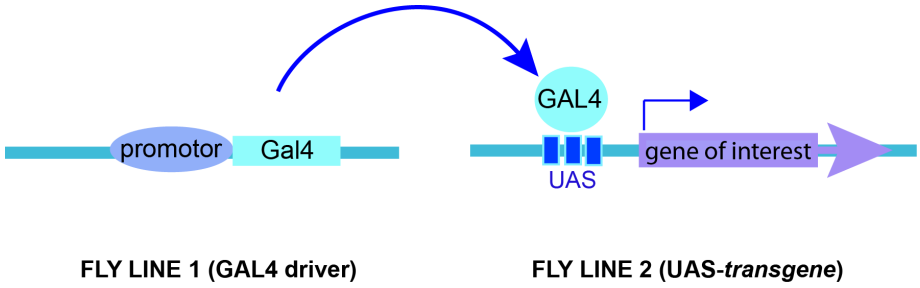

Figure 3: A powerful genetic tool to study neurodegeneration in flies. The GAL4/UAS system allows spatial and temporal gene expression in flies. In fly disease models a human pathogenic transgene is fused to a sequence (UAS) recognized by the transcription factor GAL4 (UAS-transgene). The fly line carrying such transgene is crossed to another one carrying GAL4 under the control of a specific promotor (GAL4 driver). The resulting progeny will express the gene in a specific cell or tissue type, depending on the GAL4 driver. This system also serves to knockdown the expression of an endogenous gene in selected cell types or tissues. In this case, a line expressing RNA interference (RNAi) against a specific gene under the control of UAS (*UAS-RNAi)* is crossed to the GAL4 driver line.

C. elegans can also be genetically manipulated to express human transgenes associated with neurodegenerative diseases. Generation of transgenic worms is relatively simple, low-cost and quick. It usually involves injection of transgenes into the gonad of hermaphrodite adults or bombardment with DNA-coated microparticles [39]. The *Mos*TIC technique provides a means to engineer single copy transgenes at a defined locus in the genome [40].

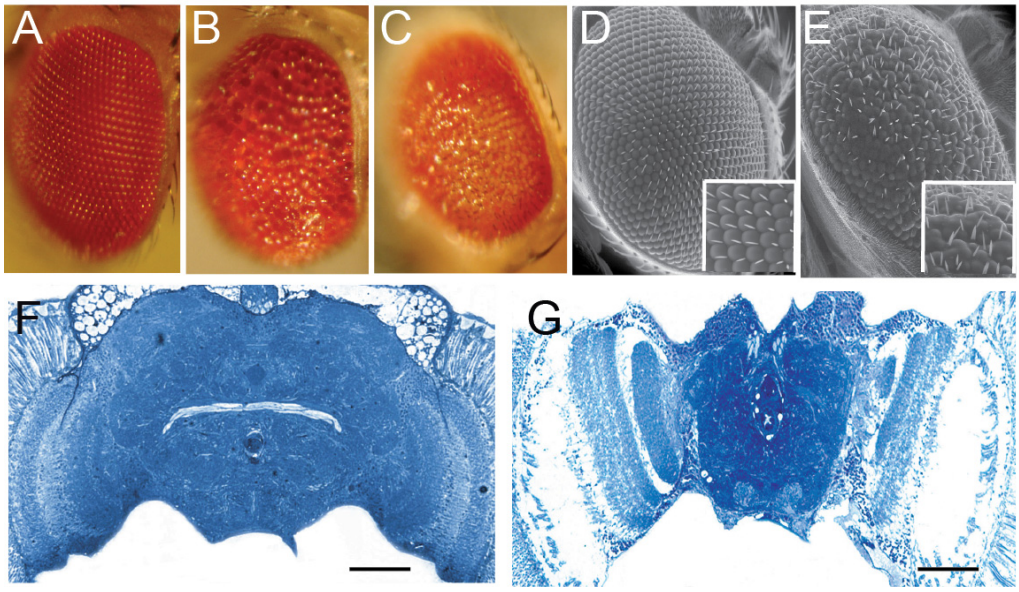

Figure 4: Neurodegenerative phenotypes in *Drosophila melanogaster*. The fly compound eye is a highly organized structure that allows detecting toxicity very easily under standard light microscopy (A-C) or scanning electron microscopy (D-E). Downregulation of the cytoskeleton gene *enabled* (*ena*) in the eye leads to disorganization of photoreceptor neurons and retinal degeneration in *GMR-GAL4>ena rev* flies (B,C,E). Images of wild-type flies showing normal eye morphology are shown in A and D. Neurodegeneration triggered by decreased *enabled* levels can be also detected in adult fly head semi-thin sections of *elav-GAL4>ena*^{rev} flies stained with methylene blue by light microscopy. Young (0–3 days) and old (30 days) *elav-GAL4>ena*^{rev} flies are shown in F and G, respectively. While the nervous system of young flies is well preserved (F), age-dependent degeneration characterized by the occurrence of vacuoles in specific areas of the brain is observed in old flies (G). Scale bar: 50 µm. Images (D-G) taken from: Rezaval *et al.*, 2008. PLoS One 3, e3332.

Gene expression can be modulated by promoter-driven expression in worms; for example, transgenes can be directed to muscle cells (*via* the unc-54 promoter) or particular subsets of neurons, such as dopaminergic neurons (*via* the dopamine transporter dat-1 promoter), or touch neurons (*via* the mec-7 promoter). The transparent nature of *C. elegans* facilitates *in vivo* visualization of neurons throughout the lifetime of the animal, using fluorescent marker genes, such as the jellyfish Green Fluorescent Protein (GFP) [41, 42]. Thus, the effects of genetic and pharmacological modulators on neuronal viability can be easily evaluated in living worms by detecting signs of cell dystrophy, such as vacuolization and protein aggregation [3, 43, 44] (Fig. **5**).

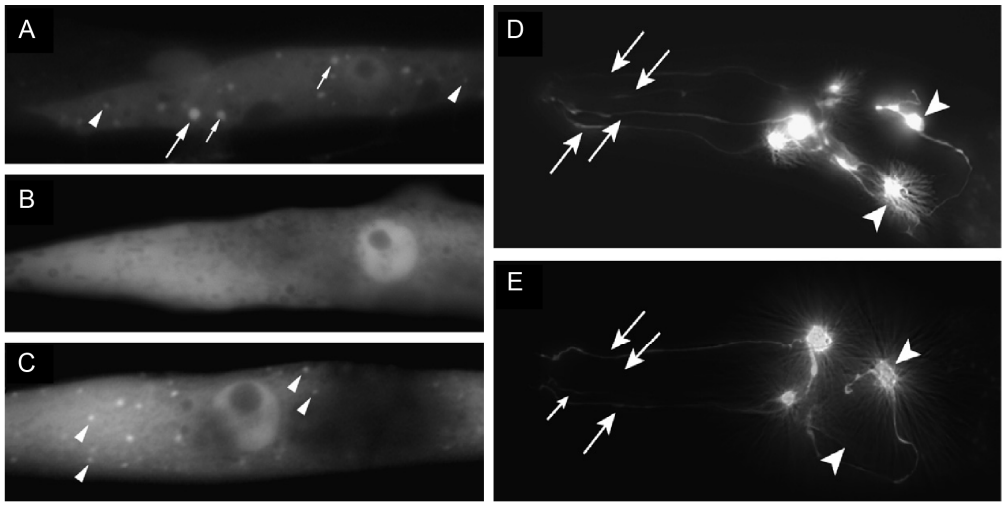

Figure 5: Neurodegenerative phenotypes in *C. elegans*. (A-C) Misfolding and aggregation of α-synuclein in nematodes. Photomicrographs of transgenic *C. elegans* displaying muscle-specific expression of α-synuclein: GFP. (A) Misfolded α-synuclein forms small (arrowhead), medium (small arrow) and large aggregates (arrow) in body wall muscle cells. (B) Decreased α- synuclein: GFP misfolding is observed when the chaperone TOR-2 is simultaneously expressed, demonstrating the utility of this transgenic strain in screening for enhancers/suppressors of misfolding. (C) Following RNAi of specific gene targets in worms expressing TOR-2 + α synuclein: GFP misfolded α-synuclein is again detected (arrows). (D-E) α-synuclein-induced toxicity in dopaminergic neurons in *C.elegans*. (D) The six dopaminergic neurons in the anterior region of *C. elegans* (four CEP and two ADE neurons) are visualized in a transgenic Pdat-1: GFP worm where GFP is under the control of the dopamine transporter promoter. The dendrites of the CEPs are indicated with large arrows and the cell bodies of the ADEs are shown with arrowheads. (E) CEP and ADE age-dependent degeneration is observed in worms expressing both Pdat-1: GFP and Pdat-1: α-synuclein. A 7-day old worm displays two intact CEP neurons (large arrows), one retracting (degenerating) CEP dendrite (small arrow), and one intact ADE neuron (arrowhead). The additional CEP and ADE neurons have degenerated and are no longer visible (grey arrow and arrowhead represent normal locations of the CEP and ADE, respectively). Images taken from: Harrington *et al*., 2011. Methods 53, 220-225.

ii) Loss-of-Function of fly or Worm Homologs of Human Disease Genes

Following a reverse genetic approach, fly or worm genes with similarity to specific human familial neurodegenerative disease-associated genes can be disrupted and the resulting mutant phenotype investigated. In *Drosophila*, total or partial genetic inactivation can be achieved *via* (1) transposon-mediated mutagenesis [9, 45-49], (2) GAL4/UAS–mediated RNA interference (RNAi) [48, 50] and (3) homologous recombination-based gene knockout [51-53]. Several large-scale P element gene disruption projects have generated thousands of stocks

of flies containing single P elements insertions at known locations in the genome. Many of these are available in fly stock centers (http://www.flybase.org). P elements have become key genetic tools in *Drosophila*, used not only as mutagens but also for *in vivo* gene tagging and inserting transgenes [9, 48, 49].

The second mutational strategy is based on RNA interference (RNAi), the gene-specific degradation or inhibition of an mRNA that prevents the encoded protein from being synthesized. This evolutionarily conserved mechanism was first characterized in *C. elegans*, and is triggered by double stranded RNA (dsRNA) that shares sequence identity to a specific mRNA [54]. RNAi is cleaved *in vivo* into short fragments that guide sequence-specific mRNA degradation or translational repression. The technique of RNAi, coupled with the availability of the complete genomic sequences of *Drosophila* and *C. elegans* has made possible the rapid study of gene function, both on a single gene level and at a global scale. In flies, specific RNAi targeting can be achieved *via* the GAL4/UAS system: a UAS line expressing an RNAi sequence (*UAS-RNAi*) is used in conjunction with a specific GAL4 line to knockdown the expression of the gene in selected cell types or tissues. Collections of RNAi knockdown strains targeting ~90% of the entire *Drosophila melanogaster* genome are available to the research community [50]. It should be noted, however, that RNAi often results in only partial gene inactivation or, in some cases, no inactivation at all. In this regard, the use of the enzyme Dicer has greatly improved the efficiency of the RNAi methodology in *Drosophila*, such that dsRNAs are better processed in the presence of this enzyme [55].

In the third mutational strategy, homologous recombination-based gene knockout allows precise gene targeting to eliminate a specific gene [51, 52, 56]. Gene targeting is the modification of an endogenous gene sequence by recombination between an introduced DNA fragment and the homologous target gene [57]. This method has proven to be a valuable tool for altering genes in mice [58] as well in *Drosophila* [51, 56].

Currently, several methods are available for genetic inactivation of genes in *C. elegans,* including i) transposon-mediated mutagenesis and ii) use of RNAi [54, 59]. As in *Drosophila*, transposon-mediated mutagenesis in *C. elegans* is based on active transposable elements to inactivate gene function. In such

approaches, transposons are mobilized randomly in a *Drosophila* or *C. elegans* strain and independent lines are then screened for the presence of a transposon insertion in the gene of interest [60, 61].

RNAi-based approaches to inactivate gene function have been utilized extensively in worms [54, 59]. Specific dsRNA can be delivered into the worm either by microinjection, at the single worm level, or by uptake of dsRNA by worms in solution (soaking) or feeding worms bacteria engineered to synthesize the specific dsRNA. The most reliable method for causing severe gene inhibition is microinjection; however, it is more labor intensive than other approaches. Soaking and feeding are more suitable methods for high-throughput genomic analyses, as large numbers of worms can be treated at once. Since *C. elegans* neurons are refractive to RNAi and not efficiently targeted by bacterial feeding approaches [59], techniques that aim to improve RNAi efficiency in neurons have been developed, such as selecting mutant strains that are more sensitive to RNAi [62, 63]. However, it should be noted that strains with intrinsic mutant phenotypes could also interfere with the process of interest.

More recently, homologous recombination methods have been developed to specifically disrupt gene function [64]. Yet these approaches are still laborious and not amenable for large scaling.

iii) Screen of Novel Genes Involved in Neurodegeneration

Classical “forward genetic” screens (from phenotype to causal gene) involve the generation of random mutations in the genome, screening of the resulting mutants for a specific phenotype and subsequent identification of the affected gene. Compared to “reverse genetics”, this approach is an unbiased method as it does not require previous knowledge about the nature of the emerging candidates. Therefore, unexpected genes and molecular pathways involved in a disease pathogenesis can be uncovered, which can be further studied in more complex animals. Large collections of mutants can be anatomically analyzed in search for “neurodegeneration hallmarks”, such as abnormal accumulation of pathogenic proteins and age-dependent neuronal death that can be identified by direct examination of the brain. Genetic screens can also be based on the observation that neurodegeneration is often associated with neuronal dysfunction that results

in altered physiology or behavior. Hence, these approaches select for mutations causing neuronal death, reduced lifespan or abnormal behaviors, such as progressive incoordination or paralysis [24].

How difficult is to produce *Drosophila* mutations in a large-scale? Ionizing radiation and chemical mutagens, such as ethyl methane sulphonate (EMS) or N-ethyl-N-nitrosourea (ENU) are used to produce mutations in *Drosophila*. However, the identification of the resulting mutations at the DNA level is labor intensive, time consuming and consequently not practical on a genome-wide basis. In contrast, P element-induced mutations can be rapidly identified on a large scale. However, P elements tend to integrate preferentially into specific hotspots, thus reducing the proportion of the genome that can be randomly targeted. Several genome-wide collections of chemical or transposon-induced mutants are currently available from *Drosophila* stock centers. Alternatively, large collections of transgenic RNAi strains are also available and can be employed in combination with GAL4 lines to screen for mutant phenotypes [50, 65]. The RNAi technique overcomes a clear limitation of traditional chemical or radiation mutagenesis screens in that it allows the identification of genes that generate lethality earlier in development, through restricting gene inactivation in a temporal and/or spatial fashion. In addition, the gene responsible for the phenotype of interest is already known, making it possible to establish a connection between a phenotype and the affected gene rapidly.

In *C. elegans*, large mutant libraries are obtained by either (1) chemical mutagenesis, such as EMS, diethyl sulfate (DES) or N-nitroso-N-ethylurea (ENU); (2) irradiation with X-rays, γ-rays or UV light; (3) transposable element movement. Since chemical mutagens are easy to use, efficient and create a wide range of genetic lesions, they have been successfully used to generate mutant libraries for PCR-based identification of deletions in genes of interest [66]. The *C. elegans* Gene Knockout Consortium (GKC: http://celeganskoconsortium.omrf.org/) and the National Bioresource Project (NBRP: http://www.shigen.nig.ac.jp/c.elegans/index.jsp) have been established to isolate deletion mutants for all *C. elegans* genes. As in *Drosophila*, a critical bottleneck for chemical mutagenesis resides in the arduous task of genetic mapping and identification of the mutant gene. Transposon-based insertional mutagenesis is a strategy complementary to chemical mutagenesis and

greatly facilitates identification of mutant genes. However, the identification of mutagenic insertions can be complicated by the presence of multiple endogenous transposons in the worm genome. In this regard, mobilization of heterologous transposons such as the *Drosophila* mariner transposon *Mos1,* has provided a more efficient way to generate mutations. These mariner elements constitute unique transposon insertions in a *C. elegans* mutant strain and are thus more readily localized [67]. Unfortunately, such transposons are not as versatile as P elements in *Drosophila* and are not useful for introducing large DNA fragments into the worm genome. The fact that there is also a bias in the sites of mariner insertions in the worm's genome represents an additional caveat. In this regard, the NemaGENETAG project (http://elegans.imbb.forth.gr/nemagenetag/) is contributing to expand the library containing transposon-tagged mutants in *C. elegans*. Emerging technologies, such as Mos TIC approach for genome engineering, allow the introduction of exogenous DNA sequences into predetermined genomic locations [40, 68]. Alternative approaches for gene inactivation include the use of RNAi in worms (*e.g.* [69]). RNAi by feeding is the least labor intensive and the most economical method used for high throughput genome-wide screenings, allowing large numbers of genes to be evaluated simultaneously [70]. Lethal phenotypes at the embryonic stage can be avoided by delivering dsRNA into the worms at the first larval stage. The use of RNAi libraries has been very effective in screening for a variety of worm phenotypes/genes [70]. There are currently two RNAi feeding libraries for *C. elegans*: known as the Ahringer and ORFeome libraries. These libraries are available to the public and together, can target about 94% of *C. elegans* genes. Importantly, WormBase contains valuable information about published large-scale RNAi data, including genes affected and all RNAi phenotypes.

iv) Identification of Modifiers of Neurodegenerative Phenotypes

Once a neurodegenerative phenotype caused by, for example, expression of a human disease-associated gene or inactivation of a given disease -associated gene ortholog is established, it is possible to perform genetic modifier screens to identify genetic enhancers and suppressors that modify the original neuropathology. In addition, pharmacological screens can be used to identify drugs that modulate the neurodegenerative phenotype and their associated molecular pathways. Therapeutic compounds that have been already identified in mammalian systems can also be

tested in invertebrate models, allowing for validation of hits and exclusion of compounds with unfavorable properties [71-73].

Moderate ‘‘high-throughput’’ screens are possible in *Drosophila* by feeding flies with food mixed with different concentrations of neuroactive compounds. The lack of a stringent blood-brain barrier in flies simplifies the access of pharmacological compounds to the nervous system. An alternative approach to deliver pharmacological compounds in a more efficient way consists of direct intra-abdominal or intra-thoracic injection into adults. However, this method is more laborious and time-consuming [74]. In this regard, microfluidic devices in combination with computer-controlled injection systems provide a more systematic methodology for injections in fly embryos [75, 76].

C. elegans also lacks a functional blood-brain barrier and are sensitive to a wide range of human neuroactive drugs [77]. Worms can be grown in liquid medium in 96-well microtiter plates containing different drugs and rapidly screened for novel compounds. Thus nematodes are even more amenable for large therapeutic screens than *Drosophila* [77]. Neuronal viability can be measured with GFP-based tags in a fluorescent plate reader or, alternatively, worm motor activity can be automatically monitored in real time [78]. Both these approaches are amenable to high throughput screens and allow the identification of neurotoxic or neuroprotective compounds.

Once a therapeutic drug has been identified in either *Drosophila* or *C. elegans* model systems, suppressor screens can be carried out to identify molecular partners involved in the compound-mediated protection of neurons. Drugs with efficacy in invertebrate models, however, need to be validated in mammalian whole-animal disease models to be considered as candidates for clinical trials [73].

3.5. CONTRIBUTIONS OF FLY AND WORM STUDIES TO UNDERSTANDING THE MECHANISMS UNDERLYING NEURODEGENERATION

3.5.1. Insights from Transgenic and Mutant Models of Human Diseases

Examples of findings associated with pathogenic mechanisms and therapeutic implications in flies and worms are offered here to illustrate the value of invertebrate models in the study of neurodegeneration.

3.5.1.1. Alzheimer's Disease

Patients with Alzheimer's disease suffer from age-dependent memory loss, deterioration of cognitive functions and dementia. Progressive neuronal degeneration affects specific areas of the brain, such as the frontal cerebral cortex and hippocampus [79]. The pathological hallmarks of Alzheimer's disease are the accumulation of extracellular senile plaques (composed mainly of the amyloid Aβ42 peptide) and intracellular neurofibrillary tangles (composed of aggregated, hyperphosphorylated forms of the microtubule-associated protein Tau) [79]. Aβ42 peptides are produced by proteolytic cleavage of the amyloid precursor protein (APP) transmembrane receptor *via* the action of β and χ-secretases. The ß-site APP-cleaving enzyme (BACE) cleaves APP at the beta site, and the presenilins, PS1 and PS2, participate in APP cleavage at the ɣ site [80]. Genetic analyses of familial Alzheimer's disease identified mutations in the APP, PS1 and PS2 genes; these mutations are all associated with abnormal APP processing and Aβ42 aggregation. Hence, it is believed that Aß42 peptide overproduction is the initial trigger of a series of pathogenic events that result in Tau hyperphosphorylation, abnormal cellular signaling, and synaptic failure, ultimately leading to neuronal death [81-83].

Different approaches have been utilized to study the normal function of APP, as well as the mechanisms by which APP dysfunction might lead to neurodegeneration in flies [4, 72]. Flies carrying loss-of-function mutations of the fly homolog of the human APP (dAPPL) display no neurodegeneration but show abnormal behaviors that are rescued by the transgenic introduction of human APP gene, indicating functional conservation between fly APPL and human APP [84]. Additional studies have linked dAPPL with physiological functions such as neuronal development and synapse formation [85-87]. Transgenic flies expressing wild type and Alzheimer's disease mutant forms of APP in the fly nervous system and retina have revealed interesting findings. APP and dAPPL overexpression leads to axonal transport defects [85, 88] that appear to correlate with impaired synaptic plasticity [89]. Evidence for dysfunction in axonal transport has also been found in other neurodegenerative disease fly models, such as Huntington's disease [90, 91], suggesting a common mechanism of neurodegeneration in different diseases.

Flies have orthologs of α-secretases [92, 93] and γ-secretase components, such as PSN and Nicastrin [94-96]. Although dAPPL lacks the Aβ domain [96b], it has been recently shown that processing of dAPPL by the fly β-secretase BACE (dBACE) results in neurotoxic Aβ-like fragments, amyloid deposits and neurodegeneration [97]. Aβ42 formation and neurotoxicity can be achieved by simultaneous expression of human BACE and APP in either the fly retina or nervous system [98, 99]. Interestingly, direct expression of human Aβ42 in the fly recapitulates aspects of the Alzheimer's disease pathology, such as age-dependent neurodegeneration and accumulation of amyloid plaques [100, 101]. Moreover, generation of Aβ42 leads to defective axonal transport [85, 88], mitochondrial mislocalisation [102] and synaptic plasticity defects [89]. Aβ42 accumulation has been also found to trigger progressive locomotor deficits, abnormal learning and reduced lifespan [99, 100, 103, 104]. On the other hand, different studies in flies have confirmed experimentally that Aß42 aggregation propensity correlates with neurotoxicity [72, 99, 105, 106] and several modifiers of Aβ42 aggregation-related toxicity have been identified. Some of these include genetic regulators of proteolytic processing of APP, such as modulators of PSN activity [107, 108] or compounds that interact with amyloid structure and reduce aggregation properties of Aβ [103, 109]. These findings highlight the utility of *Drosophila* models in providing indications of pathogenic mechanisms and identifying Alzheimer's disease compounds that target Aß42 aggregation to reduce toxicity.

As in humans, several studies in *Drosophila* have implicated heavy metals in the development of Aβ-induced pathological processes [110]. One study shows that inhibition of zinc transporters reduces Zn^{++} accumulation in the fly brain, which in turn reduces Aβ42 deposits [111]. Thus, manipulation of zinc transporters in Alzheimer's disease brains may represent a novel therapeutic strategy.

C. elegans contains one APP-related gene (*apl-1*) that similarly to dAPPL, lacks a region equivalent to the Aβ-peptide [112]. Knockout of *apl-1* causes developmental defects and larval lethality while overexpression produces movement defects and reduces viability. Interestingly, RNAi knockdown studies revealed a role for apl-1 in synaptic transmission [113]. Similarly to *Drosophila,* Aβ42 peptides have been expressed in *C. elegans* to study different aspects of Alzheimer's disease. Thus, worms carrying transgenes that drive Aβ42 peptide

expression in body wall muscles or neurons have been created [114, 115]. Aβ42 expression under the control of a muscle-specific promoter leads to intracellular amyloid deposits in the muscles, in addition to progressive paralysis and reduced lifespan in worms [44, 114-116]. This model has been subsequently used to identify binding partners of Aβ that contribute or respond to Aβ toxicity [117-119]. Interestingly, Aβ deposits induce stress response in worms by promoting expression of heat shock proteins. Thus, heat shock chaperone function might play a role in modulating intracellular Aβ42 metabolism and toxicity [116-118]. Aβ42 accumulation has been also found to induce increased iron levels and oxidative stress in human cell and worm models of Alzheimer's disease [120, 121]. Carbonyl accumulation, an oxidative damage indicator, also correlates with Aβ42 expression in worms. This phenomenon has also been observed in human neuronal cultures exposed to Aβ42 and brain tissue from Alzheimer's disease patients [122]. Aβ-expressing nematodes have also served to identify potential therapeutic reagents of the Aβ42-related toxicity [23]. Tetracyclines have been found to interact with Aβ42 oligomers and prevent their aggregation in Aβ transgenic worms [123]. Moreover, these compounds decrease superoxide production, and thus oxidative stress, in Alzheimer's disease nematode models. These findings suggest a potential use of these drugs for reducing Aβ aggregates. Additional compounds useful for preventing Aβ42 toxicity include coffee extracts [124] and extracts from the *Ginkgo biloba* that have been shown to decrease reactive oxygen species (ROS) generated by oxidative stress [125, 126].

In alternative approaches, *Drosophila* and *C. elegans* models for tauopathy have been established to study the pathological properties of intracellular neurofibrillary tangles and Tau associated with Alzheimer's disease. These strategies have been recently reviewed and therefore will not be discussed here [3, 21, 23, 127, 128].

3.5.1.2. Parkinson's Disease

Parkinson's disease is characterized by age-dependent loss of dopaminergic neurons in the brain, resulting in loss of motor capacity, involving tremors, rigidity and bradykinesia, as well as cognitive disorders [129]. The progressive degeneration of dopamine neurons has been associated with the formation of inclusion bodies called Lewy bodies, containing misfolded and aggregated α-synuclein protein [130, 131].

Familial forms of Parkinson's disease have been linked to mutations in *α-synuclein, ubiquitin carboxy-terminal hydrolase-L1* and *Parkin* genes. These findings implicate Lewy body components and defects in the degradation of misfolded/aggregated proteins in the mechanism of the pathology [131, 132].

Expression of wild type and mutant forms of human *α-synuclein* in flies recapitulate key features of Parkinson's disease such as inclusions of α-synuclein reminiscent of Lewy bodies, age-dependent degeneration of dopaminergic neurons and progressive motor [133-137]. Expression of Hsp70, a highly conserved molecular chaperone involved in refolding of misfolded proteins, can prevent the pathology of α-synuclein Parkinson's disease models in flies [134] and mice [138]. Moreover, pharmacological treatments involving geldanamycin suppress α-synuclein toxicity in flies by inducing the heat shock response [139], suggesting a potential therapy for Parkinson's disease. Notably, human genetic data shows that some polymorphisms in Hsp70 are genetic risk factors for Parkinson's disease [140]. These findings reveal a role for abnormal protein folding and aggregation in the disease pathogenesis [134, 141]. Interestingly, superoxide dismutase activity prevents the death of dopaminergic neurons in flies, highlighting the importance of oxidative stress in the α-synuclein pathogenesis [142]. In addition to abnormal protein aggregation and oxidative damage, altered histone acetylation is involved in Parkinson's pathogenesis. α-synuclein inhibits histone acetylation in the nucleus and induces neurotoxicity that can be reverted by histone deacetylase inhibitors [143]. Other studies have focused on the role of phosphorylation in the generation of neurotoxic isoforms of α-synuclein [144-147], providing new insight into the signaling pathways underlying Parkinson's disease.

Several genes have been associated with autosomal recessive juvenile parkinsonism, including *DJ-1*, *Pink1* and *Parkin*. *Drosophila* homologs of these genes exist and have been mutated. These mutants are associated with mitochondrial disruptions [148], supporting the notion that mitochondrial dysfunction is an important factor underlying the pathogenesis [4]. The *Parkin* gene, an E3 ubiquitin protein ligase, is involved in proteasomal degradation of damaged proteins. *Drosophila PARK2* null mutants exhibit increased oxidative stress, reduced lifespan, behavioral defects and age-dependent muscle degeneration associated with neuronal apoptosis and mitochondrial pathology [149-151]. Other studies suggest that PINK1 and Parkin act

together in a common pathway regulating mitochondrial morphology and function, including mitochondrial fission/fusion [152-156]. New *Drosophila* models of mitochondrial dysfunction are emerging to specifically study the mechanisms responsible for mitochondria-mediated dopaminergic neuronal loss [157]. Biochemical approaches have revealed genetics modulators of Parkin-mediated toxicity. For example, glutathione S-transferase (GST) S1 activity is sufficient to rescue dopaminergic neuronal death in Parkin mutants, likely by modulating cellular response to oxidative stress [151, 158, 159]. In addition, another member of the GST family in *Drosophila* (glutathione S-transferase Omega 1) suppresses the phenotypes of *parkin* and *α-synuclein* mutants by regulating mitochondrial ATP synthase activity [160].

Environmental toxins, such as the herbicide paraquat and the pesticide rotenone, appear to be risk factors for sporadic Parkinson's disease. Exposure of flies to such environmental contaminants leads to increased oxidative stress through mitochondrial pathways. These treatments induce parkinsonian-like symptoms in *Drosophila*, including dopaminergic neuronal death and behavioral abnormalities that can be mitigated by adding the antioxidant melatonin. These findings suggest that antioxidants may be helpful in the treatment of the Parkinson's disease pathology [161, 162].

C. elegans models of Parkinson's disease have also been generated that examine α-synuclein toxicity. α-synuclein::GFP or α-synuclein::YFP (Yellow Fluorescent Protein) fusion proteins were expressed specifically in either body-wall muscles, the nervous system or specific subsets of neurons, such as motor neurons or dopaminergic neurons. Overexpression of wild type and mutant forms of human α-synuclein in dopaminergic neurons has been shown to trigger neuronal loss accompanied by accumulation of misfolded α-synuclein aggregates in *C. elegans* [163, 164]. Since nematodes have only eight dopaminergic neurons, it is straightfoward to examine their integrity over time. Interestingly, α-synuclein overexpression renders these worms incapable of reducing their locomotion upon the presence of food, a behavioral response controlled by dopaminergic neurons. This phenotype can be rescued by re-establishing normal dopamine levels [164]. High throughput genomic approaches using α-synuclein transgenic worms have uncovered changes in expression of genes associated with components of

ubiquitin-proteasomal and mitochondrial systems that may arise as a consequence of mitochondrial dysfunction in the Parkinson's pathology [165].

Large-scale RNAi screens in worms have permitted identification of modulators of the neurodegenerative phenotype triggered by α-synuclein expression *via* the premature formation of aggregates of fluorescently labelled misfolded proteins *in vivo* [166]. Other studies have identified modifier genes associated with protein degradation, lipid metabolism, RNA metabolism, vesicular trafficking and endocytosis pathways, in addition to aging-associated genes [166-172]. Several of these modifiers have been successfully validated as neuroprotective in mammalian systems. An interesting example is VPS-41, a protein involved in lysosomal trafficking of Golgi-derived vesicles [173]. The human ortholog of VPS-41 (hVPS41) also protects *C. elegans* neurons and mammalian neuroblastoma cells from the toxic effects produced by Parkinson's disease-associated toxins [174]. Recent studies suggest that hVPS41 prevents α-synuclein toxicity by facilitating clearance of misfolded and aggregated proteins [175].

MicroRNAs (miRNAs) are short ribonucleic acid (RNA) molecules that repress mRNA translation or mediate mRNA degradation in a sequence-specific manner in animals and plants [176]. Interestingly, microRNA regulation has been linked to Parkinson's pathogenic mechanisms in disease worm models, as is the case in mammalian systems, suggesting a conserved pathological mechanism across species [177].

C. elegans have orthologs for various human genes linked to familial Parkinson's disease, including parkin (pdr-1), PINK1 (pink-1) and DJ-1 (djr-1.1, 1.2). Mutations in these genes can cause loss of dopaminergic neurons or mitochondrial pathology [178, 179]. pdr-1 null mutants exhibit lower levels of ubiquitin conjugates, suggesting that alterations in the ubiquitin proteasome system may be a causative factor for the pathogenesis of Parkinson's disease [178].

In addition to genetic models of Parkinson's pathology in worms, the effects of environmental agents have also been evaluated. For example, worms exposed to either 6-OHDA or rotenone (neurotoxins with harmful effects in rodents [180]), show selective degeneration of dopaminergic neurons [167, 181, 182]. Studies

using these models demonstrate that restricted diet can prevent dopaminergic neuron degeneration [183, 184], suggesting a link between Parkinson's and metabolism. Neurotoxicity models of dopaminergic neuron degeneration have also revealed oxidative stress and the protein misfolding are major contributing factors in neurodegeneration and disease progression [183, 185].

3.5.2. Insights from Neurodegenerative Mutations

Forward genetic screens have identified a number of interesting mutations that cause neuronal dysfunction and death. This strategy has been enormously successful in flies. Pioneering studies in neurodegeneration were carried out in Seymour Benzer's laboratory, one of the most influential laboratories in the history of *Drosophila* neurogenetics. Fly mutants such as *bubblegum*, *swiss cheese* and *drop-dead* were first isolated in screens selecting for flies with defective phototaxis behavior or reduced lifespan, followed by histological examination that revealed vacuolization in the brain [186-189]. Bubblegum is considered an interesting candidate to study "human-like neurodegeneration processes". It has a mutation in the *VLCFA acyl coenzyme A synthetase* gene that leads to abnormal accumulation of very long chain fatty acids, as it is observed in patients with adrenoleukodystrophy (ALD). Moreover, such neurodegenerative phenotype can be alleviated by feeding the flies with 'Lorenzo's oil', a treatment based on monounsaturated fatty acids used to lower VLCFA levels in ALD patients [189]. Other studies have isolated fly mutants on the basis of additional neurodegenerative phenotypes, including paralysis induced by high temperature or mechanical stress [190, 191] and abnormal circadian rhythms [192]. These and additional large-scale genetic screens have identified mutations that interfere with mitochondrial function, signal transduction, lipid homeostasis, protein homeostasis, channel function, cytoskeleton, oxidative stress response and glial-neuronal signaling in *Drosophila* [24, 28]. The characterization of these mutants has shown that many of them recapitulate important features of human neurodegenerative diseases, *i.e.*, vulnerability of specific neuronal populations and progressive degeneration. Interestingly, the importance of some of these genes in neurodegeneration has been validated in mammalian disease models (*e.g.*[193, 194]). This suggests that this approach may identify novel genes important for conserved mechanisms that maintain nervous system integrity.

CONCLUDING REMARKS

The generation of animal models that recapitulate physiologic and pathologic conditions in humans are key for biomedical and scientific progress. They provide an opportunity to explore the mechanisms underlying disease pathogenesis as well as develop effective preventive measures and therapies. During the past decades, invertebrate models of neurodegeneration have emerged as successful complementary systems to mammalian models, facilitating identification of relevant pathways and novel disease-associated genes. It is important to bear in mind, however, that invertebrate models have potential caveats and limitations in studying the function of human disease genes. They lack a number of disease-related factors and biophysical processes that may influence specific pathologies. It follows that observations in invertebrates should be subsequently validated in mammals to determine their relevance to human diseases. Nevertheless, the conservation of important basic biological processes in *Drosophila, C. elegans* and mammals have permitted the recreation of essential pathological features observed in human patients, substantiating their enormous potential for dissecting conserved pathogenetic mechanisms [3, 4, 20-26]. Moreover, these simple models offer extraordinary genetic tools to decipher genetic pathways of disease-genes and to discover genetic factors that modulate the neurodegenerative phenotype. Notably, findings from worm and fly models of Aβ42 toxicity, polyglutamine repeat proteins and α-synuclein have identified conserved chaperone proteins as important suppressors of neurotoxicity [117, 118, 134, 195]. These findings suggest that some toxic mechanisms might be common to different neurodegenerative diseases. The fact that overexpression of *glutathione-S-transferase* can suppress the toxicity associated with either long polyglutamine repeat proteins, *α-synuclein* overexpression or mutations in *Parkin* also implicates oxidative stress as playing a role in different neurodegenerative processes [3, 4, 151, 158-160]. Importantly, several genetic modulators identified in enhancer/suppressor screens have been validated in mammalian systems [174, 175, 196-200]. In addition, loss-of-function studies of endogenous genes homologous to human disease genes in *Drosophila and C. elegans* have yielded new clues to pathogenic mechanisms. For example, mitochondrial dysfunction was first linked to defective PINK1/Parkin signalling in *Drosophila* [152, 153, 156]. On the other hand, invertebrate neurodegenerative

mutants isolated in forward screens that show characteristic features of progressive neurodegeneration have provided valuable insights into conserved genetic pathways and mechanisms required for maintaining the structural integrity of the nervous system. Indeed, this approach has allowed researchers to identify novel neurodegeneration genes, suggesting their human orthologs may also be involved in pathological processes [24, 28]. Since collections of genetic mutations in flies and worms are constantly expanding, it is reasonable to anticipate that additional disease genes will be identified.

Pharmacological screens in worms and flies have identified potential therapeutic compounds. For example, the identification of chaperones and histone deacetylase inhibitors as suppressors of neurodegenerative phenotypes in flies has led, in some cases, to validation in mouse models and human clinical trials [34, 35, 72, 134, 139, 141]. Sophisticated and automated techniques that are impractical in mammals continue to emerge in invertebrates. These will increasingly facilitate high-throughput screens for candidate therapeutic reagents.

Given the rapid advances in the field of neurodegeneration in *Drosophila* and *C. elegans,* it is logical to expect an increasing number of high-quality studies that will continue to enrich the study of neurodegenerative diseases and complement studies in mammalian systems. Futures studies in invertebrates will focus on understanding key aspects of the neurodegenerative pathology, including ageing and disease susceptibility. Illumination of these processes is essential for diminishing events that promote age-associated neuronal decline and disease. Such studies may ultimately provide a molecular link between ageing and neurodegeneration.

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CONFLICT OF INTEREST

The author confirms that this chapter contents have no conflict of interest.

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