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Editors

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About the Book

This book is aimed at generating an updated reservoir of scientific endeavors undertaken to unravel the complicated yet intriguing topic of neurodegeneration. The fruit fly, *Drosophila melanogaster*, has been utilized as a model organism to study a number of human neurodegenerative and neuromuscular diseases for more than two decades. The fruit fly offers multiple advantages for the investigation of the molecular mechanisms of diseases. Short life cycle, high offspring numbers, low cost of maintenance, simple yet powerful genetic manipulation techniques, annotated genome and availability of mutants, are some of the attractive features of *Drosophila* as a model organism. *Drosophila* has orthologs of about 75% of human disease causing genes, thus making it one of the most suitable model organisms to understand the molecular basis of neurodegeneration. This book will help readers gain insight into the classical as well as the recent knowledge obtained from *Drosophila* that aids to dissect the molecular mechanisms underlying different neurodegenerative disorders and unravel new scopes for therapeutic interventions. To begin with, the readers will be acquainted with the different methodologies available to create humanized fly models that faithfully reflect the pathogenicities associated with various disorders. A brief discussion on neurofibrillary tangles, a characteristic phenotype associated with common neurodegenerative disorders, precedes the elaborate description of lessons learned from *Drosophila* about Alzheimer's, Parkinson's, Huntington's diseases, RNA expansion disorders, and hereditary spastic paraplegia disease. This book also includes the contribution of stem cell biology, metabolic processes and developmentally critical signaling pathways in neuronal development and degeneration. The book concludes with the use of *Drosophila* for identifying pharmacological therapies for neurodegenerative disorders. The wide range of topics covered here will not only be relevant for beginners who are new to the imperative role of *Drosophila* as a model to study human disorders, but will also be a major contribution to the scientific community, giving an insight into the paradigm shift in our understanding of neurodegenerative disorders in an interesting and awe-inspiring manner. The editors have attempted to comprehensively anthologize the lessons on neurodegeneration learned from *Drosophila* and guide the readers to gain insight into the multidimensional aspects of disease pathogenesis of human neurodegenerative disorders.

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About the Editors

Mousumi Mutsuddi is currently a faculty in the Department of Molecular and Human Genetics, Banaras Hindu University. Before joining Banaras Hindu University, she was a scientist at Broad Institute, Massachusetts Institute of Technology, USA. She has been a visiting scientist at Whitehead Institute for Biomedical Research, Massachusetts Institute of Technology, USA; National Institute of Health, USA; and the University of Valencia, Spain. Her group is currently working on the molecular genetics of neurodegenerative diseases, genetic basis of ocular disorders, apoptosis, and neuronal development. She spearheads the research group that works on congenital retinal disorders in the north Indian population. Her group has identified a new causal genetic variant for infantile nystagmus in *FRMD7*, and has also used whole-exome sequencing to identify rare variants causing congenital ocular disorders. In addition, she has also identified the molecular mechanisms underlying motor neurodegenerative disorder of Spinocerebellar Ataxia 8 using the *Drosophila* model and has characterized novel genes involved in neuronal development, like *maheshvara*. Her research findings have been published in reputed journals like *Current Biology, Genetics, American Journal of Human Genetics, Development, Human Mutation*, etc. Dr. Mutsuddi's research contributions have won her numerous recognitions and awards from apical organizations of India like the Department of Biotechnology and Indian National Science Academy as well as invitations and collaborations from research institutes in the USA and Europe.

Ashim Mukherjee is working as a professor in the Department of Molecular and Human Genetics, Banaras Hindu University, India. He has almost 30 years of research experience in the field of *Drosophila* genetics and molecular biology. He has worked at premier research institutes in India and abroad and as a postdoctoral research associate at the University of Massachusetts, Amherst, and the Harvard Medical School, Boston, USA. He has decade-long teaching experience at the postgraduate level. His research interest is to decipher the molecular mechanism involved in the regulation of Notch signaling. He has identified several novel interacting partners involved in different aspects of Notch signaling and has published more than 30 papers in renowned journals like *Nature Cell Biology*, *Genome Research*, *Current Biology*, *Genetics*, *Developmental Biology*, *Cellular Signalling*,

Journal of Neuroscience, etc. The Department of Science and Technology and the Department of Biotechnology, Government of India, have funded his research work. He is member of the Drosophila Board of India and life member of Indian Society of Cell Biology and the Indian Society of Developmental Biology. He is also member in the editorial board of several journals and also serves as reviewer for many journals.

Mighty Fly: An Introduction to *Drosophila*

Vartika Sharma, Abhinava K. Mishra, Mousumi Mutsuddi, and Ashim Mukherjee

Abstract

Model organisms have been a key prerequisite in uncovering the mechanisms governing various aspects of development and disease. In the era of deep sequencing, multi-omics data integration, high-throughput screening, and personalized medicines, researchers are constantly exploring new avenues to address the biological problems in living organisms. Ease of handling, availability of genetic toolset to carry out functional studies, and relevance to human health make a model organism the default choice to perform experiments. *Drosophila* has been an instrumental model organism to study the mechanisms of development for several decades. The striking similarity between the fly and human disease genes also makes it an appropriate system to study the disease etiology and screen for therapeutic targets. Here, we describe the use of the *Drosophila* model in understanding the organism development and design principles based on these studies that provide significant insights into mechanisms of human disease. We discuss the choice of *Drosophila* as a model system, various genetic toolkits available in the fly, and attempts to use *Drosophila* in developing human disease model and drug discovery. Finally, we discuss the importance of *Drosophila* in stem cell studies and catalog the resources available to the *Drosophila* research community. We conclude the chapter with the discussion of new approaches to utilize the power of *Drosophila* as a model organism.

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Keywords
Drosophila · Model organism · Balancer chromosomes · GAL4/UAS system · FLP-FRT system · CRISPR/Cas9 · Disease model · FlyBase

Introduction

Am not I
A fly like thee?
Or art not thou
A man like me?
-"*The Fly*", William Blake

With these lines, William Blake posed a question around 200 years ago, which science proved probably correct in the subsequent years. Fruit fly or *Drosophila* is a dipteran found all over the globe. During the course of evolution, these arthropods diverged from the vertebrate lineage approximately 600 million years ago (Adoutte et al. 2000; Peterson et al. 2004), indicating that this tiny creature may be totally unrelated to humans. However, research in the last few decades revealed a striking similarity of this tiny marvelous creature with humans and portrayed it as an organism that can be used as a model to study eukaryotic biology. *Drosophila* (*drósos* = Dew, *phílos* = loving), at a taxonomic scale, occupies a position in the family Drosophilidae. Apart from the common denotation of fruit flies, they are also known as pomace flies, vinegar flies, or wine flies because of their general tendency of lingering around rotten overripe fruits. The entire genus of *Drosophila* contains around 1500 species, and of these, *Drosophila melanogaster* species stands apart as it is extensively used in research, particularly in genetics and developmental biology.

How useful this tiny creature is in field of Biology can be understood with the fact that till date it has bagged Nobel Prize six times in its name. From its very first use in the laboratory in the early 1900s until the present day, *Drosophila* has been at the center to many genetic breakthroughs. The pioneering work of Thomas Hunt Morgan, in which the foundation stone of heredity was laid, was done using *Drosophila* as a model organism. In 1910, Thomas Hunt Morgan was rewarded for discovering the very first mutation, a white-eyed fly. Morgan with his three students, A. H. Sturtevant, C. B. Bridges, and H. J. Muller, formulated the chromosomal theory of inheritance (Sturtevant 1965). This theory, proposed by Morgan, fetched him the very first Nobel in 1933 in Medicine or Physiology and so was the first Nobel Prize to *Drosophila*. In the next few decades, *Drosophila* studies at Columbia University by Morgan and his students laid the experimental foundation for genetics. Alfred H. Sturtevant constructed the first genetic map by measuring recombination frequencies and showed that genes are arranged in a linear order, for the very first time in 1913 (Sturtevant 1913). T. S. Painter at the University of Texas published the first drawings of *Drosophila melanogaster* polytene chromosomes, which included the chromosomal localization of several genes, thereby giving the idea of physical mapping (Painter 1934).

The legacy was continued by Morgan's eminent student Herman Joseph Muller, who received the Nobel in 1946 for the discovery of heritable mutation by means of X-ray irradiation. Muller, in 1927, proposed the idea that dose-dependent X-ray can generate mutations in genes, and the higher the dose of X-ray, the higher will be the frequency of mutation (Muller 1927). This report was enthralling, as it raised the possibility that desirable mutations can be generated in the near future. The generation of "Balancer Chromosomes" (specialized chromosomes with multiple inversions that prevent recombination) also came forward after Muller's invention.

In 1995, the *Drosophila* researchers, Edward B Lewis, Christiane Nüsslein-Volhard, and Eric F Wieschaus shared the prize "for their discoveries concerning the genetic control of early embryonic development." At that time (in the late 1970s and the early 1980s), little was known about developmental biology and embryogenesis. Nusslein-Volhard and Wieschaus then were able to identify the genes that play a pivotal role in the body segment formation in *Drosophila* (Nüsslein-Volhard and Wieschaus 1980). Edward Lewis in another independent study showed how the development of specialized organs from these body segments was regulated by specific genes (Lewis 1978). These findings laid the foundation stone of developmental biology and genes involved in congenital abnormalities in the coming future.

Another revolution in *Drosophila* research took place in the 1980s when Allan Spradling and Gerry Rubin discovered the methods for generating transgenic flies (Rubin and Spradling 1982). This major breakthrough gave researchers a strong genetic tool that has the potential to increase the research capacity. Further, Berkeley *Drosophila* Genome Project, in collaboration with the company Celera, achieved a rare feat in 2000 with the sequencing of the fly genome.

Richard Axel and Linda B. Buck were jointly awarded the Nobel Prize in 2004 for their discoveries of odorant receptors and the organization of the olfactory system (https://www.nobelprize.org/prizes/medicine/2004/summary/). Here again, *Drosophila* confirmed its indispensable role as a biological model system.

This tiny creature has not only revolutionized genetics but also played a promising role in the field of immunology. Jules Hoffman in an accidental discovery found that flies with mutations in the Toll gene died when infected with bacteria and fungi due to lack of an innate immune system (Hoffmann 2007). In an independent study, Bruce Beutler discovered that Toll-like receptors (TLRs) were also present in mice, showing a striking similarity between mammals and their fly counterpart (Beutler 2004). Ralph Steinman discovered dendritic cells and their ability to activate T cells (Bashyam 2007). These parallel lines of work were jointly awarded Nobel in 2011.

The most recent Nobel to "*Drosophila*" was awarded in 2017. The sleep–wake cycle or the circadian rhythm was decoded using this tiny little creature by Jeffrey C. Hall, Michael Rosbash, and Michael W. Young. Jeffrey Hall and Michael Rosbash discovered that PER, the protein encoded by the *period* gene, accumulated during the night and degraded during the day. PER protein levels thus oscillate over a 24-h cycle to synchronize the circadian rhythm. Michael Young, in an independent study, discovered a second clock gene, *timeless*, encoding the TIM protein that was required for a normal circadian rhythm. In elegant work, it was shown that TIM

binds to PER and acts as a transcription factor, thereby blocking the *period* gene activity to close the inhibitory feedback loop (https://www.nobelprize.org/prizes/medicine/2017/). The key developments in *Drosophila* research has also been highlighted in Table 1.

Table 1 Key developments in fly research
Bellen et al. (2010)

Year	Major genes and Methodologies discovered in *Drosophila*
1910	***white*** gene discovered
1915	Chromosome theory of inheritance First ***Notch*** Mutation
1918	First ***achaete*** mutation
1923	First ***ultrabithorax*** mutation
1927	Discovery of X-rays as mutagen First Balancer Chromosome
1935	Physical mapping using Polytene Chromosome
1936	Discovery of Mitotic Recombination in Flies
1939	Discovery of ***Notch*** as a neurogenic gene **(1939-1950)**
1968	Ethyl Methane Sulfonate Mutagenesis
1969	Discovery of ***shaker*** Discovery of ***ether a go-go*** Discovery of ***transient receptor potential***
1971	Discovery of ***period***
1976	Discovery of ***dunce***
1978	***bithorax*** characterized as homeotic genes ***achaete*** and ***scute*** are proneural genes
1982	P-element mediated transformation
1984	Cloning of ***period*** Cloning of ***dunce***
1985	Cloning of ***Notch*** Cloning of ***transient receptor potential***
1987	P-element enhancer detectors Cloning of ***shaker***
1989	FLP/FRT Method Discovered
1991	Cloning of ***ether a go-go***
1993	GAL4/UAS System
1999	MARCM analysis
2000	*Drosophila* Genome Sequence announced
2006	P[acman] BAC transgenic flies
2007	Transgenic RNAi Library

(Bellen, Tong et al. 2010)

During the course of time, fruit flies have presented themselves as an excellent model organism to work on. From human disease modeling to the dissection of cellular morphogenesis and to behavior and aging, *Drosophila* has revolutionized every aspect of modern Biology, and the accelerating pace of *Drosophila* genetics suggests that the fruit fly will remain a key model organism for the foreseeable future.

WHY *Drosophila*?

The reason for using *Drosophila* as a model system is manifold. The fruit fly offers multiple advantages for the exploration of the molecular mechanism of diseases. A short life cycle of fly, high offspring numbers, low maintenance cost, availability of simple and powerful genetic manipulation tools, and availability of mutants are some of the many attractive features of why *Drosophila* is used as a model system. In addition to it, sequencing of the *Drosophila* and the human genomes revealed the strikingly enormous similarity between fly and humans, with ~75% of the genes involved in human disease showing a minimum of one homolog in *Drosophila* (Rubin et al. 2000). Moreover, the core cell biology operating in fly and humans is evolutionarily conserved, including the regulation of gene expression, synaptogenesis, cell proliferation, cell differentiation, cell signaling, and cell death. Several pathways and their components have been originally identified in *Drosophila* that led to the discovery of their mammalian counterparts. One such example includes the discovery of *wingless* (*wg*) in *Drosophila* that put forth the basis for the identification of the mammalian *Wnt* gene (Sharma and Chopra 1976). Wnt/wingless, since then, has been studied extensively along with its roles in a range of cellular processes and human disease (Korkut and Budnik 2009).

Structure and Organization of the *Drosophila* Genome

The entire *Drosophila* genome size is about ~180 Mb and comprises 13,600 genes, which is about 5% of the size of human genome with a more compact genetic organization. This can be compared with the 6000–6500 genes of yeast, 18,425 genes of nematodes, and 40,000–60,000 of humans. The average gene density in *Drosophila* is about one gene for every 9 kb. Irrespective of the compactness, about one-third of the fly genome consists of repetitive sequences that do not encode proteins and/or that act as transposable elements. These highly repetitive sequences is concentrated in the Y chromosome and centromeric heterochromatin regions of the autosomes (Adams et al. 2000; Bosco et al. 2007).

Interestingly, the *Drosophila* gene set shows more similarity to that of mammals when a comparison was made with nematodes. About half of the fly proteins show homology with mammalian proteins, whereas nematodes show only a third of homology with flies (Adams et al. 2000).

Chromosomes

The haploid genome of *Drosophila melanogaster* contains four chromosomes, which is much smaller in comparison to mouse (20) and human (23). The X and Y sex chromosomes, two larger autosomal elements of chromosomes 2 and 3, and the small dot fourth chromosome (Metz 1914; Deng et al. 2007). Chromosome X is acrocentric with a large left arm (XL) and a short right arm (XR). Y is also acrocentric with a slightly longer long arm (YL) and a short arm (YS). The two larger autosomal chromosomes, 2 and 3, are metacentric, with the centromere residing in the center of two roughly equal left and right arms. The fourth dot chromosome is acrocentric, similar to the X, and is only about 2% of the size of the major autosomes (Fig. 1). The low chromosome number in *Drosophila* simplifies most of the genetic manipulations and hence is a key advantage for genetic studies. In *Drosophila*, sex determination is of the XY type, with females being XX and males XY. Unlike the situation in mammals, however, Y plays no role in sex determination; sex is instead determined solely by the ratio of the number of X chromosomes to the number of copies of each autosome (the X:A ratio) (Erickson and Quintero 2007). The Y chromosome is required only to confer male fertility. Thus, normal female flies are XX and males XY. In addition, XXY flies tend to develop as normal females, as they have an X:A ratio of 1, and on the contrary, XO flies develop as males due to the decreased X dosage.

At the molecular level, sex determination in *Drosophila*, is however, controlled by activation of the *sxl* gene in females (Verhulst et al. 2010). The early expression of Sxl in females initiates a cascade of alternative splicing events that ultimately regulate differential splicing of the transcription factors *doublesex (dsx)* and *fruitless (fru)*. Sex-specific isoforms of Dsx and Fru then mediate the expression of downstream effectors that govern sexual morphology and behavior (Demir and Dickson 2005).

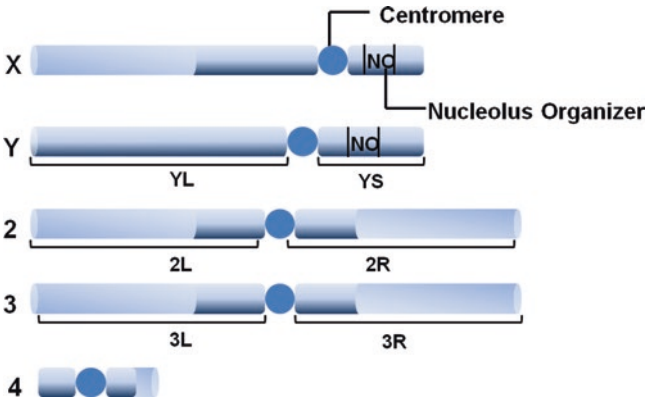

Fig. 1 The *Drosophila* chromosome complement. YL and YS, the long and short arms of the Y chromosome; 2 L, 2R, 3 L, and 3R, the left (L) and right (R) arms of chromosomes 2 and 3. The dark blue region denotes the heterochromatin region close to centromere, whereas the light blue region shows the euchromatin region

Polytene Chromosomes

Polytene chromosomes were originally observed in the larval salivary glands of *Chironomus* larvae by Édouard-Gérard Balbiani in 1881 (Balbiani 1881). The hereditary nature of these structures was, however, confirmed when they were studied in *Drosophila* in the early 1930s by the German biologists Emil Heitz and Hans Bauer. Heitz and Bauer in their studies discovered that the tangled chromosomes having distinct bands are unique to the cells of the salivary glands, midgut, Malpighian tubules and brain (D'ANGELO 1946). The most striking feature of the polytene chromosome is their capacity to endoreduplicate. These cells undergo several rounds of division in which the S phase is repeated with no subsequent mitosis. In the case of the third larval instar, the ploidy level reaches 1024–2048 after 10–11 rounds of successive cell division (Rodman 1967). This level of ploidy is reached mainly by the euchromatic portions of the genome, while the heterochromatin region is majorly under-replicated. The most unique feature of polytene chromosome is that the homologous chromosomes are tightly synapsed. The combined effect of polyploidy and pairing is that the DNA strands of each euchromatic chromosome arm form a coherent coil showing five large arms (the left and right arms of chromosomes 2 and 3, and the X chromosome) radiating out from the chromocenter. The much smaller chromosome 4 also associates with the chromocenter. Each of the euchromatic arms has a unique banding pattern caused by the differential condensation of the chromatin into darkly stained bands and less dense interbands. The polytene chromosomes have provided *Drosophila* geneticists with a readymade detailed physical map of the fly genome.

Life Cycle

A major advantage of using *Drosophila* as a model system is their short life cycle. This allows for the rapid generation of large numbers of progeny in a short time to use in genetic crosses. A single female can produce 3000 progeny in her lifetime, where a single male can sire well over 10,000 offspring (Ashburner 1989). Female flies have a special sperm storage organ, the spermatheca that enables her to lay several hundred eggs after a single mating.

Like butterflies and moths, *Drosophila* is also a holometabolous insect and completes its life cycle in four successive stages: egg, larvae, pupa and adult. The egg of *Drosophila* is about half a millimeter long and is well supplied with the yolk that eventually supports the proper development of the organism. The process of development, from a fertilized egg to adult, requires on average only 9–10 days at 25 °C. However, the development of this tiny fruit fly is highly influenced by temperature. Lowering of temperature in general lengthens the development period of the fly. *Drosophila*, when reared at 18 °C, requires an average of 19 days to turn into an adult from the egg. The shortest life cycle of 7 days is achieved by maintaining the flies at 28 °C. However, exposure to higher temperatures for a longer duration may render the fly sterile.

Upon fertilization, embryogenesis is completed in a 24-h period followed by three larval stages, namely, first, second, and third instar, with a molting event at each stage transition. The first two instars last for about 24 h, whereas the third instar typically requires 2 days for its completion. After completion of larval development, the animals metamorphose within a hard, protective chitin-based pupal case (or puparium) that forms from the outer larval cuticle. The steroid hormone ecdysone plays a key role in the metamorphosis of *Drosophila*, which shifts the gene expression from the larval to the adult fly pattern (Yamanaka et al. 2013). The animal remains in the pupal case for 4–5 days, during which most larval tissues break down and adult structures develop from a group of imaginal discs present in the larvae. These imaginal discs are flattened, sac-like epithelial structures that develop from small groups of cells set aside in the early embryo. Most structures specific to adults, such as the wings, legs, eyes, and genitalia, are generated from these imaginal discs. Adult flies emerge from the pupal case in a process termed "eclosion" and become sexually mature in 8–12 h, allowing the life cycle to repeat itself (Fig. 2).

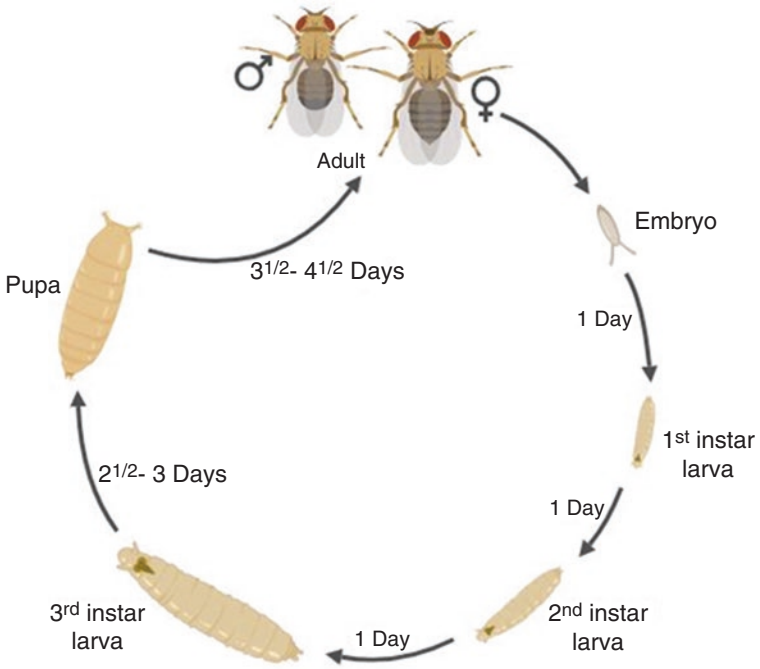

Fig. 2 ***Drosophila* life cycle**. *Drosophila* embryo hatches into the first instar larva. The transition between successive larval stages is referred to as molting. The third instar larva converts into a pupa through a process called as pupariation. The fly ecloses into an adult after the completion of the pupal stage that lasts for about 3.5–4.5 days

Drosophila: A Genetic Toolkit

A wide range of genetic manipulation techniques has been developed in *Drosophila,* making it an excellent model system to work on. *Drosophila* aids the researchers to answer a wide array of questions: What is the role of a particular gene in the development and function of an organism? Which genes are involved in the development of particular organs or tissue and what is the underlying molecular mechanism? Where is a particular gene expressed in the animal during or after development? What are the genes that mediate basic cell biological events within a specific cell type? Can the expression of a particular gene be controlled in a desired fashion?

Drosophila provides insightful genetic tools that address all of the above-mentioned and many more answers to the researchers, thus revolutionizing our understanding of basic cell biology and development.

(i) *Genetic Crosses*: The key feature that makes *Drosophila* an excellent model organism is its ability to create stable inbred stocks carrying mutations or other genetic manipulations and the ability to generate desired genotypes with the aid of suitable genetic crosses. In *Drosophila*, multigenerational crossing schemes can easily be generated with virgin females and males of desired genotypes. Males and female flies are distinguished primarily by abdominal pigmentation patterns as well as genital structures and presence of "sex combs" on the first tarsus region of males. Accurate genetic crosses are aided by visible "marker" mutations that help the geneticist in the selection of offspring. These stable visible markers like *CyO* and *Sb* exclude the possibility of choosing the other chromosome instead of the inherited one.

Balancer Chromosomes

Drosophila provides another extremely valuable tool to the geneticist: the balancer chromosomes. The term "balancer" is derived from the extensive use of these chromosomes in stock keeping. These chromosomes serve two important purposes. They maintain the lethal and sterile mutations in stock without selection and they can be used in screens for mutations by maintaining the linear integrity of a mutagenized homolog. These engineered chromosomes contain multiple inverted sequences, relative to a normal chromosome that prevents the event of recombination between two homologous chromosomes. Most balancers also contain a dominant marker that enables the researchers to track these chromosomes in the event of single- or multigenerational crosses. Balancer chromosomes have a third feature as well, that is, they carry recessive lethal mutations, and this feature enables the fly to prevent mutations of interest from being selected out of an inbred population (Kaufman 2017).

P-Element Transposons: A Critical Tool in *Drosophila* Genetics

The identification and development of the P-element as a germline transformation vector literally revolutionized the *Drosophila* genetics. P-element is a classic transposable element with a gene encoding the transposase enzyme. This enzyme acts on the 31 bp inverted repeats at P-element ends to catalyze transposition within the genome. The idea of replacing the transposase enzyme with gene of interest was hypothesized by Rubin and Spradling in 1982 to produce an ideal system for inserting DNA into the fly genome. The P-element construct with the desired gene in a frame (transformation plasmid), when co-injected with another independent source of transposase enzyme (helper plasmid), inserts the transposable element into the developing germline. A stable and heritable insertion in the fly genome is assessed with the aid of visible markers (Rubin and Spradling 1982).

In addition, the nature of P-element mobilization is too imprecise; when they excise, they take with them the adjacent genomic sequence leaving behind a deletion. These fly lines thus provide a means to generate excision mutants. The P-element insertion is not entirely random, and large collections of mobilized P inserts have shown that they favor landing in specific genes. In order to generate precise chromosomal excisions, flipase recombination targets were engineered into P-elements (Brand and Perrimon 1993).

The development of these genetic tools has greatly enhanced the process of genome editing in *Drosophila*.

GAL4/UAS System

Another popular technique used in *Drosophila* research is the *GAL4/UAS* binary transgene overexpression system, which is the most versatile expression system ever developed in *Drosophila* (Duffy 2002). The P-element transformation vector was further engineered by Brand and Perrimon to generate an expression system for tissue-specific studies of a particular gene of interest (Brand and Perrimon 1993). *GAL4* is a yeast transcription factor that drives the expression of the transgene downstream to Upstream Activator Sequence (UAS), *GAL4* insertion alone though has no effect on its own in *Drosophila.* A *GAL4* fly line expresses *GAL4* under the control of a tissue-specific promoter. This is achieved by the fusion of the identified tissue-specific promoters with the *GAL4* gene. These constructs are subsequently microinjected into fly embryos to generate the desired tissue-specific *GAL4* lines.

Likewise, the UAS lines are generated, where the cDNA of desired genes are cloned downstream of UAS and a promoter sequence, followed by its introduction into the germline by P-element-mediated transformation.

The UAS transgenes are not transcribed in the absence of the GAL4 protein and hence the flies that carry the transgenes have no effect on them. To ectopically express the transgene in a tissue-specific manner, UAS-cDNA flies are crossed with a GAL4 driver line. This enables the transgene to get expressed in the F1 generation in those cells that make the GAL4 protein (Fig. 3).

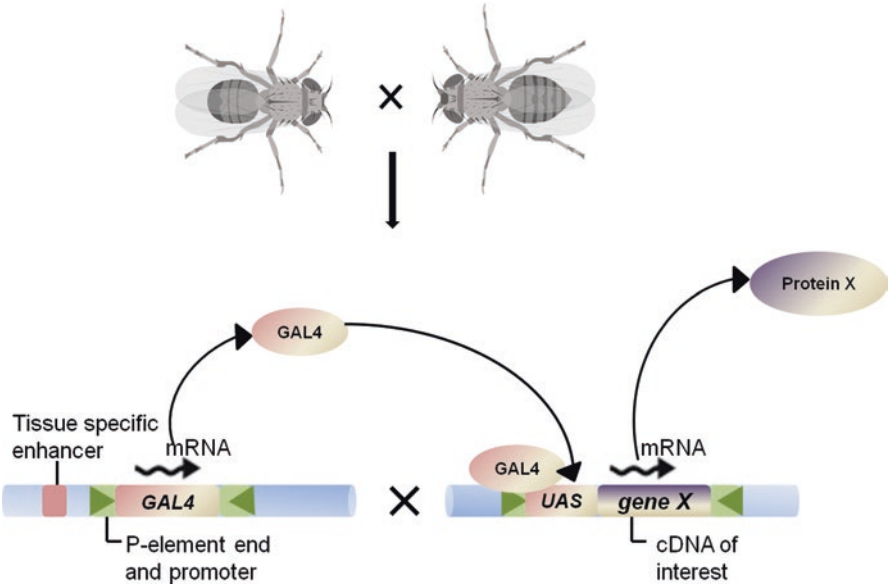

Fig. 3 GAL4/UAS system. The enhancer construct expresses the GAL4 protein in tissues dictated by nearby enhancers. The UAS construct contains cDNA of interest under the control of *UAS* promoter. Ectopic protein expression will occur in a tissue-specific manner

The GAL4-UAS system for ectopic expression is a highly versatile tool for studies of *Drosophila* development.

FLP/FRT System: Technique to Generate Somatic Mosaics

Mutations in genes that play a critical role in the developmental, cellular, or behavioral process can lead to devastating consequences, and at times, the outcome may be as severe as lethality. If mutations in a gene inhibit the completion of early embryonic development, then analysis of the gene's role in adult tissue is impeded. To overcome these challenges, geneticists came forward with a more advanced tool that allows them to study gene function irrespective of their role in early developmental stages. Homozygous mutant patches of cells, in an otherwise heterozygous background, can be generated with the help of mitotic recombination. The idea rescues the organism from lethality, as a very minute clonal region is subjected to null mutation.

Golic and Lindquist harnessed the FLP recombinase and its site-specific recombination sites (FRTs) from yeast, *Saccharomyces cerevisiae* for use in *Drosophila* (Golic and Lindquist 1989). The FLP catalyzes reciprocal crossing-over at specific recombination targets (FRTs) contained within inverted repeats of a yeast DNA plasmid.

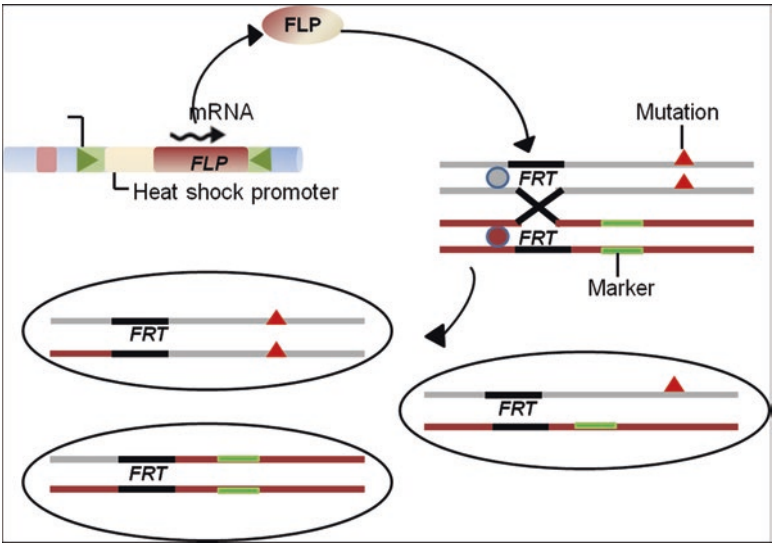

Fig. 4 FLP/FRT system. FLP recombinase is produced when a fly is subjected to elevated temperature. The flippase enzyme catalyzes recombination between homologous FRT sites in mitotic cells resulting in homozygous mutant somatic clones

The FLP from yeast was cloned downstream of and under the control of a heat-shock promoter and was introduced into the fly through a P-element-mediated transformation. Likewise, the FRT sequence was also introduced close to the centromeric region of *Drosophila*. Brief exposure of heat shock induces the FLP recombinase to catalyze recombination between the two FRT sites, resulting in the generation of patches of homozygous mutant cells, typically identified by a linked recessive marker or loss of a linked fluorescent gene product (Fig. 4).

Several modifications have been created in the FLP–FRT system including incorporation of a range of promoters for precise control of mitotic recombination. To study the effects of homozygous lethal mutations in a particular tissue, a more sophisticated system, Mosaic Analysis with a Repressible Cell Marker (MARCM), was designed. This allows analysis of individually marked mutant cells in an otherwise heterozygous background (Lee and Luo 2001). This innovation has contributed immensely in studying the in-depth role of a single gene.

RNA Interference (RNAi)

Andrew Fire and Craig C. Mello shared the 2006 Nobel Prize in Physiology or Medicine for their work on RNA interference in *C. elegans*. Since then, the regulatory role of RNA was highlighted. Small RNA molecules (miRNA and siRNA) inhibit gene expression or translation by targeting specific mRNA molecules, and they have evolved as a stable technology for gene suppression (Fig. 5). This, in

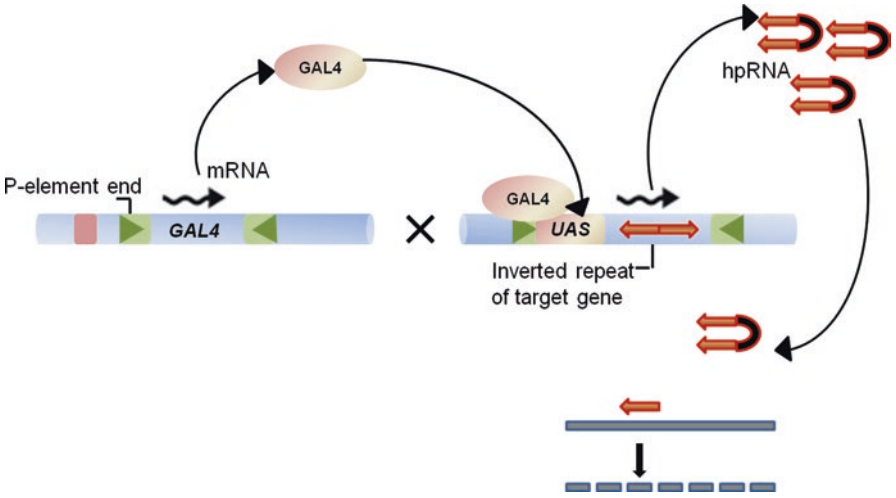

Fig. 5 RNA interference (RNAi). The *UAS*–IR line has a transgene containing an inverted repeat (IR) of the target gene under the control of UAS, a target of GAL4; the dsRNA of the target gene is expressed in a tissue-specific manner and induces gene silencing

addition to the classic GAL4/UAS system in *Drosophila*, eliminates a gene's function by reducing mRNA levels from that gene (Kennerdell and Carthew 2000). These are, however, not a permanent alteration in the gene's coding sequence and initially produced many off-target effects. Also, these RNAi lines were inefficient in their ability to knockdown RNA expression to null levels.

Few of these issues have been addressed with the new AttP-specific integration system for UAS-transgene insertion that ensures high levels of expression and with least effect on other genes due to insertion sites (Ni et al. 2008). The libraries at the *Drosophila* RNAi Screening Center and Vienna *Drosophila* Research Centre provide huge RNAi stocks that can be used to screen the majority of protein-coding genes. Researchers can elucidate the function of particular genes in cellular and developmental processes from embryo to adult by expressing the RNAi hairpin construct along with the well-characterized GAL4 line to knockdown the gene of interest (Dietzl et al. 2007).

TALEN and CRISPR/Cas9-Based Genome Editing

The off-target effects of RNAi lines forced geneticists to develop some of the more promising and precise genome editing tools. Recently, the development and application of the sequence-specific endonucleases, **T**ranscription **A**ctivator-**L**ike **E**ffector **N**ucleases (TALENs) (Beumer and Carroll 2014), and the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) system have made a revolutionary contribution to the genome editing toolbox (Gratz et al. 2013).

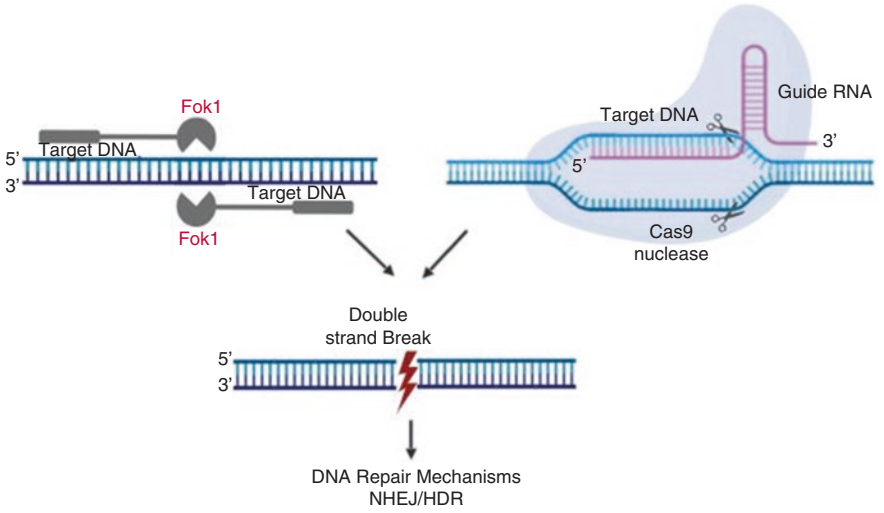

Fig. 6 TALEN and CRISPR/Cas9 system of gene editing. The gene of interest can be targeted and cut to produce a double-strand break (DSB) with the aid of transcription activator-like effector nuclease **(TALEN)** mRNA or Cas9 mRNA/single-guide RNA. DNA repair mechanisms repair the DSB by either NHEJ or HDR

TALENs consist of repeats of DNA-binding domains and a Fok I nuclease domain. Since dimerization of the catalytic domain of FokI is mandatory for nuclease activity, a pair of TALENs is designed in such a way that it recognizes the DNA sequences to the left and right of the intended cut site. Thus, TALENs can be utilized to generate site-specific double-strand breaks to assist genome editing through non-homologous end joining (NHEJ) or homology-directed repair (HDR).

The CRISPR/Cas9 system in a similar fashion introduces a double-strand break (DSB) at a specific location based on a gRNA-defined target sequence. Modification of the guide RNA is the key, as it allows the specificity to the target gene to induce a double-strand break. The double-strand breaks provide sites for creation of short insertions/deletions and large deletions in a gene of interest through non-homologous end joining repair (Fig. 6).

TALENs and CRISPR/Cas9 genome editing technologies have dramatically boosted the ability to manipulate a diverse set of genomes. Development of these editing technologies in flies has created an efficient mechanism by which a complete loss-of-function/null mutant can be generated.

Disease Models in *Drosophila*

A high degree of evolutionary conservation among genes that control the basic developmental and metabolic processes between *Drosophila* and humans provides a good reason to study *Drosophila* for heritable diseases in humans. In addition, the

availability of genome sequences of human and fruit fly has provided a good opportunity for researchers to explore this conservation further. Analysis based on the interactive cross-genomic database Homophila revealed that 75% of all human disease genes have related sequences in *D. melanogaster* (Adams et al. 2000; Fortini et al. 2000). Out of the list of thousands of human disease gene entries in the database, ~700 human disease genes have well-conserved homologs in *Drosophila.* These human homologs in *Drosophila*, when disrupted, cause a broad spectrum of human diseases such as neurological disorders, cancer, developmental disorders, metabolic and storage disorders and cardiovascular disease. Here, few of the many human diseases have been discussed in detail where *Drosophila* aids the researchers to exploit its human homology.

Drosophila as a Model for Diabetes

Drosophila shares many of the basic fundamental metabolic function with vertebrates. Like humans, the fly maintains an appropriate sugar-level circulation that compensates for changing environmental conditions and stores excess energy in the form of glycogen and lipid. These glycogen reserves are mobilized during periods of energy need, such as exercise and/or nutrient depletion (Rusten et al. 2004; Scott et al. 2004). The organ systems that control nutrient uptake, storage, and metabolism in humans, although differ from flies, show a close analogy with those of humans. In *Drosophila,* digestion and nutrient absorption occur in the midgut, which is equivalent to the stomach and intestine of humans. The fat body of *Drosophila* acts like the mammalian liver. Like humans, in *Drosophila,* the lipid particles are carried through the circulatory system as either high-density and or low-density lipophorin particles (Canavoso et al. 2001). Like hepatocytes in the human liver, specialized oenocytes are present in *Drosophila* that accumulate lipid upon starvation and function in lipid processing (Gutierrez et al. 2007). In addition, separate, discrete clusters of cells maintain fly carbohydrate homeostasis in a manner analogous to the pancreatic alpha and beta cells in humans. The antagonistic action of insulin and glucagon in humans is replicated in fly as well. In *Drosophila,* insulin-like proteins (Ilps) are released in response to high levels of circulating sugar, and a glucagon-like molecule, adipokinetic hormone (AKH), is released in response to low levels of circulating sugar (Lee and Park 2004). These striking similarities posed *Drosophila* as an excellent model to study diabetes.

The conserved insulin/IGF pathways play a central role in growth and metabolism in both humans and *Drosophila.* The genome of *Drosophila* codes for eight insulin-like peptides (ILPs or dILPs). These ILPs are secreted from the insulin-producing cells (IPCs) of the brain and transported via hemolymph to cells. Like human insulin, these dILPs 1–7 bind to the insulin receptor (InR) and activates it. The activated insulin receptor in turn autophosphorylates, allowing the binding and phosphorylation of the Insulin Receptor Substrate (IRS)-like proteins Chico and Lnk. This, in turn, triggers a cascade of intracellular events mediated by conserved components of the insulin/IGF pathway (Oldham and Hafen 2003).

Different studies further revealed the indispensable role of *Drosophila* in studying type I diabetes. Rulifson's group, at the beginning of the twentieth century, in

their studies, reported that ablation of IPC results in flies that displayed the type 1 diabetes-associated phenotype. The experimental flies showed an elevated circulating sugar compared to that of wild-type controls. Further, their studies also revealed that an increase in sugar levels after IPC ablation was rescued by expression of *Drosophila* insulin-like peptide (DILP) (Rulifson et al. 2002). This study further postulated that insulin-producing cells can be equivalent to the β-cells of pancreatic islets that produce insulin in mammals.

Haselton and group in 2010 tried to modulate *Drosophila* feeding habit and clumped it with IPC ablation. In a classic set of experiment, the adult feeding behavior was manipulated using Oral Glucose Tolerance Test (OGTT), a test used to diagnose human diabetes. This test follows a series of steps where flies were starved initially and then fed on glucose solution. The circulating sugar levels were measured over time. Wild-type flies mimicked mammalian response, where a low circulating sugar level was recorded upon starvation. The flies upon glucose feeding showed an initial rise in sugar levels, which gradually declines with time.

Ablation of IPCs contrastingly showed higher circulating sugar levels and slower clearance. This response was abrogated by artificial supply of bovine insulin (Haselton et al. 2010). This study further supports *Drosophila* as a type I diabetes study model.

Apart from type I diabetes, *Drosophila* has been implicated in type II diabetes studies as well. It was shown in a report that larvae reared on a high-sugar diet on hatching into flies showed a higher level of circulating sugar (Musselman et al. 2013). This finding was consistent with the earlier reports in honeybees and other insects (Lee and Park 2004). A report from Musselman and group reported that flies reared on a high-sugar diet showed higher expression of *dlip* transcripts. Despite higher circulating dlip levels, circulating sugar levels remained high, which resembles with the mammalian insulin resistance. A decreased level of phospho-Akt was also observed in response to exogenous insulin administration in flies reared on HSD, suggesting a weakened ability to respond to insulin signaling after chronic levels of high sugar in the diet (Musselman et al. 2013).

These many of the few examples, pictures *Drosophila* as a unique poised model to study the insulin pathway and chronic aspects of diabetes. A very well-developed genetic toolkit, higher genetic background homogeneity, a very highly polished sequenced genome and the simplified insulin cascade showing minimum redundancy are few of the favorable traits exhibited by flies in their support to be modeled for diabetes-related studies.

Drosophila as a Model for Cancer

Organisms with short life span, as is *Drosophila*, generally do not develop cancer. The number of cell divisions these organisms undergo in their whole lifespan is much lower than those of humans. Despite these limitations, *Drosophila* exhibits all the classic hallmarks of cancer such as evasion of apoptosis, sustained proliferation, metastasis, prolonged survival, genome instability, and metabolic reprogramming on perturbation of cancer-associated genes (Hanahan and Weinberg 2011; Perrimon et al. 2012). The GAL4/UAS system, the FLP/FRT recombinase system, the

availability of RNAi transgenic animals and the CISPR/Cas system all these powerful tools make *Drosophila* a powerful organism for tumorigenesis study. In addition, the majority of human cancer-causing genes have orthologs in *Drosophila* (Adams et al. 2000), and in some cases, the conservation is to the extent that the corresponding human genes can rescue the loss of function of their *D. melanogaster* orthologs. In addition, some of the most highly implicated pathways in human tumorigenesis were first identified in the flies, prior to its link to cancer in humans.

For instance, *Notch* was identified in the first half of the twentieth century as a gene, which when gets mutated results in a mutant fly with notched wings. The genetic and molecular studies in flies further revealed the evolutionarily conserved nature of the gene and the cascade. Decades after its identification in flies, the aberrant expression of human NOTCH1 was found to be a causative factor for T cell acute lymphoblastic leukemia (Ellisen et al. 1991). Notch signaling aberration has further been implicated in many of the hematopoietic and solid tumors (Pancewicz and Nicot 2011; Ranganathan et al. 2011). Like Notch, the segment polarity gene *hedgehog* (*hh*) finds its roots in flies. Mutations that disrupt the HH signaling are directly implicated in basal cell carcinoma and medulloblastoma (Barakat et al. 2010). The same applies to the Salvador–Warts–Hippo pathway that has been extensively studied in *D. melanogaster* and that is also involved in human tumorigenesis (Staley and Irvine 2012). In addition, the JAK/STAT pathway was observed to cause overgrowth in fly hemocytes prior to the discovery of its role in human leukemia (Harrison et al. 1995). The phenomenon of cell competition discovered in *Drosophila* showed that imaginal disc cells with higher fitness survive and proliferate at the expense of neighboring cells with lower fitness (Morata and Ripoll 1975). The same cell competition phenomenon operates in between wild-type and cancerous cells during tumor growth (Baker 2011), and *Drosophila* continues to uncover the mechanism underlying the process. These are few of the many examples that vividly portray the close association of *Drosophila* to human malignancy.

A recent advance in *Drosophila* techniques has enabled researchers to recreate human cancer in flies with the combination of loss- and gain-of-function conditions that are causative of certain human cancer types. The initial attempts made in this direction successfully created tumorigenesis models in flies using the expression on oncogenic version of *Ras (RasV12)* together with the mutants that disrupt cell polarity such as *scrib* or *discs large* (*dlg*). These mutations were able to create invasive tumors in the imaginal discs of fly larvae (Brumby and Richardson 2003; Pagliarini and Xu 2003; Wu et al. 2010).

In human prostate cancer, SCRIB expression is found to be downregulated, and the mouse model shows neoplastic growth in the absence of *scrib* (Elsum et al. 2012). Tumors in the *Drosophila* model can also be generated by activating the RAS pathway in synergism with mitochondrial dysfunction; this, in turn, triggers the production of reactive oxygen species (ROS), thus activating Jun N-terminal kinase (JNK) signaling (Ohsawa et al. 2012), a key pathway that regulates proliferation, metastasis and cell death. By blocking the apoptosis induced by stress (X-rays, heat shock, etc.) using a caspase inhibitor (p35), the cooperative behavior of this

hyperplastic tumor can be further aggravated in wing imaginal discs (Pérez-Garijo et al. 2009).

Another example where *Drosophil*a has been modeled and extensively used in cancer studies comes from glioblastoma. The most common and most malignant human brain tumor is widely studied in flies by manipulating the pathways that are known to be affected in human glioblastoma. The constitutive co-activation of the epidermal growth factor receptor (EGFR)–RAS and the PI3K signaling pathway is the hallmark of human brain tumors, and their constant activation in larval glial cells mimics the proliferative neoplastic growths (Read et al. 2009).

Rhabdomyosarcoma, the most common form of soft tissue sarcomas in humans, has also been successfully modeled and studied in flies. Human rhabdomyosarcomas show the expression of fused transcription factors paired box 3 (PAX3)–forkhead box O1 (FOXO1) or PAX7–FOXO1, which, when expressed in flies, results in cells that detach from myofibrils and invade nonmuscular tissue compartments, thereby imitating the human disease (Wang et al. 2008).

A screen to identify the suppressors of this phenotype revealed *rolling pebbles* (*rols)* as a downstream effector of PAX7–FOXO1 (Avirneni-Vadlamudi et al. 2012). These studies further paved the way to identify the role of TANC1 (mammalian ortholog of *rols*) in rhabdomyosarcoma (Avirneni-Vadlamudi et al. 2012). These are few of the many examples where the fly model pictures its indispensable role in cancer research.

Drosophila as a Model to Study Cardiovascular Diseases

Initial attempts to utilize the fly model in studies of cardiovascular diseases were made around 20 years ago with the advent of techniques to study heart development and function (Ocorr et al. 2014). *Drosophila* has an open circulatory system with a simple heart comprising a hollow muscular tube closed at the posterior end and the vessels run from the posterior abdomen into the thorax. Though the fly heart differs from the human heart in a majority of aspects, they show morphological similarities as well. The fly heart, similar to that of humans, is divided into distinct chambers. The fly heart contains four chambers separated by small valve-like openings through which the hemolymph (analogous to blood) enters the heart (Lehmacher et al. 2012). The simplicity of *Drosophila* circulatory system aids the researchers with a major advantage. Since heart function is not tightly coupled with survival in *Drosophila*; the researchers can examine the severe effect of genetic manipulations in flies than in the vertebrates.

Further, the molecular pathways underlying the development of the *Drosophila* heart show striking similarity with their human counterpart. *Tinman*, a homeobox transcription factor identified in flies, was reported to be crucial for heart development (Bodmer 1993). Mutations in the human homolog of this gene, *Nkx2–5*, was later shown to be associated with congenital heart disease and cardiac arrest (Schott et al. 1998). Discovery of transcription factors like *pannier (GATA4)* and *neuromancer* (Tbx20) uncovers a well-conserved cardiogenic network. This is of great importance in studying factors important in human heart development and function (Qian and Bodmer 2012).

The fly heart thus proves to be a convenient disease model owing to conserved molecular pathways and the variety of assays to study different aspects of heart disease.

Drosophila in Neurodegeneration

For nearly a couple of decades, the fruit fly has been utilized as a model organism to study a number of human neurodegenerative and neuromuscular diseases (McGurk et al. 2015). Apart from the conserved genetic circuitry, the fly brain is estimated to have 300,000 neurons, and like mammals, it is organized into areas with separated specialized functions (Rubin et al. 2000). This composite nervous system of *Drosophila* also displays complex behaviors such as learning and memory, making it an attractive system for the study of neuronal dysfunction and memory loss.

Multiple neurodegenerative diseases ranging from dominant polyglutamine-repeat diseases, tauopathies, Parkinson's disease (PD), Alzheimer's disease (AD), and triplet-repeat expansion diseases in noncoding DNA like SCA8 has been modeled and studied in *Drosophila* (Bilen and Bonini 2005). Of the many neurodegenerative diseases reported, Alzheimer's disease is the most common and accounts for almost 60–70% cases of dementia (Burns and Iliffe 2009). In humans, it is characterized by the presence of extracellular amyloid plaques and intracellular neurofibrillary tangles accompanied by neuronal loss. Neurofibrillary tangles are composed of aggregated, hyper-phosphorylated forms of the microtubule-associated protein TAU (Hashimoto et al. 2003). To create a *Drosophila* model for Alzheimer's study, wild-type and mutant forms of human TAU in fruit flies were expressed. This fly model in turn mimics several features of the human disease like progressive neurodegeneration, age-dependent neuronal loss, premature death, and neuronal accumulation of abnormally phosphorylated forms of TAU (Wittmann et al. 2001). Further, in reports, hyperphosphorylation of tau by *shaggy*, the *Drosophila* GSK3β homolog aggravates neurodegeneration. This, in consistency, ameliorates the development of the tau phenotype when the GSK3β was inhibited, thereby establishing a novel therapeutic strategy for Alzheimer's (Mudher et al. 2004).

The other distinctive neuropathological feature of AD is the formation of neuritic plaques composed primarily of the Aβ peptide. Aβ peptides are produced by proteolytic cleavage of the Amyloid Precursor Protein (APP) transmembrane receptor at the β and γ sites. In normal physiological condition, γ secretase cleaves APP in a heterogeneous fashion resulting in a major proportion of Aβ40 and a small proportion of Aβ42. In reported cases of familial AD mutations in APP, γ secretase shows pathogenically high levels of the Aβ42 peptide, revealing the primary culprit in AD pathogenesis (Nussbaum and Ellis 2003). Since the Aβ domain in the *Drosophila* APP-like protein (APPL) is not conserved and also the flies lack β-secretase activity (Fossgreen et al. 1998), an alternative strategy was deployed to model AD in flies. The transgenes encoding the human Aβ40 and Aβ42 peptides were delivered in flies. When specifically expressed in the brain, both Aβ40 and Aβ42 led to age-dependent learning defects, but only Aβ42 was capable of causing the formation of

diffused amyloid deposits in the fly's mushroom bodies. Also the life span of flies with the Aβ42 transgene was also severely shortened (Iijima et al. 2004). In addition to it, when the expression of the Aβ42 transgene was directed in the *Drosophila* eye, progressive eye disorganization was seen. Further, on screening, the genetic modifiers of the Aβ42-induced rough-eye phenotype in the *Drosophila, neprilysin* gene was found to suppress the Aβ42 phenotypes by lowering the levels of the peptide (Finelli et al. 2004). This study highlighted the potential of neprilysin upregulation to be used as a novel therapeutic approach to AD.

Another neurodegenerative disorder, Parkinson's disease (PD), is characterized by severe motor symptoms, including uncontrollable tremor, imbalance, slowness of movement and rigidity. Neuropathological hallmarks of this condition show progressive degeneration of dopamine neurons in the substantia nigra and the presence of cytoplasmic neuronal inclusions, the Lewy bodies (Nussbaum and Ellis 2003). Missense mutation in the α-synuclein gene has been associated with familial and sporadic cases of PD, indicating that accumulation of Lewy bodies might play a central role in the pathogenesis of both familial and sporadic forms of Parkinson (Krüger et al. 1998). Like the Alzheimer model, the *Drosophila* model of PD has been produced by expressing wild-type and mutant forms of human α-synuclein in flies. Human α-synuclein in flies recapitulates the neuropathological features of PD, showing progressive degeneration of dopaminergic neurons along with the accumulation of α-synuclein aggregates (Feany and Bender 2000). Further, α-synuclein modifiers were screened in the fly model and human molecular chaperone Hsp70 (Heat shock protein 70) was found to prevent dopaminergic neuronal loss. Synergistically, an interference with the endogenous chaperone protein aggravates the disease phenotype (Auluck et al. 2002).

The expansion of CAG repeats within the open reading frame (ORF) of the disease-causing gene has been implicated in a variety of human neurogenerative diseases such as Huntington's disease, spinobulbar muscular atrophy, spinocerebellar ataxia (SCAs), collectively known as polyQ diseases. Glutamines (translated by expanded repeats) cause dominant toxicity leading to late onset of neurodegeneration. Expanded polyQ chains, when expressed in *Drosophila* neurons, produce cytotoxic aggregates, followed by neuronal degeneration (Marsh et al. 2000). These transgenic flies uncovered a variety of genetic modifiers including Hsp40/HDJ1, tetratricopeptide repeat protein 2 and human myeloid leukemia factor as a suppressor for polyQ-mediated neurodegeneration in *Drosophila* eye (Kazemi-Esfarjani and Benzer 2000).

These are few of the many examples where *Drosophila* models for a range of human neurodegenerative diseases. In addition to these few diseases discussed, there are many more diseases listed in Table 2, where *Drosophila* serves as a model to study human diseases. In this book, the comprehensive genetic analysis of pathways that mediate neuronal degeneration, the mechanisms involved in pathogenicity, and the role of *Drosophila* in pathological amelioration of various neurodegenerative diseases has been discussed in detail.

Table 2 *Drosophila* as a human disease model

S. no.	Disease category	Disease	Reference
1.	Neurodegenerative disease	(i) Adrenoleukodystrophy	Sivachenko et al. (2016)
		(ii) Alzheimer's disease	Fernandez-Funez et al. (2015)
			Helmfors (2015)
		(iii) Amyotrophic lateral sclerosis	Romano et al. (2015)
			Machamer et al. (2014)
		(iv) Angelman's syndrome	Valdez et al. (2015)
			Lee et al. (2014)
		(v) Ataxia telangiectasia	Rimkus and Wassarman (2018)
		(vi) Charcot–Marie–tooth disease	Bharadwaj et al. (2016)
			El Fissi et al. (2018)
		(vii) Fragile X syndrome	Oh et al. (2015)
			Greenblatt and Spradling (2018)
		(viii) Friedrich's ataxia	Chen et al. (2016)
		(ix) Huntington's disease	Babcock and Ganetzky (2015)
			El-Daher et al. (2015)
		(x) Parkinson's disease	Suzuki et al. (2015)
			Wang et al. (2011)
		(xi) PolyQ disorder	Yadav and Tapadia (2016)
			Chen et al. (2019)
2.	Metabolic disorders	(i) Barth syndrome	Xu et al. (2015)
			Malhotra et al. (2009)
		(ii) Diabetes	Barry and Thummel (2016)
			Park et al. (2014)
		(iii) Galactosemia	Jumbo-Lucioni et al. (2017)
3.	Cardiac disease	(i) Cardiomyopathy	Bogatan et al. (2015)
			Walls et al. (2018)
4.	Cancer	(i) Colorectal cancer	Bangi et al. (2016)
		(ii) Squamous cell carcinoma	Fu et al. (2016)
		(iii) Rhabdomyosarcoma	Galindo et al. (2015)
5.	Miscellaneous	(i) Retinitis pigmentosa	Chow et al. (2016)
		(ii) Mitochondrial disease	Fogle et al. (2016)
			Foriel et al. (2018)
		(iii) Nephrotic syndrome	Hermle et al. (2017)

Source: http://www.sdbonline.org/sites/fly/modelsystem/aamodelsystem.htm

Drosophila in Drug Discovery

In vitro approaches such as cell culture and biochemical assays show contrasting effects of drug administration in comparison to in vivo studies. In the course of identifying treatment for Huntington's disease, researchers came across this

disparity. A multiple lead compounds, including benzathiazole was identified to inhibit polyglutamine-mediated aggregation of toxic and misfolded proteins, the primary cause of HD (Heiser et al. 2002). Riluzole, a closely related compound to benzothiazole, with previously reported therapeutic benefit in patients with amyotrophic lateral sclerosis (Lacomblez et al. 1996) was hence selected for HD treatment. Interestingly, these primary hits were all found to be toxic to cells in the culture model of aggregation as well as animal model of HD, and none had any therapeutic value (Hockly et al. 2006). To overcome these in vitro screening barriers, drug testing on whole animals with all relevant organ systems is preferred. Traditional animal models such as mice are a good choice but fail on a primary screening platform where hundreds or thousands of drug efficacies need to be tested.

The fruit fly is hence a valid alternative in the drug discovery process. The numerous advantages that *Drosophila* offer include low maintenance and screening cost as well as rapid result analyses. Other advantages that *Drosophila* offers have been discussed in detail in the earlier section of this chapter. One of the key advantages the fly model in drug discovery provides is that *Drosophila* offers multiple routes for drug administration. For embryos, drug administration can be done via permeabilization; larvae can be fed on solid food mixed with desired drugs. Adult flies offer even more routes for drug administration. Drugs can be administered via injection, through food or sucrose/drug-saturated filter paper. Drug can also be injected directly on exposed nerve cord of decapitated flies or injected into the abdomen as per the nature of drug and demand of experiment (Pandey and Nichols 2011).

Earlier reports elucidate that *Drosophila* have been successfully used in primary as well as secondary screening of a variety of drugs for the therapeutic discovery of a wide range of human diseases. Many forms of cancer, as already discussed in the *Drosophila* disease model section, have been developed in flies with the aid of specific genetic manipulations. The oncogenic isoform of Ras1 expression alone or in combination with PTEN RNAi mimics cancer like overgrowth in the fly tracheal system. Ras1, PTENi mutant, flies die as larvae, and this lethal phenotype was deployed to screen 1192 FDA-approved drugs. Two hits from this screen, trametinib and fluvastatin, were able to synergistically rescue the lethality and suppressed tumor formation. This was consistent with the data in human A549 adenocarcinoma cells (Levine and Cagan 2016).

Likewise, the expression of human Raf oncogene generates intestinal tumors in *Drosophila*. A large screen of 6100 compounds and 88 FDA-approved drugs identified 14 approved chemotherapy drugs as strong inhibitors of tumor growth. Furthermore, 10 uncharacterized small molecules were also able to inhibit tumor growth. Some of these drugs paradoxically induce proliferation of intestinal stem cells (ISC) by activating the conserved JAK–STAT pathway. Thus, tumor recurrence is possibly induced by these chemotherapeutic agents by inducing stem cell proliferation. Key findings from this study suggest that recurrence of tumor might be reduced by a combination of certain chemotherapeutics with anti-inflammatory drugs that inhibit the JAK–STAT pathway (Markstein et al. 2014).

In addition, the most remarkable drug screening tools *Drosophila* offers is the development of personalized fly avatars. *Drosophila* avatars cost far less than the mice avatar, which requires a lot more expense and maintenance. Cagan and group came forward with this fly avatar where they have developed a method for creating patient-specific thyroid and colorectal tumors in flies (Rozehnal et al. 2016). In this version of personalized medicine approach, identification of gene variants for prediction of tumor was made possible with the analysis of gene sequence data from a patient's tumor. Fly avatars were then created by introducing several gene variants in fly gut or eye. These transgenic flies were further utilized for screening against either single or a combination of 1200 FDA-approved drugs. Drugs or combinations with the highest efficacy and lowest toxicity were then used for clinical trials (Strange 2016).

Drosophila in Stem Cell Research

Drosophila stem cells (SC) have striking resemblance to mammalian stem cells; this highlighted the role of flies in SC research. The extraordinary property of stem cells to differentiate into various cell types in addition to their own self-renewal intrigued researchers to explore further, and *Drosophila* model aids indispensably. Prior to stepping into stem cells and *Drosophila*, the discussion of the stem cell niche is mandatory. The concept of the niche was originally proposed by Schofield in 1978 (Schofield 1978). Niche plays a crucial role in understanding the key concepts of stem cell self-renewal (Nystul and Spradling 2006).

A stem cell niche can be defined as the specific location where stem cells can reside for an indefinite period of time and produce progeny cells while self-renewing (Ohlstein et al. 2004). A region that is stably maintained is of utmost importance for stem cells to have their renewal property. The cells forced to leave this "specific location" or "niche" losses the factors needed for self-renewal and ultimately differentiate. Thus, stem cell niche provides the adequate microenvironment for stem cells to not only differentiate into different kinds of cells but also self-renew and maintain their own population. Regenerative therapies hugely rely on this property of stem cells; hence, studying the mechanisms that govern stem cell differentiation is very important to advance our knowledge base for stem cell-based therapy development.

Fruit fly harbors a range of stem cell populations, including germline stem cells (GSC) in testes and ovary, somatic stem cells (SSC) in ovary, mid gut and hind gut, stem cells in developing larval brain (neuroblast), hematopoietic precursor cells and renal and nephric stem cells (RNSCs) in Malpighian tubules (Micchelli and Perrimon 2006; Ohlstein and Spradling 2006; Yu et al. 2006; Fuller and Spradling 2007; Kirilly and Xie 2007; Mandal et al. 2007; Singh et al. 2007; Pearson et al. 2009). Each one of these has been studied extensively and provided key insights into mechanisms that regulate differentiation and self-renewal. Development of genetic tools for lineage tracing and functional analyses has helped enormously to understand the similarities and differences in stem cell populations across tissues in *Drosophila* and mammalian cells. Here, we will describe a few of these

tissue-specific stem cell and niche interactions and their roles in development in the fly model.

(a) Stem Cells in *Drosophila* Gonads:

Both *Drosophila* testes and ovary contain two distinct populations of stem cells called "germline stem cells" and "somatic stem cells". In testes, a non-proliferative population of somatic cells known as "Hub cells" (HC) decorate the stem cell niche, whereas in ovary, the stem cell niche includes three distinct types of somatic cell populations, namely, terminal filament cells (TFC), cap cells (CS), and escort cells (EC) (La Marca and Somers 2014). While there are differences in mechanisms by which stem cells and their niche interact, there is a general mechanism that relies on adhesive interactions and asymmetric signaling (Losick et al. 2011). In *Drosophila* ovary, cap cells hold the germline stem cells via adhesive interactions while adhesion between hub cells and germline stem cell in testes aids in proper asymmetric cell division. These asymmetric divisions ensure that one of the daughter cells remains in niche and the other exits and differentiates (Hardy et al. 1979; Wieschaus and Szabad 1979; Yamashita et al. 2003; Sheng and Matunis 2011). Somatic stem cells in the ovary, however, do not depend on asymmetric signaling, and their differentiation depends on a precise spatiotemporal regulation of several signaling pathways such as Notch, Wingless, Hedgehog and JAK-STAT (Kirilly and Xie 2007; Nystul and Spradling 2007; Dai et al. 2017).

(b) Stem Cells in *Drosophila* Gut:

Midgut of adult *Drosophila* is analogous to the mammalian small intestine and the hindgut is equivalent to the large intestine. Over the last decade, several studies have substantiated the presence of stem cells in *Drosophila* midgut (Micchelli and Perrimon 2006; Ohlstein and Spradling 2007; Sahai-Hernandez et al. 2012). This discovery of intestinal stem cells places *Drosophila* as a very powerful in vivo model to study the components of epithelial stem cells during infection, stress, or aging. ISC division results in self-renewal of ISC and a daughter cell named "enteroblast" (EB). This is brought about by asymmetric Notch signaling. Transcriptional repression of Notch maintains the ISC fate, and activated Notch promotes EB daughter fate. EB can further differentiate into two types of cells, enteroendocrine (EE) cells and enterocyte (EC) cells, depending on level of Notch signaling. A strong Notch signaling promotes EC daughter fate, whereas a weak Notch signal results in EE daughter (Micchelli and Perrimon 2006; Ohlstein and Spradling 2007; Bardin et al. 2010; Lucchetta and Ohlstein 2012).

In addition to Notch signaling, EBs also require Janus Kinase–Signal Transducer and Activator of Transcription (JAK–STAT) activity for a multicompetent lineage (Takashima et al. 2008; Jiang et al. 2009; Beebe et al. 2010). Another population of ISC has been identified in the anterior region of hindgut that also requires Wingless and Hedgehog signaling (Takashima et al. 2008). The interaction of ISC to its niche is still an active area of investigation that demands further studies.

(c) Stem Cells in *Drosophila* Brain:

Populations of neural stem cells called "neuroblasts" (NB) have been identified during embryonic and larval brain development. Embryonic neuroblasts form most of the larval central nervous system (CNS) (Prokop and Technau 1991). These NBs undergo rounds of asymmetric divisions that produce another NB and a smaller ganglion mother cell (GMC). GMCs further divide to produce distinct populations of neurons or glial cells. Several other neuroblasts, known as type II neuroblasts, divide and give rise to intermediate precursors before producing GMCs (Homem and Knoblich 2012). Unlike GSCs, several intrinsic factors such as polarity and mitotic apparatus are sufficient to guide self-renewal and differentiation events. This highlights the dispensable nature of stem cell–niche interaction and offers a plasticity that is specific to the microenvironment (Yu et al. 2006).

(d) Hematopoietic Precursor Cells:

A few recent studies have identified stem cell population called hematopoietic precursor cells (HP) in the lymph gland, which is the source of adult blood cells (hemocytes in *Drosophila*). Like the other stem cell and niche interactions, HP cells interact with a group of cells known as posterior signaling center (PSC) and this interaction is required for their maintenance (Krzemień et al. 2007; Mandal et al. 2007). New studies using lineage analysis are now attempting to identify bona fide hematopoietic stem cells (HSC) in embryonic and larval lymph gland and their interactions with niche (Minakhina and Steward 2010; Dey et al. 2016).

Stem cell and niche interaction is an active area of investigation that is constantly using *Drosophila* to identify new stem cell populations. One such study to identify stem cell population in flight muscles of *Drosophila* is particularly interesting (Gunage et al. 2014)**.** This further highlights the untapped potential of *Drosophila* model for stem cell research. The examples discussed above highlight the role of *Drosophila* in studying the basic stem cell biology and mechanisms governing their renewal and differentiation. While this expands our knowledge base to a great deal, the attempts are now being made to explore the potential of *Drosophila* in vivo stem cell models to screen for potential chemotherapeutic drugs that inhibit *Drosophila* tumor (Markstein et al. 2014). One of the advantages of such screenings is that it utilizes in vivo tissue environment and will provide greater insights into interactions of tumor stem cells with their microenvironment.

Limitations of Using *Drosophila* as a Model Organism

Undoubtedly, *Drosophila* provides an unbiased approach to gain insights into human biology and diseases associated with it. The mutations in fly, though mimic many of the human diseases, are not their precise representatives. One of the reasons behind this lies in the fact that most of the classical fly mutants from forward genetic screens are typically loss-of-function alleles. Human development and disease manifestations are more complex and require a much more complex system to understand it precisely. Human disease mutations have complex presentations,

including both loss-of-function of the wild-type allele and gain-of-function of the mutant allele. This gain of function can still be modeled in *Drosophila* using the GAL4/UAS system. There are certain diseases where loss-of-function of a wild-type protein has a major role. The unavailability of *Drosophila* orthologs of such corresponding human disease-associated genes limits the use of a fly model for studying such diseases.

Irrespective of the versatility of the GAL4/UAS system, the extent of overexpression of a particular gene is questionable. The magnitude of overexpression can hugely differ from the exact clinical situation (Floresco et al. 2005). In cases, overexpression of a wild-type gene can have a disease phenotype (Prelich 2012). Excess GAL4 protein, on the other hand, can have their own phenotypes that can create confusions at instances. *GMR–GAL4* itself has an eye-roughening phenotype primarily associated with ommatidial degeneration and apoptosis. Hence, a proper control is mandatory to exclude the phenotype disparity for the *Drosophila* researchers.

Lastly, it is a concern whether the fly model can faithfully recapitulate human biology. Although the majority of signaling cascades that operate in *Drosophila* show a close homology to humans, their exact mimicry cannot be done, how precise the model system may be. The manifestation of a particular phenotype in flies can be a cause of multiple reasons, and at times, specificity may lack to a greater extent. For instance, the *Drosophila* photoreceptor degeneration provides a convenient readout; it mostly reflects generic neurotoxicity instead of selective neurotoxicity that is disease specific. Thus, before coming to a particular conclusion, the researchers need to verify relevant phenotypes using other systems that are more specific for the study of particular signaling cascade/disease.

Despite few of these limitations, the fly model, so far, has been widely used and contributed enormously in understanding the etiology of human diseases and identifying targets for therapeutic interventions. The "good" and "bad" about fruit fly is briefly summarized in Table 3 of this chapter.

Table 3 *Drosophila* at a glance: the 'good' and the 'bad' about fly

The good:	
1.	Small size, short generation time, simple husbandry
2.	Shares key features with higher organisms: Segmented body plan, sensory and motor systems, sexual behavior, learning and memory ability, innate immunity
3.	Simple karyotype, giant polytene chromosomes, synthetic balancer chromosome availability
4.	High extent of homology to the human genome
5.	Availability of mutants, enhancer and protein traps, and RNAi lines
6.	Public stock centers and databases
The bad:	
1.	Absence of tissue types that are present in mammals, such as cartilage, bone, and blood
2.	Lack of an adaptive immune response
3.	Open circulatory system. The absence of veins and arteries precludes the modeling of some important processes

Gonzalez (2013)

Drosophila Resources

There are several online resources available for the fly geneticist to obtain crucial information about different *Drosophila* strains, molecular reagent availability, and data on genomes, genes, proteins and molecular interactions. The most useful and extensive resource for the fly community is the FlyBase http://flybase.org (St. Pierre et al. 2013).

FlyBase is an eminent resource that in addition to the gene information also provides links to other stock centers containing relevant *Drosophila* information. It is a "one-stop-shop" for all the data and information a researcher needs regarding *Drosophila*. It provides a very user-friendly interface with access to genome data and annotations from multiple *Drosophila* species. FlyBase also aids researchers to search batches of genes based on expression pattern or other specific criteria. A gene entry provides all the information regarding gene structure, genomic neighborhood, protein sequence, homologs, known alleles, and phenotypes. In addition, FlyBase also serves as a major source of references to information cited in the literature.

The other important *Drosophila*-related websites that provide data regarding fly genetics are as follows:

- Berkeley *Drosophila* Genome Project (BDGP) http://www.fruitfly.org.
- This utilizes the genome data and annotations available via FlyBase to refine and update it.
- *Drosophila* Interaction Database (CuraGen) http://www.droidb.org/DBdescription.jsp.
- *Drosophila* Interaction Database (DroID) provides easy access to gene and protein interaction data available across platforms into one location.
- *Drosophila* Polymorphism Database http://dpdb.uab.es/DPDB/dpdb.asp.
- This database provides access to a collection of all the existing polymorphic sequences available in the *Drosophila* genus.
- Drosophila melanogaster Exon Database http://proline.bic.nus.edu.sg/dedb.
- Database that contains information on D. melanogaster exons presented in a splicing graph form
- *Drosophila* Population Genome Project http://www.dpgp.org.
- Dataset enriched in population-level data on transcriptomes for studying gene regulatory evolution and de novo genes.
- Interactive Fly http://www.sdbonline.org/fly/aimain/1aahome.htm.
- Guide to information regarding aspects of fly development with links to other important resources.
- *Drosophila* Genomics Resource Center http://dgrc.cgb.indiana.edu.
- A resource center that provides cellular and molecular reagents, in particular, cDNA clones, vectors and cell lines.
- Flybrain http://flybrain.neurobio.arizona.edu.

- Flybrain is an online atlas and database of the *Drosophila* nervous system that provides specific information concerning different anatomical structures, developmental stages and visualization techniques of fly brain.
- Virtual Fly brain http://www.virtualflybrain.org/site/vfb_site/home.htm
- This is a hub for *Drosophila melanogaster* neural anatomy and imaging data.
- FlyMove http://flymove.uni-muenster.de.
- FlyMove is an internet resource to study the development of the fruit fly with the aid of images and movies.
- Fly Atlas http://flyatlas.gla.ac.uk/FlyAtlas2/index.html
- Based on microarray data, Fly Atlas catalogs gene expression at the level of mRNA enrichment across multiple tissues for genes.
- The WWW Virtual Library–*Drosophila* http://www.ceolas.org/fly.
- The directory points to various internet resources for research on the fruit fly

In addition to these informative resources, *Drosophila* public stock centers serve as an indispensable resource for obtaining a variety of lines. Amongst these, https://bdsc.indiana.edu/ the Bloomington *Drosophila* Stock Centre at Indiana University, is the largest and most widely used stock center by fly researchers. Other commonly used stock centers are as follows:

- Kyoto *Drosophila* Genetic Resource Centre https://kyotofly.kit.jp/cgibin/stocks/index.cgi
- Vienna *Drosophila* resource Centre https://stockcenter.vdrc.at/
- National Institute of Genetics-FLY https://shigen.nig.ac.jp/fly/nigfly/
- The Exelixis Collection at the Harvard Medical School https://drosophila.med.harvard.edu/
- Gene Disruption Project Database http://flypush.imgen.bcm.tmc.edu/pscreen/index.php

Lessons from the Past and Future Directions

Ever since Morgan identified the *white* gene in *Drosophila*, most of the early twentieth-century studies in *Drosophila* focused in uncovering the genetics and development of the fly. *Drosophila* continues to be a very powerful system to identify mutations, carry out screenings and uncover the biology of uncharacterized genes and proteins. However, over the last decade, with the advent of interdisciplinary approaches like quantitative live imaging, computational modeling, robotics and artificial intelligence, our understanding of the biology has improved like never before. *Drosophila* has become a very instrumental model for scientists to harness the power of interdisciplinary techniques and ask the questions that are otherwise very difficult to address in other systems. In fact, now scientists are revisiting our current understanding of several pathways and developmental processes known for several decades, using mathematical modeling to gain a wealth of new information (Kulasiri and Xie 2008; Jaeger 2009; Ziraldo and Ma 2015; Lazopulo and Syed

2016; Liu et al. 2016; de Andres-Bragado et al. 2018). An interesting study using the live imaging approaches in *Drosophila* ovary provided insights into mechanisms of collective cooperative cell migration (Prasad et al. 2007). This led to the identification of another very interesting biological phenomenon of global tissue-scale revolutions during egg chamber elongation (Haigo and Bilder 2011). Integrating systems biology to our existing knowledge of development is the way forward to understand the complex tissue behaviors. Several attempts are being made to create platforms to accommodate such modeling studies and create database for open access. A smart computer program, Janelia Automatic Animal Behavior Annotator, JABA (http://jaaba.sourceforge.net/), has helped in creating a brain-wide atlas of fruit fly behavior. Not surprisingly, NASA has established a fruit fly lab (https://www.nasa.gov/ames/research/space-biosciences/drosophila-containers-and-platforms) to explore the power of this humble organism in helping to understand the complex biological behaviors in extraterrestrial territories.

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Methods for Creating Fly Models to Understand the Molecular Mechanisms Underlying Neurological Diseases

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Abstract

With global incidences of neurological disorders surpassing the one billion mark, the study of these disorders and the development of suitable therapeutic remedies have become increasingly important. Such studies are contingent upon the availability of suitable model systems that recapitulate all the major hallmarks of these disorders as seen in humans. As significant homology exists between humans and *Drosophila melanogaster*, flies have proved to be one of the most suitable model organisms for the study of neurological disorders and their underlying molecular mechanisms. Additionally, the availability of a vast array of genetic tools renders *Drosophila* a very versatile model system. Here we discuss some of the most widely used techniques for the development of *Drosophila* models for neurological disorders and to assess the function of fly homologues of disease-causing genes.

Keywords

Drosophila · UAS/GAL4 · Protein tagging · Gene editing · MiMIC · CRISPR

Introduction

Neurological diseases are those that affect the central and peripheral nervous systems. To date, more than 600 neurological disorders have been reported including microcephaly, epilepsy, behavioural disorders like autism, infections like meningitis, brain and spinal cord trauma, gliomas and degenerative diseases that lead to

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demise of neurons as in Alzheimer's and Parkinson's diseases. Therapeutic interventions for most of these disorders are symptomatic treatments invlolving lifestyle changes or drugs that reduce the severity of the disease. Targeted treatment for non-infectious and non-cancerous neurological disorders remain elusive due to a lack of understanding of the underlying mechanisms and disease progression. Recently, progress has been made in understanding the biological mechanisms underlying neurodevelopmental and neurodegenerative diseases through the use of model organisms such as *Drosophila*. The central and peripheral nervous systems of *Drosophila* are well characterised, rendering them an easy-to-use genetic model organism for the study of genes involved in development and disease. The use of *Drosophila* as a model system affords several advantages: First, many of the basic biological pathways and their molecular players are conserved between flies and humans. Nearly 75% of human disease-causing genes have a functional orthologue in flies which allows study of human disease-associated genes and underlying pathogenic mechanisms in flies (Adams et al. 2000; Rubin et al. 2000; Yamamoto et al. 2014). Second, flies have a shorter life cycle that permits expeditious study of genes, which would take a significantly longer time in vertebrate models. Third, the fly genome is very amenable to manipulation, rendering the generation of desired mutations in the gene(s) of interest simple and straightforward. In addition, large collections of fly lines exist including loss of function alleles, RNAi lines, protein overexpression lines and tools for genetic manipulation available at fly stock centres (Bloomington Drosophila Stock Center, Vienna Drosophila Resource Center and Kyoto Drosophila Genomics and Genetic Resources Center) that are easily obtained. Apart from this, due to the availability of a variety of genome engineering tools, fly avatars that carry specific disease-associated mutants can be generated at will to support detailed mechanistic studies of rare neurological disorders.

Despite the anatomical differences between the fly and human brains, most of the cellular and molecular mechanisms underlying brain development including self-renewal of neuronal stem cells and cell fate decisions are conserved from flies to mammals (Homem and Knoblich 2012; Homem et al. 2015). Fly neurodevelopment and physiology are also very similar to those seen in higher organisms. For example, processes like axon guidance, circuit formation, synaptic plasticity, and neurotransmission are mediated by the same or a similar set of receptors/ligands, synaptic proteins and neurotransmitters (Yoshihara et al. 2001). In addition, numerous assays have been developed in flies to study neuronal disorders, for example: (1) the rough eye assay has been extensively used to study tauopathy and Huntington's disease (Jackson et al. 1998, 2002); (2) electroretinogram (Jaiswal et al. 2015) and pseudopupil assay are used to study progressive photoreceptor degeneration (Steffan et al. 2001); (3) giant fibre system (GFS) recordings and neuromuscular electrophysiology to elucidate the pathogenic mechanism underlying amyotrophic lateral sclerosis (ALS) and spinal muscular atrophy diseases (Pennetta et al. 2002; West et al. 2015); (4) adult climbing and flight assay for Parkinson's disease (Feany and Bender 2000; Greene et al. 2003); (5) learning and memory assays and adult brain histology for Alzheimer's disease (Chakraborty et al. 2011; Finelli et al. 2004).

Two major strategies have been used to model human diseases in flies: the forward genetic approach and the reverse genetic approach. The forward genetic approach is unbiased and involves isolation of random mutations based on the phenotype of interest, induced using strategies like chemical mutagenesis and transposon-mediated mutagenesis. On the other hand, the reverse genetic approach begins with the identification of a fly orthologue of the gene of interest. Once a suitable gene has been identified, desirable mutations can be introduced, and the ensuing pathology is studied. The reverse genetic approach is suitable when genes involved in the pathogenesis of the disease are known and a targeted approach is warranted. Several databases exist that can be utilized for the identification of fly homologues such as FlyBase (http://flybase.org/), MARVEL (http://marrvel.org/) and Gene2Function (http://www.gene2function.org). In flies, there are four major ways to supress/obliterate gene function: gene knockdown by RNA interference/RNAi (Mohr and Perrimon 2012), protein knockdown by deGradFP (Caussinus et al. 2011), Clustered Regularly Interspaced Short Palindromic Repeats/Cas9 (CRISPR/Cas9)-mediated targeted gene disruption by introducing point mutations (Şahin et al. 2017) or insertion of a gene trap cassette as in CRIMIC (Lee et al. 2018). In this chapter, we shall describe the techniques that are used to model human neurological diseases in flies and gain insight into the pathogenic mechanisms underlying these diseases.

Chemical Mutagenesis

Several chemical agents are commonly used for mutagenesis, such as hexamethylphosphoramide (Nairz et al. 2004), N-ethyl-N-nitrosourea (Ashburner 1989) and ethyl methanesulfonate (EMS) (Yamamoto et al. 2014). EMS is the most commonly used agent to mutagenize flies. It is an alkylating agent that produces random GC to AT substitution throughout the genome (Blumenstiel et al. 2009; Bökel 2008). EMS is easy to administer and induces a high number of mutations at low concentrations in the range 7.5–10 mM (Yamamoto et al. 2014). EMS-induced forward genetic screens are generally designed in four steps: First, a biological phenomenon and a related phenotype are decided for which the screen is to be performed. Second, the flies are subjected to mutagenesis resulting in random mutations. Third, one or two rounds of screening are performed to isolate the desired mutants based on their phenotype. The primary screen is the most tedious part of an EMS screen involving screening of hundreds to thousands of flies. Therefore, it is essential that the phenotype assay be simple and quick; this will narrow down the number of flies that can then be further subjected to a more detailed secondary screen. After the secondary screen, in the fourth step, the mutations are mapped to genes with standard complementation assays using large deletions and whole-genome sequencing (Yamamoto et al. 2014), and the molecular mechanisms underlying the processes are determined. Numerous EMS screens have been performed in flies utilising various phenotypic assays, which have led to the discovery of several genes involved in neuronal development and function (Hotta and Benzer 1972; Min and Benzer 1997;

Yamamoto et al. 2014). An in-depth description of this topic can be found in the following chapter.

Transposable Element-Mediated Mutagenesis

Transposable elements (TE) are mobile DNA sequences that move from one location to another within the genome, which often results in gene disruption. In flies, the most commonly used TEs for genetic manipulations are *P-element* and *piggyBac*. Both share certain common features such as two terminal inverted repeats and a coding sequence for their respective transposase, an enzyme that recognizes specific inverted repeats and catalyses translocation (Castro and Carareto 2004). However, both *P-element* and *piggyBac* show insertional bias and varied translocation behaviour: *P-elements* tend to insert close to transcription sites (Liao 2000; Spradling et al. 2011; Bellen et al. 2011). *piggyBac* shows less insertional bias towards transcription sites but preferentially inserts at TTAA sequences and excises precisely unlike *P-elements* (Witsell et al. 2009). These properties of TEs affect their efficacy as mutagens. For example, *P-elements* tend to insert near transcription sites, and often these insertions do not or only partially disrupt the genes in question. This is supported by the observation that only about 25% of the total genes in *Drosophila* are disrupted by *P-element* insertions (Bellen et al. 2011). However, *P-elements* confer certain advantages such as their imprecise excision. This helps in the generation of mutant variants of the gene of interest. *piggyBac,* on the other hand, excises precisely, making it unsuitable for the generation of new alleles. These limitations led to the exploration of other TEs such as *Minos*, a naturally occurring TE from *Drosophila hydei*.

As *Minos* has no insertion bias and excises imprecisely, it serves as a much more effective tool for genetic manipulation (Metaxakis et al. 2005).

TE-based mutagenesis is possible using two different approaches. The first approach involves the generation of TE insertions. This requires controlled translocation that can be achieved by deleting the transposase coding sequence from the TE backbone. The transposase can then be introduced in trans, either with a helper plasmid using microinjection or with a transgene by genetic crossing (Nagarkar-Jaiswal et al. 2015; Rubin and Spradling 1982; Venken et al. 2011). The second approach utilizes a pre-existing TE insertion (see below). In both approaches, TE insertions are screened for a specific phenotype, and once the insertion(s) resulting in the desired phenotype are obtained, their locations are mapped using inverse PCR and the gene is identified. A crucial step in TE-based mutagenesis is to confirm that the phenotype arises as a consequence of TE insertion. This can be achieved by one of the following strategies: First, by precise excision of the TE, which should result in a reversion of the mutant phenotype; second, complementation tests using null/deletion mutants for the gene of interest; and third, by rescuing the phenotype with a transgene carrying a genomic fragment spanning the TE insertion site. Although a TE-based approach is less labour intensive and TE insertions are easily mapped, it is less efficient as compared to EMS mutagenesis. In addition, this approach hinges

upon the selection of a suitable TE, as they show insertional bias and different translocation behaviours as described above.

P-element and *piggyBac* insertions have been extensively used for forward mutagenesis in flies for the identification of genes involved in various biological processes, for example, bristle number variation (Norga et al. 2003), olfactory behaviour (Tunstall et al. 2012), synaptic transmission (Liebl et al. 2006) and neurodegeneration (Tschäpe et al. 2002). *Minos,* on the other hand, has recently been utilized for generating genome-wide insertions using an artificially engineered TE called MiMIC (Minos Mediated Integration Cassette; Fig. 1a) (Nagarkar-Jaiswal et al. 2015; Venken et al. 2011). MiMIC carries a gene trap cassette that is flanked by two *attP* sites, which are nested next to the two terminal *Minos* inverted repeats. The cassette consists of a Splice Acceptor (SA) followed by three stop codons for all three possible reading frames, an SV40 polyA signal (pA) for transcription termination and the *yellow*$^{+}$ marker to screen for transformed flies (Venken et al. 2011). The MiMIC collection contains about 7434 insertions covering about 4367 genes that are inserted at various locations in genes including 5′ UTRs, 3′ UTRs, exons, coding introns (introns flanked by two coding exons) and intergenic regions. MiMIC insertions within coding exons and coding introns in the gene trap (GT) orientation (see below) can be used for TE-based forward mutagenesis screening. Coding intronic insertions that are not in the gene trap orientation can be used for several other applications, which are described in the following section.

MiMIC-Derived Strategies

MiMiC is a *Minos*-based TE that can be inserted randomly throughout the genome at different locations. Depending on the insertion location, they can be used for various applications. For example, 5′ UTR insertions can be used to express binary factors like GAL4, LexA or QF, and insertions in intergenic regions can be used for the introduction of FRT sites (Venken et al. 2011). However, the most useful insertions are those within coding intronic regions. MiMIC insertions can function as a gene trap (GT) when MiMIC is inserted in the same direction as that of the gene. In this situation, the pA signal within MiMIC results in precocious transcription termination, and when the resulting truncated transcript is translated, the premature stop codons effectively results in the generation of a deletion mutant. Currently, there are about 2854 MiMIC coding intronic insertions that cover 1862 genes (Nagarkar-Jaiswal et al. 2015). These can be used for three major applications: endogenous protein tagging, generation of endogenous GAL4 driver lines and conditional gene inactivation.

The MiMIC gene trap cassette is flanked by two inverted *attP* sites which allows for the exchange of MiMIC with any other DNA cassette placed between two *attB* sites by recombination-mediated cassette exchange (RMCE) utilising *phiC31* integrase. For endogenous tagging of proteins, one can swap the gene trap cassette with a protein trap (PT) cassette (Venken et al. 2011). The PT cassette consists of an in-frame EGFP coding sequence flanked by two 4xGGS linkers between a splice

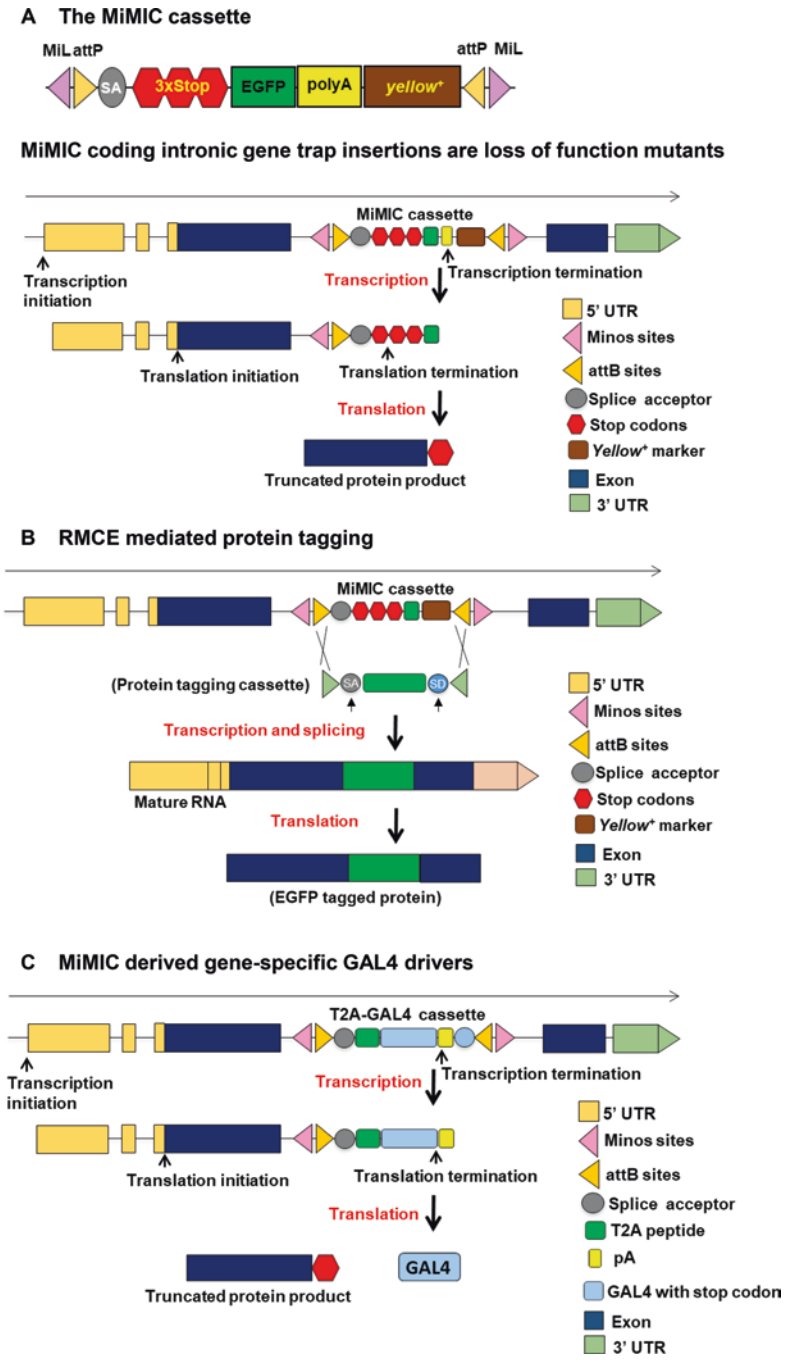

Fig. 1 MiMIC-derived strategies. Schematic showing (**a**) the MiMIC cassette and MiMIC-mediated gene disruption: When the MiMIC cassette is inserted into a coding intron in the same

acceptor (SA) and a splice donor (SD) (Fig. 1b). Once the PT cassette is inserted into a coding intron, the SA and SD within the cassette will result in the incorporation of the EFGP coding sequence into the mature mRNA as an artificial exon, results in the expression of an internally EGFP tagged protein. The flexible linkers flanking the EGFP sequence helps prevent any disruption in the folding of the tagged protein. Endogenously tagged proteins have several advantages: They can be used to determine protein expression patterns, subcellular localization and interacting partners by immunoprecipitation. Such information will be valuable for deciphering the roles of the genes of interest in neuronal development and maintenance. These lines can also be used for deGradFP-mediated conditional protein knockdown at various stages of brain development in larvae and adults using the temperature-sensitive UAS/GAL4 binary system (Caussinus et al. 2011; Nagarkar-Jaiswal et al. 2015).

The coding intronic insertions can also be used to generate endogenous GAL4 driver lines. This involves swapping the gene trap cassette with a Trojan GAL4 exon carrying an SA followed by the coding sequences for *Thosea asigna virus 2A*-like peptide (T2A), GAL4 coding sequence and the SV40 polyA signal (Fig. 1c) (Diao et al. 2015). The T2A peptide – during translation – results in ribosomal skipping from Gly to Pro present in the T2A peptide (Donnelly et al. 2001). This leads to the expression of a truncated native protein and a GAL4 protein as two independent peptide chains. As a result, a GT line is generated, which exhibits a GAL4 expression pattern corresponding to that of the native gene. Since the gene trap cassette is flanked by two *attPs*, the orientation of the insert by RMCE is random. This can lead to insertion of the PT cassette or the T2A–GAL4 cassette in either orientation. As a result, only one of the two possible insertion events will generate the required insertion. Therefore, a new swappable cassette termed 'double header' was generated, which carries a PT cassette in one orientation and the T2A–GAL4 in the opposing orientation (Li-Kroeger et al. 2018). Insertion of the double header in either direction is useful, as it generates an EGFP-tagged protein in one direction and a GT-expressing GAL4 in the other (Li-Kroeger et al. 2018).

Two MiMIC- and FRT-based strategies, namely, Flip-flop and FlpStop, respectively, have been developed for conditional gene inactivation (Fisher et al. 2017; Nagarkar-Jaiswal et al. 2017). Both of these techniques utilize insertions within coding intronic regions and involve cassette exchange via RMCE, as the constructs are nested between two inverted *attB* sites. The Flip-flop cassette carries a GT and a PT module oriented in opposite directions flanked by two inverted canonical FRT

Fig. 1 (continued) orientation as that of the gene, pA will cause precocious termination of transcription leading to truncation of gene product, creating a deletion mutant. (**b**) MiMIC-mediated protein tagging: The SA and SD present in the MiMIC cassette introduce the EGFP coding sequence into mature RNA as an artificial exon, which is then incorporated into protein upon translation (**c**) MiMIC-derived GAL4 driver lines: The MiMIC gene trap cassette can be replaced with an in-frame T2A-GAL4 cassette via RMCE. The pA signal present in the MiMIC cassette causes precocious termination of transcription, and SA insures incorporation of the T2A-GAL4 into the gene's mRNA while splicing. Upon translation, the T2A sequence causes truncation of the native protein product and expression of the individual GAL4 protein

and FRT14 sites forming a flip-excision switch (FLEx) (Schnütgen et al. 2003). The FLEx switch leads to cassette inversion upon *flippase* (FLP) expression. The PT module carries an in-frame EGFP coding sequence flanked by an SA and an SD, whereas the GT module consists of an SA followed by an in-frame T2A peptide, mCherry coding sequence, stop codon and an SV40 pA (Fig. 2a). When Flip-flop inserts in the PT orientation, it results in the expression of an endogenously EGFP-tagged protein, and when FLP is expressed, the cassette inverts from a PT to a GT orientation leading to truncation of the native protein and expression of mCherry, marking the mutant cells with red in a background of wild-type cells (cells in which the cassette inversion has not occurred) expressing EGFP-tagged protein (Nagarkar-Jaiswal et al. 2017).

The FlpStop cassette consists of a GT module and a UAS sequence oriented in opposite directions nested between two *attB* sites. The GT module carries the tdTOM sequence followed by the Tubα1 transcription terminator, SA, stop codons for all three reading frames and an SV40 polyA signal, which are flanked by the FLEx switch (Fig. 2b, (Fisher et al. 2017). FlpStop can be inserted in one of the two orientations: non-disruptive (ND) and disruptive (D). In the ND orientation, the FlpStop cassette is inserted in the same direction as that of the gene, while in the D orientation, it is inserted in the opposite direction. When FLP is expressed in ND insertions, the cassette will be inverted creating a D insertion, which will bring about two changes. First, the pA, which is located right after the SA, will terminate transcription resulting in gene trapping. Second, the tdTOM will be placed downstream of the UAS sequence leading to the expression of tdTOM when combined with GAL4, resulting in the labelling of mutant cells with tdTOM. The Flip-flop and FlpStop strategies do not rely on mitosis; therefore, they can be used for conditional gene inactivation in post-mitotic cells like neurons.

Binary Systems

Some of the most prominent neurodegenerative disorders such as Alzheimer's, Parkinson's, Huntington's and prion diseases develop as a result of toxic gain-of-function mutations expressing proteins that tend to accumulate as aggregates in

Fig. 2 (continued) is inserted in a protein trap orientation, the SA and SD will insure insertion of the EGFP coding sequence into the mature mRNA. This will result in the expression of EGFP-tagged protein. Upon FLP expression, the cassette will be flipped, which will bring the gene trap cassette into the coding frame, leading to inactivation of the gene and simultaneous expression of mCherry in the mutant cells under the control of native gene's regulatory elements. (**b**) Schematic showing the FLPStop cassette (top): The FLP Stop cassette consists of a UAS sequence, followed by the tdTOM sequence, Tubα1 transcription terminator, SA, stop codons for all three reading frames and a pA signal, which are flanked by two FRT and two F3 sequences forming a FLEx switch. The whole cassette is flanked by two inverted *attB* sites. FLPStop can be inserted in one of two orientations: nondisruptive (ND) and disruptive (D). In the ND orientation, gene is intact, while upon expression of FLP, the cassette will be inversed creating a D insertion leading to gene trapping concurrently expressing tdTomato (when combined with a GAL4)

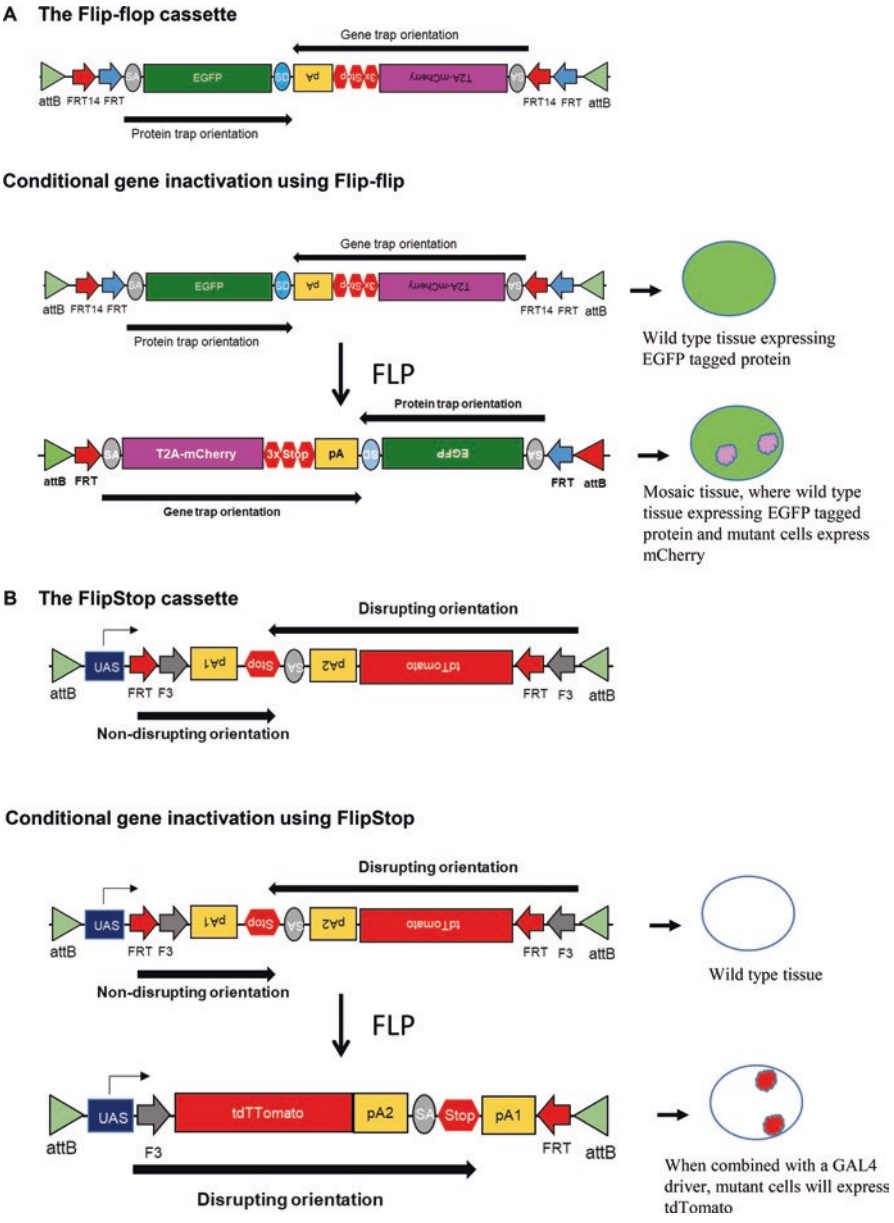

Fig. 2 Conditional gene inactivation. (**a**) Schematic showing the Flip-Flop cassette (top): The cassette has two modules – the PT and GT module. The PT module contains an SA, followed by an EGFP tag and an SD. The GT module contains the SA sequence, followed by a T2A peptide coding sequence, mCherry tag with stop codon, and an SV40 pA transcriptional termination signal. The GT and the PT modules are oriented in opposite directions and are flanked by two inverted FRT sequences and two FRT14 sequences that are nested in two inverted *attB* sequences that permit phiC31-mediated RMCE between Flip-Flop cassette and MiMIC elements. When the cassette

neurons and their surroundings. These toxic aggregates can be simulated in flies by overexpressing these mutant proteins with the use of binary expression systems. Several binary systems have been developed in flies such as the *UAS/GAL4* system (Brand and Perrimon 1993), LexA/LexO (Lai and Lee 2006) and the Q system (Potter et al. 2010). Among these the *GAL4/UAS* system is the most commonly used. GAL4 is a yeast protein that functions as a transcription regulator, controlling the expression of genes induced by the presence of galactose. GAL4 activates the transcription of genes by binding to upstream regulatory elements termed as upstream activation sequences (UAS). These sequences are cognate to enhancer elements seen in higher eukaryotes. GAL4 expression has been shown to be capable of initiating transcription of sequences placed downstream of the UAS element in *Drosophila* with no major deleterious effects (Fischer et al. 1988).

For generating fly models using the GAL4/UAS system, a bipartite approach is utilized to create transgenic flies (Brand and Perrimon 1993). A fly line is established such that the coding sequence of the protein (wild-type or with specific mutations) of interest is placed under the control of a UAS element along with a suitable reporter. These flies are termed responder lines. Since these flies do not express GAL4, it is introduced by crossing them with driver lines, which express GAL4 using gene/tissue-specific promoters. Progeny from such crosses express the protein of interest in a pattern congruent with the expression of GAL4. The tissue-specific promoters control the spatiotemporal expression of GAL4, which can be further fine-tuned using the GAL4 inhibitor GAL80 (Pilauri et al. 2005). A large collection of GAL4 drivers specific to the developing and adult nervous systems are available that can be used for neuron-specific overexpression of proteins. These lines can be obtained from BDSC (Jenett et al. 2012). One can also use the T2A-GAL4 library (Diao et al. 2015; Lee et al. 2018), a MiMIC-based tool (T2A-GAL4) that creates gene-specific GAL4 driver in which the expression of GAL4 is regulated by the native gene's own regulatory elements (described in the previous section).

The GAL4/UAS approach provides the simplest and most efficient means for the development of fly-models for neurodegenerative diseases that result from toxic gain-of-function mechanisms. For example, Alzheimer's disease, which is the most famed neurodegenerative disease, affecting nearly 29.8 million individuals worldwide (Dementia fact sheet, WHO, 2017). It is characterized by the appearance of extracellular amyloid plaques consisting of aggregated amyloid-β (Aβ) and intracellular neurofibrillary tangles composed of aggregates formed by hyper-phosphorylated tau protein. A fly model of Alzheimer's disease has been developed utilizing the GAL4/UAS system to express the human variant of $A\beta_{1-42}$ in the fly neurons using the *Elav–GAL4* driver (Crowther et al. 2005). These flies display progressive neuronal degeneration concomitant with accumulation of Aβ. The model has proved useful in significantly bolstering evidence for Aβ oligomers as being the primary toxic agent rather than mature fibrils. Similarly, fly models have been developed for Parkinson's disease by overexpressing wild-type or mutant α-synuclein in neurons (Feany and Bender 2000), PolyQ diseases like spinocerebellar ataxia type 3 (SCA3) and Huntington's disease by overexpressing mutant ATXN3 (Ellis et al. 1993) and

Huntingtin in eyes using *GMR–Gal4* (Jackson et al. 1998) and several other diseases.

The GAL4/UAS system can also be used to determine conserved biological functionality between a fly protein and its human homologue by rescuing loss-of-function phenotypes in flies by complementation with human cDNA. Similarly, one can assess the impact of disease-causing mutations on protein function by expressing disease variants in flies. The GAL4/UAS binary system also permits the construction of disease models using UAS-RNAi lines that can achieve cell/tissue-specific gene knockdown. There are several models that have been established using this approach, for example, Friedreich's ataxia (FA), which is the most common form of inherited ataxia. The disease occurs due to a reduction in the expression of the frataxin protein. Therefore, GAL4/UAS-mediated RNAi knockdown provides a convenient method for modelling this disease in *Drosophila.* Utilizing this method, the group Llorens et al. generated several lines of RNAi frataxin flies (Llorens et al. 2007). This method has been useful in studying several other disease-associated genes such as *Parkin* (Parkinson's (Yang et al. 2006)), *Sox5* (ALS (Li et al. 2017)), *VCP* (ALS (Johnson et al. 2015) and *Marf* (Charcot–Marie–Tooth disease (Sandoval et al. 2014)). However, there are two major limitations: First, they are not as effective as null mutants. Second, they often have off targets, which requires rescue of the phenotype in question with a RNAi-resistant cDNA construct to ensure that the phenotype is not due to off-target effects.

Apart from the GAL4/UAS system, two other commonly used binary systems exist that can be used in a similar fashion: LexA/LexO system and the Q system. The LexA/LexO system utilizes a bacterial transcription factor (LexA), which binds to the specific operator sequence (LexO) and drives the expression of a downstream gene. In flies, LexA is fused to an activator domain (either VP16 from herpes simplex virus or GAD from yeast gal4), which then binds to the LexO sequence that precedes the protein coding sequence (Lai and Lee 2006). On the other hand, the Q-system (QF/QA/QS) is adapted from *Neurospora crassa qa. N. crassa*, has a transcription activator (QF) that binds to a specific sequence upstream of the qa gene (QA) and activates its transcription, whereas QS, a repressor, blocks the transcription activity of QF. The activity of QS can be blocked by quinic acid (Potter et al. 2010). The advantage of these systems is that they do not interfere with each other and therefore can be used in parallel to manipulate different genes at the same time.

Genome Editing Using CRISPR/Cas9

The discovery of the CRISPR/Cas9 system has afforded a powerful tool for making very specific edits in the genomic DNA of a wide variety of model organisms including *Drosophila* (Doudna and Charpentier 2014; Mali et al. 2013; Port et al. 2014; Şahin et al. 2017; Xue et al. 2014). CRISPR/Cas9 is a naturally occurring DNA editing system – adapted from bacteria – that was originally identified as a defence mechanism against invading viruses (Barrangou et al. 2007; Garneau et al. 2010). CRISPR stands for Clustered Regularly Interspaced Short Palindromic

Repeat DNA sequences. Bacteria capture and incorporate short stretches of DNA termed 'protospacer' from invading viruses and create a specific array of these sequences termed 'CRISPR arrays' as a defence against subsequent infections. Upon infection with the same virus, this protospacer DNA is transcribed into RNA, which is called crRNA (CRISPR RNA). crRNA then combines with another CRISPR-associated RNA called tracrRNA (trans-activating CRISPR RNA), which is then bound by the CAS9 protein (CRISPR-associated endonuclease) in order to form an active ternary complex (Gasiunas et al. 2012). This complex then locates and binds to its target sequence (viral DNA), following which CAS9 induces a double-strand break. The binding of the CAS9 complex to the target DNA requires the presence of a Protospacer adjacent motif (PAM) sequence next to the crRNA target sequence (Gasiunas et al. 2012). The most widely used CAS9 for editing is from *Streptococcus pyogenes* and its associated PAM sequence is 5′-NGG-3′.

The above mechanism has been widely exploited as a genome editing tool in flies, as it is easy to use and relatively inexpensive (Ṣahin et al. 2017; Xue et al. 2014). For CRISPR/Cas9-mediated mutagenesis, the most important step is to identify a specific target sequence that is followed by the appropriate PAM sequence. Once the target sequence is identified, a suitable 'guideRNA' (gRNA) plasmid is created, which expresses a sequence complementary to the target (approximately 20 nt) followed by the sequence for the tracrRNA using a specific promoter. The most commonly used promoter for expression of gRNA in flies is the U6B promoter, which ubiquitously expresses the gRNA in the fly (Xue et al. 2014). This construct can then be introduced into flies that express Cas9 by microinjection or by genetic crossing (Gratz et al. 2015). Cas9 can be expressed in a tissue specific manner using the *GAL4/UAS* system (Port et al. 2014; Xue et al. 2014) or by directly placing Cas9 under regulatory sequences from a suitable gene. For example, in *act–Cas9* flies, Cas9 is placed under the *actin5C* promoter sequence, which is expressed ubiquitously. Similarly for germ line expression, Cas9 can be placed under *nos* 3' UTR (Port et al. 2014). Once the gRNA is introduced into the host cells, the gRNA complexes with CAS9 and brings about a double-stranded break in the target sequence. Once the double-strand break is induced, the host cell's repair mechanism functions to repair the break quickly by non-homologous end joining (NHEJ). Since NHEJ is error prone, it often results in frame shifts, leading to disruption of the gene being targeted. If specific mutations, insertions or deletions in the target sequence are desired, they can be achieved by the use of a suitable template sequence to induce homologous recombination. When a homologous DNA template is available, the host mechanism will utilize it and repair the CRISPR/CAS9-induced double-stranded break using homology-directed repair (HDR). This results in the incorporation of the desired specific change into the target sequence (Fig. 3).

In flies, two types of DNA donors have been used for genetic manipulation: small single-stranded DNA donors (ssDNA) and double-stranded DNA donors (dsDNA) (Fig. 3). ssDNA donors are short oligonucleotide sequences that require fairly short homology arms on either side (about 50 bases) and can be used for the

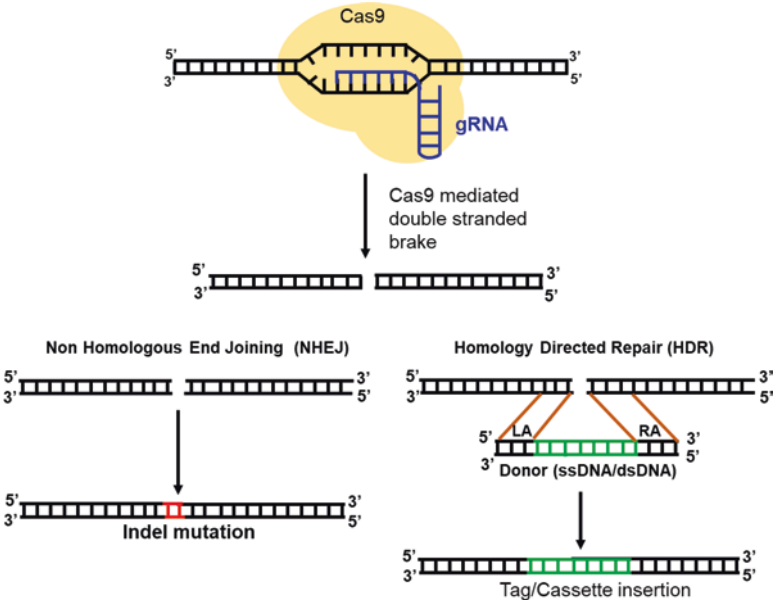

Fig. 3 CRISPR-based genome editing. In the nucleus, Cas9 associates with the gRNA and binds to the target sequence guided by the PAM sequence. Upon binding, it creates a double-stranded break and activates the host cell's DNA repair mechanism. If a repair template is not available, DNA strands are repaired by NHEJ, an error-prone process that leads to small inDel mutations that disrupt the gene function. If the donor DNA template, for example, a small epitope or a construct with a suitable screening marker and homology arms (LA – left arm and RA – right arm), is provided, then the break is repaired through HDR. This results in insertion of the desired DNA sequence at a specific genome locus

insertion of small epitopes such as HA and V5, and *attP* landing sites (Gratz et al. 2015). These insertions are then screened for using PCR. Inserts longer than 100 bp are introduced using dsDNA donors in circular plasmids bearing homology arms (0.5 - 1 kb) on either side of the sequence to be inserted. In this approach, convinient markers such as 3XP3-DsRed or 3XP3-EGFP for expression in eyes or *yellow* + body markers are used for screening. They are introduced along with the insert, which allows for the rapid identification of transformed flies (Li-Kroeger et al. 2018). These markers can be flanked by two FRT or LoxP sites so that they can be excised using FLP or Cre (Reisch and Prather 2015). One can also introduce an *attP*-flanked DNA cassette such as the CRISPR-mediated integration cassette (CRIMIC), which is a gene trap cassette that was used to generate a gene-specific T2A–GAL4 library (Lee et al. 2018). These cassettes can then be exchanged via RMCE with other cassettes that are flanked by *attB* sites such as a PT cassette, Flip-flop or FlpStop.

Using CAS9-expressing fly lines, the CRISPR/CAS9 system can be used for the generation of disease models in a simple and efficient manner. There are several

web-based resources available that provide step-by-step instructions on how to perform Cas9-mediated genome-editing in *Drosophila*, including BDSC – *flystocks.bio.indiana.edu/Browse/misc-browse/CRISPR*; CRISPR fly design – *crisprflydesign.org, flyCRISPR: flycrispr.molbio.wisc.edu;* NIG-FLY FlyCas9 – *shigen.nig.ac.jp/fly/nigfly/cas9* (Housden and Perrimon 2016a, b; Housden et al. 2016). Additionally, a large repertoire of genetic tools are available from BDSC for manipulation of flies, such as TRiP-CRISPR Knockout (TRiP-KO) transgenic lines that express gRNA for specific genes under the U6B promoter and TRiP-CRISPR Overexpression (TRiP-OE) lines that lead to transcriptional activation of the gene of interest (Transgenic RNAi Project, 2017) and nervous system-specific GAL4 lines that can specifically edit genome of neuronal cells (Jenett et al. 2012).

Conclusion

Drosophila has risen to become an exceptional model organism for the study of human neurological disorders due to the significantly high conservation of genes and their associated functions between humans and fruit flies. The availability of various tools and techniques for genetic manipulation described here allows for the establishment of fly models for various human neurological disorders and helps to decipher the pathogenic mechanisms underlying these diseases.

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Understanding Neurodegeneration and Neuroprotection Through Genetic Screens in *Drosophila*

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Abstract

Drosophila genetic screens have been invaluable in understanding neurodegenerative diseases (NDD) and neuronal maintenance. The modeling of several human neurodegenerative diseases such as Alzheimer's and Parkinson's in *Drosophila* and subsequent modifier genetic screens for neurodegenerative phenotypes have been instrumental in identifying the molecular mechanisms of neurodegeneration as well as the cellular function of genes implicated in neurodegeneration. For instance, studies on *Drosophila* homologs of *PINK1* and *PARKIN*, genes implicated in Parkinson's disease, identified their roles in mitochondrial quality control. Interestingly, unbiased genetic screens for fly mutants with neurodegenerative phenotypes have also identified many genes implicated in neurodegenerative diseases and have led to the discovery of novel players regulating neuronal health and maintenance. *Drosophila* has emerged as a valuable screening platform for validating the pathogenicity of variants identified through whole-genome sequencing of patients with neurodegenerative diseases and has thus fast-tracked the identification of causative mutations. With rapid and consistent development of genome editing technologies, together with amenability for genetic screens, *Drosophila* will continue to serve as a great system to study neurodegeneration.

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Forward genetic screen · Genetic modifier screen · Alzheimer's disease · ALS · Huntington's disease · Lipid droplet · Metabolic dysfunction · Autophagy · Whole-genome sequencing

Abbreviations

ALS	Amyotrophic lateral sclerosis
APOE	Apolipoprotein E
APP	Amyloid precursor protein
CMT	Charcot-Marie-Tooth disease
EMS	Ethyl methyl sulfonate
ER	Endoplasmic reticulum
ERG	Electroretinogram
FATP	Fatty acid transport proteins
FHM	Familial hemiplegic migraine
iPSC	Induced pluripotent stem cells
LD	Lipid droplet
MCTs	Monocarboxylate transporters
ND	Neurodegeneration/neurodegenerative
NDD	Neurodegenerative diseases
NGS	Next-generation sequencing
NTE	Neuropathy target esterase
PD	Parkinson's disease
PDF	Pigment-dispersing factor
RDP	Rapid-onset dystonia-parkinsonism
TCA Cycle	Tricarboxylic acid cycle
UAS	Upstream activating sequence
UPR^{mt}	Mitochondrial unfolded protein response
WES	Whole exome sequencing
WGS	Whole-genome sequencing

Introduction

Neurodegenerative diseases (NDD) are characterized by progressive loss of neuronal function and structure. To date mutations in approximately 195 genes have been implicated in NDD (OMIM; SysID Database). Neurodegenerative (ND) phenotypes have been well recapitulated in model organisms such as flies (McGurk et al. 2015; Jaiswal et al. 2012; Mutsuddi and Nambu 1998; Fortini and Bonini 2000), worms (Wang et al. 2017), and mice (Kreiner 2018). Mutations in the fly homologs of human NDD-linked genes often display ND phenotypes in flies validating its use as a model for NDDs (Mutsuddi and Nambu 1998; Yamamoto et al. 2014; Casci and

Pandey 2015; Singhal and Jaiswal 2018; Hales et al. 2015; Kasture et al. 2018). These studies have contributed greatly to our understanding of the endogenous function of genes linked to NDD and have provided a framework to decipher the pathogenic mechanisms that underlie NDD (Gitler et al. 2017; Taylor et al. 2016; Abeliovich and Gitler 2016; Jovičić et al. 2015; Auluck et al. 2002). A substantial advantage of using the fruit fly, *Drosophila*, to study ND, is that it displays a variety of complex behaviors governed by over 100,000 neurons (Hales et al. 2015) and has unparalleled availability of tools for genetic manipulation (Şentürk and Bellen 2018; Venken et al. 2011; Nandan J and Nagarkar-Jaiswal 2019).

Large-scale genetic screens in flies, which consist of an unbiased search for a specific phenotype in a collection of mutants, have been the driving force for understanding numerous biological processes such as development, physiology, and behavior. Forward genetic screens that were designed to identify neuronal loss or a decline in neuronal function have isolated a series of so-called ND mutants (Singhal and Jaiswal 2018). Subsequent mapping of ND mutant genes and follow-up mechanistic studies have identified a plethora of novel genes important for maintaining neuronal function and have also advanced our understanding of ND (Jaiswal et al. 2012; Lessing and Bonini 2009). Besides forward genetic screens, reverse genetic studies and modifier screens on models derived from reverse genetic studies have been prevalent in the studies of NDD in flies. In these studies, the homolog of the gene that is linked to NDD in humans is disrupted in flies to study the gene's molecular function and identify its interactors. Modifier screens involve the search for other genes that can suppress or enhance the phenotype exhibited by a mutant of interest. These studies have been instrumental in studying the molecular mechanisms causing NDD. Flies have also been utilized for functional screening of variants in a human gene identified through whole genome or exome sequencing (WGS/WES) from NDD patients. Such screens involve genetic manipulation of fly homologs of suspected human gene variants identified and further, their phenotypic analysis. This essentially, provides a glimpse into the pathophysiology of the disease and aids in giving biological sense to WES/WGS data of patients (Marcogliese et al. 2018). In this chapter, we will highlight various *Drosophila* screens that have paved the way to an understanding of neuronal maintenance and ND.

Forward Genetic Screens

The health and survival of a neuron depend on many different factors. Hence, ND can be caused by a wide range of processes such as failure of cellular protective mechanisms, unusual activation of stress-induced pathways, or toxic gain of function mutations (Hussain et al. 2018; Jellinger 2010). In this section, we will briefly discuss several ND-related genes, identified in benchmark forward genetic screens, and their mechanisms of pathology.

Screen Design and Mutant Isolation

The success of a genetic screen depends on two major factors: the method of generating random mutations and the discriminatory phenotype of the mutants. By far, the most preferred means of randomized mutagenesis have been P-element transposon-based insertions and the use of chemical mutagens such as ethyl methyl sulfonate (EMS). Mutagenesis using EMS, a chemical mutagen, involves feeding flies a low dose of EMS. EMS-induced mutagenesis has been popular due to its inherent simplicity and high probability of achieving random point mutations as compared to other mutagens such as gamma rays and X-rays (Bökel 2008). Insertional mutagenesis is based on the random insertion of an engineered transposon in the genome to generate and screen for mutants with specific phenotypes (Cooley et al. 1988; Bellen et al. 2011). One of the first ND mutants *drop-dead* (*drd)* was discovered through an EMS screen. *drd* was isolated in a screen for impaired phototransduction and reduced lifespan. Interestingly, *drd* mutants also exhibit motor defects and cerebral vacuolization upon aging (Hotta and Benzer 1972; Hotta and Benzer 1969; Buchanan and Benzer 1993), indicating that impaired phototransduction is an effective readout for ND. Below we introduce major forward genetic screens for a variety of ND phenotypes that have identified numerous novel genes.

Defective Brain Histology or Anatomy In an EMS-based screen to isolate fly mutants based on anatomical defects such as brain size, shape, and vacuolization, *swiss cheese* (*sws*) mutant was identified. These mutants exhibited reduced lifespan and age-dependent ND (Heisenberg and Böhl 1979; Kretzschmar et al. 1997). In a separate screen for shortened lifespan and altered brain histology in aged flies, 60 mutant lines were identified (Min and Benzer 1997). Among these mutants, *spongecake* and *eggroll* show vacuolization in the central nervous system upon aging and show remarkable similarities to particular human ND conditions. For example, *spongecake* displays membrane-bound vacuoles at axonal terminals upon aging, resembling Creutzfeldt-Jakob disease (Min and Benzer 1997), while *eggroll* displays dense, multilamellated structures, similar to lysosomal storage diseases like Tay-Sachs and Niemann-Pick diseases (Ferreira and Gahl 2017). Although these mutants, including *drd*, are yet to be mapped, the study of such mutants reflects the importance of fly ND mutants to understand human NDDs.

Insertional mutagenesis A P-element-based insertional mutagenesis screen for reduced lifespan and brain degeneration upon aging, identified *bubblegum (bgm*) (Min and Benzer 1999). *bgm* mutant exhibits optic lobe degeneration upon aging. *bgm* encodes VLCFA acyl-CoA synthetase, the fly homolog of human *ACSBG2*. The *bgm* mutants show elevated levels of very long-chain fatty acids (VLCFAs), a phenotype also observed in adrenoleukodystrophy (ALD) patients, wherein VLCFAs accumulate to result in progressive neuronal symptoms including seizure and paralysis (Wiesinger et al. 2015). A dietary supplement of a mixture of unsaturated fatty acids (Lorenzo's oil) was shown to ameliorate ALD symptoms by nor-

malizing the levels of saturated fatty acids (Rizzo et al. 1987; Moser et al. 2007). Similarly, the ALD-like phenotypes in flies were partially rescued by feeding them glyceryl trioleate oil, a component of Lorenzo's oil (Min and Benzer 1999). Furthermore, WES of a patient with ALD revealed a mutation in *SLC27a6*, a putative homolog of *bgm* and *double bubble (dbb)* (Sivachenko et al. 2016). This study verified the functional link identified in flies and implicated the acyl-CoA synthetase (ACS) family of genes in the pathogenesis of ALD in humans. Such studies greatly exemplify the conserved nature of fundamental molecular mechanisms, underpinning the importance of *Drosophila* models for understanding the pathogenesis of complex NDD such as ALD.

In another histology-based forward genetic screen, a collection of third chromosome insertion lines were screened. This screen identified a mutation in *löchrig* (*loe*), which displayed vacuolization of the adult brain (Tschäpe et al. 2002). *loe* encodes AMPK-gamma subunit isoform, and *loe* mutants display reduced amyloid precursor protein (APP) processing as well as increased cholesterol ester levels. APP, canonically, on proteolysis, gives rise amyloid-β peptides, and the aggregation of these peptides has been linked to Alzheimer's disease (AD) pathogenesis (Müller et al. 2017). A comparable increase in cholesterol ester levels was also found due to a mutation in the fly homolog of APP. In fact, thanks to this study and others, AMPK is being avidly pursued as a drug target to ameliorate ND in vivo (Marín-Aguilar et al. 2017).

Temperature Sensitivity, Seizure, and Paralysis Numerous genetic screens have isolated mutants that exhibit phenotypes such as temperature sensitivity, seizure, and paralysis. Many of these mutants were found to show ND phenotype and have been a great source to study ND (Palladino et al. 2003; Suzuki et al. 1971; Grigliatti et al. 1973; Siddiqi and Benzer 1976; Homyk and Sheppard 1977; Palladino et al. 2002; Wu et al. 1978). For example, two temperature-sensitive paralytic mutants, which were mapped to the α-subunit of the Na^+, K^+-ATPase, show extensive ND with aging (Palladino et al. 2003). Its human homolog ATPα was later found to be associated with NDD like rapid-onset dystonia-parkinsonism (RDP), familial hemiplegic migraine (FHM), and alternating hemiplegia of childhood (Ashmore et al. 2009; Gallardo et al. 2014; De Carvalho et al. 2004; Heinzen et al. 2014). Similarly, mutations in *mitochondrial ATPase subunit 6 (MT-ATP6, ANT1)* (Homyk and Sheppard 1977) cause progressive muscular atrophy and neuromuscular dysfunction in flies (Celotto et al. 2006). The human homolog MT-ATP6 has been linked to several human conditions, namely, childhood-onset Leigh syndrome (Wagner et al. 2017), familial infantile bilateral striatal necrosis (#500003), and NARP (neuropathy, ataxia, and retinitis pigmentosa)(Thorburn et al. 1993). Another seizure-sensitive, paralytic mutant, *easily shocked (eas)* (Ganetzky and Wu 1982), shows lipid disequilibrium and a reduction in phosphoethanolamine (PE) (Witt 2014). The mutant is defective in gene encoding *ethanolamine kinase* (Human ENTK-1) required for PE synthesis (Pavlidis et al. 1994), which is important for neuronal maintenance (Montaner et al. 2018). Such studies hint at the relevance of phospho-

lipid homeostasis in neural maintenance. Identification of temperature-sensitive and paralytic mutants, such as the ones mentioned, have been valuable in understanding symptoms like seizures, which often accompany NDDs and hence shed light on the potential process of pathogenesis.

Electroretinograms Defects Histology-based screens are very tedious and time-consuming, and hence cannot be used to interrogate many mutants. A more rapid and straightforward way to screen for ND mutants is to look for defects in photoreceptor activity, which can be assessed by recording electroretinograms (ERG) at several time points throughout the fly's life (Yamamoto et al. 2014; Hotta and Benzer 1969; Stowers et al. 2002; Fergestad et al. 2010). A large-scale unbiased genetic mosaic screen based on progressive ERG defects was conducted to systematically screen a collection of over 6000 EMS-induced lethal mutants on the X-chromosome. In parallel, this mutant collection was also screened for morphological phenotypes. This screen has led the identification of 165 genes required for neuronal development, function, and maintenance (Yamamoto et al. 2014). While homologs of 92% of the genes identified in this screen are conserved in humans, 30% of them had homologs already linked to neurological diseases, including human NDDs. For example, this screen identified mutations in the fly homologs of *MFN2*, *C8ORF38*, *CACNA1A*, and *LRPPRC*. Mutations in these genes are known to cause human NDDs (Table1). Another ERG-based screen, primarily designed to study neuronal transmission, isolated mutations in *nmnat* and *aats-met* causing degeneration of photoreceptor neurons (Bayat et al. 2012; Zhai et al. 2006). Mutations in human homolog of both *nmnat* and *aats-met* were later found to cause NDDs (Bayat et al. 2012; Chiang et al. 2012). These studies reflect that the ERG-based screens can facilitate large-scale forward genetic screens to identify new ND mutants.

Fluorescence-Based, Real-Time Retinal Degeneration To screen a large number of mutants causing degeneration of photoreceptor neurons through live imaging, two very elegant fluorescence-based methods were developed. First, a "Tomato/GFP-FLP/FRT" method (Gambis et al. 2011) and second, "*Rh1*::*GFP ey-flp/hid*" method (Huang et al. 2015). In principle, these systems utilize an Flp-FRT system to generate mutant patches in the eye of heterozygous flies, and GFP marked photoreceptors allow assessment of their integrity. This allows the screening of mutants based on a progressive reduction in fluorescence to monitor photoreceptor degeneration in real time. Using "Tomato/GFP-FLP/FRT" method, Gambis et al. identified mutations in *fatty acid transport protein* (*fatp*) gene causing photoreceptor degeneration (Dourlen et al. 2012). Huang et al. identified mutations in 18 genes that are required for viability of photoreceptors. Some of the examples are porin (human *VDAC* ortholog), *socx* (human *SOC1*), and *aats-val* (human *VARS*). Among these, VARS has been implicated in "neurodevelopmental disorder with microcephaly, seizures, and cortical atrophy" (OMIM#617802). Use of these fluorescence-

based methods of tracking degenerating in live animals can substantially speed up the screening process.

Arrhythmicity Another screen by (Rezával et al. 2008) utilized insertional mutagenesis in a misexpression screen. A misexpression screen involves overexpressing, ectopically misexpressing or downregulating genes in a restrictive manner to screen for specific phenotypes (Rørth 1996). In this study the authors examined disrupted neuronal rhythms (arrhythmicity) in PDF (pigment-dispersing factor) neurons using a *pdf*-GAL4 by disrupting genes implicated in locomotor behavior, to identify ND-related genes. Circadian rhythm aberrations, including disrupted neuronal signal oscillations such as arrhythmicity, occur routinely in NDD (Musiek 2015). To this end, they screened through a collection of P-element insertion lines from 1000 genes implicated in locomotor behavior, by misexpressing them in PDF neurons. This screen identified a gene called *enabled (ena)*, loss of which causes adult-onset progressive ND within the optic lobe, along with age-dependent vacuolization. Overall, such studies underpin the relevance of forward genetic screens in discovering new genes associated with ND.

Lessons Learnt from Forward Genetic Screens

Discovery of ND mutants through forward genetics screens has encouraged numerous studies to gain mechanistic insight into the various pathways which lead to ND. In this section, we will briefly discuss a few studies on some of the genes identified through *Drosophila* forward genetic screens that have provided novel insight into our understanding of ND.

Glial Defects and Neurodegeneration

Glia are the non-neuronal tissue found in the animal nervous system. They contribute greatly to neuroprotection and maintenance (Verkhratsky et al. 2017). A forward genetic screen identified mutations in *swiss cheese* (*sws*), which is required for glial maintenance (Kretzschmar et al. 1997). *sws* mutations result in reduced lifespan and ND. *sws* codes for lysophospholipase that deacetylates phosphatidylinositol, which in turn regulates glial wrapping around the neurons. Loss of *sws* in glia results in disrupted myelin wrapping causing glial and neuronal degeneration (Dutta et al. 2016). The human ortholog of *sws* is *PNPLA6 (patatin-like phospholipase domain-containing protein 6)*, which encodes neuropathy target esterase (NTE) and has been implicated in several neurological disorders such as spastic paraplegia (OMIM #612020), (Bettencourt da Cruz et al. 2008), and Boucher-Neuhäuser syndrome, Gordon-Holmes syndrome (OMIM #215470), Oliver-McFarlane syndrome (OMIM #275400), and Laurence-Moon syndrome (OMIM #245800) NTE in mice was recently shown to be important for Schwann cell maintenance and might play a role in the neuropathic pain response and associated neuropathies (Dutta et al. 2016; McFerrin et al. 2017). Taken together, these finding suggests a conserved role of glial maintenance in neuronal maintenance.

Proinflammatory Response and Neurodegeneration

Proinflammatory responses involve activation of the innate immune system in response to an injury or infection. Misdirected proinflammatory responses have been implicated in diseases like Alzheimer's disease (AD), Parkinson's disease (PD), and amyotrophic lateral sclerosis (ALS) (Petersen et al. 2012; Zhan et al. 2015; Deczkowska and Schwartz 2018). Although innate immune responses are usually pathogen-induced, it can also be triggered due to endogenous molecules expressed specifically during cellular stress (Matzinger 2002). In a p-element-based forward genetic screen to identify mutants causing vacuolar lesions in the aging fly brain (Cao et al. 2013), mutations in *dnr1 (defense repressor 1)* gene, which encodes for an E3 ubiquitin ligase, were identified. Its mammalian homolog, *MYLIP* encodes for the inducible degrader of the LDL (IDOL) receptor, which functions as a negative regulator of Dredd caspase (death-related ced-3/Nedd2-like protein). Dredd is responsible for the activation of Relish (Rel), an NF-κB transcription factor (Cao et al. 2013; Meinander et al. 2012; Stoven et al. 2003). Detailed studies on fly *dnr1* showed that the aberrant activation of NF-κB induces innate immune response leading to ND (Cao et al. 2013; Shih et al. 2015). In humans, IDOL regulates the degradation of low-density lipoprotein receptor (LDLR) through its ubiquitination function (Zelcer et al. 2009). In mice, loss of *idol (*murine homolog of *dnr1)* also results in a proinflammatory response (Gao et al. 2017; Hong et al. 2010), through impaired cholesterol homeostasis. Interestingly, most cases of AD also feature impaired cholesterol homeostasis (Chang et al. 2017), and hence *idol* is also being studied as a potential drug target for AD (Choi et al. 2015).

Neurodegeneration Due to Metabolic Dysfunction

Metabolic defects have a wide range of consequences in the cell, and neurons being metabolically quite active, they are highly susceptible to such an insult. To this end, most NDDs are characterized by a metabolic disorder of some kind or another (Procaccini et al. 2016)**.** Numerous fly mutants displaying ND were found to have defective cellular metabolism. For example, rescreening of temperature-sensitive paralytic mutants for degenerative brain defects identified *wasted away (tpi)*, which codes for triosephosphate isomerase (Tpi)(Palladino et al. 2002). Loss of Tpi leads to accumulation of dihydroxyacetone phosphate (DHAP) (Palladino et al. 2002; Gnerer et al. 2006). Subsequently, DHAP decomposes nonenzymatically to form methylglyoxal, which can modify protein and DNA molecules to form advanced glycation end products (AGEs), leading to oxidative damage and neurotoxicity. Additionally, *tpi* mutants also exhibit impaired synaptic vesicle recycling (Roland et al. 2016). Increased AGE and glyoxalase, along with defective synaptic recycling, has been a recurring feature in many NDDs (Gnerer et al. 2006; Juranek et al. 2015; Esposito et al. 2012; Medeiros et al. 2018). In sum, ND as a result of the loss of *tpi* seems to be an example of substrate accumulation-induced neurotoxicity.

An ERG-based screen for ND phenotypes in flies identified mutations in several X-chromosome genes encoding mitochondrial proteins [Table 1, (Yamamoto et al. 2014)]. Many of their human homologs are known to be linked to NDDs. The genes include *marf* (*Mfn2* in human), *sicily* (human NDUFAF6), *frataxin homolog* (human

Table 1 Genes identified in forward screens

Gene	Human homolog	Human ND association	References
drd	–		Hotta and Benzer (1972), Buchanan and Benzer (1993), and Hotta and Benzer (1970)
Spongecake	–		Min and Benzer (1997)
eggroll	–		Min and Benzer (1997)
löchrig/AMPKγ	*PRKAG2*	#600858, #261740, #194200	Tschäpe et al. (2002)
bubblegum/ Acyl-CoA Synthetase family	*ACSBG1, ACSBG2*	# 300100	Min and Benzer (1999)
highwire	*MYCBP2*		Neukomm et al. (2014)
nmnat	*NMNAT1,NMNAT2,NMNAT3*	# 608553	(Zhai et al. 2006)
dsarm/Ect4	*SARM1*		Neukomm et al. (2014)
eas	*ETNK1,ETNK2*		Suzuki et al. (1971) and Grigliatti et al. (1973)
tko	*MRPS12*		Ganetzky and Wu (1982) and Royden et al. (1987)
swiss cheese	*PNPLA6, PNPLA7*	#245800, #275400, #215470, #612020	Kretzschmar et al. (1997)
ANT1/ATP6/ sesB	*MT-ATP6*	#551500, #256000, #535000	Homyk and Sheppard (1977) and Celotto et al. (2006)
aats-met	*MARS2*	#616430, #611390	Bayat et al. (2012)
aats-val	*VARS*	#617802	Huang et al. (2015)
porin	*VDAC1, VDAC2,VDAC3*		Huang et al. (2015)
socx	*SOC1*		Huang et al. (2015)
drn1	*MYLIP*		Cao et al. (2013)
tpi	*TPI1*	#615512	Palladino et al. (2002) and Gnerer et al. (2006)
dNrd1	*NRD1*		Yamamoto et al. (2014) and Yoon et al. (2017)
enabled	*ENAH*		Rezával et al. (2008)
lrpprc2	*LRPPRC*	# 220111	Yamamoto et al. (2014) and Jaiswal et al. (2015)

(continued)

Table 1 (continued)

Gene	Human homolog	Human ND association	References
ocelliless	*OTX1, OTX2, CRX*	#608051, #604393, #602225, #608133, #607640, #610125	Yamamoto et al. (2014)
vps26	*VPS26A,VPS26B*		Yamamoto et al. (2014)
dankle2	*ANKLE2*	# 616681	Yamamoto et al. (2014)
marf	*MFN2*	#609260, #617087, #601152	Yamamoto et al. (2014) and Zhang et al. (2013)
cacophony	*CACNA1A, CACNA1B, CACNA1E*	#183086, #614860, #141500, #617106, #108500	Heisenberg and Böhl (1979) and Yamamoto et al. (2014)
sicily	*NDUFAF6*	#612392	Yamamoto et al. (2014) and Zhang et al. (2013)
para	*SCN8A, SCN2A*	#614306, #614558, #617080, #613721, #607745	Yamamoto et al. (2014), Suzuki et al. (1971), and Siddiqi and Benzer (1976)
comatose	*NSF*		Suzuki et al. (1971) and Siddiqi and Benzer (1976)
shi	*DNM1,DNM2,DNM3*	#616346, #118200	Suzuki et al. (1971) and Siddiqi and Benzer (1976)
dfh	*FXN*	#229300	Yamamoto et al. (2014)
ND23	*NDUFS8*	#256000	Loewen and Ganetzky (2018)
ubiquilin	*UBQLN2.UBQLN2*	#300857	Yamamoto et al. (2014) and Şentürk et al. (2019)
Brat (cheesehead allele)	*TRIM3*		Loewen et al. (2018)
pirouette	–	–	Eberl et al. (1997)

FXN), and *lrpprc2* (LRPPRC). It is important to note here that the mutations in about 200 human genes that encode mitochondrial proteins are linked to a variety of human metabolic and neurological and NDDs; however, very little is known about their pathogenic mechanisms. Therefore, interrogation of fly mutants for mitochondrial proteins can provide novel insight into the mechanism of ND underlying mitochondrial dysfunction. We will discuss a few cases below.

Studies of *dNrd1(Drosophila nardilysin1)* mutants, isolated in an ERG-based screen, identified the role of NRD in the folding of α-ketoglutarate dehydrogenase (OGDH), a tricarboxylic acid cycle (TCA) enzyme that converts α-ketoglutarate to succinyl-CoA (Yoon et al. 2017). Loss of *dNrd1* results in accumulation of α-ketoglutarate, which in turn induces mammalian target of rapamycin complex 1 (mTORC1) and thereby suppresses autophagy. A similar phenotype was also observed due to the loss of *dogdh*, which encodes OGDH, confirming implications of altered α-ketoglutarate metabolism in ND. Interestingly, rapamycin can suppress the ND phenotype of *dNrd1* mutants. This study, therefore, identified a novel mitochondrial signaling mechanism involved in ND.

Several mutations in nuclear genes encoding mitochondrial proteins, such as *lrpprc2*, were shown to cause light-induced photoreceptor (PR) degeneration. *lrpprc2* is the *Drosophila* homolog of the human *LRPPRC*, which is associated with Leigh syndrome, French-Canadian type (OMIM # 220111). It was found that the impaired phototransduction due to an ATP deficit in *lrpprc2* mutants results in excessive endocytosis of rhodopsin1 (Rh1) causing light-dependent PR degeneration (Jaiswal et al. 2015). Rh1-mediated toxicity was also observed in mutants such as *sicily* and *frataxin homolog (dfh)*, although in these mutants degeneration has other contributors such as increased oxidative stress (in *sicily*) or increased iron toxicity (in *dfh*), and therefore the degeneration manifests in a light-independent manner.

Mutations in *Frataxin (FXN)* causes Friedreich's ataxia (OMIM #229300) (Bradley et al. 2000). *FXN* encodes for a mitochondrial protein that is required for iron-sulfur cluster assembly. Mutations in *dfh*, the fly homolog of FXN, isolated through an ERG-based screen, causes ROS-independent ND. *dfh* mutants show an increased accumulation of iron, which induces sphingolipid biosynthesis that in turn causes aberrant activation of the Pdk1/Mef2 pathway, resulting in PR degeneration. These findings were further confirmed in a mouse model for Friedreich's ataxia (Chen et al. 2016a; Chen et al. 2016b). Collectively, these studies uncovered various novel mechanisms of ND underlying metabolic defects.

Lipids and Neurodegeneration

A detailed electron microscopic observation of a subset of mutants which cause mitochondrial dysfunction and ND also showed accumulation of lipid droplets. These include mutations in *sicily*, *marf*, *and aats-met (*Zhang et al. 2013*;* Liu et al. 2015*;* Sandoval et al. 2014*)*. It was found that increased ROS levels due to mitochondrial dysfunction alters electron transport chain activity is the primary cause of a dramatic activation of lipid synthesis. It was found that increased levels of ROS activate c-Jun-N-terminal kinase (JNK), which in turn activates SREBP and thereby increased lipogenesis. It was also found that the excess of lipid is

transported from neuron to the glia, where they form lipid droplets. Lipids also get highly peroxidated in presence of ROS, which causes lipotoxicity and ND. In a recent study, monocarboxylate transporters (MCTs), fatty acid transport proteins (FATP), and apolipoproteins were found to affect LD formation and accumulation in glial cells (Liu et al. 2017). Knocking down these transport proteins in LD-accumulated cells reduced LD accumulation, suggesting that glia-neuron lactate shuttle is important for neuroprotection. Hence, glial lactate is shuttled to the neurons via MCTs, which increases lipogenesis within the neuron. Lipids are then transported back to glia through FAT proteins and apolipoproteins. This study elucidates the importance of lipid homeostasis between neurons and glia in order to sustain neuronal health. The study also established functional homology between human APOE and two fly apolipoproteins (*Glaz* and *naz)*, indicating a conserved mechanism of lipid homeostasis in the brain and its importance in neuronal maintenance and survival.

In a recent forward genetic screen based on impaired visual behavior, mutations in the gene *pect* was identified (Tsai et al. 2019). *pect* is involved in the biosynthesis of specific species of phosphatidylcholine (PC) and phosphatidylethanolamine (PE); *pect* mutants exhibited progressive axonal degeneration and loss of synaptic markers. Mutations in *pect also* activate SREBP, which is a transcription factor with many effectors including lipid synthesis and synaptic function markers. Impaired lipid biosynthesis leads to impaired autophagy and hence results in bloated axon terminals and light-dependent axon degeneration. The activation of SREBP also represses transcription of several genes involved in synaptic function, which accounts for the loss of synaptic markers (Tsai et al. 2019). This study hints at a novel function for SREBP as a feedback mediator for altered phospholipid levels in the cell, to reduce synaptic vesicle pool at the axon terminal by affecting synaptic vesicle biosynthesis or utilization. These studies effectively reflect that relevance of lipid homeostasis in neuroprotection.

Intracellular Transport Defects and Neurodegeneration

Yamamoto et al. 2014 also identified, mutants of genes which affect intracellular vesicle transport such as *Crag* and *Vps26* were also isolated. Similar to *lrpprc2*, mutations in *crag* also lead to light-dependent PR degeneration (Xiong et al. 2012). The human homolog of *crag* is *DENND4*, which has been implicated in Usher syndrome (OMIM #276900, #276901). Crag is a GTP exchange factor (GEF) for Rab11 and was found to be required for the transport of newly synthesized Rh1 to the rhabdomeres. Importantly, expressing DENND4 in *crag* mutants rescues PR degeneration, suggesting *crag*'s function is conserved in humans. Loss of *vps26* also causes light-dependent PR degeneration due to Rh1 recycling defects, and expressing its human homologs can rescue the degeneration phenotype (Wang et al. 2014a). *vps26* forms a retromer with *vps35*, to promote protein and lipid recycling from the plasma membrane. iPLA2-VIA, a phospholipase, binds to the retromer and enhances its function. Thus, loss of iPLA2-VIA disrupts the retromer function, inducing ceramide accumulation along with reduced membrane fluidity, leading to ND. Interestingly, several types of Parkinson's disease (PD) show elevated levels of

ceramides (Brodowicz et al. 2018), and *vps35* has been directly implicated in multiple cases of PD (Williams et al. 2017). Interestingly, loss of *vps26* also displays synaptic dysfunction and has been also linked to late-onset Alzheimer's disease (Muhammad et al. 2008). These studies collectively reflect that there is a conserved, mechanistic link between sphingomyelin metabolism and neural maintenance.

Proteotoxicity in Neurodegeneration

Proteotoxicity can be induced in a condition where misfolded proteins are not cleared by cellular machinery (Morimoto 2008). Several mutants identified through genetic screens for ND phenotypes in flies were found to cause proteotoxicity leading to neuronal demise. For example, NMNAT was found to act as a chaperone to protect cells from proteotoxicity, and mutations in *nmnat*, the gene identified in a forward genetic screen, resulted in ND in flies (Zhai et al. 2006). *nmnat* (human, *NMNAT1,-2,-3*) encodes *Nicotinamide mononucleotide adenylyltransferase*, an enzyme involved in NAD synthesis. Overexpressing the enzymatically dead form of NMNAT was shown to be protective against neuronal-activity-induced ND and spinocerebellar ataxia 1 (SCA1)-induced ND (Zhai et al. 2008). Interestingly, under conditions of heat stress, an alternatively spliced protein form of *nmnat* is expressed, which has a protein refolding activity. Hence, the expression of the alternatively spliced NMNAT reduces proteotoxicity, and this specific function of the protein is neuroprotective (Ruan et al. 2015). These are few of the initial studies which highlighted neuroprotective mechanisms against proteotoxicity.

Identification of Novel Regulators of Autophagy in Neurons

Autophagy is a protective mechanism in place to maintain cellular homeostasis and regulate various signaling pathways. It is a bulk degradation system, and defects in this system is a hallmark of many NDDs (Kim et al. 2017; Menzies et al. 2017). We will discuss below a few mutants which impair autophagy and thus exhibit ND. *cac* encodes the α1 subunit of a *Drosophila* voltage-gated calcium channel (VGCC). Mutations in *cac* lead to defects in synaptic transmission and photoreceptor degeneration (Yamamoto et al. 2014; Tian et al. 2015), and the human homolog of *cac*, *CACNA1A*, has been implicated in multiple NDDs including SCA6 (OMIM #183086). It was found that *cacophony (cac)*, as well as its mouse homolog CACNA1A, localizes on the lysosome and mediates fusion of the lysosome with autophagic vacuoles. Consequently, loss of Cac/*CACNA1A* results in autophagic defects, accumulation of autophagic vacuoles, and induces degeneration in both neurons and synaptic glial cells.

wacky, the fly homolog of *WAC* (WW domain-containing adapter with coiled coil), is essential for energy homeostasis in the cell and, thereby, neuronal survival. *wacky* regulates the assembly of the TTT-pontin/reptin complex, and in the absence of Wacky, TTT fails to activate mTOR which leads to elevated levels of autophagy, culminating in cell death and ND (David-Morrison et al. 2016). Interestingly, loss of *WAC* has a similar effect in mammalian cell lines and has been implicated in DeSanto-Shinawi syndrome (OMIM #616708), wherein all patients with mutations in *WAC* displayed intellectual disability along with dysmorphic facial features.

ubiquilin gene *(ubqn)* codes for a ubiquitin-binding protein, and its human homolog (*UBQLN4*, *UBQLN2*) has been implicated in ALS (Teyssou et al. 2017). In flies, *ubqn* mutants show progressive neuronal and glial degradation, along with mitochondrial accumulation (Şentürk et al. 2019). Interestingly, *ubqn* mutants show suppression of TOR activity but reduced autophagic flux, which is counterintuitive since suppressing mTOR increases autophagy (Lin et al. 2015). Şentürk et al. 2019 focused on explaining this perplexing phenomenon and were able to delineate an alternative role for *ubqn* in lysosomal function. Ubqn was found to interact with the V-100 subunit of the V-ATPase on the lysosomal membrane, and aid in its clearance. Consequently, loss of *ubqn* leads to the accumulation of the V-100 and altered ATPase activity in the lysosome, which in turn reduces lysosomal acidification and hence reduced autophagic flux. Accumulation of V-100 also induces an unfolded protein response (UPR) stress and endoplasmic reticulum (ER) stress. These studies shed light on the relevance of autophagy and organelle clearance in cellular quality control and neuroprotection. Thus, forward genetic screens have not only helped us understand the function of newly discovered disease-associated genes but have also played an essential role in improving our understanding of different pathological mechanisms underpinning the complex phenomena of ND.

Modifier Screen on *Drosophila* NDD Models

Genetic modifier screens in *Drosophila* have been instrumental in identifying new genes in a pathway or assigning cellular processes/pathways to the gene under investigation (St Johnston 2002). Such screens aim to identify dominant enhancers or suppressors of phenotypes caused by manipulation of the gene of interest. In order to identify pathogenic mechanisms linked to mutations in human genes, reverse genetics studies have been successful in developing fly models with ND phenotypes. Genetic modifier screens in these NDD models have further helped to identify novel genetic interactors. In this section, we will discuss a few examples of the genetic modifier screens which give us a better understanding of the mechanism of ND in Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), Alzheimer disease (AD), and PolyQ disorders.

Genetic Modifier Screens for PD

PD is characterized by the loss of dopaminergic neurons in the substantia nigra, leading to various locomotor and cognitive defects (Poewe et al. 2017). Mutations in *SCNA*, *LRRK2*, *PINK*, *PARK*, *ATP13A2*, *GBA*, *Omi/HtrA2*, *PLA2G6*, *FBXO7*, *GIGYF2*, *VPS35*, and *UCHL1* are linked to PD (Dung and Thao 2018; Genetic Home Reference, NIH https://ghr.nlm.nih.gov/condition/parkinson-disease). Except for *FBXO7*, *ATP13A2*, and *SNCA*, other PD genes are conserved in flies. Reverse genetics studies and genetic modifier screens for PD associated genes in flies have linked mitochondrial dysfunction, aberrant lysosomal activity, and

synaptic dysfunction to PD pathogenesis (Hewitt and Whitworth 2017; Xiong and Yu 2018; Dawson et al. 2018; Nguyen et al. 2018; Karimi-Moghadam et al. 2018).

LRRK2

Mutations in the fly homolog of *LRRK2* causes ND, as does overexpression of either wild-type or mutant form of human *LRRK2* (Lee et al. 2007; Liu et al. 2008). Genetic modifier screens for *LRRK2*-mediated phenotypes have identified its role in several biological processes such as vesicular trafficking, TOR pathway, protein translation, and apoptosis (Linhart et al. 2014; Marcogliese et al. 2017; Lavoy et al. 2018; Matta et al. 2012; Gehrke et al. 2010; Chuang et al. 2014). For example, a small-scale modifier screen identified that the removal of one copy of *endophilinA* can rescue endocytosis phenotype of *LRRK2* mutant. It was further found that LRRK2 mediates phosphorylation of endophilinA and thereby regulates vesicular trafficking (Matta et al. 2012). In an independent study, induced pluripotent stem cells (iPSC) derived from patients with *LRRK2* mutation were found to accumulate autophagic vesicles among other defects (Sánchez-Danés et al. 2012). Although the role of LRRK2 in endocytosis, vesicular transport, and autophagy has been shown in several studies (Manzoni 2017; Pan et al. 2017; Migheli et al. 2013; Kim et al. 2018), its implications in PD are not clear. Another modifier screen identified the genetic interaction of *LRRK2* with Akt. Further, it was shown that LRRK2 protects DA neurons in flies by phosphorylating Akt at Ser473 to inhibit FOXO1-mediated apoptosis (Chuang et al. 2014). Later studies in patient-derived iPSCs with *LRRK2* mutations found that the low Akt phosphorylation affects neuronal health (Ohta et al. 2015; Lin et al. 2016), indicating the significance of such modifier screens in flies.

Pink1 and Park

Flies lacking *park* or *Pink1* exhibit ND phenotypes such as reduction of DA neurons, motor dysfunction, and altered wing posture (Whitworth 2011; Haelterman et al. 2014; Nagoshi 2018). Genetic interaction studies revealed that *park* and *Pink1* act in a common pathway to regulate mitochondrial dynamics (Clark et al. 2006; Park et al. 2006). A number of modifier screens have been conducted to study functions of *Pink1* and *park* and the mechanism of degeneration due to their loss (Liu and Lu 2010; Liu et al. 2012; Vos et al. 2012; Esposito et al. 2013; Fernandes and Rao 2011). For instance, a genetic modifier screen identified that the reduced protein translation or increased autophagy can suppress phenotypes caused due to *Pink1* knockdown (Liu and Lu 2010). Another screen identified that the knockdown of *miro*, *milton*, or *kinesin heavy chain* (*khc*) can rescue the muscle phenotypes in *Pink1* null mutants. The study also showed that Pink1 is required for mitochondrial mobility in motor neurons, and knockdown of *miro*, a protein involved in mitochondrial transport, results in increased mitophagy in a Park-dependent manner (Liu et al. 2012). A dominant modifier screen identified *heix* as a strong enhancer of motor defects of *pink1* mutants. It was found that Heix is required for the synthesis of vitamin K2, which is important for mitochondrial electron transport chain activity. This study implicated Pink1 as a regulator of electron transport chain activity.

By showing that vitamin K2 supplementation can rescue the *pink1* null mutant phenotypes, a putative role for vitamin K2 as a therapeutic drug for *pink-* and *park*-mediated PD was also suggested (Vos et al. 2012).

α-Synuclein (SNCA)

Although flies do not have a ***α***-synuclein (*SCNA*) homolog, overexpression of human SNCA mutant protein in flies can cause loss of DA neurons, aggregated SCNA inclusions, progressive locomotor dysfunction, and retinal degeneration (Feany and Bender 2000; Chen and Feany 2005). A genetic modifier screen for SCNA-mediated phenotypes found that the knockdown of *trap1*, a mitochondrial chaperone, can accelerate the loss of DA neurons and motor dysfunction. The study further showed that the co-expression of *trap1* with mutant *SNCA* (*SNCA*^{A52T}) in human cells rescues mitochondrial morphology defects, while knockdown of *trap1* increases sensitivity to oxidative stress and reduces complex I activity. These results suggest that *SNCA* mutation can induce mitochondrial dysfunction (Butler et al. 2012). Another screen identified *fhos*, which regulates actin-dependent remodeling of the cytoskeleton, as a suppressor of SCNA-mediated mitochondrial defects and ND. Further, it was found that SCNA disrupts the spectrin cytoskeleton, which results in impaired actin dynamics. This subsequently causes actin-mediated Drp1 mislocalization leading to impaired mitochondrial dynamics and ND (Ordonez et al. 2018). These findings point to mitochondrial dysfunction being the central cause of PD.

Modifiers of ALS Linked Genes

Amyotrophic lateral sclerosis (ALS) or Lou Gehrig's disease is an NDD arising from the loss of upper and lower motor neuron function and most often leading to fatality due to respiratory failure. Genes linked to familial ALS such as *SOD1, VAPB, TDP-43, FUS, TAF15, EWSR1, C9orf72,* and *hnRNPA2* have been extensively studied in flies to provide mechanistic insight into their cellular function as well as ALS pathogenesis (Van Damme et al. 2017; Zhang et al. 2018). Key findings of modifier screens for *FUS, SOD1, TDP-43,* and *C9orf72* are discussed in the following sections.

FUS (Fused in Sarcoma)

Dysfunction of *cabeza* (*caz*), the fly homolog of *FUS*, leads to a variety of defects such as retinal degeneration, altered synaptic function, and abnormal locomotor behavior leading to eclosion defects (Frickenhaus et al. 2015; Baldwin et al. 2016; Shahidullah et al. 2013). A dominant suppressor screen identified that the loss of one copy of *xrp1*, a chromatin binding protein, partially rescues motor performance in *caz* mutants (Mallik et al. 2018). The study further revealed that the loss of *caz* causes upregulation of *xrp1*, which leads to a dramatic change in gene expression. This dysregulation in gene expression as well as motor functional defects were

substantially rescued by heterozygous loss of one copy of *xrp1*, suggesting that the gene dysregulation due to FUS dysfunction is critical for ALS pathogenesis.

C9orf72 with Hexanucleotide (G4C2)

Expression of human *C9orf72* with hexanucleotide (G4C2) repeat expansion, the most common cause of ALS and frontotemporal dementia (FTD), also results in ND phenotypes in *Drosophila* (Mizielinska et al. 2014; Stepto et al. 2014). A modifier screen for retinal degeneration caused by G4C2 repeats identified that the ND phenotype can be suppressed by expressing RanGAP, a regulator of nucleocytoplasmic transport (Zhang et al. 2015). Another independent large-scale screen for modifiers of ND phenotype in flies expressing G4C2 repeats identified 18 genes that encode proteins required for the nuclear pore complex, the export of nuclear RNA, and the import of nuclear proteins (Freibaum et al. 2015). The data gleaned from these screens suggest that the nucleocytoplasmic transport defects are a recurring feature in ALS.

VAPB

ALS8 locus was mapped to *VAMP-associated protein B* (*VAPB*) gene. A dominant missense mutation P56S in *VAPB* causes the protein to misfold and form cellular aggregates along with wild-type *VAPB*, causing cytoplasmic inclusions resulting in ND (Nishimura et al. 2004). A genetic screen to identify interactors of *Drosophila VAPB* identified a number of genes involved in cellular energetics and homeostasis (Deivasigamani et al. 2014). The study found that the knockdown of *TOR* can suppress the phenotype caused by overexpression of mutant *VAPB* (VAP^{P58S}). More recently, it was further shown that the aggregation caused by VAP^{P58S} can be suppressed by TOR knockdown, which resulted in increased proteasomal activity (Chaplot et al. 2018). These findings indicate that the altered TOR activity may be involved in pathogenic mechanisms in ALS8.

TDP-43

TDP-43, which is mapped to ASL10, is a nuclear ribonucleoprotein that functions in RNA processing and metabolism, including RNA transcription, splicing, transport, and stability. TDP-43 dysfunction in *Drosophila* leads to motor neuron and muscle degeneration (Diaper et al. 2013; Feiguin et al. 2009). Through a genetic screen, mutations in *GSK3*, *hat-trick*, and *xmas-2* were found to suppress age-dependent ND caused by expression of mutant *TDP43* (Sreedharan et al. 2015). Interestingly, pharmacological inhibition of GSK3 was independently found to improve the survival of human motor neurons derived from ALS-patient-iPSCs (Yang et al. 2013). In another modifier screen for ND phenotype exhibited by mutant *VCP* (also linked to ALS), it was identified that the dose reduction of *TDP-43* suppresses the phenotype. It was further shown in human and mouse cells that the cytoplasmic translocation of *TDP-43* is enhanced in the *VCP* mutant and thus mediates the pathogenesis. This finding further validates the results of the modifier screen (Ritson et al. 2010).

Modifier of Alzheimer Disease Linked Genes

AD is an autosomal dominant disorder, which is characterized by the presence of intracellular neurofibrillary tangles (NFT) and extracellular amyloid plaques in the brain causing progressive dementia. AD has been mapped to more than 10 genes, of which mutations in *amyloid precursor protein (APP*), *presenilin 1 (PSEN1)*, *presenilin 2 (PSEN2)*, and *apolipoprotein E* (*APOE*) are the most common genetic cause of AD. Fly models for AD have shown to exhibit ND, Aβ aggregation, as well as synaptic and behavioral impairments (Greeve et al. 2004; Tabuchi et al. 2015; Gerstner et al. 2017). Modifier screens using AD fly models have paved the way to understand AD pathogenesis in human. One such screen to identify the different players involved in retinal degeneration caused by overexpression of Aβ implicated 23 genes linked to a number of cellular processes such as secretion, cholesterol homeostasis, and regulation of chromatin structure (Cao et al. 2008). In a similar screen identified 53 genes that modify ND phenotype caused by Psn (*Drosophila* homolog of PSEN1 and PSEN2) and APP (Van de Hoef et al. 2009). This screen identified a fly homolog of ACE, which was previously found to be linked to AD (Elkins et al. 2004; Narain et al. 2000). This screen also identified genes involved in calcium homeostasis, which has been increasingly studied in the context of AD. A subsequent study found that mutations in Psn can cause calmodulin-dependent depletion of intracellular calcium, reiterating the role of Psn in calcium homeostasis (Michno et al. 2009). Recently, altered calcium homeostasis was also seen in both the AD mouse model and human cell culture experiments (Lerdkrai et al. 2018; Popugaeva et al. 2017). Increased apoptosis has been documented in AD patients and animal models (Obulesu and Lakshmi 2014; Zhao et al. 2016; Jeong 2017), suggesting apoptosis to be one of the major characteristics of AD pathology. In a screen to identify modifiers of APP-induced cell death, *dfoxo* downregulation was found to suppress APP-induced (Wang et al. 2014b). Further experiments in human cells found that the intracellular domain of APP physically interacts with FOXO. This interaction leads to nuclear translocation of FOXO and promotes transcription of a pro-apoptotic gene, *bim*. These results suggest a potential role of APP in the increased apoptotic phenotype seen in AD.

Modifier of PolyQ Disorder-Related Genes

The family of PolyQ expansion diseases includes Huntington's disease, spinocerebellar ataxias (SCA type 1, 2, 3, 6, 7, 17), dentatorubral-pallidoluysian atrophy, and spinal and bulbar muscular atrophy. Overexpression of different proteins with PolyQ expansion in flies has been shown to cause ND, and these serve as models to study their pathogenic mechanisms (Xu et al. 2015; Koon and Chan 2017). The modifier screens for pathogenic PolyQ stretches have identified several genes involved in protein folding, protein degradation, histone deacetylation, as well as vesicular transport (Kazemi-Esfarjani and Benzer 2000; Branco et al. 2008; Cohen-Carmon and Meshorer 2012; Bilen and Bonini 2007; VoSSfeldt et al. 2012). A

recent approach, which combines cell-based and *Drosophila*-based modifier screens, found that the downregulation of RAS-MAPK-MSK1 can suppress ATXN1(82Q)-induced ND phenotype. Further, MSK1, a kinase involved in the MAPK pathway, was shown to phosphorylate ATXN1(82Q) at S776 leading to aggregate formation (Park et al. 2013). This is in accordance with studies which show several posttranslational modifications involved in PolyQ aggregate formation and disease progression (Wan et al. 2018).

Drug Screens

NDDs are still incurable, and the treatment has been mostly symptomatic. Therefore, there is an urgent need to dish out therapeutic strategies to tackle them. The most common way to find lead compounds for therapy is a small molecule screen on animal models, which involves treating animals with different compounds to find the ones which can ameliorate the disease symptoms. The success of fly models in recapitulating human ND phenotypes has allowed several unbiased drug screens on *Drosophila* NDD models. These screens have led to the identification of either potential drug targets (Rajendran et al. 2008; Jimenez-Sanchez et al. 2015; Aperia 2007) or suitable drugs to treat an ND (Outeiro et al. 2007; Qurashi et al. 2012; Lawal et al. 2014). In addition to the convenience of fly models, the concept of drug repurposing has also accelerated the drug discovery process. For instance, in a *Drosophila* model for *LRRK2*-associated Parkinsonism, Lovastatin was identified as the most effective FDA-approved drug to modify and ameliorate the disease symptoms (Lin et al. 2016). Lovastatin significantly rescued neurite degeneration by inhibiting GSK3β activity and restored motor disability in the Parkinson's model. Lovastatin, along with its therapeutic effects, also had the highest lipophilicity among its contenders and hence, has a potential pharmacotherapeutic application in Parkinsonism. In another study, a siRNA screen conducted in HEK293 cells expressing human huntingtin (HTT) with 138 PolyQ identified 257 modifiers of mutant HTT toxicity (Jimenez-Sanchez et al. 2015). A secondary screen was further conducted in *Drosophila* for in vivo validation, which led to the identification of glutaminyl cyclase (QPCT) as a good druggable candidate. Hence, a series of compounds were developed to inhibit QPCT and thus rescue the Huntington's disease phenotype in flies and human cell lines. Although several drug screens have conferred a large body of druggable targets and small molecule inhibitors, the real challenge lies in prioritizing and filtering them for clinical trials. It is in such scenarios fly models greatly aid in rapidly delivering the optimal compound for clinical trials and fast-tracking the drug discovery process.

Human Genetics on the Fly

Fly Screens and the Discovery of New Human NDD Gene

While genes identified through forward genetic screens have helped in the understanding of pathogenic mechanisms, they have also aided in the identification of mutations in human genes causing NDD. One such example is the identification of *MARS2* mutations, which cause *ARSAL* (autosomal recessive spastic ataxia with leukoencephalopathy, OMIM #611390), characterized by cerebral atrophy along with spasticity. Mutations in *aats-met*, the *MARS2* homolog in flies, were identified through a forward genetic screen, and the mutant displayed mitochondrial dysfunction and ND (Bayat et al. 2012). *MARS2* is located on chromosomal interval 2q33.1, a locus which was previously associated with *ARSAL* (Thiffault et al. 2006). Consequently, patients with *ARSAL* were tested, and they all had complex rearrangements in *MARS2* locus (Bayat et al. 2012). Hence, identification of *aats-met* successfully implicated *MARS2* with *ARSAL*. Similarly, in another screen for ND phenotypes, mutations in *Drosophila nardilysin1* (*dNrd1)* were identified. *dNrd1* was found to be important for proper refolding of a-ketoglutarate dehydrogenase (OGDH), which is required for converting a-ketoglutarate to succinyl-CoA in the tricarboxylic acid cycle (TCA) cycle. An inquiry into whole exome sequencing data, in collaboration with clinical geneticists, revealed mutations in *NRD1* and *OGDH* in two families with patients suffering from NDD and ataxia (Yoon et al. 2017). To test whether the variants identified from patients are disease-causing, they were tested in flies. Nrd mutant flies could be rescued by the expression of wild-type NRD1 or OGDH cDNA but not by overexpressing the cDNA carrying patient-specific variants, suggesting that the variants are deleterious and likely to cause disease. Among the other genes identified in the same screen, *CRX (Drosophila ocelliless)* and *DNM2 (Drosophila shibire)* were found to be associated with bull's eye maculopathy and Charcot-Marie-Tooth disease (CMT) respectively, by WES analysis of patients. These studies are excellent examples of the discovery of novel human NDD genes using phenotypic studies in flies and have potentially paved the way for similar studies in the future. The relevance of such studies lies in aiding clinicians and human geneticists to understand complex ND traits by prioritizing the array of genetic variants identified in WGS studies. Therefore, genetic screens have not only contributed to understanding certain fundamental pathways involved in neural maintenance and protection, but they have also resulted in the successful association of mutation-carrying genes to undiagnosed NDD.

Validation Screens for Big Data

Recent technological advancements such as whole-genome sequencing, transcriptomics, and proteomics have allowed quick generation of big data. For example, in recent years whole-genome sequencing (WGS) is being increasingly used to identify disease-causing mutations, especially for discovering mutations causing rare

genetic disorders (Brown and Meloche 2016; Pang et al. 2017; D'Argenio 2018). WGS/WES of a patient results in a large number of variants, which may be deemed deleterious by bioinformatic analysis. Moreover, the lack of functional understanding of majority of the human genes, as well as the difficulties in assaying the deleterious mutation, is a major challenge to identify a disease-causing variant accurately and, hence, avoid misdiagnosis (Manrai et al. 2016; Molster et al. 2018). The solution lies in a multi-way approach, wherein the variants from WGS are used to diagnose an ND but the burden to prove causality is shared by molecular geneticists using model organisms(Chakravarti et al. 2013; Chong et al. 2015; Richards et al. 2015). As established in earlier sections, *Drosophila* models can be used to effectively study gene variants and establish causality (Chakravarti et al. 2013; Bilder and Irvine 2017; Edwards et al. 2013; Lehner 2013; Fernius et al. 2017; Langellotti et al. 2018; Oriel and Lasko 2018). For instance, WES of seven individuals with epilepsy and a variety of neurological defects led to the identification of variants in IRF2BPL. These variants were then tested in *Drosophila*, and strikingly, all variants behaved like loss-of-function alleles causing ND in flies (Marcogliese et al. 2018). The fly homolog of *IRF2BPL*, *pits*, was found to be important for neural development and maintenance. Further studies can shed light on the molecular mechanism of *IRF2BPL/pits* and also aid in treating such devastating disorders. In another study, variants of the gene *RHOBTB2* were identified. All the patients carrying the mutations had developmental and epileptic encephalopathy (Straub et al. 2018). The mutations in RHOBTB2 impair its proteasomal degradation in vitro and its overexpression in the fly brain results in bang sensitivity, seizure susceptibility, and locomotor defects. Such studies successfully establish a relationship between a phenotype and its causal gene using *Drosophila*. Moreover, a large number of studies wherein several genes have been identified by WGS/WES (Makrythanasis et al. 2016; Martin et al. 2017; Moskowitz et al. 2016) can be validated using *Drosophila*. Genome-wide association studies have implicated a large number of common alleles to NDDs (Shulman 2015; Wangler et al. 2017). But they fail to account for many sporadic cases of NDD in the population. This calls for the potential role of rare gene variants in the population to affect pathogenesis. For instance, in a large-scale WES study of patients with sporadic PD, 27 gene variants were identified (Jansen et al. 2017). Using functional screening in flies and co-expression analysis with publicly available WES data, the study implicated several rare gene variants including *VPS13C, PTPRH,* and *ARSB*, which enhance alpha-synuclein-mediated toxicity, and several other variants such as *GPATCH2L, PTCHD3, SVOPL,* and *ZNF543,* which affect mitochondrial morphology. A similar study with AD patients implicated a variant of *TM2D3* with late-onset AD and revealed the gene's functional conservation in flies (Jakobsdottir et al. 2016). Strikingly, expressing *TM2D3* in flies with a mutation in *amx* (fly homolog of *TM2D3*) rescued neurogenic phenotypes and lethality. This trend toward whole-genome reverse genetics studies, through a quality alliance between human and *Drosophila* geneticists to discover new genes linked to ND, will make exploration of the genetic landscape of any disorder more convenient and efficient.

Conclusion

The brain requires a large and complex repertoire of genes for optimal function, and hence pathogenesis of ND can be very diverse. NDDs have been for a very long time, largely incomprehensible, and finding therapeutic solutions for them has been tedious and mostly ineffective in long term. Development of different animal models of ND has improved our understanding of neural maintenance. Fly models have been quite valuable for the discovery of novel genes linked to NDDs and in understanding their associated molecular mechanisms. With the constant evolution of intellectual and technical advances in *Drosophila* genetics, it continues to be an extremely powerful model system to address pertinent biological questions.

Genetic screens designed to tackle ND-associated phenotypes in *Drosophila* have been a great source to understand the genetic complexity that we harbor and has encouraged the identification of several potential targets for therapy. Additionally, the discovery of new pathogenic mechanisms of ND and the diagnosis of new NDDs have contributed hugely to the translational quality of *Drosophila* as a model system. With the advent of high-throughput screening methods, multilayered screens involving different "omics" approaches along with in vivo validation screens in *Drosophila* through collaborative efforts, involving clinical geneticists and pharmacologists, will be crucial for a greater understanding of NDD and drug discovery.

Important Resources

MARRVEL
Flybase
SysID database
NeuroX
ExAC
CHARGE
InterMine
Genematcher
Phenodb
denovo-db

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Drosophila Neural Stem Cells: A Primer for Understanding Mammalian Neural Development and Disease

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Abstract

Drosophila is an established model for over a century to study the genetic, epigenetic, and molecular aspects of various cellular processes. Conservation of gene regulatory mechanisms, signaling pathways, and homology of over 75% of fly genes with mammals have helped us understand diverse aspects of human biology. *Drosophila* neural stem cells (NSCs) or neuroblasts (NBs) were first identified in the nineteenth century; since then, they are being used as a model to understand the underlying mechanisms of the stem cell fate determination. The countless possibilities to manipulate the fly genome prove advantageous to address complicated questions of stem cell biology.

Stem cell lifecycle is dynamically regulated and is far more intricate than the normal cells. Stem cells have the property to self-renew, differentiate, or undergo dormancy until they are required again. Moreover, NSCs generate diverse progeny to perform specialized functions and are capable to end their life either by apoptosis or exit the cell cycle after fulfilling the required purpose. How are these complicated and yet organized cellular processes to make a functional nervous system regulated? What are the cues involved in the process? Are they all intrinsic to NSCs or does the stem cell environment have a role to play as well? In this chapter, we have discussed and summarized the information available to address these questions. We have reviewed and compared various conserved aspects of fly NSC biology with mammalian NSC behaviors. It is interesting to learn that the stem cells do not function in isolation and the systemic signaling and cues from its micro and macro environments play distinct roles to regulate the NSC

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fate decisions. Thus, a better understanding of cell intrinsic and cell extrinsic signaling and how they communicate and function in sync with the environment is necessary for effective use of stem cells in translational research.

Keywords
Neuroblast · NSCs · Quiescence · Apoptosis · Asymmetric division

Introduction

Stem cells are characterized by their extraordinary capability to self-renew through cell division and simultaneous production of a pool of differentiated cell types. The pluripotent early embryonic stem cells have enormous plasticity and generate all the three germ layers (Temple 2001). On the contrary, more specialized tissue-specific stem cells or adult stem cells that can develop only into definite progenitors are required for tissue homeostasis. The embryonic neural stem cells (NSCs) are ectodermal in origin and differentiate into a wide array of neurons, astrocytes, and gangliocytes (Kintner 2002). Neurogenesis was reported to be lacking in the adult life; however, recently, the adult nervous system (NS) has also been shown to possess NSCs. These findings have raised hopes and expectations to use NSCs for tissue repair and regeneration in diseased or damaged tissue (Ma et al. 2009; Morshead et al. 1994).

Regenerative medicines and tissue engineering advancements have created immense possibilities to treat several human ailments. There are earnest expectations to harness the therapeutic potential of NSCs for repair and regeneration of various nervous system-related disorders ranging from cancer to neural loss. However, our knowledge of the basic biology of NSC is still limited. We know less about the factors that regulate the fate of NSC and their progeny. Therefore, to win the marathon of translational research, it is simultaneously important to study the language of the cells. The primary goal is to understand how the multipotent state of NSC is controlled and how their progeny undergoes distinct cellular fates. The fundamental understanding of what is "normal" to the cell would help us appreciate what went wrong under disease conditions.

Due to ethical issues and limited availability of human samples, various model organisms are used to study the intricate processes related to human development and multiple disorders. Several milestone discoveries using a vast array of model organisms ranging from bacteria to mammals have enriched our knowledge about human biology. Here we have reviewed the insights of NSC biology from *Drosophila* and drawn parallels with the mammalian systems. The *Drosophila* nervous system has been intensively used to understand the cellular and molecular mechanism of NSC proliferation, their temporal and spatial specification, quiescence, and death. The fundamental similarities between the organization of neuroepithelium of *Drosophila* and mammalian cerebral cortex have again prompted the use of *Drosophila* as a model to study NSC biology. There are several excellent

publications on the individual areas related to NSC fate determination and behavior. Thus, for the easy accessibility, a list of reviews which appeared in the last 5 years, is made and we highly recommend them for further in-depth understanding (Table 1). The deep fundamental insights from *Drosophila* NSC biology would be of enormous significance to understand human neural health and disorders.

In this chapter, we will discuss the life of an NSC from its birth to the final fate determination and will shed some lights on its interaction with the surrounding environment during the journey. We will highlight the known cell intrinsic molecular signaling pathways that influence NSC behavior and also emphasize how the communication between NSC and its niche performs the instrumental role to control NSC fate in the central nervous system (CNS). Thus, an integrated view with available information of the mammalian system in a similar context will be provided.

Neural Stem Cells in *Drosophila* Nervous System: Their Types and Behavior

The fly central nervous system (CNS) consists of the central brain, optic lobes, and ventral nerve cord (VNC) and comprises nearly 200,000–300,000 neurons (Fig. 1a). The NSCs in fly CNS are more popularly known as neuroblasts (NBs) (Wheeler 1891; Wheeler 1893); thus, we are using NSCs/NBs synonymously throughout the text. Most of the NBs in *Drosophila* CNS are embryonic in origin (Hakes et al. 2018). Each central brain lobe has about 105 NBs, whereas 800 NBs are there per optic lobe (Egger et al. 2007). Besides, there are four mushroom body NBs (mbNBs) in the central brain (Fig. 1a). The long elongated tapering structure of CNS is the VNC, which can be further divided into segmental units called neuromeres. The VNC possesses three gnathal, three thoracic, and seven abdominal segments (Egger et al. 2008; Nériec and Desplan 2016; Skeath and Thor 2003) along with a non-segmented telson in the posterior end (Jorgens, G. 1987). In the embryo, each of these VNC neuromeres is further subdivided into two hemi-neuromeres, comprising about 30 NBs per hemi-neuromere in the thoracic and abdominal parts, while the gnathal and the telson neuromeres carry a reduced NB-set (Birkholz et al. 2013). Remarkably, each *Drosophila* NB can be individually identified based on its position, time of birth, and transcriptional expression pattern (Schmidt et al. 1997a; b).

Years of research on *Drosophila* have deciphered the molecular mechanism by which an individual NB in the CNS always gives rise to a stereotyped family of neurons and glia. Detailed discussion on the lineage identity is beyond the scope of this chapter, and the references mentioned here can be consulted for exceptionally meticulous description (Ito et al. 2013; Ming and Song 2011; Schmid et al. 1999, 1997a, b; Yu et al. 2013). The majority of *Drosophila* NBs divides asymmetrically, a process in which a stem cell self-renews and produces intermediate progenitor cells (discussion in a separate section later). All NSCs express the Dpn (Deadpan) protein, a mammalian ortholog of HES helix-loop-helix transcription factor (Vaessin et al. 2007). The asymmetrically dividing NBs are further subdivided into three groups based on their cell division patterns (Fig. 1b–d). Type I *Drosophila* NBs are

Table 1 Relevant reviews in the last 5 years

S.No.	Area	Year	Animal	Title of the paper
1	Self-renewal	2014	*Drosophila*	Stem cell decisions: A twist of fate or a niche market? (Arya and White 2015)
2	Self-renewal	2014	*Drosophila*	It takes two to tango, a dance between the cells of origin and cancer stem cells in the Drosophila larval brain (Januschke and Näthke 2014).
3	Self-renewal	2015	*Drosophila*	Control of neural stem cell self-renewal and differentiation in Drosophila (Janssens and Lee 2014).
4	Self-renewal	2015	*Drosophila,* mammals	Proliferation control in neural stem and progenitor cells (Kang and Reichert 2015).
5	Self-renewal	2017	*Drosophila*	Tissue growth and tumorigenesis in Drosophila: cell polarity and the Hippo pathway (Homem et al. 2015)
16	Self-renewal	2019	*Drosophila*	Polarity in stem cell division: Asymmetric stem cell division in tissue homeostasis (Richardson and Portela 2017)
7	TTFs	2017	*Drosophila*	Temporal patterning in the *Drosophila* CNS (Yamashita et al. 2010)
8	TTFs	2017	*Drosophila*	Playing well with others: Extrinsic cues regulate neural progenitor temporal identity to generate neuronal diversity (Doe 2017)
9	TTFs	2019	*Drosophila*	Temporal control of Drosophila central nervous system development (Syed et al. 2017b).
10	TTFs	2019	*Drosophila*	Temporal patterning of neurogenesis and neural wiring in the fly visual system (Miyares and Lee 2019).
11	Apoptosis	2015	*Drosophila*	Programmed cell death in neurodevelopment (Sato et al. 2019)
12	Apoptosis	2016	*Drosophila*	Programmed cell death acts at different stages of Drosophila neurodevelopment to shape the central nervous system (Yamaguchi and Miura 2015)
14	Apoptosis	2016	*Drosophila*	Control of adult neurogenesis by programmed cell death in the mammalian brain (Pinto-Teixeira et al. 2016).
15	General	2014	*Drosophila*	Drosophila neuroblasts as a new model for the study of stem cell self-renewal and tumor formation (Ryu et al. 2016)
17	General	2014	Mammals	Neural progenitors, neurogenesis and the evolution of the neocortex (Li et al. 2014).
18	General	2015	*Drosophila*	Drosophila central nervous system glia (Florio and Huttner 2014).
21	General	2016	Mammal	Neural stem cells to cerebral cortex: Emerging mechanisms regulating progenitor behavior and productivity (Freeman 2016)
22	General	2016	*Drosophila*	Stepwise progression of embryonic patterning (Dwyer et al. 2016).
23	General	2018	*Drosophila*	Drosophila as a model for developmental biology: Stem cell-fate decisions in the developing nervous system (Sandler and Stathopoulos 2016).
24	General	2019	*Drosophila*	From early to late neurogenesis: Neural progenitors and the glial niche from a fly's point of view (Harding and White 2018).

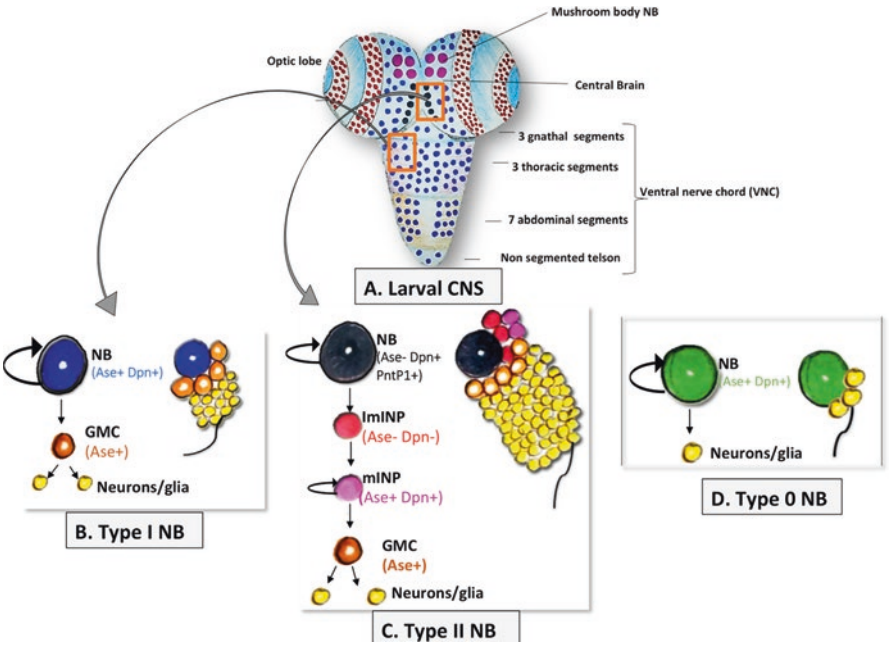

Fig. 1 Different types of neuroblasts (NBs/NSCs) in *Drosophila* larval CNS and their mode of division. (**a**) Schematic of larval CNS showing positions of different types of NBs. Asymmetric division in (**b**) type I NB self-renews and produces GMCs, which divide once and produce progeny neuron/glia (**c**) Type II NB self-renews and produces immature INPs (imINPs), mature INPs (mINPs), and GMCs. The GMCs divide symmetrically and differentiate into two progeny cells (neurons/glia). (**d**) The type 0 NBs also divide asymmetrically to self-renew and the daughter cell directly differentiates into a neuron. The known markers of type I, II, and 0 NBs and their progeny GMC or INP types are mentioned in the parentheses next to them

the most prominent NSC class among all the types. Following asymmetric cell division, the NSCs self-renew and produce a daughter known as ganglion mother cells (GMCs) (Fig. 1b and Fig. 2d) (Bauer 1904). The GMC divides only once and produces two post-mitotic cells that could terminally differentiate into a pair of neurons or glial cells (Fig. 1b). The type I stem cells can be uniquely identified by the presence of Dpn and Ase (Asense) in their nucleus but lack PntP1 (PointedP1) expression, in short can be marked as Dpn + Ase + PntP1- (Fig. 1b) (Jan et al. 2011; Weng et al. 2012).

Another class of NSCs comprises 16 type II NBs that are found exclusively in the central brain and makes adult central complex (Bello et al. 2008; Boone and Doe 2008; Homem and Knoblich 2012; Ito et al. 2013; Izergina et al. 2009; Riebli et al. 2013; Rolland et al. 2008; Viktorin et al. 2011; Yang et al. 2013; Yu et al. 2013). These cells are known to generate different lineages of neural cells in large numbers. Type II NBs can be invariably distinguished from type I NBs, as they express the transcription factors Dpn and PntP1 (PointedP1) but not Ase (Dpn + PntP1 + Ase-) (Jan et al. 2011; Komori et al. 2014; Xiao et al. 2012) (Fig. 1c). Ectopic expression

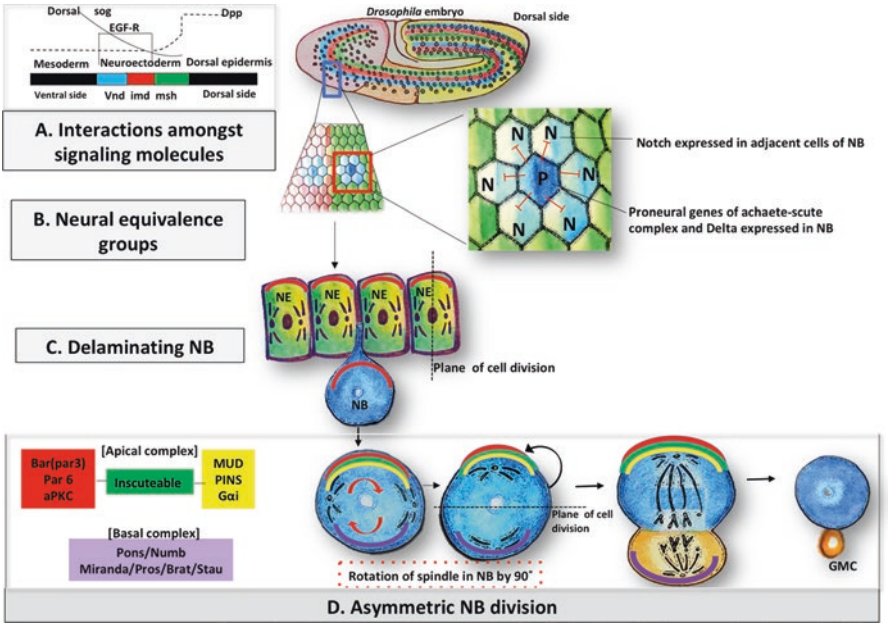

Fig. 2 Neuroblast delamination and asymmetric cell division. (**a**) Specification of neuroectoderm is mediated by the interaction of various signaling molecules. Dorsal activity is high in the mesoderm, and its moderate levels define the neuroectoderm. Dpp is high in the dorsal region, and in neuroectoderm, low activity of Dpp is maintained by its antagonist Sog. EGFR expression maintains the imd and vnd columns in the neuroectoderm. (**b**) The neural equivalence group is specified by different signaling cues that pattern the embryo into dorsal/ventral and anterior/posterior axes and, finally, the columnar patterning mediated by imd, vnd, and msh genes. Delamination of one NB from each neural equivalence group is controlled by Notch, which restricts the high levels of pro-neural gene expression in the delaminating NB only. (**c**) Polarized distribution of the fate determinants and mitotic spindle rotation in the NB guide its asymmetric division. (**d**) The apical complex segregates with the self-renewing NB, whereas the basal complex in the daughter cell promotes its differentiation into GMC

of Ase in type II NBs converts them to type I NBs (Rolland et al. 2008). Similarly, PntP1 misexpression in type I transforms them to type II NB (Jan et al. 2011). Interestingly, instead of producing GMCs immediately after the division, the type II NBs first generate intermediate neural progenitors (INPs) (Bello et al. 2008; Boone and Doe 2008). The newly born immature INPs (imINP) make a quick transition from a Dpn-Ase- to a Dpn-Ase + state, which, upon maturation (mINP), starts expressing Dpn as well as Asense (Dpn + Ase+). Thus, mINPs, more alike type I NBs, asymmetrically divide for quite a few rounds to produce four to six GMCs, which eventually form neurons or glia (Fig. 1c) (Bello et al. 2008; Boone and Doe 2008; Walsh and Doe 2017). Therefore, despite being very less in number, the type II NBs produce many more progeny neurons through intermediates (INPs) and contribute to the complexity of the central complex of the brain. Type II NBs are more similar to the primate cortical lineages and may be a good model to study the mechanism of cortical complexity (Walsh and Doe 2017).

A recently identified class of NSCs is type 0 NBs seen during late-embryonic and early larval optic lobe neurogenesis (Baumgardt et al. 2009; Baumgardt et al. 2014; Desplan et al. 2014; Karcavich and Doe 2005). Interestingly, these type 0 NBs undergo asymmetric division and self-renewal as other NB types do, but the progeny directly differentiates as a neuron without having any intermediate (Fig. 1d). In the CNS patterning from flies to mammals, a larger brain and progressively thinner VNC is a well-conserved feature. Consistently, it is shown that the progenitors proliferate longer and take a shorter time to divide in the brain than the VNC. The conserved PcG/Hox protein plays a vital role in governing the proliferative potential of progenitor and their daughters in CNS of flies and mouse (Yaghmaeian Salmani et al. 2018). The proliferation potential of type I and type 0 NBs and type I > 0 or 0 > I switch in VNC is temporally controlled by the overlapping expression of the Hox gene along the A-P axis (Monedero Cobeta et al. 2017).

Interestingly, there are many similarities between mammalian and fly neural progenitors (Brand and Livesey 2011; Fish et al. 2008; Kriegstein et al. 2006; Lui et al. 2011). The mammalian neocortex possesses different types of neural progenitors or NSCs, such as radial glia (RG), short neural precursors (SNPs), and outer radial glial cells (oRG) (Brand and Livesey 2011; Homem et al. 2015). The radial glia (RGs) is a large class of neural progenitors that give rise to several types of cortical neurons (Franco and Müller 2013; Malatesta et al. 2000; Miyata et al. 2001; Noctor et al. 2001). The RG divides largely as type I *Drosophila* NBs, where it self-renews and makes a localized intermediate progenitor cell (IPC), similar to fly GMC intermediate (Haubensak et al. 2004; Miyata et al. 2001; Noctor et al. 2004). On the other hand, short neural precursors (SNPs) are closer to the type 0 *Drosophila* NBs in their division pattern (Holguera and Desplan 2018). Interestingly, outer radial glial (oRG) cells are very abundant in the primate brain compared to that in the rodent brain and behave more like the type II NBs. The oRG, arise from RG (Hansen et al. 2010) and divide asymmetrically to produce IPCs and a large number of diverse neuronal types sequentially (Hansen et al. 2010; Homem et al., 2015; Kelava et al. 2012).

Neural Stem Cell Birth: Involvement of Notch and Other Cellular Signaling Pathways

Birth of NSCs and their fate determination are extensively studied in the embryonic nervous system of flies. The *Drosophila* NBs are developed from the single layer of neuroectoderm during early embryonic development. The interplay of intricate signaling and dorsal–ventral (D–V) and anterior–posterior (A–P) patterning subdivides the embryo into a chequerboard of unique "neural equivalence groups" (Fig. 2a, b). The cells in the equivalence group get unique transcriptional identity due to the interaction among signaling factors, which in turn decide the fate of these cells. The D–V and A–P patterning of the embryo are regulated by several transcription factors, which form gradients throughout the embryo and regulate gene expression. For example, dorsal (Dl), one of the D–V determinants, expresses at the ventral

side and restricts the decapentaplegic (Dpp) to the dorsal domain and thus prevents the dorsalization of the embryo. Short gastrulation (Sog) protein also forms a gradient defined by low to moderate expression of Dl on the ventral side and antagonizes the Dpp activity in the presumptive neuroectoderm (Fig. 2a) (Francois et al. 1994; Zusman et al. 1988).

On the other hand, localized expression of epidermal growth factor receptor (EGFR) in the neuroectoderm is required for regulation of columnar gene expression and thus columnar patterning of the neuroectoderm along the dorsal–ventral axis. EGFR maintains the expression of ventral nervous system defective (*Vvnd*) and triggers the expression of intermediate neuroblast defective (*Iind*) (Fig. 2a) (Skeath 1998; Von Ohlen and Doe 2000; Zhao et al. 2002). Vnd expresses in the ventral column, Ind expresses in intermediate, and the third columnar gene muscle segment homeobox (*msh*) expresses in the dorsal column. Besides the D–V polarity, the embryo is further divided into segments along the A–P axis by segment polarity proteins such as Engrailed, Wingless, Hedgehog, Gooseberry Distal, and Mirror (Hartenstein and Wodarz 2013).

Each equivalence group contains about five to six cells, but only one of them can become the stem cell (Fig. 2b, c) (Egger et al. 2008; Skeath and Thor 2003). Initially, all the cells in an equivalence group have the potency to become a stem cell, since they all express proneural genes of the achaete–scute (ac–sc) complex (reviewed in Bertrand et al. 2002; Campuzano and Modolell 1992; Cubas et al. 1991; Ghysen and Dambly-Chaudiere 1989; Skeath and Carroll 1992; Skeath and Carroll 1994). However, only the cell that expresses the proneural genes to the highest level becomes an NSC and delaminates from the monolayer of neuroectodermal cells (Fig. 2b, c), while the other cells take the epidermal fate. Interestingly, Notch signaling plays a critical role in restricting the stem fate to one per equivalence group by a mechanism very well known as lateral inhibition (Fig. 2b) (Artavanis-Tsakonas and Simpson 1991; Bray 1998). In a neural equivalence group, the cell that has highest levels of Delta (ligand of the Notch receptor) activates Notch signaling in the neighboring cells to repress the proneural gene expression in those cells. Finally, the lateral inhibition in an equivalence group results in the selection of only one cell as NB holding higher levels of proneural genes (Fig. 2b). As expected, loss of Notch function in the CNS results in a severe neurogenic phenotype, where supernumerary cells adopt an NSC fate (F., P. D. 1939; Greenwald 2012; Struhl et al. 1993).

Various members of the Notch signaling pathway are expressed in embryonic neuroepithelial and radial glial stem cells (RG), as well as in the adult NSC (Durrer et al. 2002; Tokunaga et al. 2007). Although, in mammals, the generation of NSCs from the germ layers does not depend on the Notch signaling, Notch is required in a later window for NSC maintenance and fate determination (Arya and White 2015; Hitoshi et al. 2002). Notch activation is required for the maintenance of neural progenitor character in radial glia, which is widely considered as NSC in mammalian neocortex and outer subventricular zone (OSVZ) (Campos et al. 2001; Pierfelice et al. 2011). High levels of Notch are also shown to maintain the undifferentiated state of NSCs by repressing proneural gene expression (Papers et al. 2001). The Notch signaling acts as a binary switch in many fate decisions, and thus, it is

perhaps not surprising to see that it also plays an instrumental role at various levels during nervous system development even after NB birth. Notch levels have a more profound effect on the survival of type II NBs and the NBs in the central brain and reduced Notch levels lead to a complete loss of these NB types (Bowman et al. 2006; Wang et al. 2006). Contrary to this, the type I NBs in the abdominal region survive longer upon notch knockdown (Arya et al. 2015). Similarly, in the mammalian brain, the Notch signaling also acts as a binary fate selector, which impact various cellular processes in a context-dependent manner during nervous system development. Studies with Notch knockouts suggest that it is implicated both as pro-survival and pro-death signals for neural precursor or progenitors (Mason et al. 2006; Yang et al. 2004).

Key Aspects of Neural Stem Cell Polarity and Asymmetric Cell Division: A Slip Could Lead to Tumor Formation

The most striking aspect of stem cell behavior is its asymmetric division, which results in the formation of two cells of distinct size and fate. The large cell retains stemness, whereas the smaller one soon proceeds to make terminally differentiated progeny (Fig. 2b–d) (Chia et al. 2008; Sousa-Nunes and Somers 2013). What determined the complex nature of asymmetric cell division (ACD) of NBs? During cell division (a) the orientation and asymmetric positioning of the spindle fibers; and (b) polarized distributions of certain protein complexes along the anterior–posterior axis of the cell are some of the major determinants, which creates the asymmetry. ACD and its implications in cancer biology have been extensively researched and reviewed independently (Doe 2008; Li et al. 2014; Sousa-Nunes and Somers 2013; Wodarz and Huttner 2003). In the present context, we are summarizing selected aspects of stem cell proliferation by asymmetric cell division, mainly to discuss the similarities between fly and mammalian systems (Bardin et al. 2004; Betschinger and Knoblich 2004; Chia et al. 2008; Wang and Chia 2005).

The NSCs are derived from the symmetrically dividing neuroepithelium (NE) cells, and the same apico–basal polarity, as seen in the NE cells, is retained in the potential NSCs following its delamination (Fig. 2c). Then why do the NSCs behave differently than the NE cells and divides asymmetrically? There are two aspects to it: One is the plane of cell division, while the other one is the asymmetric length of the spindle fibers and location of the cleavage furrow. While one results in the asymmetric segregation of protein complexes in daughter cells, the other one creates the difference in their size (Fig. 2c–d).

The apical cortex of the NE and NB cells possess Par complex, consisting of Baz (Bazooka), Par6 (partitioning-defective 6), and an atypical protein kinase (aPKC), frequently referred as Baz–Par6–aPKC complex (Fig. 2c) (Kuchinke et al. 1998; Lu et al. 2001; Petronczki and Knoblich 2001; Tepass et al. 1990; Wodarz et al. 2000). Along with the Baz–-Par6-aPKC complex, Lgl (lethal giant larvae), Dlg (discs large), and Scrib (scribble) also localize apically (Albertson and Doe 2003; Ohshiro et al. 2000; Peng et al. 2000). The basally localizing cell fate-determining complex

comprises Pros (Prospero) (Choksi et al. 2006; Hirata et al. 1995; Knoblich et al. 1995; Spana and Doe 1995), Brat (Brain tumor) (Bello 2006; Betschinger et al. 2006; Lee et al. 2006a; Marin et al. 2013), and Numb (Notch inhibitor) (Guo et al. 1996; Knoblich et al. 1995; Spana and Doe 1996), which segregates into the GMCs (Fig. 2c). After ACD of the NB, the apical cortex determines a self-renewal fate, whereas the basal region receives factors, which control the differentiation of fate.

It is fascinating to see that within the epithelial plane, the NE cells divide horizontally, which result in the symmetric distribution of apico-basal fate determinant, contrary to this the respective delaminating NSCs divide perpendicular to the NE plane (Fig. 2c, d). The initial step for the switch from symmetric to asymmetric division involves a 90° rotation of the mitotic spindles (Kaltschmidt et al. 2000; Rebollo et al. 2009) (Fig. 2d). This rotation places spindles to apical-basal orientation, which is always perpendicular to the overlying NE. It allows a polarized distribution of the cell fate determinants in the daughter cells pushing them toward either self-renewal or differentiation pathways (Fig. 2c, d). In the NBs, another apically localizing protein complex, which is in the center to the correct spindle orientation, consist of Pins (Partners of ins), G-protein Gαi complex, Cno (Canoe/Afadin) and Mud (Mushroom body defect) proteins (Izumi et al. 2006; Knoblich et al. 2006; Schaefer et al. 2000; Schaefer et al. 2004; Siller et al. 2006). The Par complex in the apical cortex binds with Inscute (Insc), together they unite with Pins, which is finally activated by the binding of G-protein Gαi complex. Subsequently, the complex of Mud with astral microtubules ensures the proper alignment of spindles with the axis of cortical polarity (Fig. 2d) (reviewed in Hartenstein and Wodarz 2013; Li et al. 2014). Additionally, the Pins- Gαi-Mud complex also stimulate a basal shift in cleavage furrow, thus generating daughters of different sizes (Cabernard et al. 2010; Knoblich 2010). During cell division, several cell cycle regulatory complexes such as Cdc2/Cyclin-B, B, and Aur-A kinase are described to work further in the maintenance of the apical complexes (Li et al. 2014; Tio et al. 2001; Wang et al. 2006). These complexes segregate into the renewing NB, whereas the basal cortex segregates as GMC is destined to differentiate.

The differentiation-inducing determinants such as Brat, Pros, Numb, and the RNA-binding protein Stau are anchored with Mira and Pon that finally direct their localization toward the basal cortex of the progenitor cells (Sousa-Nunes and Somers 2013) (Fig. 2d). Mira interacts and holds the Brat, Pros, Numb, and the RNA-binding protein Stau to suppress stem cell fate in potential GMCs, whereas Pon anchors Numb, which turns off Notch signaling in prospective GMC (Betschinger et al. 2006; Cai et al. 2004; Fuerstenberg et al. 1998; Ikeshima-Kataoka et al. 1997; Jiang et al. 2008; Lee et al. 2006a; Matsuzaki et al. 1998; Schuldt et al. 1998; Shen et al. 1997). Thus, the segregation of Numb in the basal compartment after cell division distinguishes the two prospective cells in terms of notch signaling where the apical cell, which devoid of Numb will have Notch on status and undergoes self-renewal, whereas the other cell on the basal side, which retains Numb, will have Notch off state and follow differentiation fate. Subsequently, in the newly formed GMC (Notch off), nuclear Pros also favors the differentiation fate by

suppressing the genes, which are required for proliferation and self-renewal (Cabernard and Doe 2009; Choksi et al. 2006; Li and Vaessin 2000).

Recently, the importance of extrinsic signals in NSC polarization is also highlighted (Loyer and Januschke 2017). The signals, of yet unknown nature, from stem cell niche help in proper stem cell polarization and act upstream of apical-basal polarity signals in the larval brain. The plane of cell division in the self-renewing NSCs is maintained by the immediate GMC. Disruption of the NB/GMC interface integrity disturbs the "memory" of the axis of polarity and thus the division plane of the stem cell. However, the exact nature of these extrinsic signals provided through the NB/GMC interface still needs to be elucidated (Loyer and Januschke 2017).

Correspondingly, in mammals, the switching of cell polarity in response to spindle orientation and asymmetric distribution of fate determinants allows asymmetric division of NSCs. Most of the genes involved in the apical–basal polarity and asymmetric division of the NSCs in flies have mammalian orthologs with similar evolutionary conserved roles, defects in their functions result in tumor formation in fly and mammalian brain. For example, Par complex is a pioneer apical polarity determinant; disruption of Par complex in flies makes two self-renewing daughter cells, instead of just one, and forms a tumor in the larval brain. Similarly, mutations in mammalian Par complex results in brain tumors as well as metastasis in human cancers (Martin-Belmonte and Perez-Moreno 2012; Wang et al. 2006). Insc, another potential player of spindle orientation, also plays a conserved role in flies and mammals. In fly brain, misexpression of Insc is enough to reorient the axis of the spindle and convert symmetric division to asymmetric division (Schober et al. 1999).

Similarly, Insc in mouse neocortex regulates spindle orientation, and mutations in Insc affect the number of progeny produced (Lancaster and Knoblich 2012; Postiglione et al. 2011). Likewise, mutations in Aur-A kinase form supernumerary stem cells at the cost of neurons in flies (Lee et al. 2006b). Aur-A kinase is a regulator of cell cycle progression, which ensures proper localization of aPKC and numb in the apical and basal cortex, respectively (Lee et al. 2006b; Wang et al. 2006). Consistently, loss of numb leading to induction of Notch signaling in the larval brain leads to severe hyperplasia of NSCs (Lee et al. 2006b; Wang et al. 2006). Activation of Notch signaling in mammals blocks the neuronal differentiation in the embryonic cortex and is shown to be associated with brain tumors (Dirks 2008; Fan and Eberhart 2008; Peters 2010).

Homeodomain transcription factor Pros (Prospero) is one of the critical fate determinants in GMC that is required for cell cycle exit and neural differentiation. Although transcribed and translated in NB Pros is transported to GMC nucleus during asymmetric division (Choksi et al. 2006; Demidenko et al. 2001). Several type I and II NB-specific signaling cues converge on to Pros to regulate the fate of GMCs and neurons. As expected deregulation of Pros or mutation in the gene results in tumorous growth in the larval brain (Choksi et al. 2006). Although Prox1, the mammalian ortholog of Pros, does not segregate asymmetrically, still it restrains proliferation in mammalian retina and might have an analogous role in the neural cortex (Dyer 2003; Li and Vaessin 2000). Similar to Pros, Brat, a TRIM-NHL family protein, also segregates to the GMCs where it acts as a translational inhibitor of

self-renewal genes and acts as a tumor suppressor (Trunova et al. 2006). Dpn and Zld (Zelda) transcription factors are shown to be a direct target of Brat (Reichardt et al. 2018). Defect in Brat function specifically affects type II NBs. Loss of Brat transforms the intermediated progenitor neural progenitors (INPs) toward more stem cell-like fate and formation of neoplastic brain tumors (Chang et al. 2012; Harris et al. 2011; Marchetti et al. 2014). Likewise, TRIM32, the mammalian Brat ortholog, also segregates asymmetrically and promotes neural fate in mouse neocortex. Loss of TRIM32 leads to progenitor over-proliferation by degrading c-Myc and an array of microRNAs including Let-7a, which is known to control proliferation in cancer (Schwamborn et al. 2009).

Type II NBs are marked by the expression of Ets domain transcription factor Pointed (PntP1) that is required for its specification. The immediate progenitors that arise by asymmetric cell division in type II NBs are immature INPs (imINPs). Intriguingly, the INPs are very similar to type I NBs, since they also express stem cell-specific self-renewing factors, yet their fate is restricted and the imINPs are programmed for maturation guided by PntP1 (Jan et al. 2011; Peng et al. 2016). Failing this leads to an increased number of type II NBs along with the loss of imINPs. PntP1 in early imINPs suppresses Pros expression that allows its maturation instead of differentiation (Peng et al. 2016). The INP maturation also involves inhibition of Notch by Numb and suppression of Armadillo (Arm)/β-catenin and thus Wnt signaling (vertebrate homolog) by Brat (Komori et al. 2014; Rolland et al. 2008). The late imINPs also exclusively expresses Erm (Earmuff), a Zn-finger transcription factor. Erm in the INPs has been shown to repress Notch signaling to check the dedifferentiation of these cells and also activate Pros that restricts the proliferation potential of the INPs (Li et al. 2016; Weng et al. 2010). Moreover, Erm interacts genetically with the SWI/SNF chromatin remodeling complex and histone deacetylase 3 (HDAC-3) and plays a significant role in locking the INP identity and its potential to proliferate. Nonetheless, a mammalian homolog of Erm, Fez1, is implicated in several cancer types. Alteration in Fez1 leads to chromosomal instability and aneuploidy; however, a detailed molecular mechanism of which is yet to be discovered (Vecchione et al. 2007). Thus, fly NSCs emerge as an excellent model to reveal the underlying mechanism of stem cell–related cancers (Li et al. 2014).

Temporal Transcriptional Series: A Heritable Molecular Identity That Links Various Fates of NSCs and Their Progeny

The NSCs give rise to incredibly diverse neural cells in the CNS. Individual NSC produces a distinct subset of neurons that are specialized in performing distinct functions. How this diversity arises is intriguing. We have started understanding various critically important events, which decide the fate and identity of NSCs and their progeny. The NSC/NBs are stereotypically born in five sequential waves during embryonic stage 8–11 (4 hours into development) and express genes that define their spatial identity (discussed earlier in Fig. 3) (Campos-Ortega and Knust 2003; Doe and Technau 1993; Hartenstein and Campos-Ortega 1984; Truman and Bate

1988). Subsequent expression of the temporal factors add another dimension to the NB identity and behavior (Fig. 3). An essential characteristic of the *Drosophila* NBs and their immediate progenitors, born in a specific time window, is the expression of a unique and temporally controlled series of transcription factors (Odenwald et al. 2008). Thus, the spatial elements and temporal transcription factors (TTFs)

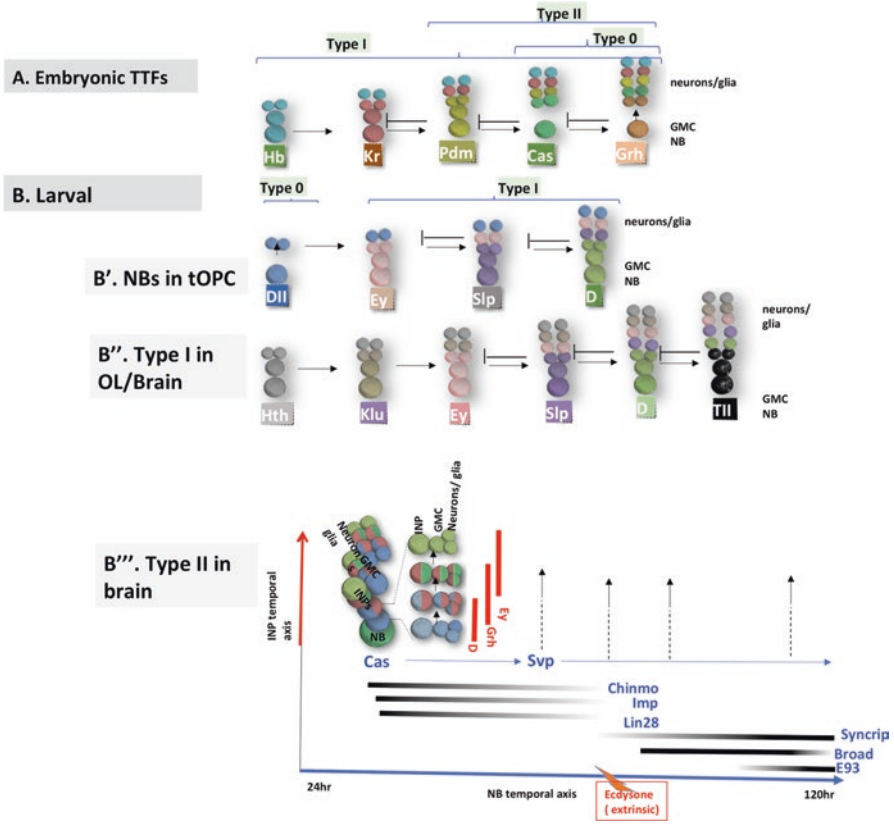

Fig. 3 Distinct temporal transcriptional factor series (TTFs) define molecular and temporal patterning of neuroblasts (NBs). (A) Various members of Temporal Transcription factor series (TTF) are expressed sequentially in type I, type II, and type 0 NBs in the embryo. Some of the type I switch to type 0 in the Cas expression window. (B) TTF series followed by different NB types in larvae. B') In tOPC (tip of outer proliferation center), Dll expressing type 0 NBs switch to type I and sequentially express Ey, Slp, and D TFs. B") type I NBs in OL (optic lobes) and brain follow a different TTF series than that in the type I in VNC. B"'. The type II NBs start the series with Cas and subsequently switch to Svp. Type II NBs also express Chinmo, Imp, and Lin28 (early TFs) until mid-larval life and then changes to Syncrip, Broad, and E93 (late TFs). Extrinsic ecdysone signaling also aids in defining the identity and behavior of NB and its progeny. The INPs born from the type II NBs also follow their own TTF series along with the parental one. The TTFs for type II NBs are represented in blue and for INPs in red. Most of these TTFs regulate each other through feed-forward activation and feedback repression. In all cases, the parental transcriptional profiles are inherited by their progenies as either the INPs or GMCs

together direct NSC identity and produce lineage-specific neurons. Similarly, in the mice neocortex, the common progenitors give rise to diverse progeny neurons in a defined temporal order. Although, in this case, the TF series might not be the same, the process is largely conserved (Barberis et al. 2016; Franco and Müller 2013; Shen et al. 2006; Tan et al. 1998; Walsh and Cepko 1992).

Interestingly, the NB expresses a "distinct" array of transcriptional factors (TFs) and switches its transcriptional profile periodically as it progresses in life (Fig. 3). These distinct TFs appear in a series widely known as "Temporal Transcription Factors series" (TTF series), which is discrete for the different classes of NBs (Fig. 3) (Allan and Thor 2015; Doe 2017; Li et al. 2013; Walsh and Doe 2017; Yasugi and Nishimura 2016). Likewise, the GMC and the progeny neuron or glia born in that window also express the same TFs as the parental NB as their birthmark (Fig. 3) (Allan and Thor 2015; Brody and Odenwald 2000; Isshiki et al. 2001; Pearson and Doe 2003; Skeath and Thor 2003). The embryonic TTF series for type I NBs at the time of delamination starts with the expression of Hunchback (Hb) (zinc finger Ikaros family), followed by Kruppel (Kr) (zinc finger Kruppel-related family), Pdm2/Nubbin (POU domain family), and Castor (Cas) (zinc finger Casz1 family) and Grainyhead (Grh) (CP2 domain family) sequentially (Fig. 3A) (Brody and Odenwald 2000; Grosskortenhaus et al. 2005; Isshiki et al. 2001; Odenwald et al. 2008; Pearson and Doe 2003). Not all the NBs delaminate at the same time, rather they are born in five sequential waves in about a 4-hour window. Thus, depending on the time of birth, the late-born type I NBs start their TTF series from Kr, Pdm, or Cas transcription factors (Tsuji et al. 2008). Most of the transcription factors in the TTF series are regulated by feedback repression. For example, Pdm represses Kr, Cas represses Pdm, and Grh suppresses Cas in type I NBs (Fig. 3A) (Baumgardt et al. 2009; Grosskortenhaus et al. 2006; Odenwald et al. 2008; Tran and Doe 2008; Tsuji et al. 2008).

At the end of embryogenesis, most of the NBs undergo quiescence and resume cycling again during larval life (Fig. 4) (discussed in the later sections). Upon reactivation, type I NBs in VNC recommences the expression of TTFs and start the series with Cas followed by Svp (seven up) (Yasugi and Nishimura 2016). Similar to type I NBs TTF series, the temporal transcriptional regulation has also been characterized for the central larval brain and optic lobe NBs (Allan and Thor 2015; Doe 2017; Li et al. 2013; Walsh and Doe 2017; Yasugi and Nishimura 2016). Type II NBs, in the central brain, are born in embryos and follow a TTF series that includes Pdm → Cas → Grh, whereas the late-born embryonic type II NBs skip the expression of Pdm and follow a truncated TTF series of Cas → Grh (Fig. 3A) (Walsh and Doe 2017). Embryonic type II INPs progenitor express Dicheate (D) only (Walsh and Doe 2017). Type II NBs and their progenitor INPs undergo quiescence and reinitiate the TTF series after larval hatching as seen in case of type I NBs.

Interestingly, in the larval central brain, the transcriptional profile of NBs and their INPs changes with age. In addition to TTFs, post-transcriptional regulators and systematic hormonal cues also regulate the early to the late molecular identity of NBs (Fig. 3B) (McDermott et al. 2012; Munro et al. 2006). During the earlier

window, the NBs express Cas, Svp (Seven-up, an orphan nuclear hormone receptor) along with Chinmo, and RNA-binding proteins Imp and Lin28 (Bayraktar and Doe 2013; Chai et al. 2013; Chen et al. 2016; Homem et al. 2014; Liu et al. 2015; Maurange et al. 2008) (Fig. 3 B"'), whereas in the latter half of larval life (~60 hours), the NBs start expressing EcR, Broad, and E93 (pipsqueak transcription factor family member (Fig. 3B"')) (Bayraktar and Doe 2013; Chai et al. 2013; Chen et al. 2016; Homem et al. 2014; Liu et al. 2015; Maurange et al. 2008; Syed et al. 2017a). In addition to TTFs, RNA-binding proteins are also shown to play a pivotal role in the regulation of the temporal fate and termination of the central brain NBs during larval/pupal life (Fu et al. 2017; Liu et al. 2015; McDermott et al. 2012; Munro et al. 2006). Syp, which codes for evolutionarily conserved mRNA-binding protein, governs the NSC competence to respond to the external hormonal signals (Syed et al. 2017b). Descending Imp and ascending Syp expression is required for ecdysone responsiveness and Pros pulse in these NBs (Liu et al. 2015). Larval INPs inherit the TTFs from their parental NBs; simultaneously, they also recruit their own TTF series, such as D → Grh → Ey (Fig.3B"') (Bayraktar and Doe 2013). Thus, type II NBs make a bigger set of remarkably distinct neurons possibly due to the highly diversified transcriptional program in them and their progenitors (Syed et al. 2017a).

Type I NBs in the optic lobe follow the TTF series Homothorax (Hth) → Klumpfuss (Klu) → Eyeless (Ey) → Sloppy paired 1 and 2 (Slp1 and Slp2) → Dichaete (D) → Tailless (Tll) where these factors are expressed in an overlapping manner, and most of them regulate each other by feed-forward activation and feedback repression as seen in the case of type I NBs (Fig. 3B') (Doe 2017; Li et al. 2013). In the tip of Outer Proliferation Center (tOPCs), the NBs follow Distalless (Dll), Eyeless (Ey), Sloppy-paired (Slp), and Dichaete (D) TF series. The NBs in Dll widow behave like type 0 and, later on, switch to type I and start the expression of Ey, Slp, and D sequentially (Fig. 3B')(Doe 2017).

The NSCs in the mammalian system are also regulated by spatial and temporal transcription factors acting in series to produce numerous types of neuronal cells. Contrary to *Drosophila*, where the early born neurons are pushed outward and late-born neuron remains closer to the parental NBs, in the mammalian cortex, the early born neurons lie in the deepest layer and the late-born neurons move away from the progenitors. Similar to *Drosophila* TTF series, Ikaros and Casz1 (mammalian ortholog of fly Hb and fly Cas, respectively) play an important role in determining the identity of early- and late-born neurons, respectively, in different neuronal layers in the mammalian retina (Alsio et al. 2013; Elliott et al. 2008; Mattar et al. 2015). Svp, a COUP-TF family member transcription factor, although is not included as TTF series in type-I lineages, but it is required for temporal repression of Hb and Cas in many lineages (Gabilondo et al. 2011; Kanai et al. 2005; Mettler 2006; Stratmann et al. 2016; Tran and Doe 2008). The mammalian system has two Svp homologs, COUP-TF1 and COUP-TF2, which act as switching factors to regulate temporal identity transitions from neuron to glia in the developing CNS (Naka et al. 2008). Similarly, Imp1, the mammalian ortholog of Imp, is required to maintain mouse NSCs (Naka et al. 2008). Thus, the evidence is compelling and indicates

that TTFs play an essential and evolutionarily conserved role in generating neural diversity in flies and mammals. More studies are needed to identify other TTFs in the mammalian system.

How various spatial, temporal, and systemic signals integrate and define the NSC competence to make diverse progeny is described. The competence of NSCs is largely governed by the selective opening of chromatin in specific regions, which facilitate the integration of various temporal signals. The chromatin accessibility in the NBs varies from cell to cell depending on their initial spatial profile, which allows the timely binding of TFs and facilitates the birth of temporal neuron (Sen et al. 2019). It would be interesting to learn if similar integration also operates in the vertebrate system to define NSC competence and generation of diverse neurons.

Quiescence and Following Reactivation of Neural Stem Cell: Cross-Talk Among Cell Intrinsic, Extrinsic, and Systemic Cues

Soon after birth, the NSCs start proliferation and experience several identity switches. *Drosophila* neurogenesis occurs in two waves, one during the embryonic period and another through the larval development (Fig. 4). Toward the end of embryogenesis, most of the NSCs shrink and become mitotically dormant, a phase where they do not proliferate for a while (Cashio et al. 2005; Datta 1995; Peterson et al. 2002; Prokop et al. 1998; Truman and Bate 1988). Almost all the NBs in *Drosophila* embryos, with a few exceptions, undergo quiescence toward the end of embryogenesis (Fig. 4) (Dumstrei et al. 2003; Hartenstein et al. 1987). Interestingly, the duration of quiescence may vary in a different part of the CNS, and finally, the dormant NBs re-enter the mitotic phase in larval life (Fig. 4). The entry followed by the timely exit of the NSCs from quiescence is a well-orchestrated cellular event regulated by cell intrinsic and extrinsic factors.

The quiescent NSCs in fly CNS and mammalian SVZ (sub-ventricular zone) and SGZ (subgranular zone) spinal cord are morphologically distinct from the cycling stem cells (Ma et al. 2009; Ming and Song 2011; Morshead et al. 1994). Instead of being more rounded as is the case for most of the actively dividing NSCs, the quiescent NSCs are elongated and extend their processes toward neuropile or other stem cells (Chell and Brand 2010; Tsuji et al. 2008). Possibly, these extensions serve as a communication string with the surrounding neighbors. However, an exact role for the extension is yet to be discovered. Many tissue-specific stem cells are found to remain quiescent or dormant and persist for an extended period in animals from flies to mammals (Coller et al. 2006; Fuentealba et al. 2012; Temple 2001). The dormant NBs in flies re-enter the mitotic phase after larval hatching (Fig.4). Similarly, in mammalian brain SVZ and hippocampal SGZ, the NSCs switch between quiescence and proliferation phase (Ahn and Joyner 2005; Ma et al. 2009; Morshead et al. 1994). Therefore, untimely loss of the dormant progenitors could affect the tissue repair and regeneration in case of any disease or damage. To understand the regulatory networks, which control the entry and exit from quiescence, is crucial for efficient usage of stem cells for regenerative and therapeutic purpose.

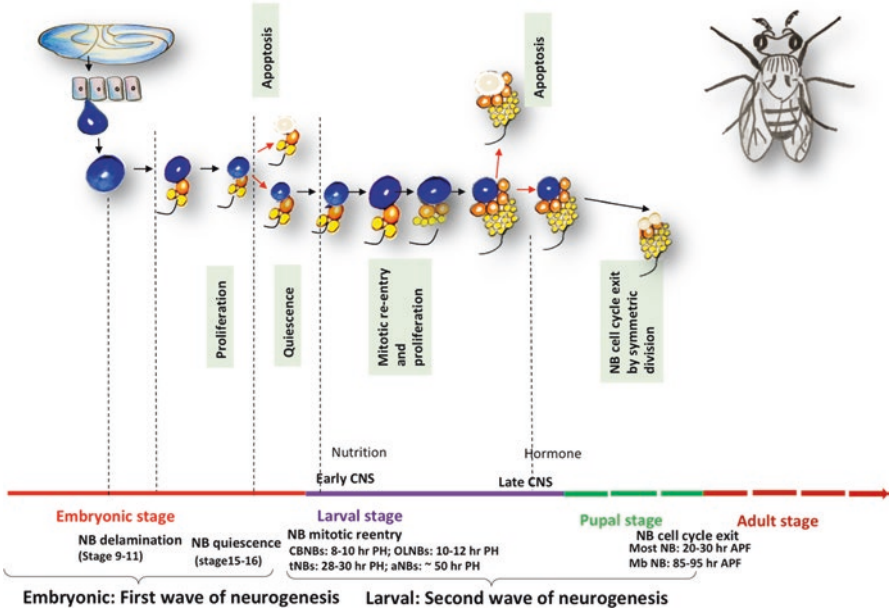

Fig. 4 *Drosophila* neurogenesis along the developmental timeline. Two waves of neurogenesis: First occurs during the embryonic development when the neuroblast (NB; blue) delaminates. The NBs proliferate and produce GMC (orange), which gives rise to numerous neurons and glia (yellow). Toward the completion of embryonic development, most of the NBs enter into a quiescent phase that lasts till larval hatching. After larval hatching, the NBs re-enter the proliferation phase and start the second wave of neurogenesis. NBs in different regions, such as CBNBs (central brain NBs), OLNBs (optic lobe NBs), tNBs (thoracic NBs), and aNBs (abdominal NBs), exit quiescence PH (post-hatching) at different time points and re-initiate proliferation. The second wave of neurogenesis proceeds through larval development and ends in pupa where they exit the cell cycle and undergo either terminal differentiation or apoptosis to shape the adult nervous system. Most of the aNBs, type II and MBNB (mushroom body NBs) end their life through apoptosis at different stages of development

Stem Cell Entry into the Quiescence and Its Maintenance

After birth, the embryonic NSCs undergo 5–12 rounds of cell division and then become quiescent. A remarkable aspect of the NSC division is the reduction of cell volume after every division and by the time the embryo is about 11–15 hours old (around staged 15–16), the NSCs become very small, stop proliferation, and soon enter into a quiescent state (Fig.4) (Hartenstein et al. 1987).

Using thoracic NB 3–3 as a model, it is shown that the entry into quiescence is controlled by cell-intrinsic signals provided by spatial and temporal identity factors (Tsuji et al. 2008). For example, perturbation of spatial identity by a mutation in Antp, a HOX protein, prolongs the proliferation of NSCs and delay, entry into quiescence. Similarly, messing up with the temporal integrity by mutations in Pdm or Cas, members of NSC TTF series, also interrupts the precise timing of quiescence.

A Pdm mutant shows premature entry into quiescence, whereas Cas mutants escape quiescence and proliferate longer possibly due to prolonged Pdm expression. Surprisingly, the atypical homeodomain transcription factor Pros, which is involved in determining the differentiation fate of GMCs (discussed above), also regulates the entry of NSC to quiescence (Lai and Doe 2014). The differential levels of Pros are shown to perform distinct functions; high Pros leads to differentiation of GMCs, whereas low levels of Pros in NSCs induce quiescence during late embryonic development. How TTFs regulate Pros pulse in NSCs is yet to be determined (Lai and Doe 2014).

A consensus regarding NSC quiescence is that the quiescent stem cells are arrested in the G0 phase (Cheung and Rando 2013). However, recently, it is shown that about 75% of quiescent fly NSCs are arrested in the G2 phase of the cell cycle, and the remaining small population is in the G0 phase of cycle (Otsuki and Brand 2018). Moreover, the NSCs in different phases of quiescence exit at different time points, the G2 NSCs are first to exit quiescence than the G0 NSCs, possibly to maintain order in neural circuit formation. Tribbles (Trbl) encodes an evolutionarily conserved pseudo kinase with known functions in insulin and mitogen-activated protein kinase signaling and is required for the entry and maintenance of G2 quiescence. Trbl regulates the quiescence of embryonic and larval NSCs through two distinct effectors. In embryos, NSC promotes quiescence through degradation of Cdc25/String, while in the larvae, the NSC quiescence is maintained by blocking the activation of Akt and thus inhibiting downstream insulin signaling (Fig. 5). In response to the nutritional stimulus, the NSC exits from quiescence and the insulin signaling is activated, which inhibits transcription of *trbl* to remove its repressive effect on proliferation (Fig. 5). Thus, the *trbl* directs both the entry of NSCs into quiescence and its subsequent exit (Otsuki and Brand 2018).

NSC microenvironment plays a crucial role in the regulation of several aspects of NSC behavior including NSC quiescence (Fuchs et al. 2004; Fuentealba et al. 2012; Riquelme et al. 2008), and neural apoptosis (Discussed later). The neurogenic niche is evident in flies and vertebrates, and the NSCs are in close contact with the niche. Several signaling pathways are implicated in establishing cross-talk between NSC and its niche, especially with glial cells (Bjornsson et al. 2015; Hoyle 1986). Communication with glia is critical for the entry and exit of NSCs into quiescence (Fig. 5C,D). Glial secretion of anachronism (Ana) glycoprotein is required for quiescence maintenance; loss of Ana initiates the entry of NSC into the S phase of cell cycle precociously (Datta 1995; Ebens et al. 1993). Terribly reduced optic lobes (Trol), which encode a heparan sulfate proteoglycan, the homolog of vertebrate Perlecan, probably acts downstream of Ana and functions antagonistically (Park et al. 2003; Voigt et al. 2002). Possibly, Trol stimulates the G1 to S transition through fibroblast growth factor (FGF) and Hedgehog (Hh) signaling (Park et al. 2003). Similarly, the mammalian neurogenic niche in the adult brain also plays important role in maintaining the quiescent state of NSCs in SEZ (subependymal zone) and hippocampal area through negative feedback from Notch and BMP signaling (Ables et al. 2010; Covic et al. 2010; Mira et al. 2010).

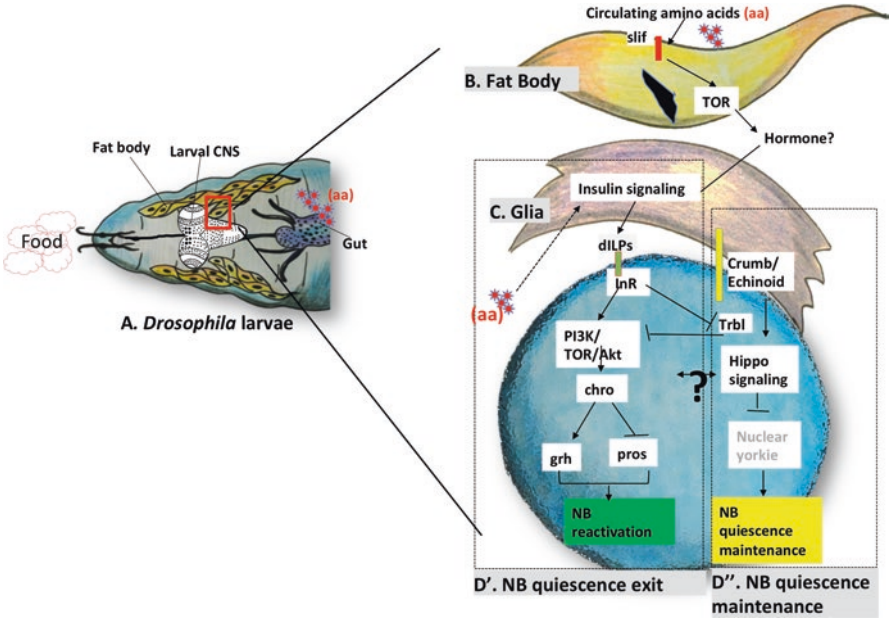

Fig. 5 Quiescence and following reactivation of neural stem cell. (A) *Drosophila* larvae showing the organization of CNS, gut, and fat bodies. Food intake increases the circulating amino acids (aa, red). (B) circulating amino acids are sensed by the transporter Slimfast (Slif, red) present on the fat body (FB) cells and the TOR pathway is activated. (C) FBs then secrete a yet-unknown signal, possibly a hormone, which activates insulin signaling and release of ILPs in the glial cells. D') Exit from quiescence: ILPs bind to the insulin receptors (InR, green) in NBs and activate the PI3K/AKT pathway and downstream TOR signaling, which result in stem cell growth and proliferation. D") NB quiescence maintenance: On the other hand, the maintenance of quiescence also depends on the glia and NB communication. Both the cells are in contact with each other through transmembrane proteins Crumb and Echinoid proteins (yellow), and quiescence is maintained by the activation of Hippo signaling. Trbl regulates NB entry, maintenance, and subsequent exit from G2 quiescence. Trbl promotes quiescence by inhibiting Akt signaling, while insulin signaling reverses the repression of akt by inhibiting trbl to resume NB proliferation

The Salvador/Hippo/Wart (SHW) signaling, a very well-known regulator of growth and cell proliferation, was also found to play a role in the maintenance of the NSC quiescent state in flies (Fig. 5D) (Weynans et al. 2016). Again, the interaction of NSC with the neural niche is vital to activate the intrinsic signaling in NSCs. The cell-to-cell contact proteins Crumbs and Echinoid that are expressed in both glial and NSCs regulate downstream Hippo signaling in a nutrition-dependent manner. Loss of communication between NSC and its niche glial cells inhibits Hippo signaling, which leads to premature nuclear localization of Yorkie and early NSC growth and exit from quiescence (Fig. 5D") (Weynans et al. 2016). The SHW pathway,

which is widely known to control organ size, was first discovered in flies and soon recognized as a highly conserved pathway in mammals to control development and cancer and is implicated in stem cell biology as well (reviewed in Gomez et al. 2014; Hansen et al. 2015; Hariharan 2015; Ramos and Camargo 2012).

Stem Cell Exit from Quiescence: Role of Insulin/PI3K/TOR Pathways to Integrate NSC Extrinsic and Intrinsic Signals

As discussed above after multiple rounds of divisions, the embryonic NSCs become very small and enter quiescence. After a prolonged quiescent phase, the same NSCs reactivate and grow significantly to increase their size and volume to resume proliferation in the larvae (Fig. 4). Likewise, the in vitro cultured mouse adult quiescent NSCs also undergo a growth phase before they actively start proliferation (Codega et al. 2014; Costa et al. 2011). A remarkable aspect of the developmental reactivation of the NSCs is their responsiveness to the nutritional status of the animal. The external environmental/nutritional cues are communicated to the deep-seated NSCs to induce their growth and subsequent activation of the second wave of neurogenesis.

Insulin signaling is in the center of NSC exit from the quiescent state. Once the larvae start feeding, the concentration of circulating amino acids increases in the body and a chain of signaling cascades are initiated in different cells, which finally terminate the NSC quiescent state (Fig. 5). The larval fat bodies, equivalent to the vertebrate liver and adipose tissue (Colombani et al. 2003), sense the presence of amino acids through a cationic amino acid transporter, the Slimfast, and activate downstream TOR signaling (Fig. 5B). Moreover, the fat bodies also release a signal, possibly a hormone or mitogen, which is received by the glial niche and median neurosecretory cells (mNsCs) in the brain (Fig. 5B). In turn, both glia and mNsCs in the brain produce distinct *Drosophila* insulin-like peptides (dILPs). In *Drosophila*, there are seven insulin/IGF-like peptides (dILPs 1–7) and a single insulin/IGF receptor (dInR). The ILPs produced by a glial subset is vital for NSC reactivation, whereas the ILPs from mNsCs are required for organ growth (Sousa-Nunes et al. 2011). ILPs bind to the InR receptors present on the NSC surface and result in the activation of PI3K/AKT signaling and NSCs exit from quiescence (Britton and Edgar 1998; Chell and Brand 2010; Sousa-Nunes et al. 2011). The gap junction proteins Innexin 1/2 and calcium wave in the glial niche are also shown to be important for NSC re-activation, though, the detailed mechanism is yet to be identified (Otsuki and Brand 2017; Spéder and Brand 2018). Insulin signaling plays a conserved role in mammalian neurogenesis also (Liu et al. 2014). Insulin growth factor-1 (IGF-1) promotes the proliferation of NSCs in the embryonic CNS and inhibits their apoptosis during postnatal development (Mairet-Coello et al. 2009; O'Kusky et al. 2004). Mutation in IGF1R leads to prenatal and postnatal growth impairment and microcephaly defects (Rivarola et al. 2014). IGF-2 regulates the proliferation of radial glial cells and its loss results in smaller brains as seen in the case of IGF-1R (Lehtinen et al. 2011).

The spindle matrix complex (SMC) in the NSCs is one of the downstream effectors of insulin signaling. The SMC, in general, localizes with spindles during mitotic progression, whereas during interphase, it stays in the nucleus (Rath et al. 2004). Chromator (Chro), a member of SMC, regulates NSC reactivation by inducing the expression of TTF *grh* (Grainyhead) to promote NSC proliferation and suppress *pros* that is required for NSC quiescence (Fig. 5) (Li et al. 2017). Since the ectopic expression of Chro under starvation condition could not induce NSC exit from quiescence, it is suggested that Chro is necessary but not sufficient for NSC reactivation, and possibly, there are other parallel mechanisms (Li et al. 2017). As mentioned in the previous section, Hippo signaling also acts in a cell intrinsic manner, helping to maintain quiescence (Weynans et al. 2016), it would be interesting to know if the SMC, Hippo, and late TTFs interact and regulate the transition between quiescence to proliferation.

Although the availability of nutrients plays a central role in the initial NSC exit from quiescence, followed by its growth and mitotic division, the same NSCs remain refractory to the nutrition availability during the late larval stage. During this phase, it is Anaplastic lymphoma kinase (Alk), which helps in continued growth and proliferation of NSCs irrespective of nutrient availability. Alk is a tyrosine kinase, which, interestingly, could activate the downstream targets of InR signaling even in the absence of the insulin pathway ligand ILPs. Alk is activated by its ligand Jelly-belly, expressed in the glial niche, and ensures the activation of Alk and downstream PI3K/Akt signaling cascades in NSCs even under starvation to safeguard the sustained growth and promote their proliferation (Cheng et al. 2011). Thus, under low nutrition condition, when the net body growth is at a halt, the activation of downstream signaling cascade through Alk could help "sparing" of the specific tissue, such as the brain, likely to ensure the survival of the animal in the long run. Nevertheless, the cancer cells mis-utilize the ALK and PI3K/Akt signaling to grow independent of nutrition, subsequent tumor growth is apparent in case of cancers such as glioblastoma and non-Hodgkin lymphoma (Bai et al. 2000; Cheng et al. 2011; Dittmer et al. 2006; Lymphomagenesis et al. 2001).

In contrast to the majority of NBs present in *Drosophila* CNS, which undergo the quiescence phase, the mushroom body NBs (MB-NBs) are an exception and maintain their large size and keep proliferating without following a quiescent period (Britton and Edgar 1998; Marin et al. 2013). These cells continue to proliferate even under dietary restriction (Sipe and Siegrist 2017). What makes these cells refractory to nutrition availability? As discussed above, the PI3K signaling is in the center of the nutrition-dependent cycling of NSCs. MB-NBs also express PI3K when larvae are actively feeding (Sipe and Siegrist 2017). Remarkably, MB-NBs switch gears to a P13K-independent mechanism and continue cycling even when the nutrition is restricted. It is recently shown that eyeless (Ey), orthologous of mammalian Pax-6, is a crucial player in uncoupling the link between NSC proliferation and nutrition status. Ey is expressed in all MB-NBs, and it is required for their proliferation independent to the availability of dietary amino acids. The Ey mutant MB-NBs behave

more like their other counterparts in the brain and stop proliferation if nutrition is withdrawn. It would be exciting to identify the downstream effectors of Ey that support MB-NB escape from the dietary constrain (Sipe and Siegrist 2017).

Methods to Eliminate the Neural Stem Cells After the Completion of Organogenesis: Cell Cycle Exit or Apoptosis

Once the organogenesis is complete, the progenitors have to be removed to avoid formation of superfluous cells or tumors (Blum and Benvenisty 2008). Barring a few exceptions, most of the NSCs in *Drosophila* CNS are eliminated during late-larval to mid-pupal life. The cellular temporal clock and memory must implement the entire developmental program at the right time and place. It is interesting to note that TTFs intersect with almost all developmental fates of NSCs including their timely demise. Similar to quiescence, the NB size plays a vital role in their death as well. All the NBs in the brain and VNC reduce their size first before they finally exit cell cycle or undergo apoptosis. Even the constantly cycling large mushroom body NBs reduce their size primarily by autophagy followed by the activation of the cell death pathway (Chell and Brand 2010; Siegrist et al. 2010; Sousa-Nunes et al. 2011; Syed et al. 2017a; Tsuji et al. 2008) (discussed in a later section). The NBs are timely eliminated from the developing NS either by undergoing symmetric division, followed by terminal differentiation or by programmed cell death mediated by caspases (Harding and White 2018; Homem et al. 2013).

Neural Stem Cell Cycle Exit by Symmetric Division and Terminal Differentiation

Most of the asymmetrically dividing NBs in the central brain and thoracic region of larval VNC end their life by undergoing a terminal symmetric division and differentiation. The size of NSC plays a vital role in its potential to proliferate. During the active proliferation phase, the NBs regrow to their original size after every division in the early larval life (Homem et al. 2013). However, later during the larval–pupal transition, the NSC stops growing and gradually reduces its size and volume (Homem et al. 2014). Multiple signaling pathways have been shown to regulate NSC size and its timely removal. Hh signaling acts downstream of cell-intrinsic post-embryonic TTF Cas and is necessary and sufficient for the NSC cell cycle exit through a burst of nuclear Prospero expression (Chai et al. 2013). Similarly, Ecdysone, a steroid hormone, also regulates the transition and acts downstream of Cas (Castor) and Sev (Seven-up) (Fig. 3B”’). The Ecdysone signaling, during the pupal life, increases oxidative phosphorylation through the genes involved in chromatin regulation and energy metabolism to reduce NSC size (Homem et al. 2014; Maurange et al. 2008). Interestingly, the competence of NBs to respond for Ecdysone signaling is decided by the mutually opposing gradients of Imp and Syp RNA-binding proteins (Liu et al. 2015; Ren et al. 2017). It is shown recently that initial

high levels of temporal factor Imp suppress the ecdysone/mediator complex-driven metabolic changes, required for NB to shrink and subsequent elimination, to ensure its continued proliferation. However, in the following window, the level of Imp declines and Syp gradually peaks, makes the NB competent for the Ecdysone signaling which schedules the end of NB life through terminal differentiation. The Syp-mediated temporal identity of NB acts upstream of the strong Pros pulse, which schedules the end of NB life through terminal differentiation. How the Cas and Imp/Syp gradient integrate with other temporal cues such as hormonal and Hh signaling would help in understanding the mechanism of timely NB elimination. Similar metabolic regulation has been shown to control the proliferation in mouse NSCs and progenitor cells as well (Kovacs et al. 2012). It would be interesting to further know if these signaling and networks are also involved in the NSC size reduction during late embryonic development when the NSCs undergo quiescence.

Apoptosis Is a Natural Way of Pruning and Homeostasis in the Nervous System: Elimination of Neural Stem Cells

The nervous system shows remarkable plasticity during development. Dynamic integration of neural cells along with the removal of redundant ones is required for the effective functioning of the nervous system, perturbation of which could cause neurodegenerative disorders, autism, mental disorders, or neural cancers (Hazlett et al. 2017; Norambuena et al. 2017; Rosoklija et al. 2018; Schoenfeld and Cameron 2015; Yaghmaeian Salmani et al. 2018). Apoptosis is a common and conserved way for sculpting nervous system. In *Drosophila* and mammalian nervous system, nearly 50% the neural cells are eliminated through caspase-mediated death (Buss et al. 2006; Cashio et al. 2005; Hamburger 1975; Harding and White 2018; Luer et al. 2006; Peterson et al. 2002; White and Steller 1995; Yalonetskaya et al. 2018). Since the canonical apoptotic pathway is highly conserved from worms to mammals, the *Drosophila* nervous system is used widely to understand the molecular mechanisms of apoptosis (reviewed in Arya and White 2015; Harding and White 2018; Pinto-Teixeira et al. 2016; Ryoo and Baehrecke 2010; Yalonetskaya et al. 2018).

Drosophila development starts as an embryo, after transitioning through various larval stages the adult fly emerges. The nervous system in *Drosophila* starts taking shape during embryonic development, where visible signs of apoptosis are evident as early as the embryonic stage 11/12 (Cashio et al. 2005; Truman et al. 1992). Similar to neurogenesis, the developmental apoptosis of NSCs and their neural progeny takes place in two waves. The first wave occurs during the embryonic life between stages 14 and 16 when the embryo prepares to become larvae. Although there is apparent apoptosis throughout the nervous system; yet most strikingly, death can be seen in the abdominal region of the ventral nerve cord (VNC). Due to the massive clearing of abdominal NSCs and their mature progeny, the embryonic VNC condenses and compacts by the time embryonic life ends (Abrams et al. 1993; Cashio et al. 2005; Page and Olofsson 2008; St Pierre et al. 2011).

The second wave of apoptosis occurs during mid- to late-larval life around the time when the larva is preparing to become adult. Indeed, the sculpting is required to remove all the stem cell progenitors that generate stage-specific neurons, and all unnecessary neural networks which are no longer needed for the next stage. This well-timed nervous system remodeling is a prominent example of how the changing needs of an organism are being taken care of during animal development. What are the molecular mechanisms behind the timely death of neural cells? The precise timing of NSC apoptosis of embryonic abdominal NBs is tightly controlled by temporal and spatial cues and signaling inputs from the neural niche. The genes at RHG locus *rpr*, *grim*, and *skl* are the key upstream activators of the canonical cell death pathway; they play a central role to integrate the upstream cues with the core apoptotic machinery and downstream caspase activation (Fig. 6). It is important to note that promiscuous activation of any of the gene at the RHG locus results in massive death in most of the tissues with few exceptions (White et al. 1996). Thus to avoid any precocious activation, the RHG locus is kept under tight transcriptional and epigenetic control (Arya et al. 2019; Harte et al. 2011; Lin et al. 2009; Zhang et al. 2008). Several tissue-specific enhancers are identified so far that regulate the

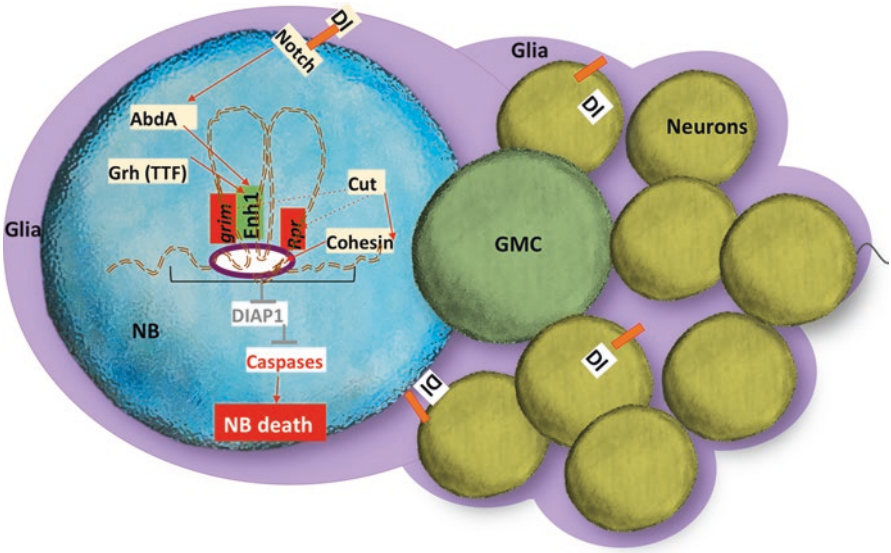

Fig. 6 NB competence to undergo apoptosis is regulated by chromatin architecture and integration of intrinsic and extrinsic transcriptional inputs. The abdominal NB lineage (neurons/glia) produces Dl (delta), which activates Notch in the NB. Successively, within NB, Notch induces a pulse of AbdA, which stimulates transcription of *rpr* and *grim* and subsequent activation of downstream canonical apoptotic signaling leading to NB death. Grh, the last member of the embryonic temporal factor series, together with AbdA, binds to the Enh1 regulatory element to activate *grim* and *rpr*. Cut, another transcription factor also regulates the pro-apoptotic genes *rpr* and *grim* by controlling their chromatin architecture (red dashed lines) at the level of enhancer-promoter interaction through the Cohesin complex (purple circle) or via histone modification at *rpr* and *grim* loci

expression of this locus in different tissues (Jiang et al. 2000; Khandelwal et al. 2017; Lohmann et al. 2002; Peterson et al. 2002; St Pierre et al. 2011; Zhang et al. 2008).

The apoptosis of a subset of NSCs in the abdominal region of VNC is controlled by an intragenic NSC-specific enhancer called the neuroblast regulatory region (NBRR) (Peterson et al. 2002; St Pierre et al. 2011). It is important to note that the RHG locus is about 300 kb long and a stem cell-specific enhancer (NBRR), which is in the middle of rpr and the grim is around 40–60 kb away from the genes on either side. Thus, a long-range enhancer-promoter interaction is required for timely activation of these genes. Interestingly, knockout of the *NBRR* enhancer alone, leaving the genes in the RHG locus intact, perturbs the timely developmental removal of NSC resulting in prolonged survival of superfluous stem cells (Tan et al. 2011). Through genetic studies using a 5 kb reporter of the NBRR region, we have shown that multiple coordinated transcriptional inputs schedule the timely death of NB (Arya et al. 2015; Tan et al. 2011). The Hox gene *AbdA* initially provides the regional identity to the NB and, later again, during stem cell death gets activated by Notch signaling (Arya et al. 2015; Prokop et al. 1998; Rogers et al. 2002). We showed that the pro-apoptotic Notch signaling is activated by the Delta ligand being expressed on the neighboring progeny of the stem cell and controls the expression of the AbdA in the following window just before death (Fig. 6) (Arya et al. 2015). It is interesting to note that although the cell death machinery is activated autonomously in the NSCs, the neighboring glial niche plays an instrumental role to activate the notch and decide the right time of stem cell death (Arya et al. 2015; Pinto-Teixeira et al. 2016) (Fig. 6). Furthering the findings, Khandelwal et al. (Khandelwal et al. 2017) have shown that the AbdA and Notch along with a late member of TTF series, Grh physically interacts with the NBRR enhancer to regulate NB death in the abdominal region of larval VNC (Khandelwal et al. 2017). Forced expression of AbdA induces apoptosis in late-stage NSCs in the larva. Interestingly, the NBs that fail to express Grh do not undergo apoptosis even if ectopic high-concentration AbdA is present. Thus, the inputs to induce death are tightly controlled by multiple factors, integration of which at the right time and place only could activate apoptosis. Most of the abdominal NBs in the *Drosophila* VNC are eliminated only when they are in a specific transcriptional state of Diachete-negative, Grh-positive, Castor-negative, and AbdA-positive (D-Grh + Cas-AbdA+) (Almeida and Bray 2005; Cenci et al. 2005; Maurange et al. 2008). Since the Hox locus is under the tight control of Polycomb-group (PcG) family of chromatin remodelers, loss of PcG genes such as Polycomb, Sex combs extra, and Enhancer of zeste also lead to ectopic death of NSC due to aberrant induction of Hox genes (Bello et al. 2007).

We have recently reported that Cut protein, which belongs to a homeodomain class of DNA-binding proteins, also regulate the death of embryonic abdominal NBs but the mechanism is different from the aforementioned regulation involving AbdA, Grh, and Notch (Arya et al. 2019). In addition to the direct transcriptional inputs from sequence-specific transcription factors, the RHG locus is also epigenetically regulated. Our Chip data show that the RHG locus is distinctly marked by the presence of repressive H3K27me3 present in the nervous system, which could

affect its accessibility for binding with other factors. We showed that Cut alters the H3K27me3 levels on the rpr and grim genes to inhibit the formation of facultative heterochromatin at the loci (Arya et al. 2019). In general, the stem cells are plastic and have more open chromatin conformation (Marshall and Brand 2017; Tee and Reinberg 2014). Interestingly, we found that the younger NBs show a shallow level of H3K27me3 histone marks; however, with age, the overall H3K27me3 level increases in stem cells indicating a gradual loss of plasticity over a period. We found that loss of the Cut protein enhances the rate of this transition and also reduces the accessibility of the genes in the RHG locus (Arya et al. 2019).

Moreover, Cut also genetically regulates stromalin (SA), a subunit of the Cohesin complex, which is required for long-range enhancer-promoter interaction. Knockdown of various cohesin complex components, including SA and Nipped-B, also result in the similar rescue of stem cell as observed in the case of cut knockdown. The chromatin architecture and long-range enhancer–promoter interactions are required to schedule the death of NSCs in the frame of right time and space. Therefore, it is likely that Cut might affect the NB cell death by altering the interaction of NBRR enhancer with the promoters of rpr and grim by cohesion tethering. However, reduced levels of cut do not affect NBRR enhancer activity when checked through reporter assay (Arya et al. 2019). Together, it distinctly shows that stem cell death is controlled at multiple levels by the integration of chromatin architecture and various sequence-specific transcription factors that regulate the timely activation of the cell death gene.

A likely cross-talk with the surrounding is also noted in case of apoptotic removal of the Mushroom Body (MB) NBs. The mushroom body is found in the brain and function in olfactory learning and memory. MB-NBs are the biggest and longest proliferating stem cells in the *Drosophila* CNS that are eliminated around the mid pupal stage through RHG-mediated apoptosis, perturbation of which leads to their extended survival (for up to a week) into the adult life (Pahl et al. 2019). It is important to note that these cells never undergo quiescence and continue proliferation from embryonic life till the late pupal stage. For the elimination, they first undergo autophagy led to significant size reduction before formal activation of the canonical apoptotic machinery. The removal to these cells depends on the cross talk between insulin/PI3K kinase signaling and the RHG-mediated apoptotic pathway. Before the activation of the apoptotic cascade, the MB-NB size and proliferation rate reduces due to a decrease in insulin/PI3K signaling and nuclear localization of FOXO, which induce autophagy (Peterson et al. 2002; Siegrist et al. 2010). The involvement of Insulin/PI3K suggests that possibly some systemic cues should link the autophagy and apoptosis to decide the correct timing of stem cell death. Indeed, recently, it is shown that the temporal expression of ecdysone-induced protein E93 (pipsqueak transcription factor family member) in MB-NBs downregulates PI3K signaling to activate autophagy (Siegrist et al. 2010). As seen in the case of cell cycle exit of type I NBs (section above), here the opposing gradients of temporal factors Imp/Syp also play an important role. In MB-NBs, the expression of E93 is negatively regulated by Imp and positively regulated by Syp. E93 levels are further enhanced by extrinsic Ecdysone signaling during late stages of pupal development.

Thus, E93 acts as an integrating link between the Imp/Syp temporal series and systemic hormonal cue (Pahl et al. 2019). Predictably, the life of mushroom body MB-NBs could be extended even further up to a month in adults if autophagy and genes at the RHG locus are simultaneously inhibited (Pahl et al. 2019). This indicates that the presence of multiple factors determines the competence of NB to undergo death in the correct space and time. Perhaps the signaling orchestrated to ensure that NSCs should be eliminated only after producing a precise array of progeny neurons and glia.

As expected, any defect in the apoptotic machinery leads to prolonged survival of NSCs, and their differentiated progeny ultimately results in massively deformed or enlarged nervous system. For example, in *Drosophila*, mutations in crucial cell death genes such as rpr and grim result in severely hypertrophic adult nervous system (Tan et al. 2011). Similarly, mice mutants of several other members of the critical apoptotic signaling pathway, including caspase-3, caspase-9, Apaf-1, and Bcl-2 family genes display numerous nervous system patterning defects at various stages of development causing subsequent animal lethality (Tan et al. 2011; Cashio et al. 2005).

Summary and Conclusion

Stem cells have the potential to be used as future therapeutics. Use of NSC to cure neurodegenerative disorders is one of the highly demanding areas of current research and therapy development. NSCs, neurons, or glial transplantation in animal models of neurodegeneration have demonstrated significant improvements in ameliorating disease symptoms. To address individual types of neurodegeneration diseases, precise programming of stem cell differentiation and proliferation is necessary. Uncontrolled proliferation of transplanted NSCs has been shown to trigger the risk of tumor formation. Thus, without a grip on the fundamental biology of NSCs along with a good understanding of the pathology of neurodegeneration, it is rather daunting to successfully achieve the goal to do translational research. Insights from *Drosophila* neurology have provided deep understandings of cellular and molecular functions in mammalian systems.

In the chapter, we have discussed various cell intrinsic and extrinsic mechanisms through which the correct fate of stem cells and its competence to respond to multiple signals is regulated in space and time. The stem cell fate determination does not solely depend on cell-autonomous signals; instead, it is an intimate interaction between the NSC with its micro- and macro-environment, which is necessary for proper nervous system development. A diverse array of cell intrinsic transcriptional regulators and cellular signaling pathways specifies the identity of stem cell and their progenitors. Several cell-autonomous factors regulate stem cell proliferation, and mutation of some of these intrinsic regulators causes uncontrolled expansion of stem cell population. Recently, the role of a neural niche in regulating NSC behavior has been much appreciated. *Drosophila* glial cells have been shown to regulate

timely death and quiescence of NSCs. Thus, a cross-talk exists between NSC and its microenvironment.

Additionally, we have learned that the NSC and their niche also communicate and receive cues from its environment. The NSCs in flies respond to long-distance signals such as Ecdysone steroid hormone. The "correct" chromatin state determines the competence of NSC to respond to various incoming cues. Many times mere forced expression of a transcriptional regulator is not sufficient to influence the stem cell fate if the stem cell is not in the "correct" competence window. Spatial factors control the chromatin conformation, which makes the chromatin accessible for other transcription factors crucial for NSC fate. In vivo model organisms are very useful to understand the biological networks. Exciting parallels determining the fly and vertebrate NSC fate and behavior would help to comprehend the neural biology and its use in therapeutics better.

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Understanding Motor Disorders Using Flies

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Abstract

The fruit fly, *Drosophila melanogaster*, is an attractive model for studying human disease. The popularity of the model is a consequence of its well-developed toolbox for genetic engineering and the finding that 75% of genes that cause human disease have orthologs in the fly. Diseases of the human nervous system have been modeled extensively in the fly, taking advantage of a complex, well mapped out nervous system. A popular strategy to model a disease is to identify the fly ortholog of a disease gene and develop an experimental model, based on the ortholog, to gain insight into the mechanisms of gene function and malfunction. The lessons learned from the fly can then be used to dissect out the cellular and molecular basis of the disease in humans.

In this chapter, we highlight research using *Drosophila* to gain insight into mechanisms that underlie neurodegenerative diseases, with a focus on amyotrophic lateral sclerosis (ALS). Till date, 31 familial genetic loci have been identified in ALS, with each gene involved in cellular processes that are widely divergent from each other. This divergence of function has hampered efforts to elucidate a common model for the initiation and progression of ALS. Here we describe well-established fly models for *C9ORF72, SOD1, TDP-43, FUS, VAP,* and *VCP*. We explore the alterations in protein and RNA homeostasis, metabolic changes, intracellular and intercellular signaling, and transport, stress, and immune response concerning each of these genetic loci as well as architectural changes that occur during development and aging of the fly. Studies that provide

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evidence for common themes between these loci through genetic, epistatic, or physical interaction have been highlighted.

Many cellular hallmarks of these diseases can be recapitulated in *Drosophila,* providing a platform to conduct further sophisticated genetic and chemical perturbations to gain a better understanding of the human disease. In this chapter, we speculate on the possibility of a gene regulatory network that underlies the breakdown in motor function in ALS, composed of ALS causative genes, which reveal critical mechanistic features that can be targeted for therapy.

Keywords

Amyotrophic lateral sclerosis · Familial · *Drosophila* · Gene regulatory network · Neurodegeneration

Introduction

In humans, a subset of neurodegenerative diseases that affect motor functions is collectively termed as motor diseases. Amyotrophic lateral sclerosis (ALS), hereditary spastic paraplegia (HSP), Charcot-Marie-Tooth (CMT) disease, and spinal muscular atrophy (SMA) are a few examples of clinically described motor diseases. Amyotrophic lateral sclerosis, also known as Lou Gehrig's disease, is a late-onset, slowly progressive disorder that culminates in the death of motor neurons of the brain cortex, brain stem, and the spinal cord. This causes loss of signaling between motor neurons and voluntary muscles, causing paralysis and subsequent death of the patient. Although the debilitating clinical features of SMA are similar to ALS, SMA is found to manifest as early as during infancy, while ALS sets in above the average age of 55 years. SMA can be classified as Type 1–4, depending on the age of onset. Similar to SMA, HSP and CMT are found in juveniles as well as in adolescents. HSP is more pleiotropic in origin characterized by corticospinal dysfunction, muscular weakness, and spasticity, which may involve cerebral atrophy, speech, and cognitive defects and even optic defects. CMT affects the peripheral nervous system, both motor and sensory, causing atrophy of long axonal projections and nerve endings, as also the protective myelin sheath, without being a fatal disorder.

Amyotrophic lateral sclerosis is a motor neuron disease first identified as a neurological condition in 1874 by Jean-Martin Charcot. The term "A-myo-trophic" (In Greek, A: not, myo: muscle, trophic: nourishment) refers to lack of nourishment to the muscle. Death of motor neurons leads to disruption of signaling to the voluntary muscles, which leads to atrophy of the muscles. The term "lateral" refers to the lateral region of the spinal cord whose motor neurons are affected. "Sclerosis" refers to the scarring caused due to the degeneration of the motor neuron. A case of

typical ALS disorder shows clinical symptoms by an average age of 55 years. The prognosis of the disease thereafter is rapid and results in death within 3–5 years of onset. The disease could commence either as "bulbar" or as "spinal," affecting upper motor neurons from the cortex or lower motor neurons from the brain stem and spinal cord, respectively. The symptoms that follow include muscular weakness, fasciculation, spasticity, speech defects, and, finally, paralysis. A common reason for death is respiratory failure owing to the loss of control over the thoracic and diaphragm muscles. In most cases, the sensory functions remain unaffected. ALS may also manifest atypically, such as in the case of juvenile ALS, which is early onset (25 years or younger), ALS with fronto-temporal dementia (FTD), and ALS with spinal muscular atrophy (Andersen and Al-Chalabi 2011).

The origin of the manifestation of ALS in the cell is hard to pinpoint. The disease works by destabilizing the general homeostasis in the motor neuron, as well as its communication with the neighboring glial cells and muscle cells that form the tripartite junction. At the cellular level, ALS is marked by a number of stereotypic hallmarks of neurodegeneration. A prime feature shown in ALS patient tissue samples is the presence of proteinaceous, ubiquitinated cellular inclusions, identified clinically as "skein-like" or "Lewy body-like," "Bunina bodies," hyaline inclusions, as well as TDP-43-positive RNA foci (Blokhuis et al. 2013). Several other homeostasis mechanisms in the cell get affected in the course of the disease, such as ER stress, unfolded protein response, mitochondrial dysfunction, oxidative stress, glutamate excitotoxicity, ubiquitin proteasomal machinery, and autophagy, to name a few. Cellular structures such as neurofilaments, microtubules, and neuromuscular junctions also become disrupted. Axonal transport, ER-to-Golgi trafficking, and vesicular trafficking are other processes that are impaired in motor neurons (Ferraiuolo et al. 2011). The disease pathogenesis has also been shown to involve cell non-autonomous factors such as crosstalk of motor neurons with voluntary muscle cells at the synapse and neuronal neighbors such as astrocytes and microglia that can signal and evoke an immune response (Boillée et al. 2006). The kind of response particularly generated at the cell autonomous and cell non-autonomous level might concur with selective susceptibility of motor neurons in this disease.

Around 90% of the cases known are sporadic, whereas around 10% of the cases are found to be familial. Thirty-one genetic loci have been linked to familial ALS with or without other associated conditions such as FTD in various cohorts of families throughout the world. Superoxide dismutase1 (SOD1) is the first known genetic loci in ALS (Rosen 1993). Since the advent of genome-wide association studies (GWAS) and linkage and sequencing studies, a variety of genetic loci with specific mutations have been identified. These mutations that are known for each of these genetic loci show different levels of prevalence and penetrance. Certain loci are also associated with other neurodegenerative diseases such as C9ORF72 with fronto-temporal dementia, VAPB with spinal muscular atrophy, and ataxin-2 with ataxia. Thus, there exists a pleiotropy in the manifestation of the disease pertaining to different loci (Andersen and Al-Chalabi 2011). The progressions of sporadic or familial cases have not been shown to be clinically different. Indeed, recent reports show that relatives of patients with sporadic ALS are susceptible to the disease and that SALS may have a

genetic basis for the pathogenesis of the disease (Andersen and Al-Chalabi 2011). Several genome-wide association studies and linkage and sequencing studies have analyzed the genetic makeup of SALS (Sporadic ALS) patients and demonstrated that around 26 susceptibility loci might be involved. Among these, several loci have been shown to be common between FALS (Familial ALS) and SALS, among which the most abundantly found in population studies in ALS are the hexanucleotide repeats at C9ORF72, superoxide dismutase1 (SOD1), TAR DNA-binding protein-43 (TDP-43), and fused in sarcoma/translocated in liposarcoma (FUS/TLS) (Renton et al. 2014).

Modeling Motor Disorders in Flies

Drosophila serves as a simple, yet elegant model to study varied aspects of human diseases ranging from genetic to cellular to phenotypic characteristics. For example, counterparts of about 75% of human disease-causing genes are found in *Drosophila*, whose functional relevance can be studied using a plethora of genetic tools developed in the fly. According to FlyBase, of the 31 loci involved in typical ALS, 15 orthologs have been identified and modeled in flies (Table 1). Additionally, transgenic flies expressing human orthologs for these genetic loci have been developed to model ALS (Table 1). This is particularly a useful strategy to study loci that are not conserved in *Drosophila*, the best example being that of the hexanucleotide expansion of C9ORF72. The UAS-GAL4 system has been extensively used for expression or knockdown of ALS loci as well as expression of its associated mutations in specific tissues. This approach allows for understanding the role of these genes and subsequent manifestation of a disease condition in a cell-specific manner. Reverse genetics screens have been designed using this strategy to study the interactors of these loci to identify genetic interactomes and gene regulatory networks (GRNs) that govern the disease. Various pathways affecting disease progression have been identified through these studies such as MAP kinases, BMP, Notch, and TOR signaling. Owing to ease of maintenance of large populations, along with genetic manipulations, *Drosophila* also serves as a platform for large-scale drug testing. A variety of phenotypic readouts such as NMJ defects, aggregation, ubiquitination, retinal degeneration, motor defects, and lifespan defects mimic classical ALS phenotypes mapping different stages of disease progression. With the advent of CRISPR-Cas9 technology, genome edited models are now being developed to study disease-causing genes that are physiologically more significant, bearing a closer resemblance to human disease initiation and progression.

SOD1

Superoxide dismutase 1 is the first known ALS locus, identified in 1993 (Rosen et al. 1993), and till date, more than 150 different mutations have been reported in both familial and sporadic cases of the disease. SOD1 is an antioxidant enzyme that is responsible for containing the ROS levels in the cell by converting superoxide species

Table 1 *Drosophila* models for human ALS. Tabulated below are lists of genes for which fly models have been generated

Label	Symbol	FlyBase ID	Name	Function	References
ALS1	*Sod1*	FBhh0000019	Superoxide dismutase 1	Cellular redox regulation	Elia (1999), Mockett et al. (2003), Watson et al. (2008), Bahadorani et al. (2013) and Şahin et al. (2017)
ALS2	*Als2*	FBhh0000033	Amyotrophic lateral sclerosis 2	Rho guanine nucleotide exchange factor for RABs	Takayama et al. (2014)
ALS4	*Hsap\SETX, CG7504*	FBhh0000611	Senataxin	DNA/RNA helicase	Mushtaq et al. (2016)
ALS6	*Hsap\FUS, caz*	FBhh0000018	Fused in sarcoma, cabeza	RNA binding and regulation	Lanson et al. (2011), Sasayama et al. (2012), Machamer et al. (2014) and Matsumoto et al. (2018)
ALS8	*Vap33*	FBhh0000020	VAMP-associated protein 33 kDa	Lipid homeostasis, vesicular trafficking	Chai et al. (2008), Ratnaparkhi et al. (2008), Chen et al. (2010), Moustaqim-barrette et al. (2013) and Sanhueza et al. (2014, 2015)
ALS10	*Hsap\ TDP-43, TBPH*	FBhh0000017	TAR DNA-binding protein-43 homolog	RNA binding and regulation	Li et al. (2010), Voigt et al. (2010), Miguel et al. (2011), Diaper et al. (2013a), Romano et al. (2015) and Chang and Morton (2017)
ALS11	*FIG4*	FBhh0000335	FIG4 phosphoinositide 5-phosphatase	Phosphoric ester hydrolase activity	Bharadwaj et al. (2016)
ALS14	*Hsap\VCP, TER94*	FBhh0000021	Valosin-containing protein	ER-associated degradation	Chang et al. (2011), Wang et al. (2016) and Kushimura et al. (2017)
ALS15	*Ubqln*	FBhh0000823	Ubiquilin	Ubiquitin chaperone	Kim et al. (2018a)
ALS17	*Hsap\ CHMP2B*	FBhh0000022	Charged multivesicular body protein 2B	Component of the heteromeric ESCRT-III complex	West et al. (2015)
ALS18	*Hsap\ Profilin, chic*	FBhh0000023	Chickadee/ Profilin	Binds to actin polymers	Matsukawa et al. (2016) and Wu et al. (2017)

(continued)

Table 1 (continued)

Label	Symbol	FlyBase ID	Name	Function	References
ALS20	*Hrb98DE*	FBhh0000034	Heterogeneous nuclear ribonucleoprotein at 98DE	RNA binding and regulation	Romano et al. (2014)
FTDALS4	*Hsap\TBK1*	FBhh0000148	TANK-binding kinase 1	Inhibition of I-kappa B	Kuranaga et al. (2006)
FTDALS1	*Zzzz\ GGGGCC*	FBhh0000024	GGGGCC hexanucleotide	–	Xu et al. (2013), Freibaum et al. (2015), Tran et al. (2015), Zhang et al. (2015), Lee et al. (2016) and Moens et al. (2018)
Postulated	*CG14718*	FBhh0000408	EWSR1/TAF15	RNA binding and regulation	Couthouis et al. (2011, 2012)
SCA/ALS13	*Hsap\ ATXN2, Atx2*	FBhh0000062	Ataxin-2	RNA binding, translational regulation	Elden et al. (2010)

to free oxygen and peroxide. SOD1-immunoreactive puncta are observed in SOD1-ALS patients. Most of the SOD1 mutations tested in model systems render the protein to form cellular oligomeric inclusions. The nature of these aggregates is shown to be variable; some mutations have been shown to form thioflavin-reactive insoluble amyloids, while others have been shown to form soluble inclusions (Sheng et al. 2012). Different mutations have been shown to render the protein to form aggregates with different propensities (Prudencio et al. 2009). The study shows that mutations that lower the net charge on SOD1 protein or increase the hydrophobicity of the molecule have an increased propensity for aggregation in comparison with wildtype (Sheng et al. 2012). The study has also correlated increased aggregation propensity to faster progression of disease and death post-diagnosis (Prudencio et al. 2009). Most of the mutations appear to functionally impair the protein. Most, but a few, SOD1 mutants lose their ability to bind to Cu and/Zn ions responsible for its catalytic activity and stability. This could be a possible reason for increased ROS levels in SOD1 patients. However, SOD1 knockdown mice have been shown to not develop ALS, and disease mutants such as SOD1-G93A and SOD1-A4V that do not lose their catalytic activity have also been identified, indicating that oxidative stress may be triggered through other homeostatic defects in the disease. Instead, this observation shows that these might be gain-of-function mutations (Prudencio et al. 2009).

Being the oldest known locus in ALS, over the last 25 years, various fly lines to model ALS1 have been generated using older techniques to generate null mutations, P-element insertions, tissue-specific inducible overexpression and knockdown, and, more recently, CRISPR-Cas9 genome editing tools. Studies performed during the 1990s have favored oxidative stress generated due to the loss of function of SOD1 in ALS1 as a disease mechanism. Indeed, SOD1 null mutation or feeding hydrogen peroxide or paraquat, a ROS-generating drug, caused a decrease in the lifespan of flies. This decrease could be rescued by motor neuron-specific overexpression of SOD1 using the UAS-GAL4 system as well as heat shock-induced expression of SOD1 using the FLIP-FRT system (Parkes et al. 1998; Elia 1999; Sun and Tower 1999; Kumimoto et al. 2013). However, while both *Drosophila* (dSOD1) and human SOD1 (hSOD1) could rescue the lifespan of null mutants, activity levels of human SOD1 were significantly lower than *Drosophila*. A number of ALS mutants of hSOD1, such as G93C and G37R, showed partial rescue, while A4V and G41D showed marginal rescue, and I113T showed no rescue of lifespan of SOD1 null mutants (Mockett et al. 2003). Glial-specific dSOD1 expression, but not hSOD1 G84R, could also rescue peroxide toxicity more prominently in older flies (Kumimoto et al. 2013). Although a sudden decline of activity levels and motor defects of these older mutant rescue lines correlated with lifespan, the activity levels and motor functions of young flies were found to be comparable with wildtype rescue line. Taken together, these results indicated that lowered function of SOD1 below a certain threshold could induce oxidative stress and subsequent death in *Drosophila* (Mockett et al. 2003). While oxidative stress is central to neurodegenerative diseases, loss of SOD1 activity itself may not be directly responsible for ROS toxicity given that other antioxidants such as SOD2 or catalase could compensate for its activity. Reciprocally, it was tested whether SOD1 and other antioxidants

could enhance the lifespan of the fly. Different reports based on the use of different transgenic flies have yielded contradictory results. Ectopic overexpression of SOD1 in motor neurons alone showed increased enzymatic activity and lifespan (Parkes et al. 1998; Elia 1999; Sun and Tower 1999). This claim was refuted in a report that compared the effect of several antioxidants in combinations from across various studies along with their experiments. Their findings indicated that overexpression of antioxidants in long-lived strains did not drastically change lifespan as compared to short-lived strains (Orr et al. 2003). More recent studies in the last ten years have detailed the motor function and lifespan changes with an expression of dSOD1 or hSOD1 or mutants of hSOD1 in different cell types. Ubiquitous expression or knockdown of SOD1 could, respectively, increase or decrease the lifespan of the flies. While overexpression did not produce a change, knockdown could drastically reduce motor function (Martin et al. 2009). Pan-neuronal, motor neuronal, and muscle-specific hSOD1 expression or knockdown only appeared to produce a minor or no improvement in lifespan with limited reduction in motor function (Watson et al. 2008; Martin et al. 2009; Bahadorani et al. 2013). Although dSOD1 and hSOD1 are evolutionarily conserved, given these motor defects, hSOD1 does not appear to be a functional equivalent when expressed in *Drosophila.* However, glial, but not motor neuron-specific, expression of dSOD1 reduced lifespan and motor function (Kumimoto et al. 2013). Zinc-deficient loss-of-function mutant of hSOD1 D83S does lead to motor defects associated with mitochondrial dysfunction with only a marginal effect on lifespan when expressed under motor neuronal, pan-neuronal, or glial promoters, but not in muscles (Bahadorani et al. 2013). On the other hand, motor neuronal expression of toxic gain-of-function mutant, hSOD1 G85R, showed reduced lifespan and motor function in an age-dependent manner (Watson et al. 2008). This was accompanied by a reduction in motor neuron number, increase in electrophysiological defects as well as accumulation and aggregation of SOD1 with an increase in age. Not only mutant but wildtype hSOD1 also showed similar defects in motor neurons. Curiously, a simultaneous increase in chaperone, HSP70 staining was observed in the surrounding glial cells, indicating a non-cell-autonomous response (Watson et al. 2008). In another study, this G85R mutant, when expressed simultaneously in glia and motor neurons, could increase the lifespan and climbing activity of the fly (Kumimoto et al. 2013). Genes involved in metabolisms such as pentose-phosphate pathway, NADP, and glutathione metabolism seem to be downregulated in G85R flies, indicating a direct effect on oxidative stress (Kumimoto et al. 2013). Given these conflicting results, the study of the importance of glia in the development of these phenotypes is crucial in the disease. Recently, a knock-in line using CRISPR cas9 strategy was created that harbored mutations such as H48R, H71Y, G85R, G51S, and G37R, and characterized (Şahin et al. 2017). These mutants showed reduced eclosion rates and lifespan, increased motor and muscle defects accompanied by reduced motor neuron number. These mutants, while forming dimers and higher molecular weight complexes, showed reduced expression in an increase in age. These knock-in lines have validated the toxic gain-of-function effects associated with these mutants in flies serving as a *Drosophila* model that most closely mimics the development of the disease (Şahin et al. 2017).

C9ORF72

In 2011, C9ORF72 was discovered as the most commonly found locus in the ALS-FTD spectrum, accompanied with increased glutamate excitotoxicity thus underlining a strong link between these diseases. The locus essentially represents the expansion of the non-coding hexanucleotide, GGGGCC, to several hundred repeats in the disease, in contrast to the 2–25 repeats found in normal conditions in the C9ORF72 gene. The pathological conditions associated with this locus are multifaceted. Reduced expression of the C9ORF72 gene owing to the presence of repeat expansions was hypothesized to lead to neurodegeneration due to haploinsufficiency. However, knockout mice models failed to develop any neurodegeneration proving haploinsufficiency to be an unlikely course of action. The hexanucleotide repeats at the molecular level acquire very stable DNA/DNA or DNA/RNA G-quadruplex conformations along with DNA/RNA hybrid R-loops. Such secondary structures have been shown to stably bind nucleolar proteins such as nucleolin (NCL) and hnRNPs in a conformation-dependent manner forming nuclear inclusions that can cause protein mislocalization and nuclear stress. It appears that disease mechanisms are centered more toward the gain-of-function phenotypes arising with the sense and anti-sense RNA quadruplexes of G4C2 repeats that lead to the formation of nuclear RNA foci that could potentially sequester RNA-binding proteins and cause nuclear toxicity. Abortive transcripts of variable lengths that get generated from this locus are translated through a non-AUG translation mechanism (Zhang et al. 2014). RNA products undergo non-AUG translation to form five different dipeptide repeats (DPRs) of polyGR and polyGA from sense RNA, polyPR and polyPA from antisense RNA, and polyGP from both, culminating in protein aggregation. A key question of what drives disease progression, RNA toxicity or DPR aggregates or both, has been addressed using flies as a model.

Since 2013, there have been several reports focused on the use of different construct designs to overexpress variable lengths of G4C2 repeats in the 5'UTR or in the intron under an upstream activating sequence (UAS) followed by a downstream SV40 3′UTR containing a polyA tail, enabling the selective expression of RNA and/or DPRs to delineate the pathological cause for the disease. Few studies have favored the RNA toxicity hypothesis leading to retinal degeneration with eye-specific expression or a reduction in the number of active zones in larval neuromuscular junctions with motor neuron-specific expression, suggesting impairment in RNA metabolism and nucleocytoplasmic transport as major causes of cellular defect in ALS (Xu et al. 2013; Zhang et al. 2015; Celona et al. 2017). In these studies, toxicity associated with RNA complexes that sequester RNA binding proteins, such as Pur alpha or RANGAP or Zfp106, could be rescued by the overexpression of these proteins. However, these studies have not accounted for the presence of DPR aggregation as a possible disease mechanism. Several studies have shown that RAN expression of DPRs, in addition to RNA repeats, but not intronically expressed RNA repeats alone, leads to retinal defects, reduced lifespan, reduced bouton number at the NMJ, and reduced muscle size along with increased nucleolar volume (Mizielinska et al. 2014, 2017; Freibaum et al. 2015; Tran et al. 2015) In fact, a recent study demonstrated that presence of

interspersed stop codons prevent the non-ATG translation of the G4C2 repeats but retain formation of cytoplasmic as well as nuclear RNA foci of around 1000 repeats, but do not show drastic lifespan defects or eye defects, proving that the effects arise from DPR pathology (Moens et al. 2018). When DPRs of 50 copies of polyGR, polyGA, polyPR, or polyPA were conventionally expressed in the eye, using a codon-optimized sequence to prevent the formation of any stable secondary structures of RNA repeats, dramatic eye degeneration was observed (Boeynaems et al. 2016). Two genetic screens have identified a number of modulators of eye degeneration phenotype involved in nucleocytoplasmic transport placing it as a core mechanism in C9ORF72-mediated pathology (Freibaum et al. 2015; Boeynaems et al. 2016). Impairment of nuclear transport allows for leakage of RNA repeats into the cytoplasm promoting the expression of toxic DPRs. Inhibition of nuclear export via SRSF1 could rescue the eye phenotype as well as motor functions in flies (Hautbergue et al. 2017). Consistently, it has been shown that arginine containing DPRs, polyGR, and polyPR appears to cause more aggressive phenotypes as compared to polyGA, polyPA, and polyGP (Mizielinska et al. 2014; Wen et al. 2014; Freibaum et al. 2015; Tran et al. 2015; Yang et al. 2015; Boeynaems et al. 2016). When 36 repeats of toxic GR/PR species were expressed in a narrow subset of neurons that are glutaminergic, NMJ phenotypes of increased synaptic vesicles and active zones, accompanied by increased glutamate excitotoxicity and intracellular calcium, were observed (Xu and Xu 2018). Inhibition of vGLUT, a glutamate transporter, in this background could rescue the associated motor defects and shortened lifespan (Xu and Xu 2018). The arginine containing DPRs has been shown to disturb the phase transition of low complexity domain (LCD) proteins into ribonucleoprotein (RNP) complexes such as nucleolus, stress granules, and Cajal bodies (Lee et al. 2016). The field currently favors DPR pathology to be the driving force in ALS/FTD via disruption of RNA bodies, RNA processing, and nucleocytoplasmic transport. Elucidating the differential outcome of C9ORF72 pathology between these two diseases remains a challenge.

TDP-43

Drosophila has been extensively used to model and study TDP-43 pathology in ALS. TAR DNA-binding protein 43 is shown to form ubiquitinated cytoplasmic inclusion in SALS and ALS linked with fronto-temporal dementia (ALS-FTD) cases (Neumann et al. 2006). It is a DNA/RNA binding protein that is usually found to be present in the nucleus. It binds to intronic and 3' UTR of RNA, thereby playing an essential role in RNA metabolisms such as processes like RNA splicing, transcriptional control, and RNA trafficking. Due to mutations in TDP-43 in a diseased condition, proteinopathy is observed in the cytoplasm of the spinal cord and brain tissue, bringing a possible loss-of-function phenotype (Blokhuis et al. 2013). This associated aggregation is conferred by the C-terminal region of TDP-43, which is a low-complexity domain that harbors most of the mutations associated with the disease. Till date, 47 missense mutations and one nonsense mutation have been

found in the TDP-43 locus. TDP-43 immunoreactivity is now being used as a clinical marker to detect ALS/FTD conditions.

The initial hypotheses to address how TDP-43 causes the disease revolved around loss-of-function versus toxic gain-of-function mechanisms. TDP-43 (TBPH in *Drosophila*) null flies generated through classical genetic methods yielded phenotypes such as lowered lifespan, motor defects, disrupted NMJ, and lowered dendrite branching (Feiguin et al. 2009; Lu et al. 2009). Flies lacking TDP-43 showed impaired mTOR signaling through its regulation of the levels of the raptor, a member of the TORC1 complex, with a direct effect on genes involved in autophagy (Xia et al. 2016). Null mutants of TDP-43 led to the increased post-synaptic accumulation of glutamate. This excitotoxicity appeared to be a result of the loss of function of glutamate acid decarboxylase (GAD1) (Romano et al. 2018). Glia-specific knockdown of TDP-43 could increase glutamate excitotoxicity by affecting axon wrapping and glutamate receptor clustering via glutamate transporter, EAAT1 (Romano et al. 2015). TDP-43 null flies also showed lowered levels of *cacophony*, a voltage-gated calcium channel, leading to loss of motor function, which could be rescued by the overexpression of *cacophony* even in a subset of motor neurons alone (Lembke et al. 2017).

Overexpression of wildtype fly or human TDP-43 gene leads to defects in NMJ, eye, locomotion, and lifespan, suggesting gain-of-function roles (Li et al. 2010; Voigt et al. 2010; Estes et al. 2011; Miguel et al. 2011). Intriguingly, despite similarities between phenotypes of null and overexpression, a high-throughput RNA sequencing has shown that there is little overlap in the gene expression patterns between these genotypes (Hazelett et al. 2012). The effect of wildtype overexpression appears to be more exacerbated than overexpression of point mutants for C-terminal, RRM, NLS, or nuclear export signal (NES) (Li et al. 2010; Voigt et al. 2010; Estes et al. 2011; Miguel et al. 2011). Surprisingly, although point mutations in the RRM cause nuclear puncta, retinal, and lifespan defects, deletion of RRM domain does not cause any neurodegeneration, but abrogates the deleterious effects of wildtype and ALS-linked mutant TDP-43 overexpression (Li et al. 2010; Ihara et al. 2013). Along with its roles in the nucleus, TDP-43 also regulates RNA packaging, splicing, and transport in the cytoplasm. It is proposed that in the presence of RRM deletion mutant, TDP-43 mutants cannot sequester RNA targets, thus preventing ALS pathology (Ihara et al. 2013). An example supporting RNA binding as a mechanism involved in ALS is the regulation of translation and localization of *futsch* mRNA by TDP-43 via a stretch of UG-rich region in its 5' UTR (Coyne et al. 2014; Romano et al. 2016). In normal conditions, TDP-43 transports *futsch* mRNA for translation at the NMJ. However, overexpressed TDP-43 or its mutant in the CTD sequesters the *futsch* mRNA into RNP complexes altering its localization and expression (Coyne et al. 2014). Overexpression of *futsch* could reverse the effects of TDP-43 pathology, including RNA transport and aggregation (Coyne et al. 2014). Another modifier of TDP-43, identified in reverse genetics screen in a mammalian cell line, is inositol-1, 4, 5-triphosphate receptor, inhibition of which could increase nuclear export of TDP-43, thereby reducing its nuclear dosage and further rescuing climbing defects and lifespan in flies (Kim et al. 2012).

Furthermore, ALS-linked mutations in TDP-43 have also been shown to impair anterograde transport of TDP-43 RNA granules and, subsequently, its mRNA targets (Alami et al. 2014). While several studies (Li et al. 2010; Voigt et al. 2010; Miguel et al. 2011; Diaper et al. 2013a) have detected nuclear accumulation but not mislocalization upon overexpression of TDP-43 in neurons, others (Estes et al. 2011, 2013; Gregory et al. 2012) have reported the presence of cytoplasmic accumulation in the eye disc and glial cells. These TDP-43 defects and aggregation could be lowered by pharmacological upregulation of heat shock response and chaperone activity (Gregory et al. 2012). A chaperone, HSPB8, in particular, has been shown to rescue against toxic aggregation of various TDP-43 mutants and truncated forms, TDP-25 and TDP-35, through autophagic degradation (Gregory et al. 2012; Crippa et al. 2016). Clusterin, an extracellular chaperone, localizes to the cytoplasm in the presence of ER stress, countering motor, and lifespan defects by aiding the clearance of cytosolic TDP-43 aggregates (Gregory et al. 2017). Peptides flanking the mutation A315T in TDP-43 have been shown to form amyloid structures in vitro that were found to be infectious and neurotoxic in *Drosophila* neuronal cells in culture. This study has demonstrated the prion-like behavior of aggregate formation and propagation of the disease (Guo et al. 2011).

The phenotypes of TDP-43 overexpression are suggested to show dose-dependent increase, implying that accumulated TDP-43 renders the protein ineffective leading to loss of function or a dominant negative effect in case of mutants, which determines the extent of neurodegeneration. The consensus in the field favoring dosage-dependent neurodegeneration was thought to be the defining factor in TDP-43 pathology in *Drosophila,* initiating with synaptic defects followed by loss of neuronal connections and neuronal death (Diaper et al. 2013b). In normal conditions, the levels of TDP-43 are maintained by an alternative splicing mechanism through the action of three splicing factors, SF2, Rbp1, and Sf3b1. This effect was inferred by expressing a transgenic construct of TDP-43 with a region of the 3'UTR responsible for autoregulation, which could reduce the TDP-43 mRNA levels and subsequent protein levels in the cell (Pons et al. 2017). With the age of the fly, it appeared that TDP-43 regulation is affected, leading to a decrease in TDP-43 levels before the onset of motor defects (Cragnaz et al. 2015). A genome-edited version in *Drosophila*, replacing the fly homolog with a human TDP-43 gene or its mutants G294A or M337 V, serves as a potential model to study the outcome of ALS pathology in flies. TDP-43 expressed under the endogenous promoter appears to be autoregulated, phosphorylated, and ubiquitinated, without drastic defects in motor function or lifespan. Further analysis would shed light on the functional aspects of human TDP-43 in flies (Chang and Morton 2017).

FUS

Fused in sarcoma was first described as a proto-oncogene involved in liposarcoma. In 2009, it was found to be another RNA-binding protein involved in ALS and FTD (Vance et al. 2009; Neumann et al. 2010). FUS binds pre-mRNA at intronic regions,

non-coding RNA, exons, and 3'UTRs, and is involved in processes such as DNA repair, miRNA processing, transcription, splicing, and mRNA transport. Mutations in FUS, mainly in the nuclear localization sequence (NLS), have been shown to cause the formation of skein-like cytoplasmic aggregates in large cohorts of ALS cases, with diffused nuclear signal causing loss of function of the protein (Vance et al. 2009). FUS consists of a prion-like sequence in its N-terminal region that has been shown to promote aggregation even in the wildtype protein in yeast (Sun et al. 2011). It is intrinsically prone to aggregate in vitro (Blokhuis et al. 2013). In patients of both sporadic and familial cases, FUS is a part of cytoplasmic aggregation that may or may not be TDP-43 positive (Neumann et al. 2010). FUS pathology does not seem to be limited to ALS/FTD as FUS-positive cellular puncta have been observed in other neurodegenerative diseases as well as Huntington's disease and spinocerebellar ataxia. FUS pathology was found to be similar to that of TDP-43 in that it affected RNA processing and nucleocytoplasmic transport. As in TDP-43, FUS consists of an RRM domain, a low-complexity domain glycine-rich region, and a zinc finger domain. Cabeza (caz) is the *Drosophila* homolog of FUS. Expression of domain deletion mutants of caz showed changes in the levels of the endogenous caz protein. Indeed, overexpression of wildtype FUS could lower the expression of the endogenous caz, emphasizing the presence of an autoregulatory function (Machamer et al. 2014). In flies, this reduction appears to be attributed to the active degradation of *caz* via the ubiquitin-proteasomal machinery (Yamamoto et al. 2018). While the complete deletion mutant of *caz* showed motor defects and reduced lifespan, neuronal knockdown of *caz* also showed NMJ disturbances and motor defects but not reduced lifespan (Sasayama et al. 2012). This loss-of-function effect could be rescued by the cell-specific overexpression of human FUS or *Drosophila caz* but not mutant FUS-P525L (Sasayama et al. 2012; Machamer et al. 2014). Motor neuron-specific overexpression of caz or FUS wildtype or disease mutants also led to phenotypes similar to loss of function such as lowered bouton number, impaired synaptic function, and motor defects (Chen et al. 2011; Lanson et al. 2011; Xia et al. 2012; Shahidullah et al. 2013). However, unlike loss of function, overexpression of FUS wildtype or mutants shows retinal degeneration and mushroom body defects with axonal degeneration as well (Chen et al. 2011; Miguel et al. 2012). While overexpression of FUS alone only showed low cytoplasmic localization, expression of the mutants such as R524S and P525L showed cytoplasmic inclusions reminiscent of the disease (Chen et al. 2011). Nuclear accumulation of the insoluble form of FUS leading to the manifestation of neurodegenerative phenotypes suggests that ALS symptoms may be triggered before cytoplasmic proteinopathy (Miguel et al. 2012). Improving the solubility of the protein using molecular chaperone HSPA1L could reverse some of the retinal degenerative effects. Alternatively, overexpressed FUS may behave in an altered manner, such as a change in post-translational modifications like phosphorylation may cause a toxic gain of function (Miguel et al. 2012). A recent screen performed to identify modulators of FUS-R521G mutant in a class of motor neurons of abdominal ganglion revealed genes involved in nucleocytoplasmic transport such as exportin-1 and Nup154 as suppressors of phenotype. Cytoplasmic aggregation of FUS mutants could be solubilized in the absence of

exportin-1, preventing its sequestration into stress granules, thus providing a neuroprotective role (Steyaert et al. 2018). These studies suggest that while perturbations of wildtype FUS or caz affect neuronal well-being, it is the mutant protein that forms persistent cellular aggregation in flies. *Drosophila* primary neuronal cell co-culture studies could be used to demonstrate a prion-like cell-to-cell transfer of FUS P525L and FUS R524S aggregates, but not of wildtype FUS (Feuillette et al. 2017). However, wildtype FUS is an intrinsically disordered nuclear protein whose expression, localization, and solubility are affected by RNA binding. One such example is hsrωb belonging to a class of long non-coding RNA called architectural RNA that forms the nucleoplasmic ω-speckles compartment (Jolly and Lakhotia 2006). Knockdown of hsrω downregulates *caz* transcription as well as leads caz protein to be mislocalized into cytoplasmic inclusions (Lo Piccolo and Yamaguchi 2017; Lo Piccolo et al. 2017). The phase separation property of FUS is essential for the formation of RNA complexes. A recent study used domain deletion mutants of FUS in an attempt to understand the property of phase separation of this RNA binding protein into stress granules. Deletion of the QGSY motif in the N-terminal LCD and the RGG2 motif in the C-terminal LCD both reduce toxicity in *Drosophila* (Bogaert et al. 2018). Mutation of QGSY to GQ in the N-terminal LCD could act as dominant active by rescuing the eye degeneration phenotype of C-terminal NLS mutant FUS P525L, without being sequestered to the cytoplasmic aggregates, upon coexpression. This proved that the N-terminal LCD was important for self-assembly of FUS (Matsumoto et al. 2018). LCDs form strong synergistic interaction in the formation of liquid droplets as well as hydrogels in vitro, suggesting that point mutations in these domains might make the protein more susceptible to phase separation leading to aggregation-induced toxicity in the disease (Bogaert et al. 2018).

VAPB

In 2004, Mayan Zats group identified another ALS locus as a point mutation, P56S, in a gene coding for VAMP-associated protein B (VAPB) in eight Brazilian families, of Portugal origin. Several members of these families harboring this mutation developed motor diseases in the form of not just ALS, but also SMA (Nishimura et al. 2004). The reason for the differential manifestation of these diseases is unknown, bearing no correlation with age or gender. Other isolated cases featuring VAPB(P56S) were found in families in Japan, Germany, and the USA (Funke et al. 2010; Millecamps et al. 2010). Since then, four more mutations, T46I, S160Δ (Landers et al. 2008; Chen et al. 2010), V234I – associated with C9ORF72 (van Blitterswijk et al. 2012), and P56H (Sun et al. 2017), have been identified through sequencing studies. VAPB is an ER membrane protein that integrates into the membrane via its C-terminal domain. The protein works as a homodimer or a heterodimer with VAPA. Through its N-terminal MSP domain, VAPB interacts with several proteins that contain an FFAT motif, displaying roles in membrane tethering between organelles, vesicular transport, and lipid transport. VAP localizes in the ER membrane as well as membrane contact sites between organelles and intracellular vesicles. VAPB

plays an important role in cellular homeostasis by regulating calcium signaling and proteostasis. VAP mutant, owing to change in conformation, leads to misfolding and aggregation of the protein. Overexpression of VAP(P58S), VAP(T48I), and VAP(V260I) in the *Drosophila* homolog, VAP33a (after that mentioned as VAP), led to the formation of cellular puncta (Ratnaparkhi et al. 2008; Chen et al. 2010; Sanhueza et al. 2014). Coexpression of tagged VAP and VAP(P58S) protein showed colocalization, suggesting the dominant negative effect of the VAP(P58S) that interacts with and sequesters the wildtype VAP into its ubiquitinated aggregates. VAP null mutation and expression of other disease-related mutants, VAP(P58S), VAP(T48I), and VAP(V260I), are accompanied with ER stress in the adult brain of the fly as suggested by aggregation and mislocalization of ER luminal resident proteins, Boca, PDI, chaoptin, SERCA, and Hsp70, and increase in puncta of chaperone upregulated in UPR, Hsc3, and XBP1-GFP (Tsuda et al. 2008; Chen et al. 2010; Sanhueza et al. 2014; Yadav et al. 2018). Upon neuronal expression, the N-terminal MSP domain of VAP can be cleaved and secreted out of the neurons possibly, as a ligand for ephrin or Robo/Lar-like receptors on the muscle, thereby affecting cytoskeleton and mitochondrial morphology (Tsuda et al. 2008; Han et al. 2012). The secretion of MSP domain does not seem to occur in the presence of VAP(P58S) aggregation, such that neuronal overexpression of VAP causes myofibril disruption in the muscle while VAP(P58S) does not (Tsuda et al. 2008). Neuronal overexpression of VAP leads to dosage-dependent changes in the NMJ, including smaller bouton size and increase in bouton number (Pennetta et al. 2002; Chai et al. 2008; Ratnaparkhi et al. 2008). Human and *Drosophila* VAP appear to be phenotypically similar at the NMJ, suggesting evolutionarily conserved functionality (Chai et al. 2008). The mutant VAP(P58S) appears to have the opposite effect with a lesser number of larger boutons similar to VAP null phenotype, showing disruption of microtubule organization, lowered number of active zones, and reduced retrograde BMP signaling (Ratnaparkhi et al. 2008; Forrest et al. 2013).VAP(V260I), on the other hand, shows an increased number of smaller boutons with an affected microtubule architecture similar to increased VAP expression (Sanhueza et al. 2014). VAP null mutants in *Drosophila* show defects in dendritic localization and axonal transport of Down syndrome cell adhesion molecule (Dscam) protein involved in self-recognition and avoidance in DA neurons (Yang et al. 2012). Phosphoinositide levels appear to be increased in ALS8, leading to axonal and synaptic defects. Sac1, the phosphoinositide phosphatase, the enzyme required to regulate phosphoinositide metabolism, was found to interact with VAP physically. Downregulation of Sac1 or expression of VAP(P58S) affects synaptic microtubule organization that could be rescued by reducing the levels of phosphoinositide (PI) (Forrest et al. 2013). A phosphatidylinositol transfer protein (PIPT) domain-containing protein, RDGBα, responsible for PIP2 metabolism, is recruited to the ER:PM contact sites via its interaction with VAP in photoreceptor cells (Yadav et al. 2018). Despite lowered synaptic function, both neuronal overexpression of VAP and VAP(P58S) could rescue VAP-deficient flies. This suggested that while VAP(P58S) appears to be dominant negative and phenocopies VAP null at the NMJ upon overexpression, it does not seem to be non-functional. While ubiquitous and muscle-specific expression of

VAP and VAP(P58S) caused lethality at 29 °C, at 25 °C, VAP, but not VAP(P58S), showed lethality (Ratnaparkhi et al. 2008). This suggested that above a certain threshold VAP protein, but not VAP(P58S), could develop toxic functions in the cell. Muscle-specific expression of VAP(V260I) showed a change in the shape, size, and position of muscle nuclei, leading to a disruption of nuclear envelop architecture (Sanhueza et al. 2014). Pan-neuronal and glial cells appeared to be more tolerant of the overexpression of these proteins, as they did not lead to the lethality of the fly at either temperature (Ratnaparkhi et al. 2008). However, neuronal VAP(P58S) overexpression did seem to cause motor defects and neuronal death in the larval brains according to one transgenic model (Chai et al. 2008). Eye-specific expression of VAP(P58S) indeed showed retinal degeneration that could be rescued by the overexpression of inhibitor of apoptosis, DIAP2 (Forrest et al. 2013; Sanhueza et al. 2015). Expression, in sensory organ precursor cells, of wildtype VAP but not mutant VAP, reduced the number of thoracic macrochaetae. Coexpression of VAP and VAP (P58S) could recover the thoracic bristle number. A reverse genetic screen designed to identify interactors of VAP using macrochaetae as a read-out helped identify 103 genes that formed a part of gene regulatory network consisting of 406 genes including physical interactors (Deivasigamani et al. 2014). This screen identified the TOR pathway as a modulator of VAP as well as VAP(P58S). Downregulation of TOR appears to rescue morphological defects at the NMJ associated with VAP(P58S), while upregulation of TOR could rescue the effects associated with VAP (Deivasigamani et al. 2014). Members of the TOR pathway were also identified as modulators of VAP(P58S) aggregation through a S2R+ cell-based screen. This interaction could be based on ROS regulation coupled with proteasomal degradation of VAP(P58S) aggregates (Chaplot et al. 2019). Another reverse genetics screen around the same time identified a large network of genes modulating of retinal degeneration associated with eye-specific expression of VAP(P58S). Genes involved in vesicular and endocytic trafficking (Rab5, Rab7), proliferation (Ric) and apoptosis (Diap2), proteolysis and lipid biogenesis were identified as a part of the network. VAP(P58S) aggregates expressed in the fly brain clustered with Rab5, similar to that found in patient motor neuron samples (Sanhueza et al. 2015). In 2013, constructs of the genomic region of VAP as well as VAP containing the P58S mutation were generated and site-specifically inserted into the third chromosome to generate transgenic flies expressing VAP or its mutation under its promoter (Moustaqim-barrette et al. 2013). Both the wildtype and the mutant genomic construct could rescue the lethality associated with VAP null mutant. While wildtype VAP could rescue the entire length of the *Drosophila* lifespan, the VAP(P58S) genomic rescued flies survived only up to 25–30 days post-eclosion. Curiously, when expressed at endogenous levels, the heterozygous combination of one copy each of wildtype and mutant construct could survive for as long as wildtype flies. The expression of VAP(P58S) at endogenous level does not compromise the functional VAP protein unlike its overexpression using the UAS-GAL4 system. It appears that the threshold of VAP(P58S), as well as VAP protein level, determines the extent to degeneration in the fly. This suggests that the reduction in lifespan of VAP(P58S) genomic-rescued flies is a result of partial loss of function of VAP(P58S)

mutant protein. Oxysterol binding protein (OSBP), a physical interactor of VAP, normally present in the ER and responsible for cholesterol transport, is mislocalized to the Golgi in VAP null flies. The shortened lifespan of the VAP(P58S) genomic-rescued flies could be increased to wildtype levels by the overexpression of human OSBP, specifically in the motor neuron. Overexpression of hOSBP restored OSBP localization to the ER in VAP null flies, lowering accumulation of ER proteins and ER stress associated with VAP loss of function (Moustaqim-barrette et al. 2013). The genomic-rescued flies display ER stress and disruption of ER quality control compartment, demonstrating the partial loss of function of VAP(P58S), which is also observed with VAP(P58S) overexpression (Tsuda et al. 2008; Moustaqim-barrette et al. 2013).

Other Genetic Loci

A set of ALS loci involved in degradative mechanisms, such as valosin-containing protein (VCP), ubiquilin-1/ubiquilin-2 (UBQLN1/2), TANK-binding kinase (TBK1), and senataxin (SETX), have been modeled in *Drosophila*. VCP is a hexameric AAA ATPase that forms a part of the ER-associated degradation complex responsible for the translocation of ER-based proteins for proteasomal degradation. Pathogenic mutations in VCP have been identified in several neurodegenerative diseases such as ALS and inclusion body myopathy with Paget's disease of bone and fronto-temporal dementia (IBMPFD). VCP is conserved in *Drosophila* as TER94. Dominant active pathogenic mutations of VCP involved in IBMPFD cause midline crossing of β/γ lobes of the mushroom body in the brain, muscle disruption, and retinal degeneration, which is sensitive to cellular ATP levels (Chang et al. 2011). It plays a role in dendritic pruning promoted by ecdysone signaling via Mical, actin-severing enzyme, in class IV DA neurons. The regulation of Mical mRNA and subsequent dendritic pruning in pupal stages is controlled by RNA-binding proteins such as TDP-43, whose localization is dependent on VCP (Rumpf et al. 2014). Stress-induced sumoylation of VCP has suggested a mechanism for its nuclear transport, stress granule recruitment and promotion of ERAD pathway; reduced sumoylation in pathogenic mutants could result in altered co-factor binding and function (Wang et al. 2016). VCP mutations associated with ALS expressed in motor neurons lead to NMJ defects such as the appearance of ghost boutons and decrease in bouton number, coupled with crawling defects. In muscles, VCP mutant protein leads to sarcomere and mitochondrial defects similar to that in *PINK* and *parkin* mutant. VCP appears to be essential for mitochondrial quality control and is recruited to the mitochondria via parkin (Kim et al. 2013b; Kimura et al. 2013).

Mutations in the proline-rich region of an X-lined ALS locus, UBQLN2, cause juvenile as well as adult-onset ALS and FTD. UBQLN1/UBQLN2 are ubiquitin chaperones that participate in both proteasomal and autophagic degradation mechanisms. UBQLN interacts with both ubiquitin ligases and the proteasome via its ubiquitin-associated domain and ubiquitin-like domain. Mutations in its proline-rich region caused misfolding and cytosolic aggregation of UBQLN2 that appear to

be both ubiquitin and p62 positive. Mutant UBQLN2 proteins showed an age-dependent decrease in solubility and increase in sensitivity to chymotryptic cleavage. Eye-specific expression of mutant UBQLN2proteins caused hyperpigmentation, while neuronal expression leads to changes in NMJ morphology and climbing defects. Proline mutants possessing enhanced binding to ubiquitin and toxic gain of function, clubbed with changes in folding and subsequent aggregation, appear to be the cause for toxicity (Kim et al. 2018a).

The *Drosophila* homolog of the recently identified gene in ALS, TBK1, has been studied previously as a regulator of an inhibitor of apoptosis, DIAP2, levels via its phosphorylation and subsequent degradation, in developing sensory organ precursor cells, thereby controlling the non-apoptotic functions of caspases (Kuranaga et al. 2006). A DNA/RNA helicase, SETX, has been shown to modulate NMJ structural organization by affecting the number of futsch loops and actin puncta associated with the boutons. The effect of SETX and its mutants at the bouton showing a decrease in several active synaptic zones could be a result of increased BMP signaling and decreased highwire activity (Mushtaq et al. 2016). Highwire is an E3 ubiquitin ligase that negatively regulates BMP signaling (Mccabe et al. 2004).

A few ALS loci are involved in endosomal trafficking such as Alsin2, FIG4, CHMP2B, and actin polymerization regulator, profilin, with essential roles in membrane remodeling. Alsin2 is a GTP exchange factor (GEF) involved in the activation of the early endosomal protein, Rab5. ALS-linked mutations in Alsin2 led to a reduction in its GEF activity in *Drosophila* S2 cells. Knockout of Alsin2 caused defects in NMJ and dendritic morphologies similar to Rab5 knockout along with climbing defects, which could be rescued by expression of Alsin2 under ubiquitin-GAL4, but not with motor neuron-specific expression (Takayama et al. 2014). FIG4, a phosphoinositide phosphatase, was found as a locus in not only ALS but also CMT and Yunis–Varon syndrome. Mutation known in CMT has been studied using *Drosophila* FIG4 protein in larval muscles. FIG4 null mutation caused an accumulation of lysosomes, which could be partially or entirely rescued by mutant FIG4 and wildtype FIG4 overexpression, respectively. This phenotype could also be rescued by inhibiting the upstream Rab7 and HOPS complex function, preventing fusion of late endosome with lysosomes. FIG4, in complex with VAC14 and FAB1, showed a non-catalytic function, involved in the maintenance of lysosomal size (Bharadwaj et al. 2016). A member of the ESCRT-III complex, CHMP2B, was found be involved in ALS-FTD. An FTD-associated mutant of CHMP2B developed NMJ and eye defects that could be modulated by members of recycling endosome machinery, RAB8, which was in turn regulated by JNK and BMP pathway (West et al. 2015). Profilin regulates actin polymerization through its interaction with formin. Neurodegenerative effects of mutant forms of human profilin expression in *Drosophila* appeared to be a result of partial loss of function in nature. These mutants do not seem to aggregate, as seen in the case of the disease and mice models. Overexpression of both wildtype and mutant forms of human profilin lowered satellite boutons along with decreased synaptic vesicles. However, wildtype overexpression led to the formation of several ghost boutons and an increased number of active zones, as compared to mutants. Nevertheless, they were able to rescue pupal

lethality associated with the knockdown of the endogenous *Drosophila* profilin, *chickadee* (Wu et al. 2017).

Studies on RNA pathology have gained momentum in the field of ALS and FTD concerning other RNA binding proteins, as well. A functional screen in yeast revealed RNA binding proteins such as EWSR1, TAF15, HNRNPA0, and DAZ1 as potential ALS loci that had a propensity for cytoplasmic aggregation similar to that seen in TDP-43 and FUS. Expression of TDP-43, FUS, HNRNPA0, and DAZ1 proved to be highly toxic to yeast, whereas EWSR1 and TAF15 showed milder toxicity. These genes, when tested in the *Drosophila* eye, developed retinal degeneration in a dose-dependent manner. RGG mutants of EWSR1 and TAF15 also showed rough eye phenotypes. Pan-neuronal overexpression of these genes lowered the lifespan and climbing ability of flies. Finally, puncta of these proteins were found in sporadic cases of ALS, further emphasizing the impact of RNA pathology in ALS (Couthouis et al. 2011, 2012). In flies, hnRNPA2 mutants have been shown to cause mild myotubule organization defects as well as cytoplasmic inclusions (Kim et al. 2013a). Like TDP-43 and FUS, EWSR1, TAF15 hnRNPA1, and A2 are examples of proteins that contain low-complexity domains or prion-like domains enabling them to phase separate into functional membrane-less organelles of RNP complexes. In ALS, mutations identified in these loci make the protein more susceptible to aggregation, altering the dynamics and function of these RNP complexes.

A Unifying Genetic Network in ALS and Other Neurodegenerative Disease

Death of motor neurons is often viewed as the core feature responsible for ALS. However, a collection of cell autonomous and non-cell autonomous events lead to the onset, progression, and death of the patient in ALS. The motor neurons engage in cell-to-cell communication with different cell types such as glial cells, intermediate neurons, and muscles. Perturbations in external cues and downstream signaling lead motor neurons to develop a higher level of susceptibility that manifests in ALS. These perturbations can be genetic as well as environmental. Several essential genes identified as ALS loci perform important housekeeping functions such as RNA processing, protein quality control, axonal transport, vesicular and endosomal trafficking, ER, mitochondrial and oxidative stress regulators. A major class of loci is the RNA binding proteins containing a prion-like domain (QGSY-rich), RRM, glycine-rich domain, RGG domains, and NLS site (PY motif). Mutations identified in ALS have been mapped to all these regions of these genes making these proteins more prone to mislocalization and aggregation. RNA-binding proteins are generally responsible for RNA packaging and trafficking. This property becomes more crucial in stress where these proteins form reversible protective RNP complexes such as P-bodies and stress granules in order to process mRNA degradation. The formation of these RNP complexes is often regulated by post-translational modifications such as phosphorylation and ubiquitination (Li et al. 2013).

Around ~95% of ALS cases appear to display TDP-43 pathology in both familial and sporadic cases. TDP-43 pathology is observed in the background of other mutations such as FUS, C9ORF72, hnRNPA1/2, VCP, and UBQLN. *Drosophila* studies have explored the interaction between RNA binding proteins that cause similar disease manifestations. Caz null flies and TBPH null flies show a shortened lifespan and motor defects with increased bouton number in larval NMJ. While Caz overexpression in motor neurons can rescue these phenotypes of these null flies, TBPH overexpression was not sufficient to rescue caz null phenotypes, suggesting a robust epistatic relationship between these genes. Expression levels of neither protein were dependent on one another. Caz and TBPH appeared to interact physically but only in the presence of RNA. Caz mutant proteins were found to be physical interactors of TBPH, even though caz mutant proteins mislocalized to the cytoplasm (Wang et al. 2011). Mutants of FUS and TDP-43, when overexpressed in the eye, show rough eye phenotypes and retinal degeneration. This effect is exacerbated when mutants of both genes are coexpressed (Lanson et al. 2011).

Similarly, a null mutant of ataxin-2, a protein containing polyglutamine (polyQ) expansion involved in spinocerebellar ataxia type 2, worsens the phenotypes associated with TDP-43 overexpression in the eye, motor function, and lifespan (Elden et al. 2010). *Drosophila* homolog of hnRNPA1, Hrp38, is also shown to be a physical interactor of TBPH as well as TDP-43, involved in processing TBPH mRNA by inhibiting the splicing of exon 3. Knockdown of Hrp38, as well as *TBPH* mutant with deletion in exon 3, caused neuropil degeneration, motor defects, and reduced lifespan, effects that are enhanced in combination (Romano et al. 2014).

Aggregation of proteins in the disease scenario can have a severe effect on the regulation of protein turnover and corresponding gene expression. As mentioned previously, overexpression of TDP-43 or FUS could downregulate the endogenous counterparts of these proteins in a feedback loop, while the protein product itself was also tagged for degradation in response to various cellular cues. TDP-43 pathology, but not FUS pathology, was induced in *Drosophila* eyes upon expression of mutants of profilin, probably owing to a shift in TDP-43 localization (Matsukawa et al. 2016). This change in localization of TDP-43 from the nucleus to the cytoplasm, coupled with the rough eye phenotype, was observed in the presence of VCP(R152H) mutant expression as well, as opposed to wildtype VCP expression. This mislocalization and subsequent degeneration was also observed with the coexpression of TDP-43(M33V) with wildtype VCP and even more prominent with VCP(R152H). This led to the hypothesis that VCP could be responsible for TDP-43 nucleocytoplasmic shuttling as well as its degradation, processes that may be stalled when either one of the proteins is mutated. This would culminate in the accumulation of TDP-43 in the cytoplasm, raising toxic gain-of-function effects, and being depleted in the nucleus, causing loss of function.

Interestingly, knockdown of *Drosophila* homolog, ter94, could rescue the degenerative eye effects of polyglutamine-induced aggregates, while overexpression could enhance it, relaying a plausible role for VCP in cell death mechanisms (Higashiyama et al. 2002). However, another study showed that VCP could be sequestered into polyQ aggregates of huntingtin and ataxin-1, preventing its nuclear

role in DNA repair. In this case, overexpression of VCP could bypass any modulation of polyQ aggregates, reaffirming its role in double-stranded break repair (Fujita et al. 2013). VCP appears to inhibit the rhodopsin (Rh) pathology in retinitis pigmentosa in another mechanism. Rh mutant P37H expressed in the eye misfolds and forms non-toxic aggregates, which in the presence of wildtype Rh promote light-sensitive retinal degeneration. It is also rescued by the knockdown of VCP that triggers the unfolded protein response in the eye, as also by chemical inhibition of the ERAD and proteasomal pathway (Griciuc et al. 2010a, b).

Another well-studied locus involved in degradation is ubiquitin, which shows genetic interactions with proteins involved in Alzheimer's disease. For instance, overexpression of presenilin (Psn), a γ-secretase protein, led to peculiar defects in the eye about the interommatidial bristles that correlated with decreased Notch signaling (Li et al. 2007). These defects were exacerbated with knockdown of UBQLN and partially rescued by its overexpression along with notch signaling (Li et al. 2007). UBQLN was found to physically interact with Psn via the UBA domain (Ganguly et al. 2008). UBQLN overexpression could, however, lead to an age-dependent degeneration in the eye. This feature could be rescued by the overexpression of Psn (Ganguly et al. 2008). UBQLN knockdown showed similar wing defects as seen in notch pathway downregulation, further corroborating a link between UBQLN and Psn (Li et al. 2007; Ganguly et al. 2008). Similar to VCP and profilin, UBQLN overexpression could also reduce the expression levels of TDP-43 in the eye. However, change in localization of TDP-43 or colocalization with UBQLN was not observed, questioning the mechanism of TDP-43 degraded in the presence of UBQLN (Hanson et al. 2010). However, another study showed that UBQLN could physically interact, alter solubility and ubiquitination, and delegate TDP-43 from nucleus to cytoplasm (Jantrapirom et al. 2018). Decreased solubility of TDP-43 with UBQLN knockdown could be recovered with VCP overexpression (Jantrapirom et al. 2018). Increased soluble ubiquitinated TDP-43 appears to be the toxic force in ALS pathology in *Drosophila* determined by motor assay (Jantrapirom et al. 2018). A *Drosophila* chaperone CG5445 could increase solubility and enhance the proteasomal degradation of TDP-43 protein (Uechi et al. 2018). It could physically interact with TDP-43, probably via its ubiquitin-associated domain. This interaction and solubilization were retained even in TDP-43(M33V) mutant, but not in FUS(R521C) mutant. A possible ortholog of this gene in humans, C6ORF106, can act as a potential therapeutic option (Uechi et al. 2018).

Mitochondrial dysfunction and oxidative stress are determining factors in ALS pathology. Mitochondrial morphology in indirect flight muscles and axons of leg motor neurons appeared to be fragmented with TDP-43, FUS, and TAF15 overexpression, an effect that could be rescued by the knockdown of mitochondrial fission proteins, Drp1 and Marf, or overexpression of the fusion protein, Opa1. This fragmentation could be a result of lowered marf levels degraded via the activity of E3 ubiquitin ligase, parkin (Altanbyek et al. 2016). In another study, the parkin-initiated degradation of TAF15 could rescue its degenerative effect by decreasing aggregation, retinal degeneration, motor defects, and shortened lifespan (Kim et al. 2018b). Proteasomal degradation of overexpressed VAP(P58S) is driven by ROS activation

via SOD1 knockdown as well as TOR downregulation in third instar larval brain. These modulators of VAP(P58S) aggregates were identified in a cell-based RNAi screen. Surprisingly, ROS could also decrease expression levels of endogenous VAP (Chaplot et al. 2019). VAP overexpression, as in the case of *sod1* and *sod2* null mutant, leads to the increase in several boutons at the NMJ, a phenotype correlated with oxidative stress (Pennetta et al. 2002; Milton et al. 2011). Indeed, VAP overexpression in *Drosophila*, in a cell-type specific manner, appears to be more toxic than VAP(P58S) and is accompanied by increased ROS (Ratnaparkhi et al. 2008; Chaplot et al. 2019). Synapse development is regulated by oxidative stress via MAP kinase pathways such as JNK and p38, as are TOR (target of rapamycin) pathway and autophagy (Collins et al. 2006; Milton et al. 2011; Deivasigamani et al. 2014). TDP-43 overexpression also caused ROS toxicity that could be attenuated by JNK signaling and accentuated via p38b signaling downstream of MAP kinase, Wallenda (Zhan et al. 2014). The extent of oxidative stress developed in the fly correlated with shortening of lifespan. In a relationship similar to the change in ROS, these signaling pathways also regulated antimicrobial peptide (AMP) production in response to the innate immune pathways, Toll/Dif and Imd/Relish, invoked by TDP-43 toxicity (Zhan et al. 2014).

Summary

ALS is a debilitating disease that occurs in 1 among 50,000 people per year. The late onset of the disease is coupled with a rapid prognosis of 3–5 years before patients succumb to death due to respiratory failure. Treatment in ALS is limited to two FDA-approved drugs, riluzole, and edaravone. Riluzole, the only approved drug for ALS in the last 20 years, acts by decreasing glutamate excitotoxicity, improving the life of the patients by only a few months. Edaravone, on the other hand, is involved in the reduction of oxidative stress and was approved as a treatment option by FDA in 2017. A large number of processes involved in the disease provide a battery of potential drug targets. Rapamycin has been shown to have beneficial effects in fly models of TDP-43 and VAP, as well as in models of zebrafish and mice. Rapamycin is now in phase II drug trial for ALS (Mandrioli et al. 2018). RNA therapy is another example that has been shown to work in animal models successfully. Its use has already helped in splicing correction in SMN2 pre-mRNA in children suffering from SMA (Chiriboga et al. 2016). A *Drosophila* study has helped validate the use of small binding molecules targeting the G-quadruplex of G2C4 repeats in C9ORF72, thereby increasing its lifespan by inhibiting RNA toxicity and DPR production (Simone et al. 2017). Use of siRNA and genome editing techniques against targets, such as SOD1, TDP-43, Ataxin-2, are the new methods of treatment currently being explored in animal models (Mathis and Le Masson 2018).

Drosophila research has shed light on certain unifying factors among ALS loci that agree with other disease models and patient data as well (Fig. 1). In most cases, overexpression, as well as knockdown of these loci, proved to cause morphological changes, motor defects, and lethality in the fly. While the use of null mutants and

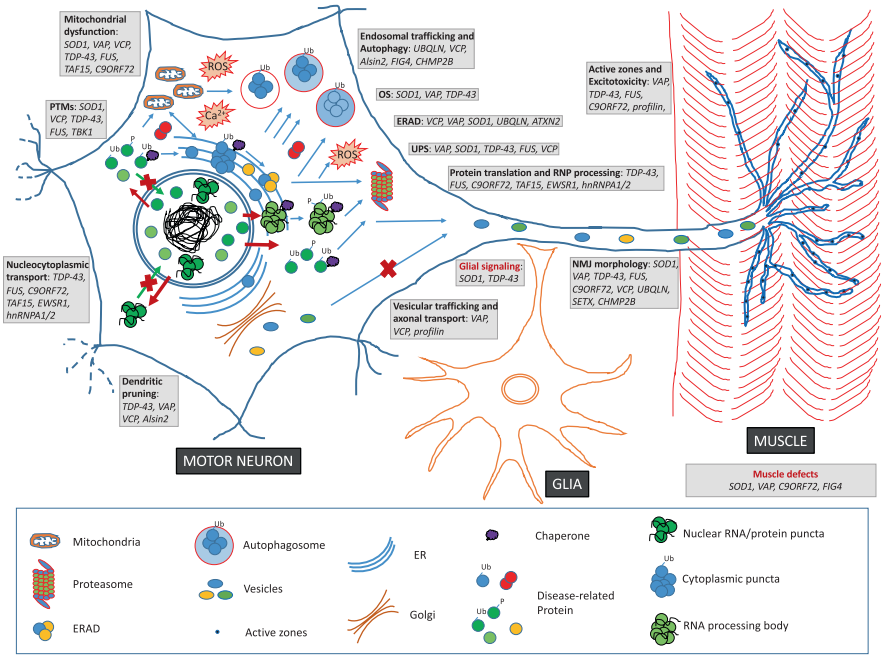

Fig. 1 Schematic representation of known functions of genes that have been identified as causative loci for motor neuron disease. Seventeen genes are listed, classified based on their function and site of action. Fifteen of these genes have *Drosophila* orthologs that have been modeled in flies (Table 1). FTDALS1 is studied using overexpression systems as G4C2 repeats and DPRs. CG14718 is identified as fly orthology of the postulated loci, EWSR1 and TAF15. Disease-causing mutations in these genes presumably cause a loss-of-function, or in some cases, a toxic gain-of-function. A class of these loci is RNA-binding proteins, DPRs, TDP-43, FUS, hnRNPA1/2, EWSR1, and TAF15. Defects associated with these loci include nuclear toxicity, impaired nucleocytoplasmic transport, altered RNA binding, and trafficking, disrupted protein translation, and toxic RNA-protein complex formation. Expression of some of these proteins such as TDP-43 and FUS also appears to be autoregulated. Another arm severely affected at the cellular level in ALS is proteostasis. This includes ER stress, unfolded protein response (URP), oxidative stress (OS), chaperone activity, ER-associated degradation (ERAD), proteasomal degradation and autophagy. Loci actively involved in these mechanisms are *SOD1, VAP, VCP,* and *UBQLN*. Mutant proteins in ALS can act as monomers with toxic gain-of-function and form toxic RNP complexes and protein aggregates. Monomeric, oligomeric, or aggregated forms of these proteins are subjected to post-translational modifications like phosphorylation and ubiquitination. Oligomers and aggregates can also be solubilized through chaperone activity. PTMs and solubilization can prime these proteins for degradation through proteasome or autophagy. Certain loci such as *SOD1, VAP, VCP, TDP-43, FUS, TAF15,* and *C9ORF72* can affect the mitochondria triggering mitochondrial fragmentation, energy imbalance, oxidative stress, autophagy, and calcium signaling defects. Transport machinery such as vesicular trafficking, endosomal recycling, and axonal trafficking can be disrupted due to microtubule disorganization along the axon and at the synapse in the case of VAP, UBQLN, VCP, Alsin2, FIG4, CHMP2B and profilin. This can lead to NMJ morphology and function defects in bouton shape and size, active zones, and glutamate release. This is directly related to perturbation of signaling across the NMJ, such as JNK, BMP, and mTOR among others. NMJ morphology is a feature most commonly affected in almost every model of ALS studied in *Drosophila*. In a few cases like TDP-43, VAP, VCP, and Alsin2, a similar disruption is also observed at dendritic nerve endings that synapse with interneurons. Although most of the functions are neuronal, a few genes contribute to the disease because of their function/malfunction in muscle and glia. Sarcomeric disorganization, myotubule disruption, and nuclear envelop defects are some of the effects accompanied by muscle expression of ALS loci such as SOD1, VAP, C9ORF72, and FIG4. Glial expression of loci such as SOD1 and TDP-43 directly affects oxidative stress, axonal wrapping, and glutamate excitotoxicity.

knockdown studies directly aid in identifying functional roles, overexpression studies demonstrate a deliberate effect of toxicity manifesting through the proteins themselves. Overexpression of these proteins could lead to degenerative phenotypes as a result of the gain-of-altered function as in the case of VAP, TDP-43, and FUS. This meant that stringent regulation of these proteins was required for their optimal function. Disease-causing mutations gave rise to a wide range of functional consequences such as dominant active, dominant negative, loss-of-function, and gain-of-function phenotypes. The mutant protein could lead to misfolding (e.g., VAP), mislocalization (TDP-43, FUS), or altered physical interaction (UBQLN), ultimately leading to aggregation. Oligomeric or aggregated forms of these proteins can tend to sequester binding partners such as proteins or RNA preventing their normal function, further adding to the toxic nature. Processes involving the movement of cellular components, such as nucleocytoplasmic transport (C9ORF72, TDP-43, FUS), vesicular trafficking (VAP, VCP, UBQLN), and axonal transport (VAP, profilin) are found to be prominently disrupted, promoting mislocalization and accumulation of mutant proteins. Thus, cytoplasmic accumulation of protein aggregates appears to act as a sink for functional protein and associated binding partners, abetting the breakdown of cellular processes. Mutant protein could also change post-translational modifications such as phosphorylation (TDP-43), ubiquitination (most loci) or sumoylation (VCP), or changes in binding proteins (VAP), proving to be more toxic as a monomer or oligomer than in an aggregated form. In response to various triggers ranging from RNA binding to ROS to chaperone activity (TDP-43, FUS, VAP), ubiquitinated mutant proteins are targeted for proteasomal degradation or autophagy. Degradation mechanisms are severely affected in cases of proteins directly involved in the process (VCP, UBQLN) as a result of ER stress and unfolded protein response.

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Metabolic Alterations Amalgamated with Huntington's Disease

Priya Lakra and Namita Agrawal

Abstract

Huntington's disease (HD) is a progressive monogenic neurodegenerative disease typified by loss of motor, psychiatric and cognitive function with no known cure. Additionally, the concomitant occurrence of metabolic disturbances including unintended weight loss has also been reported in HD patients. However, the pathophysiology remains largely unclear. The underlying pathophysiology comes further complex due to the ubiquitous expression of the causative *huntingtin* (*HTT*) gene. Research studies indicate functional changes in the peripheral organs of patients reflecting the involvement of peripheral component in metabolic disturbances observed in HD. Links between metabolic phenotype and neurodegeneration have also been suggested in HD patients. Altogether, these observations underscore the complexity of metabolic disturbances occurring in HD and accentuate the need to study this phenomenon in a combinatorial setting. Development of therapeutics targeting metabolic alterations in HD might abrogate some of the comorbidities and can substantially improve the quality of a patient's life, and might even prevent premature death.

The fruit fly, *Drosophila melanogaster,* can provide a treasured genetic system to express the human *huntingtin* gene in a temporally regulated and tissue-specific pattern. *Drosophila* can contribute to deeper mechanistic insights into the metabolic defects underlying HD due to the presence of multiple evolutionarily conserved metabolic pathways. In this chapter, we highlight the genetics, epidemiology, and metabolic disturbances manifested in HD and how *Drosophila melanogaster* can be used as a powerful genetic model for unraveling the metabolic processes and pathways that go awry in HD as a foundation for translational research and developing new therapeutics.

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Huntington's disease (HD) · Metabolic alterations · *Drosophila* · Mutant huntingtin (mHTT) · Weight loss

Introduction

Huntington's disease (HD) is a characteristic neurodegenerative disorder caused by a single genetic mutation in the *huntingtin* (*HTT*) gene that affects multiple pathways, thereby increasing the complexity of the disease with no adequate treatment till date (The HDCRG 1993). Since its first description in 1872, it was majorly described as Huntington's chorea (Huntington 1872), but multiple nonmotor signs and symptoms such as psychiatric, cognitive, and metabolic features are also attributed to this disease (Bates et al. 2015; van der Burg et al. 2009). In addition, the non-neurological metabolic phenotype of HD also includes unintended weight loss, a hypercatabolic state that can be characterized by disturbances in appetite and altered energy metabolism, and functional changes in the digestive system and other metabolic organs (Fig. 1; van der Burg et al. 2009; Carroll et al. 2015). Prior investigations into HD pathology have linked prominent neurological symptoms such as motor disabilities, behavioral abnormalities, and cognitive

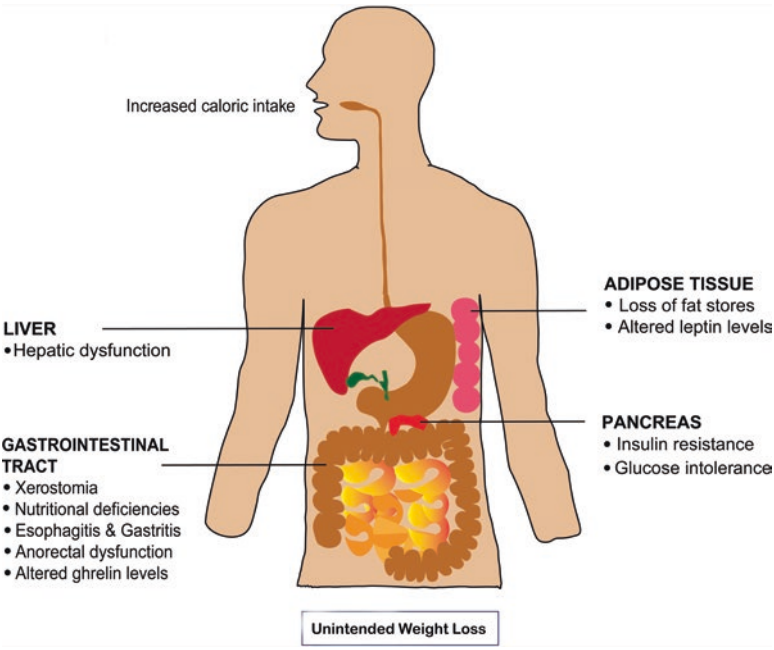

Fig. 1 Metabolic disturbances in Huntington's disease. Major phenotypic outcome as a consequence of metabolic alteration in HD is unintended weight loss despite increased caloric intake

decline to the progredient neuronal dysfunction and death of the corticostriatal circuits (Bates et al. 2015). On the contrary to the well-defined corticostriatal neuropathology, our understanding of the pathophysiological basis causing this metabolic phenotype of HD remains largely incomplete. Besides the occurrence of diverse signs and symptoms, the ubiquitous expression of *HTT* makes understanding of the entire pathophysiology of HD further complex. In the past decade, enormous data have emerged from HD patients, suggesting that these metabolic disturbances severely affect the quality of life and some of these disturbances could strongly influence the rate of the disease progression (Myers et al. 1991; van der Burg et al. 2017). Moreover, studies using mouse and fly models suggest that neurological phenotypes of HD might be improved through peripherally accessible mechanisms such as diet and modulation of circulating metabolites (Martin et al. 2009; Campesan et al. 2011; Carroll et al. 2015). These reports thus raise the enticing possibility that the metabolic interventions could have a substantial effect on alleviating neurological symptoms associated with HD and improve longevity with better endurance among HD-affected subjects. Moreover, direct measures of metabolic symptoms in premanifest HD individuals, like xerostomia (Wood et al. 2008), hepatic mitochondrial dysfunction (Hoffmann et al. 2014), and weight loss (Aziz et al. 2008; van der Burg et al. 2017), can be possibly translated into novel biochemical biomarkers of the disease. It is therefore crucial to understand the nature of the causative events resulting in metabolic alterations in HD; for example, in patients, is weight loss secondary to neurodegeneration in the CNS or general malaise, or does it have any peripheral node too?

An approach to apprehend the nature of this complex feature of HD is to model the disease in simple analogous systems like the fruit fly. The fruit fly, *Drosophila melanogaster,* was first introduced as a model system in biological research more than 100 years ago, and after that, it has been extensively employed as a valuable model system given its simple biology, which shares many similarities with the mammalian systems. Moreover, comparative analysis of the human and fly genomes unveiled remarkable conservation in genes and pathways (Rubin et al. 2000) with the presence of ~70% human disease-causing genes in the fly (Reiter et al. 2001; Chien et al. 2002). With powerful genetic techniques, human disease-causing genes can be incorporated in *Drosophila* to monitor the targeted expression of the disease-causing gene in the region of interest (Jackson et al. 1998; Marsh et al. 2003; Lewis and Smith 2015). Research to uncover the pathophysiological basis and therapeutic approaches for alleviating metabolic features in HD initially remained dependent on vertebrate model systems. However, in recent years, several evolutionarily conserved metabolic pathways are extensively identified in the flies, thereby making it a powerful genetic model to study metabolic defects underlying many diseases. Furthermore, in the past two decades, *Drosophila* models have provided significant insights into the study of human metabolic disorders like diabetes and insulin resistance, obesity, and metabolic syndrome (Baker and Thummel 2007; Leopold and Perrimon 2007; Owusu-Ansah and Perrimon 2014; Graham and Pick 2017). With these recent discoveries, it is encouraging to employ the transgenic fly in modeling metabolic alterations observed in HD.

In this chapter, we have highlighted the metabolic abnormalities associated with HD and review the potential of *Drosophila* as a model system to provide insights into the pathological basis for metabolic disturbances occurring in HD. We strongly suggest that *Drosophila* is as an excellent model system in this emerging field of research, and therefore, rapid research might help in the discovery of cost-effective novel biomarkers and testing of drugs that will alleviate metabolic alteration to relieve HD-mediated devastating symptoms.

Huntington's Disease: An Overview

The first description of this disease was given in 1841 by Charles Oscar Waters (Waters 1842). It was in 1872 when Dr. George Huntington characterized the disease known today as HD, and the report entitled "On Chorea" was published (Huntington 1872). HD is an autosomal dominant, devastating neurodegenerative disorder with no disease-modifying treatments available yet. It is caused by abnormal expansion of an unstable trinucleotide (CAG, encoding glutamine) repeat located within the exon 1 region of the *Htt* gene (OMIM: 613004) located on the short arm of chromosome 4 (4p16.3) (The HDCRG 1993). *HTT* is a ubiquitously expressed gene encoding the large 348 kDa huntingtin (HTT) protein (Hoogeveen et al. 1993; Strong et al. 1993; Marques Sousa and Humbert 2013). Although it is ubiquitously expressed throughout the body, the transcripts are present at varying levels in different cell types (Li et al. 1993; Strong et al. 1993; Marques Sousa and Humbert 2013). The mutated gene encodes a mutant form of the protein, mutant huntingtin (mHTT), harboring an amino-terminal polyglutamine (polyQ) expansion in its exon 1 region (Saudou and Humbert 2016). Notably, the HTT protein is conserved from flies to humans, with the highest identity found among mammals. Interestingly, even after two decades of the discovery of the *HTT* gene, the normal functions of wild-type (WT) HTT are still under investigation. Several studies have reported some of the key molecular functions of the wild-type HTT, for instance, transcriptional regulation, regulation of autophagy, coordination of cell division by mediating spindle orientation and aiding cellular transport including that of brain-derived neurotrophic factor (BDNF)-containing vesicles within the neurons, ciliogenesis, and endocytosis (Saudou and Humbert 2016).

HD is a rare genetic disorder with variable prevalence across different ethnic groups, and these differences are thought to be partially attributed to the genetic differences in average CAG repeats in the *HTT* locus with higher CAG repeats in higher prevalence groups (Bates et al. 2015; Kay et al. 2018). HD has a higher prevalence in western populations with 10.6–13.7 cases per 100,000 individuals (Fisher and Hayden 2014; Rawlins et al. 2016). However, Japan, Taiwan, and Hong Kong have a lower prevalence of one to seven per million individuals (Pringsheim et al. 2012; Sipilä et al. 2015; Xu and Wu 2015). HD usually affects at the mean age of 35–45 years. Nevertheless, some cases of juvenile HD are also reported as an early-onset case. The age of onset of HD is found to be inversely correlated with the length of the CAG repeat expansion in the *HTT* gene (Rubinsztein et al. 1996; Lee

et al. 2012; Kay et al. 2016); interestingly, certain additional genetic modifiers are also found to be associated with HD progression (Gusella et al. 2014; Hensman Moss et al. 2017). HD pathology is broadly marked by motor defects, cognitive impairment, and psychiatric and metabolic disturbances.

Motor dysfunctions in HD are the first to set in and give HD its characteristic appearance. Motor disturbances in HD can be divided into two components: impairment in involuntary movements like chorea, and voluntary movement defects including rigidity, bradykinesia, and incoordination. In adult-onset HD, chorea begins early in the course of the disease, eventually followed by impairment in involuntary movements. However, in early-onset HD including juvenile HD and the later stages of the adult-onset HD, the second component tends to predominate (Ross et al. 2014; Bates et al. 2015). Cognitive disabilities in HD include attention deficit, psychomotor slowing, impulsivity, and, most importantly, lack of awareness. Psychiatric features of HD are relatively less severe than cognitive and motor decline. They commonly involve depression, irritability, and apathy. Major depression is common in HD and resembles depression in individuals without this disorder, and it is often treated in a similar manner (Thompson et al. 2012; Killoran and Biglan 2014).

Interestingly, apathy is another common disabling neuropsychiatric feature of this disorder, present even in premanifest individuals, and tends to worsen gradually (Tabrizi et al. 2013). All these central neurological symptoms of HD are linked to the selective loss of neurons in the basal ganglia and cerebral cortex (Ross et al. 2014; Bates et al. 2015). The archetypal neuropathology of HD is characterized by HTT fragmentation (Lunkes and Lindenberg 2002; Landles et al. 2010; Sathasivam et al. 2013; Neueder et al. 2017), neuronal inclusions containing huntingtin aggregates (DiFiglia et al. 1997), and progressive dysfunction and death of striatal and cerebral cortex neurons (Vonsattel et al. 1985).

In addition to the extensively studied neurological symptoms of HD, patients usually exhibit a range of metabolic disturbances, especially unintended weight loss, which may appear early in the course of the disease and sometimes even before the characteristic symptoms begin. Moreover, patients with HD are noted to exhibit skeletal muscle wasting and cardiac dysfunction (van der Burg et al. 2009).

Metabolic Alterations in Huntington's Disease

The relationship between metabolic abnormalities and HD was evoked even before the discovery of the causative gene, though the exact pathophysiology is yet to unfold. Unintended weight loss is a significant metabolic manifestation in HD. Further, numerous novel findings in patients and transgenic murine models identified considerable functional changes in several organs involved in maintaining metabolic and whole-body energy homeostasis, for instance, pancreas, gut, liver, adipose tissue, and hypothalamus in the HD setting. The metabolic abnormalities in HD patients not only affect quality of life of patients but also influence the progression of the disease. Eventually, these disturbances can contribute substantially to both morbidity and mortality as well.

Weight Change: A Mystery in Huntington's Disease

Unintended weight loss is a characteristic and disabling clinical manifestation of HD that affects nearly all patients with HD. HD patients show alteration in their weight throughout the entire course of the disease; however, they become cachectic at the later stages of the disease. Even some presymptomatic carriers exhibit weight loss; therefore, possibly, it begins as a minor feature (Stoy and McKay 2000; Djousse et al. 2002; Trejo et al. 2004; Robbins et al. 2006) in asymptomatic genetic carriers, gradually exacerbates and terminates with profound emaciation in the advanced stage (Djousse et al. 2002; Mochel et al. 2007). It was presumed that higher energy expenditure occurring as a result of choreiform movements underlies weight loss in HD patients. However, such correlation is very unlikely as weight loss is present in presymptomatic carriers or in early stages of HD where chorea is absent or minimal and aggravates in later stages, where patients exhibit dystonia and rigidity (Sanberg et al. 1981). Strikingly, a recent large cohort study by van der Burg and colleagues strengthened and extended the previous studies, indicating that body weight is indeed a strong predictor of HD progression where higher body mass index (BMI) is linked to slower progression of the disease independent of CAG repeat size in *HTT* and disease stage (Myers et al. 1991; van der Burg et al. 2017).

Importantly, detailed investigations found that weight loss in patients arises despite normal to increased food intake (Morales et al. 1989; Trejo et al. 2004; Marder et al. 2009). Additionally, many patients are noted to have an insatiable appetite, and even presymptomatic genetic carriers consume more calories than controls, reflecting that HD is associated with appetite dysfunction (Mochel et al. 2007). Overt dysphagia and nutritional deficiencies are also common in the HD setting (Lanska et al. 1988; Heemskerk and Roos 2011), and undernutrition is indicated as an additional risk factor for mortality.

Systemic Energy Deficit in Huntington's Disease Patients

Besides profound emaciation, other metabolic disturbances are also noted in patients with HD. Studies in an attempt to elucidate the metabolic state of the HD patients reported significantly elevated 24-h sedentary energy expenditure (24-h EE) and waking metabolic rate (WMR) as compared to the control subjects. These findings of higher energy expenditure in HD subjects, however, correlated with the chorea scores indicate that increased involuntary movements could be one of the causes (Pratley et al. 2000; Gaba et al. 2005). In subsequent studies, HD patients at an early and an intermediate stage of the disease exhibit increased total energy expenditure (TEE) along with an increase in basal resting energy expenditure (Goodman et al. 2008; Aziz et al. 2010a).

Insulin Deficiency and Imbalance in Glucose Homeostasis

HD patients have a high tendency to develop diabetes mellitus (DM), approximately seven times more often than matched control individuals (Farrer et al. 1985; Ristow 2004; Hu et al. 2014). Some reports, however, do not document the increased risk of DM in patients with HD (Boesgaard et al. 2009; Zarowitz et al. 2014), indicating that while the prevalence of DM is high, not all patients with HD develop DM. Several studies indicate impaired glucose homeostasis as the likely cause for the higher prevalence of DM; however, the etiology is still not fully understood. Patients with HD are reported to develop impaired glucose tolerance and abnormal insulin secretion, with significantly lower insulin sensitivity than that in control subjects (Podolsky et al. 1972; Podolsky and Leopold 1977; Lalic et al. 2008). An early study showed that HD patients were glucose intolerant and their islet β-cells were hyper-responsive to glucose and arginine (Podolsky et al. 1972). Interestingly, HD patients with lower than normal BMI developed insulin resistance without lipid abnormality (Lalic et al. 2008), in contrast to individuals with pre-DM and the metabolic syndrome who exhibit obesity, low high-density lipoprotein cholesterol levels, and high triglyceride levels (Tripathy et al. 2000). Contradictory to these observations, few reports also displayed comparable levels of fasting plasma glucose and insulin in pre-manifest and manifest patients compared to those in control subjects (Boesgaard et al. 2009; Russo et al. 2013; Wang et al. 2014; Nambron et al. 2016). The disparity in these studies may stem from several factors which influence metabolic profile in humans, such as food intake, lighting conditions, sleep/wake cycle, sampling, etc. (Davies et al. 2014). Histological examination of pancreatic islet cells from nine patients with different neuropathological stages of the disease appeared normal with no reduction in islet β-cell area and no cytoplasmic or nuclear inclusions and exhibited similar levels of insulin mRNA as in control subjects (Bacos et al. 2008). In one of the studies, R6/2 mice exhibited atrophy of pancreatic islet β-cells and disturbed exocytosis of insulin along with intranuclear inclusions and, in turn, developed DM (Hurlbert et al. 1999; Björkqvist et al. 2005, Hunt and Morton 2005). These reports strongly suggest the requirement of further investigation in the insulin level and glucose homeostasis in HD using model organisms.

Gastrointestinal Dysfunction: A Contributor to Weight Loss

HD patients are reported to exhibit xerostomia and a significant increase in serum vasopressin levels (Wood et al. 2008). Xerostomia could cause mastication and swallowing difficulties. Also, a study suggested that the problem of dry mouth increases with the disease progression (Wood et al. 2008). A high prevalence of esophagitis and gastritis is also found in HD patients, and this gastroesophageal inflammation is correlated with the severity of the disease and not with the motor

disturbances (Andrich et al. 2009). A study in the R6/2 mouse of HD reveals abnormalities in the gastrointestinal tract (GIT) and reduced whole gut transit time (van der Burg et al. 2011). At the anatomical level, mucosal thickness and villus length were found to be reduced along with impairment in the enteric nervous system regulating gut motility indicated by the loss of several enteric neurons like VACht-, VIP-, and CART-producing neurons (van der Burg et al. 2011). Ghrelin-producing neurons are also reported to be reduced in the stomach of the R6/2 mouse (van der Burg et al. 2008). Similarly, few other reports suggest an alteration in gastric mucosal cells in the later stage HD subjects (McCourt et al. 2015). Plasma levels of ghrelin, an orexigenic hormone majorly of gastric origin, are found to be significantly elevated in patients with HD compared to those in controls. Nevertheless, CSF ghrelin levels tend to be higher but somehow did not reach statistical significance (Popovic et al. 2004). Moreover, a recent study reports anorectal dysfunction in presymptomatic and symptomatic HD subjects with significantly elevated anal incontinence and chronic constipation (Kobal et al. 2018). These studies indicate substantial GIT dysfunction in patients and transgenic HD mouse model.

Altered Liver Function in Huntington's Disease

Beyond that, several studies in patients with HD and transgenic HD mice models have described hepatic abnormalities. The liver plays a crucial role in maintaining systemic energy homeostasis by regulating blood glucose levels, synthesizing metabolites, and removing toxins from the body. Methyl-^{13}C-methionine breath test (MeBT) revealed a progredient abnormality in hepatic mitochondrial function despite a clinically normal liver function in 30 HD premanifest carriers and 21 manifest patients as compared to that in controls (Stuwe et al. 2013). Interestingly, there was a strong correlation between hepatic abnormality with functional tests and cognitive scores of the UHDRS that may reflect a parallel decline in functional and cognitive abilities and hepatic mitochondrial functioning (Stuwe et al. 2013). Impairment in hepatic glucose synthesis has been reported in patients with HD and R6/2 mice. During high-intensity exercise, the liver is stimulated to produce more glucose by the action of enhanced catecholamine. In healthy individuals, the increased hepatic glucose output leads to an abrupt increase in the blood glucose concentration immediately after exercise (Hespel et al. 1986). HD patients do not exhibit an increase in the arterial glucose concentration immediately after discontinuation of intense exercise, indicating abnormal hepatic glucose formation (Josefsen et al. 2010). However, no significant difference occurs in resting arterial blood lactate concentration or lactate clearance after intense exercise in HD patients (Josefsen et al. 2010). In, R6/2 mice model liver, reduced hepatic gluconeogenesis was observed along with both the enzymatic activity levels and cytosolic mRNA levels of hepatic phosphoenolpyruvate carboxykinase, a key regulatory enzyme in liver gluconeogenesis (Pilkis and Granner 1992). These results suggest that hepatic

gluconeogenesis and glycogenesis might be altered in HD liver, and as both these processes are crucial in maintaining plasma glucose levels, these alterations could have deleterious physiological consequences. Besides these metabolic functions, the liver plays a crucial role in detoxification of the body, a function which is also reported to be decreased in HD patients. In HD patients and R6/2 mouse, there are significantly higher blood citrulline levels along with urea cycle deficiency that results in high circulating concentrations of ammonia with concomitant brain damage and locomotor dysfunction. Interestingly, central signs of HD were drastically improved with a low-protein diet aimed at reducing the plasma ammonia concentrations (Chiang et al. 2007).

Anomalies in White Adipose Tissue

In addition to the aforementioned metabolic defects, abnormalities in adipose tissue have also been reported in both the patients and murine models of HD. Adipose tissue is a critical endocrine and metabolic organ which plays a vital role in the regulation of metabolic homeostasis to maintain body weight mainly through adipokines, namely, leptin and adiponectin (Ahima 2006; Trujillo and Scherer 2006). In HD patients, weight loss is accompanied by loss of fat stores from the body (Farrer et al. 1985; Farrer and Meaney 1985; Trejo et al. 2004); however, fat-free mass was similar in HD subjects and control subjects in contrast to the lower than normal BMI indicating reduced fat mass as a plausible factor for weight loss in HD (Sussmuth et al. 2015). HD patients also displayed reduced plasma leptin levels indicative of impaired adipose tissue function (Popovic et al. 2004). In one study, there was a significant increase in plasma leptin levels (Aziz et al. 2010b). However, this discrepancy in leptin levels of HD subjects can be due to the differences in CAG repeats, age, gender, BMI, and other parameters of measurements in different studies.

Moreover, lipodystrophy and loss of adipose tissue predispose patients to insulin resistance and diabetes and lead to a hypermetabolic state (Garg 2004). In agreement with this supposition, reports in R6/2 mice revealed adipose tissue dysfunction (Fain et al. 2001; Phan et al. 2009). Circulating levels of leptin and adiponectin were found to be significantly decreased in R6/2 and CAG140 knock-in mice in later stages. Also, the expression of various adipogenic and lipogenic genes in adipose tissue is substantially altered in R6/2 mice (Phan et al. 2009). Another HD mouse model, HD N171, displayed profound thermoregulatory defects and impairment in brown adipose tissue (Weydt et al. 2006). Importantly, in addition to previous studies reporting reduced levels of leptin and insulin resistance (Popovic et al. 2004; Lalic et al. 2008; Aziz et al. 2010b), a few other studies could not identify any altered metabolic markers in the patients with HD (Lazar et al. 2015; Nambron et al. 2016). Reports using transgenic *Drosophila* are not yet available. Overall, the identification of accurate biomarkers for metabolic dysfunctions in HD is still under investigation.

Pathophysiology of Metabolic Alterations

In an attempt to elucidate the nature of the pathogenesis of metabolic dysfunctions in HD, two hypothetical factors are formulated: (a) primary or direct factors that occur due to the direct detrimental effect of mHtt on peripheral tissues itself and (b) secondary factors that are directly related to hypothalamic degeneration or general malaise occurring as a result of central signs of the disease (Fig. 2).

Regulation of energy homeostasis and bodyweight is accomplished by a multifaceted system that involves the gastrointestinal tract (GIT), adipose tissue, the liver, pancreas, and the hypothalamus (Flier 2004; Badman and Flier 2005). The peripheral organs integrate the information about the energy status of the body and

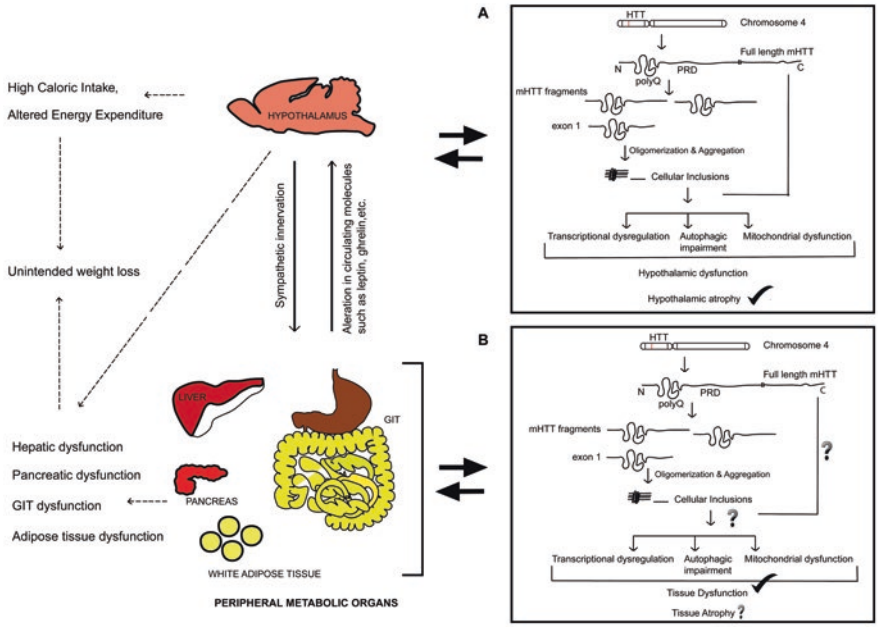

Fig. 2 Schematic representation of a hypothetical pathway underlying metabolic alterations in Huntington's disease. The metabolic features of Huntington's disease (HD) can have two components, that is, direct component involving peripheral metabolic organs and indirect component involving neurodegeneration or general malaise. Huntingtin (*HTT*) gene is expressed ubiquitously in all human tissues. (**a**) Mutant *HTT* carrying expansion of CAG repeats encodes the full-length huntingtin protein carrying polyglutamine (polyQ) stretch at its N terminal, mutant HTT protein. Mutant full-length huntingtin can also be aberrantly processed into an mRNA encoding only exon 1 and proteolytically processed into other N-terminal fragments. Some of these mHTT fragments, particularly exon 1, are reported to undergo oligomerization and aggregation, resulting in the formation of inclusion bodies in the neurons. mHTT causes alteration in cellular processes like transcriptional dysregulation, impairment in autophagy, and mitochondrial dysfunction through either loss-of-function or gain-of-function mutations. These cellular impairments lead to tissue dysfunction and atrophy of tissues such as the hypothalamus. (**b**) mHTT expression in the peripheral tissues might also be undergoing the same process as that described in (**a**). *PRP* Proline-rich domain, *GIT* Gastrointestinal tract

communicate with each other as well as with the regulatory centers in the hypothalamus by the endocrine signaling pathways. As many abnormalities have been reported in the peripheral tissues of the HD patients, it is possible that the subtle functional changes in peripheral organs cumulatively contribute to the metabolic symptoms occurring in HD. mHtt is known to affect cellular pathways and organelles which are essential to all cell types, for example, transcription, mitochondria and cellular energetics, autophagic machinery, and vesicle transport. Subsequently, peripheral cells from HD patients display mitochondrial (Panov et al. 2002, 2005), transcriptional (Luthi-Carter et al. 2002; Strand et al. 2005; Chaturvedi et al. 2009), and cholesterol defects (Valenza et al. 2005, 2007a, b), as do the neurons. Transcriptional downregulation of PPARγ-coactivator 1α (PGC-1α), which regulates mitochondrial biogenesis and cellular respiration, occurs in neurons, muscle, and fat tissue in HD. Also, it increased the occupancy of REST/NRSF in lymphocytes from HD, indicating dysfunctional activity of RE1/NRSE sites (Marullo et al. 2008); impaired macroautophagy and accumulation of lipid droplets in the cytoplasm of primary hepatocytes from a knock-in mouse model of HD have been observed (Martinez-Vicente et al. 2010). Moreover, intracellular mHTT aggregates, a presumptive pathological hallmark of HD, are found within the peripheral tissue of murine models of HD. Interestingly, in a recent report, the presence of the most pathogenic amino-terminal fragment exon 1 was found in peripheral cells of HD patients (Neueder et al. 2017).

Hypothalamus is a crucial coordinator center for various physiological processes like maintenance of body energy homeostasis, sleep-wake cycle, and regulation of several other autonomic processes (Morton et al. 2006; Hill et al. 2008). It consists of various nuclei including paraventricular nucleus (PVN), supraoptic nucleus, the suprachiasmatic nucleus (SCN), arcuate nucleus, nucleus tuberalis lateralis (NTL), mammillary bodies, and lateral hypothalamic area. In addition to the cortico-striatal atrophy (Vonsattel et al. 1985), hypothalamic atrophy and cell death also occur in HD patients (Petersén and Björkqvist 2006; Aziz et al. 2007). Hypothalamic atrophy occurs even in the early stage of HD patients (Kassubek et al. 2004); however, a comprehensive neuropathological classification of the hypothalamus is still warranted. Hypothalamic pathology involves atrophy of nucleus tuberalis lateralis (NTL), paraventricular nucleus (PVN), and lateral hypothalamic area along with the loss of orexin-, somatostatin-, and vasopressin-producing neurons and an increase in CART-producing neurons (Kremer et al. 1990, 1991; Timmers et al. 1996; Petersen et al. 2005; Gabery et al. 2010). These reports indicate that since hypothalamic nuclei have a pivotal role in the regulation of body weight and energy homeostasis, dysfunction restricted to these nuclei alone can affect the systemic metabolism and body weight of the patients (Kremer and Roos 1992; Aziz et al. 2007). However, several studies indicate that the correlation of hypothalamic dysfunction with weight loss is unlikely in HD. A study with R6/2 mice reported a progressive decline in anorectic as well as orexigenic peptides, and thereby despite having evidence for increased energy metabolism and hypothalamic signaling defects, the mechanism remained highly unclear (van der Burg et al. 2008). Another mouse model, BACHD, developed obese phenotypes, and selective expression of both short and longer

fragments of mHtt in the hypothalamus of WT mice recapitulated the metabolic abnormalities of BACHD mice, while patients are usually cachetic. Importantly, targeted inactivation of mHtt in the hypothalamus in young BACHD mice prevented the development of metabolic abnormalities, but once developed, these abnormalities could not be reversed by the targeted inactivation, indicating the involvement of multiple pathogenic processes (Hult et al. 2011).

Finally, these observations raise the enticing possibility that the metabolic abnormalities of HD can have their development trajectory. These reports strongly point toward the complexity of metabolic alterations occurring in HD and accentuate the need to study this phenomenon in a combinatorial setting. Further studies are warranted to understand the exact molecular mechanism underpinning these metabolic alterations and how metabolic alterations, in turn, affect central symptoms and disease progression in HD patients.

Drosophila: An Ideal Model to Study Metabolic Alterations in Huntington's Disease

For the past few decades, various studies inclined on the elucidation of metabolic homeostasis in *Drosophila* have amply demonstrated that the prime metabolic, nutrient-sensing, and endocrine signaling pathways of mammalian systems are well conserved in flies. Subsequently, the simple genetic system of the fly can be exploited with ease and cost-effectively to define the central metabolic pathways that are evolutionary conserved, with implications for better understanding of how metabolic homeostasis is achieved in humans and what metabolic pathways go awry in neurodegenerative diseases. Evaluation of metabolic activity in *Drosophila* includes quantification of basic metabolites, food intake, metabolomics study, starvation, lifespan assays, easy dietary paradigm shifts such as availability of high-lipid or high-sugar diets, lipid droplet staining using Nile red or Oil Red O, mitochondrial studies, and ATP measurements (Tennessen et al. 2014).

Overview of the Metabolic System of the Fly

Animals must sense the nutritional status of their body and balance energy expenditure with caloric consumption in order to coordinate growth, reproduction, and energy homeostasis. A balance between energy consumption (or caloric intake), energy expenditure, and energy storage (generally in the form of energy reserves like triglycerides and glycogen) is crucial to maintain the metabolic homeostasis. In organisms, these physiological functions are tightly regulated by concrete crosstalks of various metabolic, energy-sensing, and endocrine pathways that are evolutionarily conserved in both humans and *Drosophila*. In multicellular animals, nutritional information is typically perceived and communicated by peripheral organs. Subsequently, this information is relayed to other peripheral organs and to the specialized regions in the brain, which generates an accurate physiological and behavioral response to maintain energy

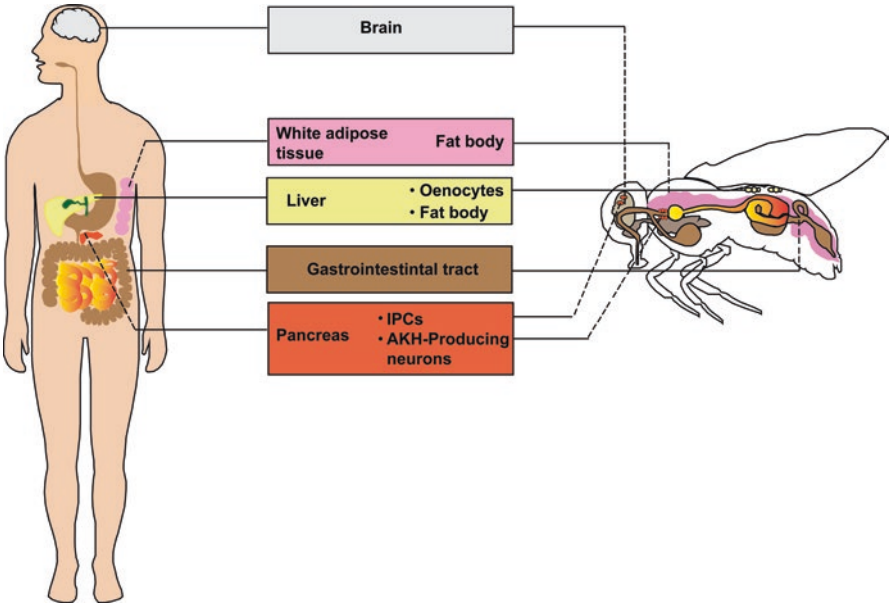

Fig. 3 Metabolic organs share an analogy between human and *Drosophila*. Various metabolic organs of the fly share analogy with those of the human. Evolutionarily conserved metabolic pathway and organs make fly an excellent choice for studying metabolism-related disorders

and metabolic homeostasis. Similar to mammals, the fly also holds an intricate signaling network between brain, endocrine glands, gut, and adipocytes that regulates metabolism and feeding. Also, the fly has various organs, paralleling those in mammals, which have a pervasive role in metabolic regulation and energy homeostasis. These organs, like in mammals, work in coordination to sense the nutritional and environmental cues and regulate nutrient uptake, storage, and mobilization in order to maintain energy homeostasis. They include a functionally segregated gut that is functionally equivalent to mammalian gastrointestinal tract (Apidianakis and Rahme 2011), the fat body, analogous to human white adipose tissue and liver (Baker and Thummel 2007), oenocytes, functionally equivalent to mammalian hepatocytes (Gutierrez et al. 2007), Malpighian tubules, analogous to kidney, neuroendocrine cells, namely, insulin-producing cells and *corpora cardiaca*, adipokinetic hormone-producing cells forming a bipartite *Drosophila* "pancreas" and certain other brain regions acting as hypothalamic nuclei (Fig. 3).

Metabolic Alterations in the Fly Model of Huntington's Disease

In the *Drosophila* model of HD, weight loss during later stages and metabolic abnormalities arise as a result of neuronal expression of exon 1 fragment of mHtt (Aditi et al. 2016). The diseased flies exhibit modulation in the systemic levels of

major biomolecules including lipids, glycogen, trehalose, and proteins. A significant defect in intracellular lipid accumulation, as evident by the size and number in lipid droplets present in the fat body, further validates variation in lipid homeostasis in the HD fly model. The diseased flies also display dysregulated feeding behavior. Further analysis revealed that the expression of the exon 1 fragment of mHtt in neuroendocrine cells, namely, insulin-producing cells and adipokinetic hormone producing neurons, results in an altered metabolic state of the fly (Aditi et al. 2016). Body weight alterations and metabolic dysfunctions occurred profoundly in the HD flies; however, in early stages of the disease, the correlation between weight loss and neurodegeneration was unlikely (Aditi et al. 2016), indicating that other peripheral mechanisms might be involved in the development of emaciation and other metabolic disturbances observed in HD. As more information is gleaned in the fly, clear links to metabolic pathophysiology in HD will certainly be uncovered.

In HD patients, the serum kynurenine-to-tryptophan ratio is found to be increased (Stoy et al. 2005; Forrest et al. 2010). Tryptophan is an essential amino acid which plays multiple physiological roles like the precursor of key neuromodulators, serotonin and tryptamine. In mammals, the majority of the tryptophan is catabolized through the kynurenine pathway within different organs such as brain, liver, and GIT (Le Floc'h et al. 2011). Various metabolites of the kynurenine pathway can readily cross the blood–brain barrier, and some of these intermediates are neurotoxic, such as 3-hydroxykynurenine, quinolinic acid, and picolinic acid, while some of them are neuroprotective such as kynurenic acid (Le Floc'h et al. 2011). Importantly, imbalance in the levels of these neuroactive metabolites has been associated with the neurodegeneration including HD (Maddison and Giorgini 2015). Under normal physiological conditions, the balance between these metabolites is maintained, but at the early stage of HD, cortex and neostriatum exhibit increase in the levels of 3-hydroxykynurenine and quinolinic acid (Guidetti et al. 2004), while levels of kynurenine acid significantly decrease in cerebrospinal fluid and the striatum (Heyes et al. 1992; Jauch et al. 1995).

In mouse and fly models of HD, genetic and pharmacological manipulation of the kynurenine pathway has been documented to ameliorate neurodegeneration (Campesan et al. 2011; Zwilling et al. 2011; Beconi et al. 2012). Neuronal expression of mHtt exon 1 in these models also induces an increase in the ratio of 3-hydroxykynurenine to kynurenic acid as in HD patients, and this increased ratio is likely to be associated with the neurodegeneration. Moreover, chemical inhibition of kynurenine-3-monooxygenase, an enzyme required for the conversion of kynurenine to 3-hydroxykynurenine in the kynurenine pathway, abrogates neurodegeneration and leads to a significant shift toward kynurenic acid synthesis in the fly model of HD. Further, administration of 3-hydroxykynurenine significantly aggravated toxicity in HD flies indicating that 3-hydroxykynurenine is pathogenic in HD flies, whereas administration of kynurenic acid was found to be neuroprotective in HD flies (Campesan et al. 2011). Therefore, with these strands of evidence, modulation of the kynurenine pathway could be valuable in the abrogation of neurodegeneration in HD, and metabolic interventions at the peripheral level may strongly influence neurodegeneration in HD.

Closing Remarks

A growing body of evidence indicates that metabolic alteration is one of the key features of HD. In addition to the selective neurodegeneration in the corticostriatal circuits, it is becoming evident that abnormalities occur in the peripheral tissues of HD patients. Therefore, finding appropriate therapeutic strategies for abrogating metabolic disturbance in HD might be the most possible way to suppress HD. However, it is important to know the root cause of these metabolic alterations and whether such anomalies occur as a result of the direct effect engendered by mHtt expression in the peripheral tissues or as an indirect effect of hypothalamic neurodegeneration or as a combinatorial effect of direct and indirect factors. Several reports in HD indicate substantial involvement of peripheral components in the pathophysiology of metabolic disturbances.

Mouse and fly model studies indicate that neurological symptoms of HD can be alleviated by the management of metabolic alterations in peripheral tissues and circulating metabolites. With the present understanding about the involvement of metabolic alteration in HD progression, it can be translated into the development of novel biomarkers of the disease. Using fly as a powerful genetic tool, an accelerated, cost-effective, and vital method to understand the pathophysiological basis of these metabolic disturbances in HD can be well understood.

This chapter provides a detailed and recent account of the metabolic symptoms in HD and emphasizes the power of fly as a model. *Drosophila* provides a powerful platform, as it exhibits remarkable similarities with mammalian metabolic and homeostatic energy pathways. The spatiotemporal regulated expression of mHtt in individual tissues or a combination of them can further expand our understanding of the toxic effects of mHtt beyond the neurons. A stake of functionally conserved molecular players and pathways and availability of a wide array of sophisticated genetic tractable tools along with power of large-scale forward genetic screens that can have metabolic endpoints makes it fairly reasonable to accept that the metabolic research using fly models can garner insights with high clinical relevance. It is very much likely that the fly is poised to unravel new mechanistic insights into the metabolic symptoms of neurodegenerative diseases like HD in the years to come.

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Notch Signaling: From Neurogenesis to Neurodegeneration

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Abstract

Notch signaling pathway plays a pivotal role during development of an organism. The Notch pathway is an evolutionarily conserved signaling system which has been shown to play a major role in cell fate determination, differentiation, proliferation and apoptotic events, as well as self-renewal processes of different tissues. The same pathway can be deployed in numerous cellular contexts to play varied and critical roles for the development of an organism. In *Drosophila* embryo, loss of Notch function produces remarkable excess of neurons at the expense of the epidermis, and hence Notch was identified as a "neurogenic gene". Several studies have revealed the importance of Notch in the nervous system, including in the maintenance of immature neurons and the control of neurite outgrowth of differentiated neurons. Notch signaling also contributes to the regulation of synaptic plasticity and olfactory functions in the adult brain. Notch signaling has been known to play a crucial role in neural stem cell maintenance and neurogenesis in embryonic as well as adult brain. Thus, it is not surprising that aberrant Notch function can lead to various neurodegenerative diseases. The wealth of genetic resources available for flies offers a unique opportunity to dissect involvement of Notch signaling in neurodegeneration. Understanding the different spatiotemporal regulatory mechanisms of Notch signaling and involvement of Notch signaling pathway in neurodegeneration will help to comprehend various underlying causes of human neurodegenerative diseases at the molecular level.

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Notch Signaling

Notch mutation was first discovered more than a century ago by Morgan and colleagues as a dominant X-linked mutation that exhibits a notched wing phenotype in *Drosophila melanogaster*, and hence the name *Notch* was given for this gene (Mohr 1919). Decades later loss of *Notch* function studies in *Drosophila* embryo revealed *Notch* as a "neurogenic gene" because it produces remarkable excess of neurons at the expense of the epidermis (Lehmann et al. 1983; Poulson 1945). The Notch pathway is an evolutionarily conserved signaling system that operates to influence an astonishing array of cell fate decisions in different developmental contexts. Notch signaling is highly pleiotropic in nature since it regulates different developmental processes such as cell fate determination, differentiation, proliferation, apoptosis, and stem cell maintenance (Andersson et al. 2011; Artavanis-Tsakonas et al. 1999; Baron et al. 2002; Fortini 2009; Liu et al. 2010). Notch is exceptionally sensitive to gene dosage that is both haplo-insufficiency and presence of extra copies of *Notch* results aberrant phenotypes. Notch signaling pathway affects cell fate determination not only across the wide spectrum of metazoan species, but also across a broad range of cell types in a single organism and at different steps during cell lineage progression (Guruharsha et al. 2012; Lai 2004). Thus, aberrant Notch function leads to many diseases in humans including neurodegenerative diseases. Notch signaling has been known to play a crucial role in neural stem cell maintenance and neurogenesis in embryonic as well as adult brain (Alberi et al. 2011; Artavanis-Tsakonas et al. 1999; Borggrefe and Oswald 2009; Lugert et al. 2010). Neuronal atrophy and eventual neuronal loss are the prevalent characteristics of several neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, etc.

The Core Notch Pathway

Notch is a single-pass transmembrane receptor that regulates diverse cellular processes during the development of an organism. Almost all core components in Notch signaling pathway are well conserved from *Drosophila* to humans, such as Notch receptors, ligands, negative and positive modifiers, and transcription factors (Gazave et al. 2009). A simple schematic of Notch signaling pathway is shown in Fig. 1. The prototype Notch receptor is synthesized as a 300 kDa polypeptide. During maturation in *trans*-Golgi network, Notch receptor is proteolytically cleaved by furin-like convertases (S1 cleavage), which give rise to a 180 kDa N-terminal extracellular subunit (Notch-ECD) and a 120 kDa C-terminal transmembrane intracellular subunit (NTM) (Blaumueller et al. 1997). These two subunits are held

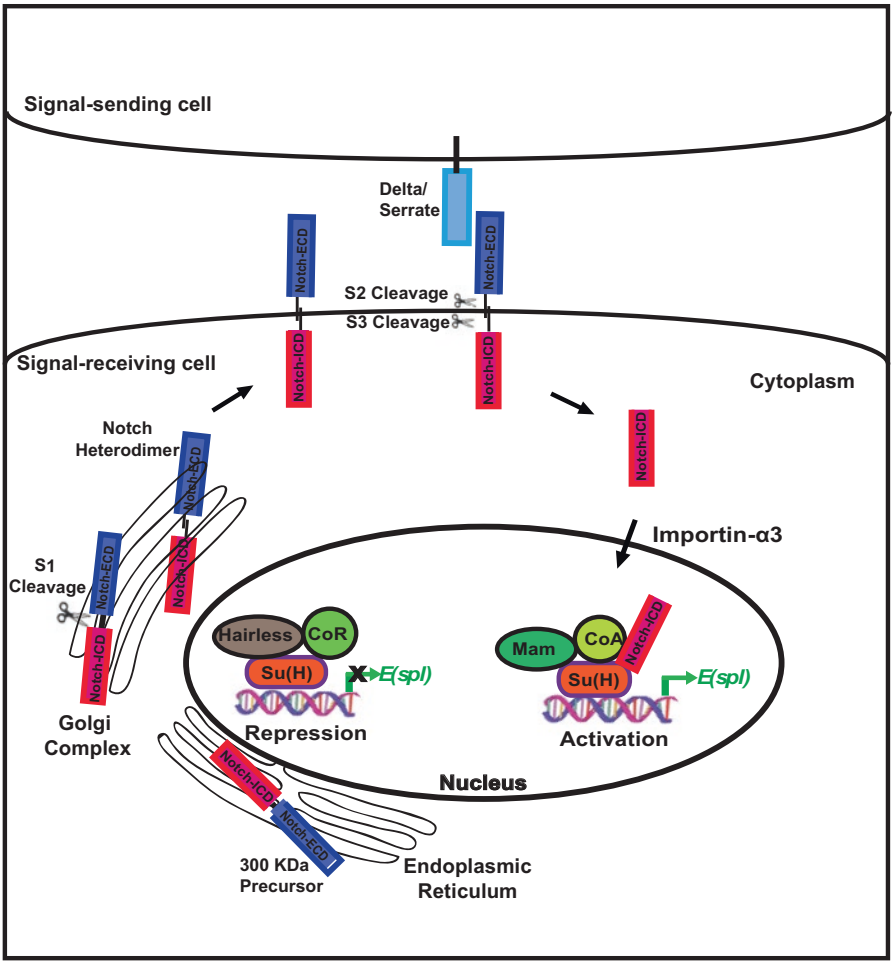

Fig. 1 Notch signaling pathway
Notch receptor (Notch1–4 in mammals) is synthesized as a 300 KDa polypeptide in the endoplasmic reticulum. During post-translational processing in the *trans*-Golgi network, Notch receptors are cleaved by furin-like convertases at site 1 (S1 Cleavage) to create Notch heterodimer (Notch-ICD and Notch-ECD). N- and C-terminal halves of the Notch heterodimers are held together by non-covalent interaction. Heterodimer of Notch receptor translocates to the cell membrane. *Trans*-activation of Notch heterodimer is mediated by the Notch ligands, Delta/Serrate (DLL1, DLL3, DLL4, Jagged1, Jagged2 in mammals) present in the neighboring cell. Ligand binding to the Notch receptor leads to the second cleavage by ADAM metalloprotease(s) at site S2 (S2 cleavage) and γ-secretase at site S3 (S3 cleavage), releasing the Notch-ICD in the cytoplasm. Notch-ICD is translocated to the nucleus with the help of Importin-α3. In the nucleus, Notch-ICD initially interacts with Su(H) DNA-binding protein (CBF1 or RBP-Jk in mammals) and then helps in the recruitment of activator, Mastermind, and other co-activators. This association turns on the transcription of Notch target genes such as *E(spl)* family genes such as *Hey* and *Hes*, whereas in the absence of Notch-ICD, Su(H) recruits repressor (Hairless) and corepressors, which turn off the transcription of Notch target genes

together non-covalently by a calcium-dependent interaction (Rand et al. 2000). This processed heterodimeric Notch receptor is then transferred to the cell membrane and it interacts with ligands of the DSL family (*Drosophila* Delta and Serrate (Jagged in mammals) and *C. elegans* LAG-2). Binding of ligands expressed in adjacent cell to Notch-ECD leads to second proteolytic cleavage (S2) by ADAM family of metalloproteases in the extracellular portion of NTM (Brou et al. 2000). This is followed by an intramembranous cleavage (S3) by γ-secretase complex (Presenilin, nicastrin, PEN-2 and APH-1) and results in the release of Notch intracellular domain (Notch-ICD) from the membrane (Brou et al. 2000; De Strooper et al. 1999; Struhl and Greenwald 1999). Then the released Notch-ICD translocates to the nucleus with the help of importin-α3/importin-β transport pathway, where it transduces Notch signals by regulating the transcription of downstream target genes (Kopan et al. 1994; Sachan et al. 2013; Struhl and Adachi 1998, 2000). This Notch-ICD is a transcriptional co-activator, and exceedingly small, histochemically invisible amount of Notch-ICD is sufficient to activate target genes. This Notch-ICD directly participates in a transcriptional complex involving CSL transcription factor (CBF1 or RBP-Jk of mammals/*Drosophila* suppressor of hairless [Su(H)]/*C. elegans* LAG-1) and transcriptional co-activators like Mastermind (Mam) in *Drosophila*/Mastermind-Like (MAML) in mammals. This ternary complex also recruits histone acetylase CBP/p300 and SKIP leading to activation of Notch target genes (Aster et al. 1997; Leong et al. 2002; Mishra et al. 2014; Oswald et al. 2001; Petcherski and Kimble 2000; Sachan et al. 2015; Singh et al. 2019; Tamura et al. 1995; Vasquez-Del Carpio et al. 2011; Wu et al. 2000). This association converts CSL transcriptional repressor to transcriptional activator, and it activates the most classical target genes, belonging to basic helix-loop-helix (bHLH) families of transcription factor, e*nhancer of split* [*E(spl)*] in *Drosophila*, *hairy/enhancer of split* (HES), and *Hrt* (Hes-related) or *hairy/enhancer of split-related with YRPW motif* (Hey, also called HESR) in mammals. These bHLH transcription factors in turn repress *achaete-scute complex* (As-C) proneural genes (Campos-Ortega 1993; Fortini and Artavanis-Tsakonas 1994; Wu et al. 2000). Thus, these factors repress the transcription of genes involved in differentiation. While in the absence of Notch-ICD, CSL recruits corepressor factors such as NCoR (nuclear receptor corepressor)/SMRT (silencing mediator of retinoid and thyroid hormone receptors), histone deacetylase (HDAC), SHARP (SMRT and HDAC-associated repressor protein)/MINT (Msx2-interacting nuclear target), CIR (CBF1 interacting corepressor), SKIP (Ski-interacting protein), and histone demethylases KDM5A/Lid (Borggrefe and Oswald 2009; Engel et al. 2010; Lai 2002; Liefke et al. 2010; Moshkin et al. 2009; Oswald et al. 2005; VanderWielen et al. 2011). Various components of Notch signaling pathway have been mentioned in Table 1. Depending upon the cellular context *wg*, *cut*, *string, c-myc, cyclin D,* etc., are also Notch target genes (reviewed in Bray and Bernard 2010). Apart from these factors, there are also specific corepressors that antagonize the gene expression engaging the Notch signaling pathway at different cellular contexts. For example, *Drosophila insensitive*, which is homolog of mammalian BEND6, has been identified as a neural-specific CSL corepressor for peripheral

Table 1 Components of Notch signaling pathway

Components	*Drosophila*	Mammals	*Caenorhabditis elegans*
Receptor	Notch	NOTCH1 NOTCH2 NOTCH3 NOTCH4	LIN-12 GLP-1
Canonical ligands	Delta	Delta-like 1 (Dll1) Delta-like 3 (Dll3) Delta-like 4 (Dll4)	APX-1 (Soluble) LAG-2 (Soluble) ARG-1 DSL1-7
	Serrate	Jagged1 Jagged2	
Non-canonical ligands	Weary (Wry) (reported in cardiomyopathy)	DLK1 (in angiogenesis) DLK2 (in preadipocytes) DNER (in cerebellar development) EGFL7 (in neurogenesis)	DOS OSM-11
Transcription factor	Su(H)	RBPjk/CBF-1	LAG-1
Transcriptional co-activators	Mastermind Chip Hat-trick	Mastermind like1 (MAML1) Mastermind like2 (MAML2) Mastermind like3 (MAML3)	LAG-3
Transcriptional corepressors	Hairless, SMRTR CtBP, CtIP, Groucho, HDAC, Sin3A, LSD1, CoREST1, Insensitive, LID	SHARP, CIR1, NCoR/ SMRT (NCoR2), HDAC, BEND6, KDM5A, Bcl-6, CtBP1, NKAP, SAP30	
S1 cleavage (furin convertase)	Furin	Furin, PC5/6	
S2 cleavage (metalloprotease)	Kuzbanian, kuzbanian-like, TACE	ADAM10, ADAM17/TACE	SUP-17/ kuzbanian, ADM4/TACE
S3 cleavage (γ-secretase)	Presenilin Nicastrin APH-1 PEN-2	Presenilin 1 Presenilin 2 Nicastrin APH1a-c PEN-2	SEL-12/ Presenilin APH-2/nicastrin APH-1 PEN-2
HECT-type E3 ubiquitin ligase (for lysosomal degradation)	dNedd4 Su(dx)	Nedd4 Itch	WWP-1
Ring finger-type E3 ubiquitin ligase (Promotes Notch towards Rab 11 vesicles)	Deltex	Deltex 1–4	

(continued)

Table 1 (continued)

Components	*Drosophila*	Mammals	*Caenorhabditis elegans*
F-box protein E3 ubiquitin ligase (Promotes degradation of Notch-ICD by phosphorylation)	Archipelago	Fbw7	SEL-10
E3 ubiquitin ligase (Targets Notch ligands Delta and Jagged/Serrate during endocytosis)	Mind bomb 1–2 Neuralized	Mind bomb, Skeletrophin, Neuralized 1–2	Y47D3A.22
DUB (Deubiquitinating enzyme)	USP12 eIF3-S5	USP12 eIF3f	
Cytoplasmic Notch inhibitor	Numb	Numb, Numb-like	
Numb-associated kinase	Numb-associated kinase	AP2-associated kinase	SEL-5
Notch target genes	*E(Spl)*-complex genes, *myc, wg, cut* etc.	*HES/HEY/ESR, Myc, p21, Bcl-2, cyclin D1*	*REF-1*
Notch nuclear transport pathway component	Importin-*α3 or* Karyopherin-*α3*	Importin subunit alpha-3 or Karyopherin subunit alpha-4	
Negative cytoplasmic regulators of Notch	DTRAF6 Deltex	TRAF6 Deltex-1 NRARP	

neurogenesis, which promotes neural differentiation and inhibits neural stem cell renewal (Dai et al. 2013).

Notch activity is regulated at multiple levels, including patterns of receptor and ligand expression, Notch-ligand interactions, trafficking of the receptor and ligands, and covalent modifications including glycosylation, phosphorylation, and ubiquitination of the receptor (reviewed in Andersson et al. 2011). In addition, Notch signaling is also modified by various cytoplasmic factors such as Deltex, a positive as well as negative modulator of Notch signaling depending on the cellular context (Matsuno et al. 1995; Mukherjee et al. 2005), Numb, negatively regulates Notch (Frise et al. 1996), and SEL10, an F-box protein that promotes Notch-ICD turnover (Gupta-Rossi et al. 2001).

Modes of Notch Action

The core Notch signaling pathway is conserved in most of the Notch-dependent processes. The Notch pathway functions in diverse developmental and physiological processes, which are broadly subdivided into three classes: lateral inhibition, cell lineage decision, and boundary formation. The first report that Notch is involved

in all the above-mentioned functions came from studies involving neurogenesis in *Drosophila* (reviewed in Artavanis-Tsakonas et al. 1999). From these studies it became evident that Notch is involved in the development of various stages of a particular tissue. For example, during the first stage of neurogenesis, Notch regulates the number of cells, which will adopt neuronal fate (through lateral inhibition); subsequently it determines whether progeny will acquire neural or glial fates (through lineage decision) (reviewed in Bray 2006).

Lateral Inhibition

In *Drosophila*, during patterning of neuroectoderm, groups of 4–7 cells termed as "proneural clusters" are defined by the expression of patterning genes. Although all these cells in a proneural cluster have equivalent potential to give rise to neural cell type, one cell will be destined to become either neuroblast for generation of neuron in central nervous system (CNS) or sensory organ precursor (SOP) cell in the peripheral nervous system (PNS) (reviewed in Furukawa et al. 2000; Gaiano and Fishell 2002; Gaiano et al. 2000). Among equivalent groups of cells, one specific cell is preferred for progenitor of CNS or PNS by lateral inhibition (Fig. 2). Constitutively in this process two kinds of genes are involved, proneural and neurogenic genes. Proneural genes of *achaete-scute* complex (*achaete*, *scute*, *asense*, *lethal of scute*), *atonal*, *Bearded,* and *SoxNeuro*, which encodes for basic helix-loop-helix transcription factors, direct the cell to acquire neural fate. In contrast, neurogenic genes such as *Notch*, *Delta*, *Serrate*, *mastermind*, *neuralized*, and *enhancer of split complex* mediate the cell to adopt epidermal fate (reviewed in Iso et al. 2003). Balance between proneural and neurogenic genes determines the fate of a specific cell in a proneural cluster to become a neuroblast or a SOP. The cell that becomes neuroblast or SOP, expresses highest levels of Notch ligand Delta, thus activating Notch in the surrounding cells, inhibiting their differentiation into neuroblasts or the SOP. Neighboring cells, which are now deprived of proneural genes due to Notch expression, convincingly adopt epidermal fate due to lateral inhibition. In Notch mutants due to deficiency of *Notch*, all cells start expressing proneural genes at the expense of epidermis resulting in the overproduction of neurons (reviewed in Gaiano and Fishell 2002).

Cell Lineage Decision

In addition to lateral inhibition, Notch also plays another vital role for cell fate diversification when cells choose between two alternative fates and this process is known as a binary fate decision. During the development of CNS, cells can opt for neuroblast fate where with each asymmetric division, it recapitulates itself and at the same time gives rise to secondary precursor cell known as ganglion mother cell (GMC). After the SOP cell of PNS is chosen, first division of SOP generates two cells, pIIa (Notch on) and pIIb (Notch off) (Fig. 2). Each of these two cells further divides and generates hair and socket from pIIa and pIIb undergoes division to form pIIIb and glial precursor cell (GP), which moves away and gives rise to many glial or adult mechanosensory bristles. The next division of pIIIb generates neuron and sheath. During PNS development, Notch plays an opposite role in glial cell

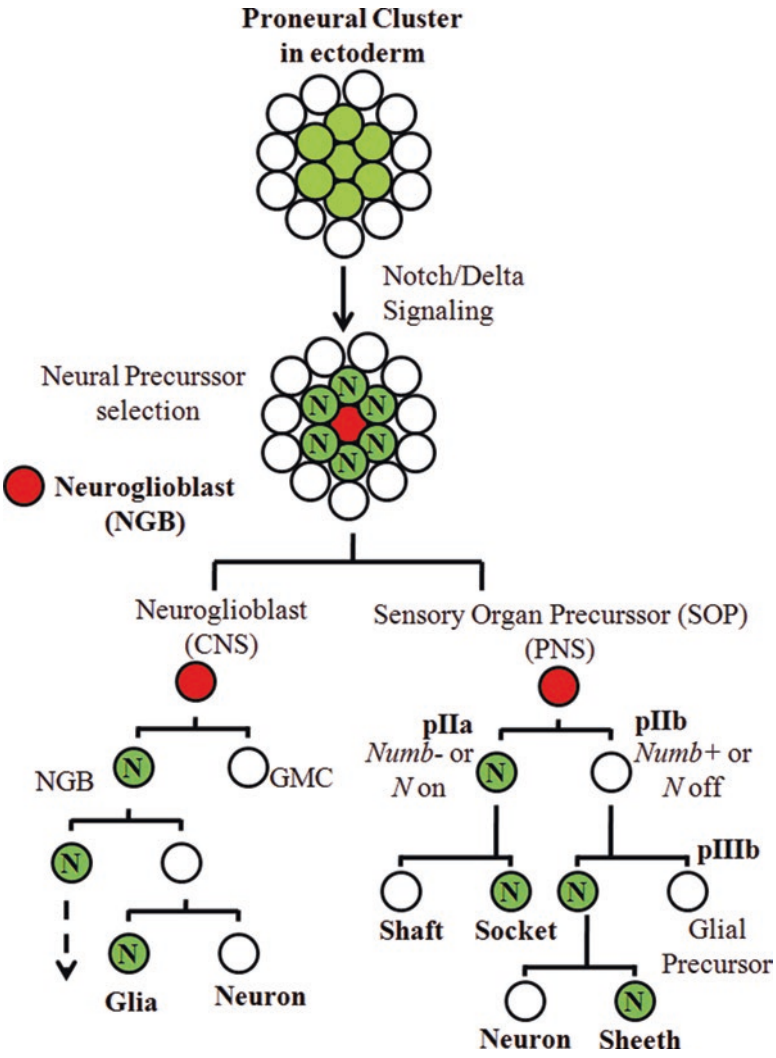

Fig. 2 Lateral inhibition mediated by Notch signaling
Schematic representation showing Notch-mediated lateral inhibition during cell-fate specification in the central nervous system (CNS) and peripheral nervous system (PNS) in *Drosophila*. A single cell within a proneural cluster will become neuroblast for CNS or SOP for PNS and inhibits other neighboring cells from acquiring a neuronal fate

development compared to CNS. At the same time, there is also some evidence where during SOP lineage a few glial cells require Notch. In these Glial cells, Numb protein accumulates, which acts as an antagonist of Notch, and physically interacts with Notch-ICD in association with α-Adaptin. α-Adaptin is a member of AP-2 complex, which acts like an adaptor molecule and binds with the Numb, which in turn is accountable for receptor-mediated endocytosis of Notch for differentiation into pIIb cells (Berdnik et al. 2002).

The role of Notch in the maintenance of stem cells is another example of binary cell fate choices. Notch plays a major role in the decision of which cell will become a stem cell to maintain the stem cell pool and which cell will differentiate (Chiba 2006). It has been reported that Notch1 regulates neural stem cell (NSC) number during development, and Notch1 signaling maintains the reservoir of undifferentiated cells in adult mice during hippocampal neurogenesis (Ables et al. 2010).

Boundary Formation

In *Drosophila*, Notch and Wingless (Wg) signaling pathways are key controllers for dorsoventral (DV) boundary formation in both developing eye, and wing imaginal discs. *apterous* (*ap*) expression in the early wing primordium induces expression of the Notch ligand Serrate in dorsal (D) cells and restricts the expression of another Notch ligand Delta to ventral (V) cells (Diaz-Benjumea and Cohen 1995). Serrate (dorsal) and Delta (Ventral) cells activate the Notch symmetrically in cells on both sides of the DV compartment boundary (de Celis et al. 1996; Doherty et al. 1996). Expression of the glycosyltransferase Fringe makes dorsal cells more sensitive to Delta and less sensitive to Serrate (Fleming et al. 1997; Moloney et al. 2000; Munro and Freeman 2000). Consequently, activated Notch induces Wg expression in cells along the DV boundary. Wg further induces the expression of Serrate and Delta in nearby dorsal and ventral cells and Serrate and Delta signal back to activate Notch, thereby maintaining Cut and Wg expression along the DV boundary (Milan and Cohen 2000, 2003) (Fig. 3).

In the vertebrate central nervous system, neural plate acts as a signaling hub for planar signals. The cells along the neural plate separate into cell population for forebrain, midbrain, hindbrain, and spinal cord (Fraser et al. 1990; Kiecker and Lumsden 2005).

Midbrain-hindbrain boundary (MHB) is the best characterized place to study the boundary formation. In a similar manner to DV boundary of *Drosophila* wing

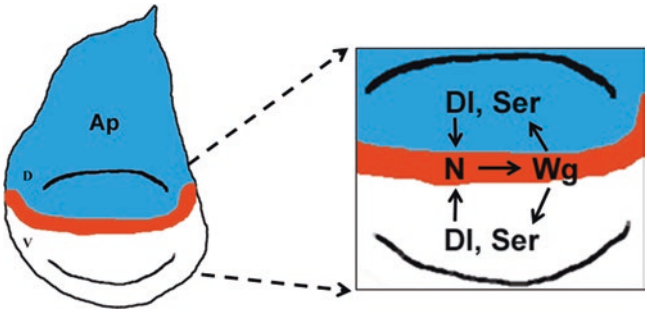

Fig. 3 Schematic representation of role of Notch in the dorsoventral (DV) boundary formation in late third instar larval wing imaginal disc
Notch is activated in DV boundary by its ligands, Dl and Ser, expressed in neighboring cells. Activated Notch turns on Wingless (Wg) expression in DV boundary cells. A positive-feedback loop between Wg expressing cells along the DV boundary and Ser- and Dl-expressing cells in adjacent cells maintain the signaling center along the DV boundary

imaginal disc, Notch is active in the narrow boundary of MHB. Blocking the Notch signaling either by inhibitor of γ-secretase activity or with truncated ligand in the MHB of neural tube in chick embryo leads to the morphologically absence of MHB in the embryos. It has been reported that differential Notch signaling stabilizes the MHB through regulating cell sorting and specifying boundary cell fate (Tossell et al. 2011). Notch also plays an important role in boundary formation in other places as well during development. For example, Notch has a profound role in boundary formation between the prospective somites during somitogenesis in vertebrates.

Notch Signaling in Neurodegeneration

Conservation of human disease genes, powerful genetic tools, and short life cycle of *Drosophila* make it an invaluable model of choice to study human diseases. Here we review the involvement of Notch signaling in the neurodegeneration process by focusing specifically on the information obtained using *Drosophila* as a model system. Notch signaling plays a critical role in brain development. Notch signaling pathway also has a profound role in adult synaptic plasticity and memory formation. Thus, it is not surprising that aberrant Notch function leads to neurodegenerative diseases in humans. Although there is plethora of information on the role of Notch signaling in neurodegeneration using different model systems, here we will restrict our discussion mainly on the information gathered about the involvement of Notch signaling in different neurodegenerative diseases using *Drosophila*.

Alzheimer's Disease (AD)

Alzheimer's disease (AD) is a widespread age-related neurodegenerative disorder that mainly affects the central nervous system of elderly population (Ferri et al. 2005). Clinically, AD is characterized by progressive memory loss and cognitive impairment because synaptic contacts are lost in the neocortex as well as in the hippocampus, which results in dementia and impaired intellectual and linguistic skills (O'Brien and Wong 2011; Scheff et al. 2006). The hallmark pathognomonic features such as senile plaques and neurofibrillary tangles (NFTs) are observed during post-mortem examination for the diagnosis of AD. In 1907, Physician Alois Alzheimer first identified these two pathological alterations in the brain of a female patient suffering from dementia (LaFerla et al. 2007). Senile plaques are formed due to the accumulation of misfolded protein that is a pathogenic form of amyloid-ß, which is derived from amyloid protein precursor (APP). In the specific regions of brain, extracellular deposition of aggregates of small peptide amyloid-ß (Aß), such as $A\beta_{40}$ and $A\beta_{42}$, generates pathogenic amyloid plaques (Hardy and Higgins 1992; Karran et al. 2011). NFTs are intraneuronal aggregation of hyperphosphorylated forms of tau, which is a microtubule-associated protein (Goedert et al. 1988; Grundke-Iqbal et al. 1986; Ihara et al. 1986; Kosik et al. 1986). According to the

data from the National Center for Health Statistics 2014, AD was the leading cause of death after heart disease in the United States (Xu 2016). Majority of known AD cases fall under sporadic category, while about 5% cases are of familial AD (FAD) (Rogaeva 2002). Due to insufficient knowledge of the cause of Alzheimer's disease, its effective treatment is unavailable.

AD state is generated due to improper cleavage of APP in the brain (Fig. 4). APP has very short half-life of ~30–60 mins and undergoes post-translational modifications (Storey et al. 1999). Mutations in either gene encoding for APP or APP processing catalytic component, Presenilin (PS), have been directly linked to AD (Goate et al. 1991; Levy-Lahad et al. 1995; Sherrington et al. 1995). In normal conditions, APP undergoes a series of proteolytic processing by α-secretase (*Drosophila* Kuzbanian) and γ-secretase. While in Alzheimer's disease state, APP undergoes sequential proteolytic processing by β and γ-secretase. β-Secretase activity is delivered by β-site APP-cleaving enzyme (BACE), while γ-secretase activity is provided by PS. These consequent cleavages lead to elevated levels of longer and pathogenic form $A\beta_{42}$ peptides compared to more benign 40-amino-acid-long amyloid ß-peptide ($A\beta_{40}$) (Selkoe 2004; Wolfe and Haass 2001).

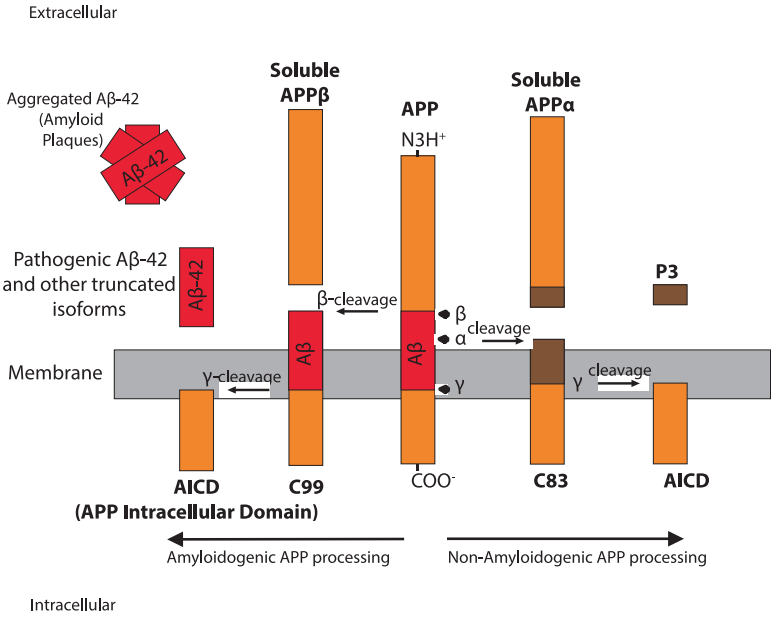

Fig. 4 APP processing by α, β and γ secretase
Cleavage of APP by α-secretase releases a soluble fragment of APPα extracellularly and a membrane-bound fragment, C83. γ-Secretase cleaves C83 to produce the P3 and APP intracellular domain (AICD) during non-amyloidogenic pathway. However, during amyloidogenic processing in Alzheimer's state, mutations in PS subtly modify the cleavage pattern. Associated mutations cause cleavage of APP by β-secretase that generates the soluble APPβ fragment extracellularly and C99 transmembrane fragment. Cleavage of C99 by γ-secretase produces longer and pathogenic form $A\beta_{42}$ peptides and AICD

Table 2 *Drosophila* models for human Alzheimer's disease

AD-associated genes	Fly models	References
APP (Alzheimer's Disease)	Overexpression APP transgenic lines (*pUAST-APP, UAS-APP695II*, *UAS-APP695III*, *UAS-APP-Swedish* (K670 N/M671 L)), *Appl* and *Aβ* mutants	Chakraborty et al. (2011), Fossgreen et al. (1998), Furotani et al. (2018), Greeve et al. (2004), Merdes et al. (2004), Mhatre et al. (2014), Muhammad et al. (2008), Rieche et al. (2018), Stokin et al. (2008) and Wentzell et al. (2012)
APP-Like (Alzheimer's disease)	*Appl* $^{-/-}$ *Appl*-42,673 RNAi line *Appl*-G3 RNAi w	Goguel et al. (2011), Luo et al. (1992)
AB Peptide (Alzheimer's disease)	Overexpression model of full length *Appl* and *dAβ*	Carmine-Simmen et al. (2009), Feng et al. (2018), Finelli et al. (2004) and Iijima et al. (2004)
	Human $A\beta_{40}$ or $A\beta_{42}$ peptide was expressed in the *Drosophila* CNS	
PSEN (Alzheimer's disease)	Overexpression of full-length *Drosophila Psn*	Seidner et al. (2006)
	14 different mutations at conserved residues in *Drosophila* Presenilin has been created corresponding to identified mammalian *Psn1*mutations	
Tau (Alzheimer's disease)	Isolation of *Tau* cDNA and generation of Tau antibodies	Heidary and Fortini (2001), Jackson et al. (2002), Passarella and Goedert (2018), Sealey et al. (2017) and Wittmann et al. (2001)
	Wild-type, mutant forms of human tau (such as Δ306-311 human Tau-383), as well as two isoforms of human Tau, 0N3R and 0N4R were expressed in *Drosophila*	
	Overexpression of *Drosophila Tau*	
Autophagy-related genes in *Drosophila*, neurodegenerative phenotypes	Studies of *ATG1*, *ATG5, ATG7*, *ATG8a* and *ATG18* genes in *Drosophila* Human $A\beta_{1\text{-}40}$ or $A\beta_{1\text{-}42}$ protein expression in *Drosophila* neurons	Juhasz et al. (2007), Kim et al. (2016), Ling et al. (2014), Omata et al. (2014) and Simonsen et al. (2008)

Similar to Notch pathway components, most of the AD-linked genes are evolutionarily conserved in *Drosophila.* Most importantly fruit flies can recapitulate the phenotypes observed in AD patients. Different *Drosophila* models generated for human AD have been mentioned in Table 2. During the process of understanding the molecular basis of this disease, Presenilin (PS) gene that encodes eight-pass transmembrane protein was identified. PS is the catalytic component of γ-secretase complex. PSs are frequently present in the endoplasmic reticulum, Golgi body, and

Table 3 Association of Notch signaling and AD

Associated AD mutants	Effect on Notch signaling
PS mutation	Impaired proteolytic release and nuclear translocation of Notch (Song et al. 1999)
PS deletion	Defective expression of Dll1 and hes5 and failure of normal embryogenesis with several neuronal defects (Donoviel et al. 1999; Saura et al. 2004)
APP interaction with Numb	Decreases Notch signaling (Kyriazis et al. 2008)

the plasma membrane and cleave the amyloid precursor protein (APP) for further processing. Mutations in this gene have been associated with early onset of AD (Table 3) (De Strooper and Woodgett 2003; Levy-Lahad et al. 1995; Mahoney et al. 2006; Ray et al. 1999; Selkoe 1998; Sherrington et al. 1995). In mammals, both Presenilins, PS1 and PS2, are also expressed throughout the development in most of the cell types, whereas compared to PS2, PS1 is mostly expressed during early development. Half of all FAD cases are associated with mutations in three known genes, APP, PS1, and PS2, which cause majority of early-onset Alzheimer's disease in humans (Berezovska et al. 1997; Lee et al. 1996). Majority of these mutations belong to missense substitutions in Presenilins (Fraser et al. 2000; Rogaeva 2002). There are a large number of known substrates of PS/γ-secretase (reviewed by Haapasalo and Kovacs 2011), but little is known about their regulation and activity due to their complex structure (Haapasalo and Kovacs 2011). For most of the substrates, the mechanism of action has not been identified. In those cases, it might act as a catalytic enzyme, which simply eliminates the transmembrane stubs of protein after extracellular membrane shedding (Mahoney et al. 2006; Struhl and Adachi 2000; Wolfe and Kopan 2004). Due to the complex nature of PSs, their full mechanism of action is not very well understood, but they have been well implicated in three processes: Notch signaling, β-amyloid deposition, and apoptosis. Mutation in PS results in the generation of neurotoxic form of β-amyloid ($A\beta_{42}$) compared to $A\beta_{40}$ (Haass 1997). However, in the case of the Notch family receptors, γ-secretase/PS controls the signaling process. Its requirement in Notch signaling cascade has been confirmed in various organisms including *Drosophila* and Human (Wolfe and Kopan 2004).

Notch signaling plays an essential role in neural stem cells (NSCs), in neural development, and in learning and memory formation (Fortini and Artavanis-Tsakonas 1994; Ge et al. 2004; Louvi and Artavanis-Tsakonas 2006; Yoon and Gaiano 2005). Loss of function of Notch in *Drosophila* generates defective long-term memory resulting in the regulatory role in neuronal plasticity (Presente et al. 2004). However, it has also been seen that enhanced Notch signaling suppresses the long-term memory formation in adult *Drosophila* (Zhang et al. 2015). Studies in mice supports the hypothesis that impaired Jagged1-Notch signaling is associated with defective spatial memory in adult mice (Sargin et al. 2013). In the context of age-related human diseases like Alzheimer's disease, various aspects of Notch signaling have been explored since PS-dependent γ-secretase cleavage is common in

processing of Notch and APP (Berezovska et al. 1998; Fraser et al. 2000). To understand the broad role of PS1, targeted null mutation has been created in PS1 locus to generate the knockout mice, but these mice are embryonically lethal and show various abnormalities including excessive neuronal loss, severe hemorrhages in the CNS, and defective skeletal formation. This finding supports the role of PS1 in neural progenitor cell and axial skeletal formation (Shen et al. 1997; Wong et al. 1997). These mice show reduced expression of Notch1 and Dll1in the presomatic mesoderm. FK506-binding proteins (FKBPs) are well-known modifiers of PS in *Drosophila*. FKBPs play an essential role in protein folding and trafficking. FKBP14 mutants genetically interact with components of Notch signaling and show reduced expression of Notch target genes, Presenilin protein levels, and gamma-secretase activity (van de Hoef et al. 2013). Studies have shown that microRNA-124 (miR-124) is highly expressed in CNS and potentially regulates the Notch ligand Delta. miR-124 mutant flies have defects in the climbing ability as well as have reduced life span. RNAi of *Delta* can also rescue the learning defect and enhance the life span of AD flies (Kong et al. 2015). Thus, it was concluded that miR-124 plays a neuroprotective role in AD *Drosophila* model by targeting Notch ligand Delta (Kong et al. 2015).

Dysregulation of microtubule stability causes impairment of axonal transport, degeneration of synaptic contact, and impairment of neuronal function, which ultimately leads to neuronal loss. Among several signaling pathways, Notch pathway also plays a major role in assembly-disassembly of microtubules. It has been demonstrated that Notch activation results in increased microtubule stability and it was proposed that Notch can be a potential target for microtubule stabilization and thus it may have therapeutic potential for the treatment of neurodegenerative diseases including Alzheimer's disease (Bonini et al. 2013). It has been demonstrated that Notch1 is significantly accumulated in the brain parenchyma of sporadic AD patients and consistent reduction of Notch1 signaling in neurons in AD patients suggests that Notch1 may potentially be considered a novel hallmark of AD (Brai et al. 2016).

Parkinson's Disease (PD)

Parkinson's disease (PD) is a very common late-onset neurodegenerative disease that affects the motor neurons and leads to progressive impairment in motor functions (Alexander et al. 1986; Konczak et al. 2009; Lang and Lozano 1998). It is characterized by two main pathological features: premature selective loss of dopamine neurons and accumulation of misfolded α-synuclein protein, known as Lewy bodies in multiple systems of the patients. Major symptoms of Parkinson patients include dementia, bradykinesia, impaired balance, sleep and mood dysfunction, loss of coordination between voluntary and reflexive motors commands, etc. (Braak et al. 2003; Rizek et al. 2016).

Leucine-rich-repeat-kinase2 (LRRK2) has been identified as a causative gene for autosomal-dominant familial and idiopathic PD. Genome-wide-association-studies

(GWAS) identified LRRK2 and SNCA/ α-synuclein as two strong risk loci for sporadic PD (Satake et al. 2009). A single LRRK gene, dLRRK, is present in *Drosophila* and dLRRK is localized in endosomes in which it regulates the function of Rab7 in the late endosomal-lysosomal pathway (Dodson et al. 2012). It has been shown that two LRRK2-binding proteins, NEURL4 [Bluestreak (Blue) in *Drosophila*] and HERC2 (dHERC2 in *Drosophila*), genetically and physically interact with Notch ligand Delta-like 1 (Dll1)/Delta (Dl). LRRK2, along with NEUR4 and HERC2, promotes the recycling of Dll1/Dl through endosomal trafficking of Dll1/Dl, and consequently levels of Dll1/Dl are increased in the plasma membrane. Higher concentration of Dll1/Dl negatively regulates Notch signaling through cis-inhibition. This effect was seen to be enhanced by PD-associated mutation of LRRK2 gene (R1441G ROC domain mutant). As a result, inhibition of Notch signaling accelerates neural stem cell differentiation and affects the function and survival of adult dopaminergic neurons (Imai et al. 2015). The alteration of Notch signaling in adult dopaminergic neurons in *Drosophila* modulates the function and survival of these cells, which may be associated with the neurodegeneration caused by LRRK2 mutations. These findings clearly show that there is a possible link between Notch signaling pathway and Parkinson's disease.

Polyglutamine Diseases (PolyQ Diseases)

A group of neurodegenerative disorders caused by abnormal trinucleotide repeat expansions of CAG that encode long chain of glutamine (Q) amino acid in the coding region of respective gene is known as polyglutamine (PolyQ) diseases. The expansion in the repeat length is directly proportional to disease severity (Table 4) (David et al. 1997; Imbert et al. 1996; La Spada and Taylor 2003; Orr and Zoghbi 2007; Ross et al. 1999). Although each disease falling under this category leads to neurodegeneration, each disease is diagnosed by a specific symptom and a specific pattern of neuronal death (Seidel et al. 2012). The pathogenesis of these set of diseases is not very well understood, and no effective treatment is available (Margulis et al. 2013). Among the PolyQ diseases, Huntington's disease and SCA3 account for the highest prevalence worldwide and Huntington's disease is the most well-studied PolyQ disease (Bauer and Nukina 2009).

Spinocerebellar Ataxia Type 1 (SCA1)

Spinocerebellar ataxia type 1 (SCA1) is a progressive neurodegenerative disease caused by the expansion of trinucleotide CAG repeat within the coding region of the *ataxin-1* (*ATXN1*) gene (Banfi and Zoghbi 1994; Orr et al. 1993). The characteristic features include progressive loss in the motor co-ordination and speech mutilation. Degeneration of specific neurons of brain stem neurons is also very common (Robitaille et al. 1995).

To explore the molecular mechanism behind SCA1-related neuronal degeneration, full-length human SCA1 gene was expressed in *Drosophila* using UAS/GAL4 system. The transgenic flies generated from the construct that encodes ataxin-1 30Q

Table 4 PolyQ diseases

PolyQ diseases	Locus in human	Affected gene	*Drosophila* homolog	Glutamine repeat size	
				Normal	Pathological
SCA1	6p23	Ataxin-1 (ATXN1)	Ataxin-1 (CG4547)	6–39	41–83
SCA2	12q24	Ataxin-2 (ATXN2)	Ataxin-2 (CG5166)	15–31	34–50
SCA3/MJD	14q24-q31	Ataxin-3 (ATXN3)	NA	12–43	60–87
SCA6	19p13	CACNA1A	α1ACT	4–18	21–30
SCA-7	3p21-p12	Ataxin-7 (ATXN7)	NA	7–18	38–200
SCA-17	6q27	TBP	Tbp (CG9874)	29–42	45–63
Huntington Disease	4p16.3	Huntingtin	Huntingtin	6–35	36–121
Spinal and bulbar muscular atrophy (SBMA)/Kennedy's disease	Xq12	Androgen receptor (AR)	Estrogen-related receptor	9–36	38–62
Dentatorubral-pallidoluysian atrophy	12p13.31	Atrophin 1	Atrophin	7–34	49–88

are known as wild-type human isoform, whereas the flies which were derived from the construct that encodes ataxin-1 82Q are termed as SCA1 expanded isoform. Expression of wild-type and expanded SCA1 with eye-specific *GMR-GAL4* produces degeneration of ommatidia in both cases; however, severity of the phenotype is proportional to the number of PolyQ repeats (Fernandez-Funez et al. 2000). This finding in *Drosophila* is very similar to dendritic arborization study of Purkinje cells in SCA1 mice. Transgenic mice for ataxin-1 82Q undergo neurodegeneration at a very early stage (12 weeks), whereas in the case of ataxin-1 30Q, mice neuronal atrophy is not visible until 59th week (Fernandez-Funez et al. 2000). Ataxin-1 30Q in humans may never reach the critical level required for pathogenesis and that may be the reason ataxin-1 30Q is not toxic in humans (Fernandez-Funez et al. 2000).

Ataxin-1 function is not limited to motor coordination and processing of β-amyloid protein (Crespo-Barreto et al. 2010; Matilla et al. 1998; Zhang et al. 2010). It interacts with members of transcriptional corepressor SMRT (silencing mediator of retinoid and thyroid hormone receptors) in *Drosophila* as well as in mammals (Tsai et al. 2004). Capicua and LANP (leucine-rich acidic nuclear protein) cofactor are other interactors of ataxin-1 involved in transcriptional repression (Cvetanovic et al. 2007; Lam et al. 2006; Riley and Orr 2006). Mizutani and colleagues characterized another protein BOAT1 (brother of ataxin-1), which was very similar to ataxin-1 (Mizutani et al. 2005). Tong and co-workers have further explored the role of ataxin-1 and BOAT1 in the Notch signaling pathway. At this end, when BOAT1 was expressed in the posterior compartment of the wing disc by *hedgehog-GAL4* (*hh-GAL4*) driver, it showed the phenotype that mimics *Notch*-mutant wing phenotype in adult flies such as thick longitudinal vein 5 (LV5) and absence of posterior crossvein. At the

same scenario, *hh-GAL4* induced overexpression of BOAT1 in *Notch* mutant background, expanded LV5 thickening phenotype in adult wing was observed. Notch regulates the wing vein thickening phenotype by lateral inhibition by activating various target genes such as *E(spl)* (De Celis and Diaz-Benjumea 2003). In BOAT1 expressing wing imaginal disc, *E(spl)* expression was fairly reduced. This experiment concludes that BOAT1 is an inhibitor of Notch activity (Tong et al. 2011). Further, *hh-GAL4* induced expression of BOAT1 in *Su(H)* mutant background can rescue the LV5 thickening phenotype because repressive effect of *Su(H)* is alleviated in this background. It has been shown through co-immunoprecipitation experiments that BOAT1 and ataxin-1 directly interact with CBF1 [mammalian homologue of *Drosophila* Su(H), also called RBP-Jk]. It has also been reported that BOAT1 and ataxin-1 compete with each other to bind with CBF1. Interestingly, presence of Notch-ICD demolishes the transcriptional repressor complex of BOAT1 or ataxin-1 along with CBF1 (Tong et al. 2011). These results conclude that BOAT1 and ataxin-1 are the components of Notch signaling pathway; hence they might play an essential role in Notch-dependent developmental processes.

Spinocerebellar Ataxia Type 2 (SCA2)

Spinocerebellar ataxia type 2 (SCA2) is one of the neurodegenerative disorders caused by expansion in the CAG nucleotide repeat in the translated sequence of the *ataxin-2* (*ATXN2*) gene. The characteristic features of the patients who carry this disorder are progressive cerebellar ataxia, oculomotor abnormalities, pyramidal and extrapyramidal features (EPS), dementia and peripheral neuropathy, and dystonia (Geschwind et al. 1997; Jhunjhunwala et al. 2014). The main function of ataxin-2 is unknown, but ataxin-2 interacting proteins provide a direction of the possible functions controlled by ataxin-2. Ataxin-2 interacts with various RNA-binding proteins, suggesting its major role in RNA metabolism. Ataxin-2 has also a wide variety of other interacting partners as shown in Table 5. It clearly demonstrates the broad mode of action of ataxin-2. Ataxin-2-binding protein 1 (A2BP1 or Rbfox1) is a nuclear RNA-binding protein and binds to C-terminus of ataxin-2. Both ataxin-2 and A2BP1 are enriched in Purkinje cells and dentate neurons (Shibata et al. 2000).

Table 5 Various Ataxin-2 interactors

Ataxin-2 interactors	Function	References
A2BP1/ RBFOX1	RNA binding	Shibata et al. (2000)
Endophilin A1 Endophilin A3	Vesicle endocytosis	Ralser et al. (2005)
DDX6 (DEAD/H-box RNA helicase)	RNA binding	Nonhoff et al. (2007)
Parkin	Ubiquitination	Huynh et al. (2007)
CIN85	Vesicle endocytosis	Nonis et al. (2008)
TDP-43	RNA binding	Elden et al. (2010)
RGS8 mRNA	Ca2+ signaling	Dansithong et al. (2015)
PABPC1(poly(A)-binding protein, cytoplasmic 1)	RNA metabolism	Yokoshi et al. (2014)

Mutation associated with A2BP1 leads to complex neuronal disorders (Bhalla et al. 2004; Martin et al. 2007; Sebat et al. 2007). A2BP1 is an important regulator of splicing of various neuronal genes that regulates synaptic activity (Lee et al. 2009; O'Brien et al. 2012; Underwood et al. 2005). RNAi knockdown of A2BP1 in *Drosophila* embryo leads to a reduction in neuronal cell number (Koizumi et al. 2007). Not surprisingly, A2BP1 has a profound role in the development of nervous system.

During neurogenesis, A2BP1 acts as a positive regulator of Notch signaling in a context-specific manner. In *Drosophila,* thoracic bristles are a part of peripheral nervous system and follow the lateral inhibition phenomenon (Heitzler and Simpson 1991; Jan and Jan 1994). Each of these thoracic bristles arises from sensory organ precursors (SOPs) that form a complete sensory organ made of shaft, socket, sheath, neuron and glia (Hartenstein and Posakony 1989; Reddy and Rodrigues 1999). A2BP1 is a nuclear protein and is broadly present in developing embryo and imaginal discs with some specificity (Koizumi et al. 2007; Usha and Shashidhara 2010). Overexpression of *Drosophila* A2BP1 in the proneural cluster results in the loss of adult sensory bristles, whereas its downregulation increases bristle number. It has been reported that A2BP1 is part of the Su(H) complex in the presence and absence of Notch and might function as a transcriptional co-factor to regulate the expression of *E(spl)-C* (Shukla et al. 2017). It has been suggested that A2BP1 is a context-specific positive regulator of Notch signaling during neurogenesis in *Drosophila* (Shukla et al. 2017). Similar to ataxin-2, its interactor protein A2BP1 has two PolyQ domains and it is involved in the regulation of Notch signaling pathway (Shukla et al. 2017). Notch protein also contains polyglutamine stretch. Significance of these PolyQ domains and the role of Notch in SCA2 pathology remain to be explored.

Spinocerebellar Ataxia Type 17 (SCA17)

Spinocerebellar ataxia type 17 (SCA17) is a late-onset, progressive neurodegenerative disease caused by an expanded CAG trinucleotide repeat in TATA-binding protein (TBP) gene (Bauer and Nukina 2009; Koide et al. 1999; Nakamura et al. 2001). The characteristic features are ataxia, dementia, seizures, and involuntary movements, including chorea and dystonia (Koide et al. 1999; Rolfs et al. 2003). The expanded PolyQ repeats in TBP modify the interaction with other cellular proteins and influence the gene expression such as downregulation of HSPB1 (heat shock protein and neuroprotective factor) due to boosted interaction between mutant TBP and TFIIB, reduced expression of TrkA (receptor for nerve growth factor) due to enhanced interaction between mutant TBP and Sp1 transcription factor, and reduced expression of Chaperone system-associated factor and MANF (mesencephalic astrocyte-derived neurotrophic factor) due to inefficient binding of mutant TBP and XBP1 transcription factor; also expanded repeats in TBP reduce the association of MyoD with TBP and DNA promoters that cause muscle degeneration (Davidson 2003; Friedman et al. 2007; Huang et al. 2015; Pugh 2000; Shah et al. 2009; Yang et al. 2014).

Notch signaling pathway plays a profound role in various developmental events such as neurogenesis and maintenance of neural stem cells (Hitoshi et al. 2002). Su(H) acts as an essential transcription factor in Notch signaling. In general, Su(H) belongs to the group of proteins that are rich in glutamine (Q) and asparagine (N) (Michelitsch and Weissman 2000). Upon ligand-induced activation, released Notch-ICD translocates to the nucleus and directly interacts with the Su(H) and promotes the transcription of downstream target genes, while in the absence of Notch-ICD, Su(H) acts as a transcriptional repressor and blocks the expression of target genes (Aster et al. 1997; Oswald et al. 2001; Petcherski and Kimble 2000; Tamura et al. 1995; Vasquez-Del Carpio et al. 2011; Wu et al. 2000). Ren and co-workers (2011) explored the importance of Su(H) in SCA17 model in *Drosophila*. TBP is a general transcription factor used by all three nuclear RNA polymerases during transcription process (Nikolov and Burley 1994). Highly conserved C-terminal domain of TBP directly binds to TATA-box (TATAAA), which is present at 25–30 base pairs upstream of transcription start site in all metazoans (Burley and Roeder 1996; Davidson 2003; Gill and Tjian 1991; Lee and Young 2000; Pugh 2000). Not surprisingly, homozygous mutant *dtbp* (*Drosophila* TBP) (piggyback insertion at 5′ of *dTBP*) allele is first instar larval lethal that suggest the importance of TBP in fly (Ren et al. 2011). Overexpression of *dTBP* or wild-type *hTBP* with *Hsp70-GAL4/*UAS system in homozygous mutant flies can partially rescue first instar larval lethality. Interestingly, overexpression of pathogenic form of TBP, such as hTBP54Q (54 glutamines) or hTBP80Q (80 glutamines), with *GMR-GAL4* produces eye-patterning defects (disorganized photoreceptor and progressive retinal degeneration) with severity depending upon Poly-Q length as compared to normal Poly-Q expressing TBP protein (hTBP34Q). Overexpression of normal and pathogenic form of TBP with panneuronal driver (*elav-GAL4*) causes age-onset locomotor impairment including early mortality in pathogenic form of TBP, which is the characteristic feature of SCA17 pathology in humans. Microarray analysis of these flies revealed differential regulation of many known candidate genes such as HSPB1 in the above-mentioned background as well as many novel candidates. Q/N--rich protein-dependent transcription regulators are one of them (Ren et al. 2011). Q/N-rich family proteins play an important role during neurogenesis (Harrison and Gerstein 2003). A genetic modifier screen in *GMR-GAL4* driven hTBP80Q expression for Q/N-rich transcription factors validated the role of Su(H) in the neuropathology of SCA17 disease. Knockdown of Su(H) in hTBP80Q background worsens the photoreceptor defects up to the level of irregular shape of ommatidia with missing bristles, necrosis, and retinal degeneration. Interestingly, overexpression of Su(H) in *GMR-GAL4*-driven hTBP80Q flies can rescue the patterning and retinal degeneration. hTBP80Q contains Su(H)-binding sites, which enhances this particular interaction, that reduces the fraction of available Su(H) for normal cell physiology. Although knockdown of *Su(H)* and *hTBP80Q*, together with *GMR-GAL4*, results in bristle loss, the role of Notch-ICD in this aspect needs to be further explored since this phenomenon can be due to Notch-dependent or Notch-independent function (Ren et al. 2011). Studies in the mammalian system suggest that Notch1 or RBP-J/Su(H) mutant mice result in learning and memory defects

(Costa et al. 2003). Altogether, studies in *Drosophila* and mammalian system suggest that Su(H)/RBP-J plays a functional key role in neuropathology of SCA17.

Huntington's Disease (HD)

Huntington's disease (HD) is an autosomal dominant neurodegenerative disorder caused by abnormal trinucleotide repeat of CAG in the exon 1 of *Huntingtin* (*Htt*) gene, which leads to accumulation of Huntingtin protein in the CNS. In contrast to a normal individual where CAG repeat varies from 6 to 34, in HD-affected patients, CAG repeats exceed from 36 to 121 (Andrew et al. 1993). HD begins usually in mid-life with the first sign of chorea (involuntary jerking or twitching movements), progressive selective neuronal loss (preferentially medium-sized, spiny, GABAergic neurons in the striatum), decreased neurogenesis, dementia, and psychological symptoms (DiFiglia 1997; DiFiglia et al. 1997; Martin and Gusella 1986; Moores et al. 2008; Petersen et al. 1999). Despite being an extensively studied disease, very little is known about cellular pathways involved in pathogenic Huntingtin protein expression, which leads to neuronal loss. There is no treatment available to increase the life expectancy of patients with this disorder. Due to limitations of human tissue, significant HD investigation has been established through model systems.

Drosophila homologue of *Htt (DmHtt)* gene shares a similar distribution pattern and sequence conservation with five different regions of human *Htt* (Li et al. 1999). Various transgenic *Drosophila* models have been generated to explore the many aspects of the HD. Table 6 includes the major contribution of *Drosophila* as a model system in solving the puzzle of the HD. In 1997, identification of Huntingtin interacting protein 1 (Hip1) has broadened the mechanistic aspect of HD. Hip1 has been identified as a strong binding partner of Htt, and Hip1 is also involved in the clathrin-mediated endocytosis and intracellular trafficking. This result signifies a functional link in the cellular mechanism underlying the HD. Above the threshold level of polyglutamines, the interaction between Htt and Hip1 diminishes as the number of polyglutamines increases (Gervais et al. 2002; Hackam et al. 2000; Kalchman et al. 1997; Legendre-Guillemin et al. 2002; Legendre-Guillemin et al. 2005; Mishra et al. 2001; Rao et al. 2003; Sun et al. 2005). In *Caenorhabditis elegans*, Hip1 mutant study reveals that during development it has a protective role against polyglutamine pathogenicity and mutants have defective pre-synaptic vesicles (Parker et al. 2007). Dysfunctions of HD-associated genes alter neurogenesis. The role of Notch-mediated neurogenesis in HD has been explored thoroughly. Notch and Hip1 both are known to be involved in endocytosis and intracellular trafficking.

Amyotrophic Lateral Sclerosis (ALS)

Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disease that severely affects the motor neurons (corticospinal or upper motor neuron and spinal or lower motor neurons). Most of the patients die within 3–5 years of symptom onset (Ince et al. 2003). The first gene associated with ALS was *SOD1*, and so far over 100 *SOD1*-associated mutations have been identified (Boillee et al. 2006).

Table 6 Different Transgenic *Drosophila* models of pathogenic HD using different tissue/neuronal subtype-specific driver lines

Transgenic *Drosophila* models	Effect	Finding	Reference
Amino-terminal fragments of human Htt containing tracts of Q2, Q75 and Q120 with *GMR-GAL4*	Nuclear accumulation of pathogenic Htt, Progressive Neurodegeneration severity increases with number of PolyQ length	Neuron loss phenotype cannot be rescued by co-expression of anti-apoptotic P35 protein	Jackson et al. (1998)
Htt Q20 or Q93 in exon 1with *Elav-Gal4*	Htt-Q93 leads to Progressive loss of rhabdomeres with age; 70% lethality with early adult death (Htt-Q93) compared to Htt-Q20 (Control) expressing flies	Identification of two binding factors of Htt: CREB-binding protein (CBP) and p300/CBP-associated factor (P/CAF); prevent the Progressive neurodegeneration can be reduced by HDAC inhibitors	Steffan et al. (2001)
Htt-Q0 and Htt-Q128 with *GMR-GAL4* and *Elav-GAL4*	Htt-Q128 leads to Reduced life span, Progressive loss of motor coordination, and formation of huntingtin aggregates	Htt-Q128 causes Photoreceptor degeneration, aggregation of pathogenic Htt in the cytoplasm and neurites, but not in the nucleus	Lee et al. (2004)
Htt-Q16 and Htt-Q128 with *GMR-GAL4* and *C164-GAL4*	Htt-Q128 leads to progressive neurodegeneration but not Q16 control	Partial loss of Synaptic Transmission genes (Snap, Syx, Rop) and voltage-gated Ca2+ channel gene (Vha100-1) can suppress the neurodegenerative phenotype in HD	Romero et al. (2008)
Htt exon 1 fused to EGFP with Q18 or Q62	Q62 leads to accumulation of mutant Htt and degeneration of eye	RNAi screening provided new modifiers of pathogenic Htt	Doumanis et al. (2009)
DmHtt knockout	Developmentally normal (Contrast to Htt KO mice)	Larval neurons show delayed transport rate of synaptic vesicles Adults show locomotor defects and reduced viability	Gunawardena et al. (2003), Li et al. (1999) and Zala et al. (2013)

Advancements in ALS genetics have identified several other ALS-associated mutations such as *TDP-43, FUS/TLS* (fused in sarcoma/translocated in liposarcoma), *C9ORF72* (chromosome 9 open reading frame 72), *MATR3 (matrin 3)*, *CCNF (cyclin F),* and *VCP* (valosin-containing protein) (Chia et al. 2018). However, the pathophysiological mechanisms that lead to ALS motor neuron dysfunction are

Table 7 *Drosophila* models of ALS

Gene	Mutation	Effect	Result	References
TDP-43	C-Terminus Gly-rich domain	cytosolic aggregation of TDP-43	Degeneration of neurons; early lethality	Cushman et al. (2010), Johnson et al. (2009), Neumann et al. (2006) and Zhan et al. (2013)
FUS/TLS	C-terminus Nuclear Localization Sequence	Cytosolic aggregation of FUS	Degeneration of Neurons, larval-crawling defect and early lethality	Fushimi et al. (2011), Lanson et al. (2011) and Sun et al. 2011)
C9ORF72	GGGGCC (G4C2) repeat expansion in the non-coding region	Presence of RNA foci and dipeptide repeat (DPR) proteins in the cytoplasm	Degeneration of neurons; reduced life span	Burguete et al. (2015), Freibaum et al. (2015), Mizielinska et al. (2014), Tran et al. (2015) and Xu et al. (2013)
Ter94/ VCP	R152H and A229E	VCP and TDP-43 genetically interact and disease-causing mutations in VCP promote reorganization of TDP-43	Degeneration of neurons; reduced life span	Ritson et al. (2010)
Hrp38 (hnRNP)	Gly-rich tract of Hrp38 (293–365) interacts with TDP-43	Hrp38 interacts with TDP-43	Hrp38 and TBPH genetically interact to prevent locomotor defects and reduce life span	Romano et al. (2014)

poorly understood. Various *Drosophila* models have been generated to explore the pathophysiological mechanisms, as mentioned in Table 7.

TDP-43 plays an important role in the regulation of mRNA splicing by binding to UG repeats in target RNAs. CFTR has been identified as the first RNA substrate for TDP-43. TDP-43 binding with CFTR intron 8 promotes the skipping of exon 9. This kind of important observation leads to a detailed study of the RNA interactome of TDP-43 (Polymenidou et al. 2011; Tollervey et al. 2011). Whole genome microarray in *GMR-GAL4*-driven *TDP-43* overexpressing flies has been performed, and, interestingly, Notch intracellular pathway component *Hey* came up as a direct target of TDP-43. In the TDP-43-associated neurodegeneration, *Hey* was upregulated. Life span of *TDP-43* mutant flies can be enhanced by mutating the Notch pathway components such as Delta and Serrate (Zhan et al. 2013). Loss of *htk* suppresses TDP-43-mediated age-dependent neurodegeneration seen in ALS in *Drosophila* model (Sreedharan et al. 2015). Recently, we have shown that Htk is a component

of Notch-Su(H) activation complex and hence positively regulates Notch signaling (Singh et al. 2019).

Genetic mutation in the *C9ORF72* repeat expansion GGGGCC (G4C2) in the non-coding region generates pathogenic dipeptide repeat proteins (DRP). They are known to be associated with ALS. To understand which nucleotide repeats of *C9ORF72* are toxic to the cells, three different genotypes of the flies were generated: flies that express 80 copies of GGXGCX (GA)80, 80 copies of GGXCGX (GR)80, or 80 copies of CCXCGX (PR)80 where the X can be randomly one out of four nucleotides. Cell type-specific overexpression of these repeats identified that only (GR)80 and (PR)80 repeats are toxic to the cells (neuronal/non-neuronal) (Kwon et al. 2014; Yang et al. 2015). Flies expressing (GR)80 results in notching in the wing margin of the adults implying that (GR)80 can suppress the Notch signaling. iPSC-derived human neurons and brain tissue of C9ORF72 patients also have lower expression of few Notch target genes (Yang et al. 2015). Thus, Notch signaling pathway is the target of Poly(GR) toxicity in C9ORF72-associated ALS (Yang et al. 2015).

Future Perspectives

Notch receptor is the central element of an evolutionarily conserved signaling mechanism which plays a fundamental role in metazoan development (Artavanis-Tsakonas et al. 1999). Notch signaling is known to affect a broad spectrum of cell-fate decisions throughout development. Thus, Notch malfunction has been associated with many diseases including neurodegeneration in humans. To allow the Notch signal to be deployed in numerous contexts, many different mechanisms have evolved to regulate the level, duration, and spatial distribution of Notch activity.

It has been reported that neurogenesis is impaired due to Notch signal suppression in mice that express AD-associated mutant Presenilin 1 (Veeraraghavalu et al. 2010). Parkinson's disease-associated mutation of LRRK2 causes inhibition of Notch signaling in adult dopaminergic neurons, which ultimately impairs their functions and survival (Imai et al. 2015). Recently it has been revealed that loss of *htk* suppresses TDP-43-mediated age-dependent neurodegeneration seen in ALS in *Drosophila* model (Sreedharan et al. 2015). Investigations on gene expression patterns in the TDP-43-associated neurodegeneration in *Drosophila* system have shown strong upregulation of Notch target genes (Zhan et al. 2013). It has also been reported that mutations in Notch pathway components extended the life span of TDP-43 transgenic lines (Zhan et al. 2013). Thus, Notch activation has a deleterious effect in TDP-43 flies. Recently, we have reported that Htk is a component of Notch-Su(H) activation complex and positively regulates Notch signaling (Singh et al. 2019). All these findings indicate a possible link between Notch pathway and the neurodegenerative diseases such as AD, Parkinson's disease, and ALS. Despite the plethora of information about Notch pathway, the involvement of Notch signaling in the neurodegeneration process remains largely uncharacterized. The wealth of genetic resources available for *Drosophila* offers a unique opportunity to dissect

involvement of Notch signaling in different neurodegenerative diseases. Due to the high degree of conservation between *Drosophila* and mammalian Notch signaling pathway, future research to explore intricate molecular mechanism of Notch function in neurodegeneration using *Drosophila* as a model system will advance search for therapies of neurodegenerative diseases targeting Notch pathway.

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Tau, Tangles and Tauopathies: Insights from *Drosophila* Disease Models

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Abstract

Tauopathies, such as Alzheimer's disease, Parkinson's disease, Pick's disease, etc., represent a group of neurodegenerative disorders which involve a microtubule-associated protein and tau-mediated pathogenesis and also exhibit tau inclusions in neurons or glia as their shared defining denominator. The tau protein, due to mutations or abnormal hyperphosphorylation, undergoes changes leading to the formation of aggregates in the form of paired helical filaments (PHFs) and subsequently neurofibrillary tangles (NFTs). A positive correlation between NFTs and neurodegeneration was noted, and such neurotoxic NFTs have been considered as a key factor in tau pathology. Due to limitations associated with human genetics, human tauopathies have been modelled in various organisms including *Drosophila* to examine the in-depths of the disease aetiology. Interestingly, brain-specific expression of the human tau-transgene in *Drosophila* recapitulates several pathological markers and key phenotypes. This chapter provides an overview of the molecular aspects of tau pathology and discusses the recent advances in dissecting the underlying molecular pathomechanisms using fly models.

Keywords

Drosophila · Tauopathy · Neurodegeneration · Phosphorylation · NFTs

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Introduction

Human neuronal tauopathies represent a group of neurodegenerative disorders which are marked by the formation of neuronal and glial inclusions primarily composed of the tau protein (Williams 2006; Ferrer et al. 2014). Tauopathies, the term coined by Bernardino Ghetti and Michel Goedert, signifies over 20 forms of different disorders such as Alzheimer's disease (AD), postencephalitic parkinsonism, amyotrophic lateral sclerosis/parkinsonism-dementia complex (ALS/PDC) of Guam, progressive supranuclear palsy (PSP), corticobasal degeneration (CBD), Pick's disease, frontotemporal dementia (FTD), Niemann-Pick Type C (NPC) disease, etc.(Ozansoy and Başak 2007). Among the above-mentioned conditions, Alzheimer's disease (AD) is the most prevalent form of tauopathies as approximately 26 million people worldwide suffer from it (Wheeler et al. 2012). The phenotypic manifestations of this class of disorders include behavioural and movement deficits and a variable degree of amnesia and anomia (Irwin 2016; Orr et al. 2017).

Microtubule-associated protein (MAP) tau (also designated as **τ**), are mainly found in the cytosol of neuronal and glial cells and in trace amounts in non-neuronal cells, whose dysfunction plays a central role in the manifestation of tauopathies (Williams 2006; Ferrer et al. 2014; Weingarten et al. 1975; Wang and Mandelkow 2016). The physiological role of tau is stabilization of the cell cytoskeleton by binding to its microtubules, axonal transport and neurogenesis (Weingarten et al. 1975; Hernández and Avila 2007; Vershinin et al. 2007). Also, its direct binding to DNA suggests an alleged role in DNA packaging and protection against DNA damage (Orr et al. 2017; Hua et al. 2003; Wei et al. 2008).

The transition from "physiological tau" to "pathogenic tau" is triggered by mutations in the MAPT gene (*microtubule-associated protein tau*), brain injury, post-translational alterations like hyper-phosphorylation, aberrant expression of its isoforms and spread from neighbouring cells (Orr et al. 2017; Hernández and Avila 2007). The above-mentioned causes drive the neuropathology of the disease/diseases, distinguished on the basis of various anatomical areas of the brain, cell types and presence of distinctive isoforms of tau in the pathological inclusions. In a general context, tauopathies lead to degeneration of the neurons in the cortex and subcortical regions of the brain, affecting areas such as frontal and temporal lobes in PiD, temporal and parietal lobes including parts of the frontal cortex and cingulate gyrus in AD, subthalamic nucleus and brainstem tegmentum in PSP, etc. (Kovacs 2015).

The first person to label tauopathy was a German psychiatrist, Alois Alzheimer, who spotted intra- and extracellular protein aggregates/inclusions in the postmortem brain of one of his patients who was complaining of loss of memory, delusion and depression (Maurer et al. 1997; Chang et al. 2018). These intracellular protein aggregates are subsequently named as neurofibrillary tangles (NFTs), whose primary component is the tau protein (Chang et al. 2018; Ihara et al. 1986; Alonso et al. 2001). Tau, which is a phosphoprotein, is negatively regulated by hyperphosphorylation as it leads to conformational changes and eventually causes the formation of oligomers and paired helical filaments (Götz et al. 2019). This suggests a positive correlation between disease pathogenesis and tau hyperphosphorylation (Chang et al. 2018; Alonso et al. 2001; Götz et al. 2019; Murray et al. 2014).

Five major classes of tauopathies have been defined till date in accordance to the different pathological tau components (with respect to phosphorylation and isoforms) found in the aggregates represented as a "bar code" (Kimura et al. 2018). The electrophoretic pattern of the tau protein stained against phospho-dependent and tau antibodies on the gel explicates five classes such as Class 0 (loss of tau protein expression), Class 1 (four components of tau at 60, 64, 69, 72/74 kDa), Class II (64 and 69 kDa), Class III (60 and 64 kDa) and Class IV (60 kDa). The above classification leads to categorization of human neuronal tauopathies at the molecular level (Goedert et al. 1992; Sergeant et al. 2005).

The tauopathy models could be broadly divided into three categories: (a) cell-free, (b) cellular and (c) transgenic animal models (Hall and Yao 2005). The cell-free models such as tau protein, purified tubulin, etc. mostly deal with the role of tau in microtubule formation and stability with the capacity of its hyper-phosphorylation to alter this ability (Hall and Yao 2005; Brandt and Lee 1993). The necessity to understand the mechanism by which tau acts together with cell-specific components shifts the usage to cellular models/cell lines leading to studies at the cellular and organism levels. It has been demonstrated that QBI-293 cells expressing tau-40 led to its aggregation on introducing preformed tau fibrils, suggesting a seeding mechanism behind NFT formation (Guo and Lee 2011). Among the well-established whole-body transgenic models, zebrafish, *Caenorhabditis elegans*, *Drosophila* and murine have been most extensively studied (Hall and Yao 2005). Formation of toxic tau aggregates and spread of such exogenous insoluble human tau (AD brain) from the area of injection in the rat model strongly correlates the role of NFT and its dispersal in disease pathogenesis (Smolek et al. 2018). Among all the above models, *Drosophila* has proved to be an excellent model with the virtue of the fact that ~77% of the genes that cause diseases in humans have homologs in *Drosophila* (Reiter et al. 2001). Intriguingly, because humans and *Drosophila* follow the comparable mode of tau pathogenesis, this has paved the way for in-depth investigations to acquire better understanding of the tauopathies and the management aspects (Chanu and Sarkar 2017). An overview of the MAPT has been provided below.

Microtubule-Associated Protein Tau (MAPT)

The very first isolation of tau (tubulin-associated unit) was made from porcine brain extracts and was proposed to be a highly soluble, heat-stable protein cardinal for microtubule (MT) assembly (Weingarten et al. 1976). Eventually, murine cDNA was utilized to determine the full-length sequence of the tau protein (Lee et al. 1988) and subsequently established as a member of the MAP 2/tau family of microtubule-associated proteins, which also includes other vertebrate homologs (Chapin and Bulinski 1991; Dehmelt and Halpain 2005). In fact, tau was one of the first and most extensively studied MAPs (microtubule associated proteins) predominantly due to its contribution in the pathogenesis of several neurodegenerative disorders (Cleveland et al. 1977a, b).

Functional orthologs of MAP 2/tau, containing microtubule-binding domains, were also found in *C. elegans* (PTL-1), *D. melanogaster* (CG31057), *Tetraodon*

(CAG09246), frogs, chicken, mouse, rat, cow and monkey (Dehmelt and Halpain 2005; Goedert et al. 1996; McDermott et al. 1996; Heidary and Fortini 2001). Phylogenetic analysis suggests that the *Drosophila* tau protein shares significant homology with human MAPT than with human MAP 2 and MAP 4 (Heidary and Fortini 2001). Interestingly, it has been noted that the coding sequence of tau in mammals have remained conserved, but the RNA splicing pattern has undergone substantial phylogenetic divergence (Andreadis et al. 1992; Janke et al. 1999).

The human tau protein is encoded by a unique gene spanning an approximately 100 kb region of the long arm of chromosome 17, 17q21.31 (Neve et al. 1986). A single *Drosophila* tau (dTau) gene maps to the 98A6 region of the third chromosome and shares about 46% identity and 66% similarity with human tau (Heidary and Fortini 2001; Adams et al. 2000).

Splicing of Tau mRNA and Its Isoforms

The human tau gene comprises of 16 exons giving rise to distinct isoforms of the tau protein with varying sizes ranging from 352 to 441 amino acids with molecular weights 45 to 65 kDa, respectively, as shown in Fig. 1. Exon 1 lies within the promoter region of the tau gene that undergoes transcription but not translation. Exons 1, 4, 5, 7, 8, 9, 11, 12 and 13 are constitutive exons and are retained in all the

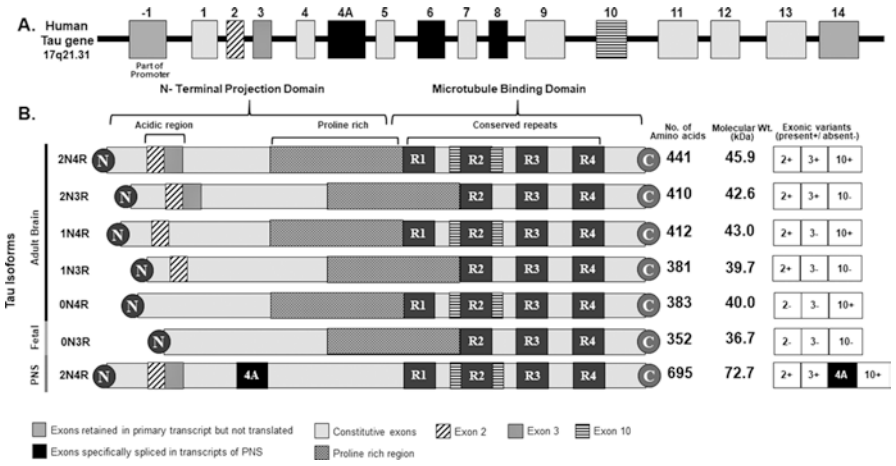

Fig. 1 Schematic representation of the human tau (MAPT) gene and various protein isoforms expressed at different developmental stages. (**a**) The MAPT gene is composed of 16 exons, of which exons 1, 4, 5, 7, 9, 11, 12 and 13 are expressed constitutively in all the isoforms. Exons 1 and 14 undergo only transcription. (**b**) Six isoforms of tau as a result of alternative splicing of exons 2, 3 and 10 characterized by presence or absence of (0N), (1N) or (2N) inserts in the amino-terminal region in combination with 3R or 4R microtubule-binding domains (R) in the carboxylic terminal region. 0N3R is regarded as the foetal tau, and expression of 2N4R, also referred to as "big tau", is restricted to the peripheral nervous system. The number of amino acids, molecular weight in kDa and exonic variants for each isoform are indicated next to every isoform

isoforms. The central nervous system nurses six isoforms of the tau protein, which are generated as a result of alternative splicing among exons 2, 3 and 10, whereas additional high-molecular-weight tau isoforms detected in the peripheral nervous system are produced by splicing of exon 4A and exon 6, also referred to as "big tau" (Goedert et al. 1992; Couchie et al. 1992; Nunez and Fischer 1997; Boyne et al. 1995; Andreadis et al. 1995; Arikan et al. 2002; Li et al. 2003; Wang et al. 2004). Polypeptide encoded by exon 2 can appear in the protein independent of exon 3, but the same is not true for exon 3 (Andreadis et al. 1995).

The tau protein exists in multiple isoforms as a result of regulated alternative splicing of primary transcript. These isoforms differ by the length of N-termini repeats and presence of three (3R) or four (4R) microtubule-binding repeats in C-termini. Human tau isoforms differ on the basis of the presence of three (3R) or four (4R) highly conserved repetitive (R) microtubule-binding domains located in the C-terminal region of the protein, encoded by exons 9–12, in conjunction with the presence or absence of one or two amino acid inserts (0N, 1N, 2N) of 29 amino acids each, localized in the amino-terminal region (Spillantini and Goedert 1998). The presence of these 3R or 4R repeats helps the tau protein in binding to the microtubules and to regulate the dynamics of neuronal cytoskeleton. Spatiotemporal expression of the various tau isoforms have been shown to harbour functional relevance in different developmental stages, such as the smallest isoform 0N3R that is expressed only in the foetal stage, whereas other isoforms appear only after the postnatal period of the human brain development. This transition in isoform expression pattern during development is in agreement with the formation of synapses, representative of the critical postnatal period for sensory and motor development (Altman and Sudarshan 1975; Simons and Land 1987). Presence of exon 10 elevates the affinity of the tau protein towards microtubules and converts a flexible foetal cytoskeleton into a stabilized adult cytoskeleton (Felgner et al. 1997). In the normal adult brain, the relative concentration of 3R-tau to 4R-tau isoforms is around 1, but 0N, 1N and 2N constitute to about 54%, 37% and 9% of the total tau, respectively (Goedert and Jakes 1990; Hong et al. 1998). Any perturbation in this ratio manifests tau-related neurodegeneration.

Regions, Domains and Motifs

Sequence and structural analyses of the human brain tau protein elucidated the presence of two large domains: amino terminal forming the projection domain (encompassing 2/3 of the entire molecule) and carboxylic terminal forming the microtubule-binding domain (covering 1/3 of the molecule). The projection domain can be further subdivided into two regions based on their amino acid composition: the amino terminal region sheltering a high proportion of acidic residues and proline-rich region. On the same lines, the microtubule-binding domain is a combination of three distinct sub-domains: basic, true-tubulin binding and acidic carboxylic terminal regions. The amino acid framework of the full-length tau protein (2N4R) proclaims the presence of 80 S or T residues, 56 negative (D + E), 58

positive (K + R) and 8 aromatic (5Y, 3F and no W) residues. This gives rise to an overall basic character to the protein, with ~120 acidic residues in the amino terminal and ~40 residues in the carboxylic terminal, which are nearly neutral in nature (Mandelkow and Mandelkow 2012). Thus, tau acts as a dipole due to the presence of two distinct domains of opposite charges (Sergeant et al. 2008). This asymmetry in charge distribution enhances its interaction with microtubules, internal folding and tau aggregation. Contrary to humans, *Drosophila* tau exhibits five putative microtubule-binding domains, highly charged N-terminal region rich in proline residues with eight serine-proline and threonine-proline potential sites for phosphorylation (Heidary and Fortini 2001).

Biophysical analysis suggests that the tau protein usually exists as a natively unfolded protein (Jeganathan et al. 2008; Mukrasch et al. 2009). The polypeptide chain of tau possesses recognizable flexibility and mobility with little secondary structures, and binding of tau to microtubules can induce conformational changes (Woody et al. 1983; Kadavath et al. 2015). Electrostatic repulsion offered by the negatively charged projection domain located in the N-terminus causes it to branch away from the microtubule surface and helps in maintaining the space between the microtubules and other components (Hirokawa et al. 1988; Chen et al. 1992; Frappier et al. 1994; Kar et al. 2003; Amos 2004).

Comparable to MAP 1 and MAP 2, tau is a phosphoprotein and its biological activity is modulated by the degree of phosphorylation (Lindwall and Cole 1984; Kopke et al. 1993; Alonso et al. 1994). Interestingly, tau can be phosphorylated at multiple sites by various protein kinases, including casein kinase type-1 and cyclic-AMP-dependent protein kinase (Pierre and Nunez 1983). Non-pathological tau contains two to three moles of phosphate per mole of protein, optimal for its interaction with tubulin and microtubule assembly (Kopke et al. 1993). The tau protein forms ~50 nm long rod-like structures that attach to microtubules as periodic and short arm-like projections forming tiny cross-bridges between the microtubules. MAP 1A has been suggested to play the role of a matrix, which helps in forming microtubule channels for the translocation of membrane organelles (Hirokawa et al. 1988).

A brief account of the biological relevance of tau in cell cytoskeleton stability and how perturbations in tau result in pathological conditions has been focused in the following sections.

Functions of Tau Protein

The most important role of tau is to promote microtubule assembly and stability. Interestingly, tau appears functionally redundant as its loss of function can be complemented by other MAPs (Qiang et al. 2006). Moeover, the tau knockout mice have been found to be viable and fertile without any sign of neurodegeneration (Gorsky et al. 2016).

The structure of tau has been found to be important for its normal functioning in a cell. Interestingly, the N-terminal domain of tau projects away from the protein

body, and hence, it does not bind to microtubules directly; however, it regulates its dynamics by influencing its attachment with other components (Chen et al. 1992). For instance, truncation in the N-terminal domain leads to vulnerable interaction between microtubules and the tau protein, irrespective of its intact microtubule-binding domain (Matsumoto et al. 2015). Also, extreme residues in the N-terminal domain have been found to modulate some signalling cascade responsible for inhibiting axonal transport in neurons (Kanaan et al. 2011).

The proline-rich domain of the tau protein harbours potential sites for interaction with proteins having Src-homology such as kinases of this family like Lck, Fgr and Fyn and other proteins like Bin1, peptidyl prolyl cis-trans isomerases, phospholipase C (PLC) γ1, PLCγ2 and growth factor receptor-bound protein 2 (Morris et al. 2011), and this collectively may regulate tau signalling functions (Guo et al. 2017). The interaction between microtubule and tau is found to be mediated by microtubule-binding repeats but regulated by neighbouring amino acids (Mukrasch et al. 2007; Sillen et al. 2007).

Other interacting proteins include presenilin 1, histone deacetylase 6 (HDAC6), apolipoprotein E, F-actin and α-synuclein (Takashima et al. 1998; Ding et al. 2008; Huang et al. 1994; Correas et al. 1990; Jensen et al. 1999). The interaction between actin and tau is mediated by a minimum of two microtubule-binding domains and allows a proper connection between microtubule and actin (Elie et al. 2015). Such interaction is important for normal axonal transport, and this has been found to be disrupted by an increased level of tau phosphorylation (Fulga et al. 2007; Minamide et al. 2000). In addition to that, the function of C-terminal and/or the information about the protein with which it interacts is not known till date (Guo et al. 2017). However, few studies have suggested that changes in this particular region might influence the domains of tau, thus altering its phosphorylation and its interaction with other proteins (Seitz et al. 2002).

Post-translational Modification of Tau

As stated earlier, the optimum level of phosphorylation is essential for the normal functioning of tau. In addition to phosphorylation, tau is also subjected to various other post-translational modifications such as acetylation, oxidation, polyamination, sumoylation, ubiquitylation, β-linked N-acetylglucosamine (*O*-GlcNAcylation), isomerization, glycation and nitration (Martin et al. 2011; Morris et al. 2015; Saha and Sen 2019). However, the most common post-translational modification is phosphorylation and *O*-GlcNAcylation (Buée et al. 2000). Enzymes like phosphatases and kinases play an important role in tau modification as they maintain a threshold level of phosphorylation (Götz et al. 2019). In view of the pivotal role of post-translational modification functioning, this aspect has been discussed in further detail below.

Tau Hyperphosphorylation

Phosphorylation plays a critical role in regulating the physiological and pathological functions of tau. The longest tau isoforms possess 85 putative phosphorylation sites, i.e. 45 serine, 35 threonine and five tyrosine residues (Crespo-Biel et al. 2014). Intriguingly, tauopathies have been shown to be associated with abnormal phosphorylation of almost 40 sites (Li et al. 2014). Pathologically, abnormal hyperphosphorylation of tau leads to the formation of predominant insoluble toxic species of paired helical filaments (PHFs) and NFTs, which denote the brain lesion hallmark of these disorders. During pathogenesis, abnormally hyperphosphorylated tau shows a two-fold to three-fold enhancement of the number of moles of phosphate per mole of protein (Kopke et al. 1993), and this declines its microtubule-binding property and makes it susceptible for aggregate formation. In agreement to the above, the extent of hyperphosphorylation, aggregate formation capability and distribution pattern of NFTs have been found to be directly associated with the disease severity and extent of cognitive decline (Serrano-Pozo et al. 2011). Interestingly, it was noted that phosphorylation increases the effective persistence length and end-to-end distance of the tau protein (Chin et al. 2016).

Tau phosphorylation is developmentally regulated. Typically, foetal tau is highly phosphorylated in contrast to adult tau. Foetal tau can be distinguished from the normal adult tau based on Ser202 site-specific phosphorylation, and interestingly, this resembles one of the abnormally phosphorylated sites during early stages of AD (Goedert et al. 1993). Foetal human brain has been found to express only a single isoform of tau, i.e. 0N3R, and two other forms were identified due to notable variation in the degree of their phosphorylation (Brion et al. 1993). It has been suggested that foetal tau hyperphosphorylation takes place in the distal region of growing axons, and when the majority of axonal terminals reach their synaptic targets, the hyperphosphorylation status of foetal tau minimizes (Jovanov-Milošević et al. 2012).

It has been elucidated that the differential level of foetal tau phosphorylation is highly regulated to meet the requirement for flexibility in the microtubule system, which is vital during nervous system development (Zhou et al. 2017). It has been noted that the majority of tau phosphorylation sites are accumulated in or adjacent to flanking regions of the MTB repeats, and this suggests a negative correlation between phosphorylation and MT-binding ability of the tau protein (Guo et al. 2017; Zhou et al. 2017). Tau phosphorylation at the amino acid position(s) Ser262, Ser293, Ser324 and Ser356, which are correspondingly located in each of the four microtubule-binding repeats of tau, results in the reduction of tau-binding affinity to microtubules (Biernat et al. 1993; Bramblett et al. 1993). Moreover, phosphorylation at other sites such as Ser396, T153, S214, T212/S214, S396/S404, etc. has been found to be associated with abnormal phosphorylation and tau aggregation in the AD brain (Augustinack et al. 2002). Similarly, phosphorylation at Thr231 induces conformational change and that at sites Ser214, Ser356, and Ser324 alters the capacity of tau to associate with microtubules (Schneider et al. 1999; Lu et al. 1999). Interestingly, similar results have been established in *Drosophila* tauopathy

models, which show neurodegeneration along with the accumulation of filamentous actin (F-actin) and formation of actin-rich rods due to abnormal tau hyperphosphorylation (Fulga et al. 2007).

Several studies have attempted to elucidate the pathogenesis associated with the increased aggregation of the tau protein (Götz et al. 2019; Avila 2006). The first such report showed that increased tau phosphorylation extricates tau from microtubules and induces mislocalization of hyperphosphorylated tau to the somatodendritic compartment of the axons, compromising axonal microtubule integrity and inducing synaptic dysfunction, which is initially independent of neurodegeneration (Hoover et al. 2010). It was also noted that tau phosphorylation is also capable of interrupting its intracellular route of degradation. For example, phosphorylation at the Ser422 site prevents caspase-3-mediated cleavage of tau (Guillozet-Bongaarts et al. 2006); however, phosphorylation at another site, Ser262/Ser356, results in inhibition of the interaction between tau and CHIP-HSP90 complex leading to its escape from proteasomal degradation (Dickey et al. 2007). Interestingly, phosphomimic tau showed their clearance by autophagy in a selective manner compared to endogenous tau (Rodríguez-Martín et al. 2013). Microinjection of tau into synaptic terminals has been demonstrated to enhance the levels of calcium, which, in turn, disrupts the synaptic transmission via a pathway that involves kinase activation (Moreno et al. 2016). Taken together, it is increasingly clear now that phosphorylation modifies the association of tau with its interacting partners such as cytoskeletal components, cytoplasmic membrane, DNA, Fyn kinase, etc. and intrudes with different functions of tau in terms of maintenance of cellular integrity and signalling pathways (Götz et al. 2019; Guo et al. 2017; Zhou et al. 2017; Li and Götz 2017).

In view of the critical involvement of protein kinases and protein phosphatases in the phosphorylation of tau and disease pathogenesis, this area has emerged as a primary area in tauopathy research. There are broad groups of tau kinases such as proline-directed serine/threonine-protein kinases including glycogen synthase kinase (GSK) 3α/β, cyclin-dependent kinase-5 (Cdk5), mitogen-activated protein kinases (MAPKs) and stress-inducible kinases, among others (Ferrer et al. 2005). Other than this, MT-affinity regulating kinases have also been found to regulate tau phosphorylation (Ferrer et al. 2005). Other groups of non-proline-directed serine/threonine-protein kinases include tau-tubulin kinase 1/2 (TTBK1/2), casein kinase 1 (CK1), dual-specificity tyrosine phosphorylation-regulated kinase 1A (DYRK1A), microtubule affinity-regulating kinases (MARKs), Akt/protein kinase B, cAMP-dependent protein kinase A (PKA), protein kinase C, protein kinase N, 5-adenosine monophosphate-activated protein kinase (AMPK), calcium−/calmodulin-dependent protein kinase II (CaMKII) and thousand-and-one amino acid protein kinases (TAOKs), protein kinases specific for tyrosine residues such as Src (LCK, Fyn, etc.) and ABL family members (ARG and ABL1) (Martin et al. 2011; Tremblay et al. 2010; Scales et al. 2001). In addition to the above, protein phosphatases such as PP1, PP2A, PP2B, PP5, etc. have shown to play an important role during regulation of the dynamic activity of tau (Liu et al. 2005; Hoffman et al. 2017).

Interestingly, in contrast to several reports which correlated tau phosphorylation with disease pathogenesis and aggregate formation, a recent report has suggested

the protective role of site-specific tau phosphorylation in AD models of mouse (Ittner et al. 2016). It has been reported that during early phases of pathogenesis, the neuronal p38 mitogen-activated protein kinase p38γ mediates site-specific phosphorylation of tau, which, in turn, intervenes in the postsynaptic excitotoxicity signalling complexes and inhibits amyloid-β toxicity (Ittner et al. 2016). In addition, site-specific tau phosphorylation at Thr205 has been shown to disrupt the assembly of PSD-95/tau/Fyn complexes and mediate Aβ toxicity (Ittner et al. 2010).

Tau Acetylation

Emerging discoveries have established the role of tau acetylation as an important post-translational modification in its physiological and pathological functions (Wang and Mandelkow 2016). Tau acetylation is largely mediated by cAMP-response element binding (CREB) protein (CBP), while SIRT1 and HDAC6 are responsible for its deacetylation (Cook et al. 2014a). Besides that, some amino acid residues, for example, cysteine residues at 291 and 322 in R2 and R3, respectively, provide an intrinsic acetyltransferase property to tau, which helps in its autoacetylation (Cohen et al. 2013). Interestingly, acetylation at these residues is dependent on the proximity of the targeted lysine residues at 274 and 340 amino acid sites (Luo et al. 2014). In addition, autoacetylation results in tau fragmentation, which may result in its increased autophagic degradation (Cohen et al. 2016; Esteves et al. 2018). CBP acetylates tau at both lysine- and proline-rich residues in microtubule-binding repeats, while autoacetylation occurs preferentially at lysine residues in microtubule-binding repeats (Cohen et al. 2016). Acetylation at some residues, i.e. 259, 290, 321 and 353 positions, has been found in control/healthy brain and is suggested to protect tau from increased phosphorylation, and thus, it suppresses its pathogenic aggregation (Cook et al. 2014a). Interestingly, this protective acetylation is reduced in the AD brain (Cook et al. 2014b). This is supported by the fact that acetylation of residues Lys174, Lys274 and Lys280 has been found in the postmortem brain of AD, FTLD-tau, PSP and Pick's disease (Irwin et al. 2013; Min et al. 2015). Also, acetylation at Lys280 along with some other sites was found to inhibit proteasomal degradation of the tau protein and also lead to increased phosphorylation (Morris et al. 2015; Min et al. 2010; Cohen et al. 2011).

Since acetylation of tau has functional significance in disease pathology, approaches like mutating lysine to other amino acids and considering its effect on tau acetylation can potentially help in understanding the role of tau acetylation in disease pathogenesis (Gorsky et al. 2016). Interestingly, acetylation at the Lys280 site has been found to intensify the neurotoxic effect of tau in *Drosophila* (Gorsky et al. 2016). In addition, tau acetylation also influences synaptic function as transgenic mice expressing tau with lysine to glutamine mutation and mimicking the acetylation of K274 and K281 show memory deficits and impaired hippocampal long-term potentiation (LTP) (Tracy et al. 2015).

The above studies suggest dynamic involvement of acetylation in normal tau functioning and also in disease pathogenesis. It would be interesting to examine how the acetylation is either protective or detrimental in a site-specific manner. Therefore, an in-depth investigation on various aspects of tau acetylation may help in designing novel therapeutic strategies against human tauopathies.

Other Post-translational Modifications

Tau has been found to be glycosylated at the N-terminal in AD patients' brain but not in the control brain, suggestive of the fact that such modification may contribute to the formation and maintenance of neurofibrillary tangles (Wang et al. 1996). In addition, glycosylation has been reported to prevent dephosphorylation, thus accelerating phosphorylation of tau (Liu et al. 2002). However, the addition of *O*-linked *N*-acetylglucosamine (*O*-GlcNAc), which occurs on serine threonine residues in tau, has been found to be protective against increasing tau phosphorylation as it competes with kinases to modify the target amino acid (Liu et al. 2004; Smet-Nocca et al. 2011). Interestingly, *O*-GlcNAcylation was found to suppress tau aggregation, and thus, its reduced level in the AD brain might be responsible for increased tau phosphorylation and aggregate formation (Liu et al. 2004; Yuzwa et al. 2014). A significantly reduced level of the enzyme responsible for *O*-GlcNAcylation, i.e. *O*-GlcNAc transferase, in the AD brain samples also suggests its protective role (Ma et al. 2017; Götz et al. 2019). Also, *O*-GlcNAc transferase knockout mice exhibit memory loss along with degeneration of neurons and the increase in tau phosphorylation (Wang and Mandelkow 2016).

Some other post-translational modifications, for instance, glycation, deamidation and isomerization, have also been observed in the AD brain in contrast to those in healthy control brain (Watanabe et al. 2004). Such modifications have been proposed to influence tau aggregation by affecting its conformation (Watanabe et al. 2004; Ledesma et al. 1995). In addition, nitration of Tyr18, Tyr29 and Tyr394 has been detected in AD and other forms of tauopathies, which have shown to influence the tau conformation and reduce its ability to bind with microtubules (Reyes et al. 2012). Besides nitration, levels of tau ubiquitinylation also increase in tauopathies. Intriguingly, a competition between acetylation and ubiquitination for specific lysine residues has been reported in neurons of wild-type mice (Morris et al. 2015; Min et al. 2010). Sumoylation has also been reported to counteract the ubiquitination effect and accelerate tau phosphorylation (Luo et al. 2014). Significance of tau methylation could not be validated yet, but it was demonstrated that the sites for lysine residues are the same for acetylation and ubiquitination (Yang and Seto 2008).

The above studies clearly indicate that tau undergoes a variety of post-translational modifications in its physiological and pathological stages. This makes the study of the native state of the tau protein complicated, as the downstream signalling cascades are affected due to these post-translational modifications and their subsequent effects on disease pathogenesis.

Mechanism of Tau Aggregation

Toxic NFTs are made of aggregates of abnormal protein filaments, predominantly composed of tau. Accumulation of NFTs is mainly perceived in neuronal perikarya, dendrites and axons (Brion 1998; Mietelska-Porowska et al. 2014). As discussed earlier, the tau protein is characteristically very soluble due to its hydrophilic nature, and it is present in a microtubule-bound state under normal physiological conditions. However, under diseased conditions, tau detaches from the microtubules and exists as free tau monomers in the cytoplasm, known as PHF-tau, the starting material for tangle formation (Barghorn and Mandelkow 2002; Serrano-Pozo et al. 2011). Mature NFTs are largely composed of PHFs and straight filaments (SFs); however, the abundance of each component differs in various forms of tauopathies; for instance, both PHFs and SFs are present in case of AD, CBD and PiD, whereas SFs are predominant in PSP (Lee et al. 2001). PHFs are fibrils of around 10 nm in diameter, which form pairs with a helical three-dimensional conformation at a regular periodicity of about 65 nm, and cross-sections of its core display two C-shaped units (Kidd 1963; Wisniewski et al. 1976; Tapia-Rojas et al. 2018). Straight filaments are almost similar to PHFs, with the only difference being the absence of the periodic twist (Crowther 1991). An abundance of NFTs in the brain is directly related to a decrease in the level of normal tau and an increase in the level of PHF-tau (Bramblett et al. 1992; Mukaetova-Ladinska et al. 1993).

NFTs are the hallmark of various tauopathies, and this feature has been replicated in mammalian models of tauopathies (Davies and Spires-Jones 2018). Some of the classical approaches to identify NFTs include silver staining, Congo Red staining and thioflavin S staining (Lamy et al. 1989). In addition to the above, transmission electron microscopy (TEM), in situ immunostaining and several biochemical assays could also detect NFTs and PHFs (Duyckaerts et al. 1987, 1990). Intriguingly, some recent studies suggest the transcellular spreading of the tau protein in tauopathies (Demaegd et al. 2018).

Figure 2 presents the cascade of events that have been suggested to ensue tangle formation: (i) an increase in tau concentration and/or disbalance in tau isoform ratio (Avila et al. 2006); (ii) a change in tau conformation (Gamblin et al. 2000a, b, 2003a) and (iii) different post-translational modifications such as phosphorylation (Grundke-Iqbal et al. 1986), glycation (Ledesma et al. 1994), truncation (Wischik 1989), etc. Although several post-translational modifications have been suggested to play an important role(s) in the formation of tau polymers, abnormal tau hyperphosphorylation is considered to be the key factor of tau aggregation, which facilitates its detachment from the microtubules and thus increases the concentration of free tau monomers and enhances the probability of its pairing and subsequent aggregation (Götz et al. 2019). However, other post-translational modifications have also been suggested to play an active role in tau aggregation; for instance, the addition of polyanions accelerates aggregation in vitro (Goedert et al. 1996; Kampers et al. 1996; Friedhoff et al. 1998), and the oxidizing environment was found to induce the formation of disulphide linkages, which, in turn, accelerates tau aggregation (Wille et al. 1992; Schweers et al. 1995). In case of FTDP-17, certain mutations such as ΔK280, P301L, and P301S

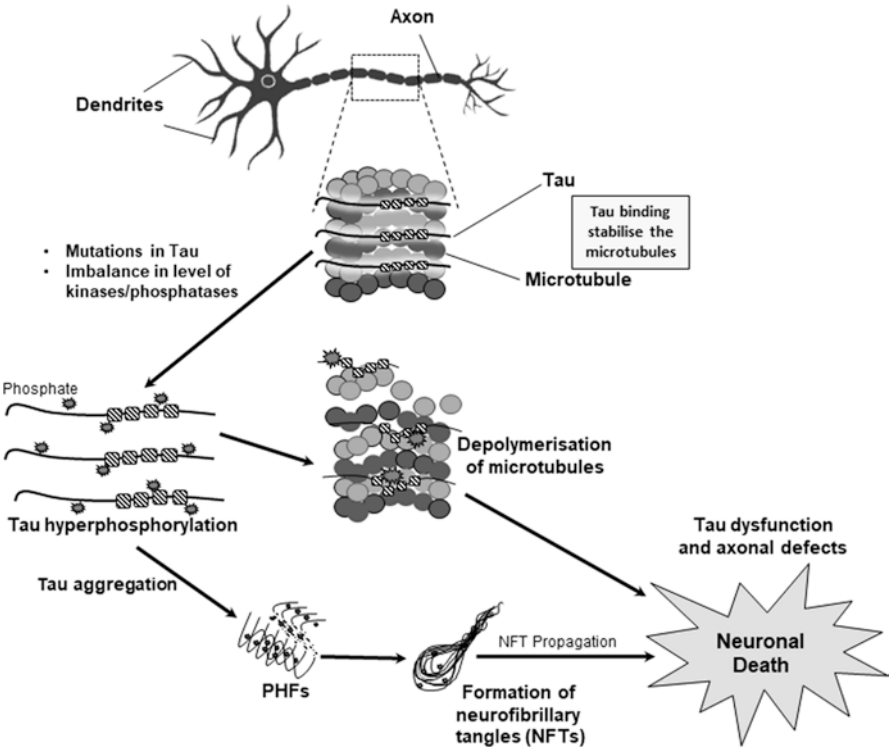

Fig. 2 Schematic representation of the key characteristic events that contribute to the formation of neurofibrillary tangles (NFTs), subsequently leading to neurodegeneration and commencement of tauopathies.

have been found to boost the rate and extent of tau fibrillization by increasing the formation of β-structure (Barghorn et al. 2000; von Bergen et al. 2001).

Biochemically, the process of tau aggregation can be defined as a nucleation elongation process (Friedhoff et al. 1998) which involves the formation of β structures around specific hexapeptide motifs in the repeat domains (von Bergen et al. 2000, 2001; Giannetti et al. 2000). The dimerization and the nucleation steps are rate-limiting and are thermodynamically not favoured during normal cellular homeostasis. However, during disease pathogenesis, the concentration of monomeric tau increases in the cytoplasm, and the tau undergoes conformational change and forms dimers (Weismiller et al. 2018). Tau monomers are then added to the nascent ends of this increasing polymer repeatedly leading to aggregate formation (von Bergen et al. 2000).

Structurally, the process of tau aggregation starts with the conformational change from a loose coil to a more compact form in which the amino terminus binds to the microtubule binding repeats (MTBR) (Mirbaha et al. 2018). Since this tau confirmation is detectable by the monoclonal antibody Alz50, it is named as Alz50 conformation (Mandelkow et al. 1996). This state is also regarded as the pre-tangle

state. After adapting this pre-tangle state, tau can change into a more compact conformation detected by Tau-66 antibody and therefore called as the tau-66 state, in which the proline-rich region binds to the MTBR (Ghoshal et al. 2001; Garcia-Sierra et al. 2003). The N-terminus of tau is cleaved in this stage. Interestingly, since the C-terminus has been shown to hinder aggregation (Abraha et al. 2000), it is cleaved at two positions, E391 (Wischik 1989) and D421, by caspase-3 (Gamblin et al. 2003b). Such truncated tau has been found to be cytotoxic and might lead to neuronal dysfunction and death. It has also been proposed that the cleavage events positively influence the process of nucleation and elongation and also help in achieving NFT stability (Binder et al. 2004).

Morphologically, NFTs can be categorized into three different developmental stages. First, pre-NFTs with the characteristic of diffuse or punctate tau are stained within the cytoplasm of normal-looking neurons and well-preserved dendrites and nucleus. These pre-NFT forms are positive for phosphorylated-tau antibodies TG3 (pT231), pS262 and pT153 (Götz et al. 2019; Augustinack et al. 2002; Kuret et al. 2005). Second, mature or fibrillar intraneuronal NFTs (iNFTs) consist of cytoplasmic filamentous aggregates of tau. Interestingly, these structures push the nucleus towards the periphery of the cell body and often extend to distorted-appearing dendrites and to the proximal segment of the axon. Such NFTs could be detected by pT175/181, 12E8 (pS262/pS356), pS422, pS46 and pS214 antibodies (Götz et al. 2019). Third, NFTs represent extra-neuronal "ghost" NFTs (eNFTs) that result from the death of tangle-bearing neurons and are identifiable by their typical flame-shaped structure and absence of nucleus and stainable cytoplasm. These mature forms of NFTs are positive for thioflavin S, Congo Red and thiazine red stains and antibodies such as AT8 (pS199/pS2002/pt205), AT100 (pT212/pS2140) and PHF-1 (pS396/pS404) (Augustinack et al. 2002; Kuret et al. 2005; Su et al. 1993; Braak et al. 1994).

A direct correlation between maturation and distribution of NFTs and the degree of cognitive decline and memory impairment in various tauopathies including AD has been observed (Braak and Braak 1991). The Braak system of NFT staging classifies the topographic progression of AD-associated NFTs into six stages: Stages I and II NFTs spread from the transentorhinal region to hippocampal formation and are associated with impairment of memory and mild spatial disorientation. Stage III and IV NFTs localize to the temporal, frontal and parietal lobes and neocortex and are linked with impaired recalling faculties, disorientation in time and space, impaired concentration, comprehension and other dementia-like symptoms. Stage V and VI NFTs are found in the unimodal and primary sensory and motor areas of the neocortex, connected with disturbances in object recognition and motor skills (Braak and Braak 1991).

Insights of Tau Pathology from *Drosophila* Models

The limitations associated with human genetics call for the use of model organisms to investigate in depth the mechanistics of disease pathogenesis and to develop effective treatment strategies. Model organisms such as mice, *Drosophila* and *C.*

elegans have been utilized to investigate the cellular and molecular mechanisms of the pathogenesis of human neurodegenerative disorders like tauopathies, poly(Q) disorders, etc. Subsequently, *Drosophila* emerged as a model of choice to investigate in-depth human tau pathology at the cellular and molecular levels. One of the most worthwhile utilizations of *Drosophila* disease models is the screening of genetic modifiers, which aim to identify second-site locus that either suppresses or enhances the disease effect.

Human tauopathies such as Alzheimer's, Parkinson's, frontotemporal dementia, etc. have been successfully modelled in *Drosophila* by expressing wild-type or mutant isoform(s) of human tau (Chanu and Sarkar 2017; Wittmann et al. 2001; Gistelinck et al. 2012; Trotter et al. 2017). Intriguingly, *Drosophila* tauopathy models duplicate the features of human neurodegenerative diseases such as degeneration of brain cells, progressive locomotor defects, cognitive impairments and reduced life span (Sarkar 2018; Sivanantharajah et al. 2019). Flexible genetic tools such as the *UAS-Gal4/Gal80* system allow the expression of a disease-causing transgene in a tissue- and a developmental time-specific manner (Chanu and Sarkar 2017; Wittmann et al. 2001; Trotter et al. 2017). In addition, expression of the disease-causing transgene in adult eyes drives easily the scorable-specific phenotype. For instance, expression of V337M human tau in the fly eyes gives a rough eye phenotype, which can be utilized to screen modifiers at a large scale within a short period of time (Shulman and Feany 2003). Similarly, targeted expression of human tau in brain or mushroom body causes degeneration of brain cells and results in locomotor and cognitive impairments (Kosmidis et al. 2010). By utilizing the fly system, it was noted that tau facilitates neurodegeneration by promoting global chromatin relaxation, and such heterochromatin loss has been proposed to act as a toxic effector of tau-mediated neurodegeneration (Frost et al. 2014). Interestingly, a positive correlation between the extent of neurodegeneration and the toxicity level of the various mutant human tau isoforms suggests a similarity between the disease pathogenic mechanisms in human and *Drosophila* (Chanu and Sarkar 2017; Wittmann et al. 2001).

It was demonstrated in fly models of tauopathies that the phosphorylation status of the tau protein increases at some specific sites, that is, AT8 and AT100 positions in an age-dependent manner, which, in turn, causes increased tau insolubility and glial tangle formation and degeneration of neuronal and dendritic cells (Colodner and Feany 2010; Lin et al. 2010). As noted earlier, fly disease models have extensively been utilized for genetic modifier screening. Extensive genetic screening performed in different laboratories has identified several serine/threonine kinases, phosphatases and the components of the cytoskeleton network as the major classes of modifiers of human neuronal tauopathies (Shulman and Feany 2003; Blard et al. 2007; Ambegaokar and Jackson 2011). By utilizing this approach, a *Drosophila* homolog of GSK-3β, shaggy, was found to modulate the tau-mediated neurodegeneration in fly models. Increased tau toxicity could be noted following the overexpression of shaggy, in which aggregated tau resembling toxic NFTs were observed (Jackson et al. 2002). In contrast, reduced level of shaggy in disease background restricts the tau pathogenesis (Jackson et al. 2002). Interestingly, genetic screenings in *Drosophila* have identified several kinases such as CamKI, Mekk1

etc. as disease enhancers, which aggravate the disease phenotype without making any impact on tau phosphorylation. Above findings contradict a direct link between the status of tau phosphorylation and disease pathogenesis (Ambegaokar and Jackson 2011). Some of the other identified tau modifiers include genes involved in cellular apoptosis, cell cycle, chromatin remodulation, ubiquitin degradation, etc. (Sarkar 2018).

Interestingly, it was initially suggested that human tau-mediated neurodegeneration and phenotypic manifestation in *Drosophila* are mediated by soluble hyperphosphorylated tau, and unlike humans, perhaps the formation of NFTs is not essential for tau pathogenesis at least in fly models (Wittmann et al. 2001; Williams et al. 2000). Although NFT-like structures were visible in *Drosophila* brain cells upon overexpression of GSK-3β in tau background, this aggravated the disease phenotypes (Jackson et al. 2002). In another study involving *Drosophila* PD models, the formation of tangle-like structures in dopaminergic neurons was reported (Wu et al. 2013). A direct correlation between the formation of intracellular NFTs and tau-induced toxicity could not be established, and hence, it was not obvious if NFTs are indeed required for tau pathogenesis. Moreover, it was also postulated that tau pathogenesis in fly models might be different from that of human disease due to the lack of neurofilaments and formation of NFTs. In another study involving *Drosophila* PD models, the formation of tangle-like structures in dopaminergic neurons has been reported (Wu et al. 2013), but the formation of NFTs in disease pathogenesis has not been shown to occur in *Drosophila* models.

Interestingly, when examining the spatial cellular distribution pattern of the phosphorylated and the unphosphorylated human tau protein (total tau) in fly models, the existence of characteristic NFTs as first reported by Alois Alzheimer in an AD patient (Maurer et al. 1997) was noticed (Fig. 3e) and subsequently validated by various methods (Chanu and Sarkar 2017). Such NFTs were consistently observed in *Drosophila* neuronal tissues upon the expression of wild-type or mutant forms of human tau-transgene (Chanu and Sarkar 2017). Here it is essential to note that since NFT formation includes both phosphorylated and unphosphorylated forms of the tau protein (Alonso et al. 1996), immunostaining with an antibody that is independent of the tau phosphorylation status and/or confirmation identifies all the available tau species and perceives the complete structure formed by tau aggregates in a smaller organism like *Drosophila*.

As observed in human and other mammalian model systems, the NFTs in fly models exhibit various morphological phases, that is, pretangles with dense cytoplasmic inclusions and relatively mature intracellular and extracellular neurofibrillary tangles comprising the filamentous aggregates with a flame-shaped structure. The relatively less toxic round- and/or globose-shaped tangles were observed under mild-diseased condition, whereas the flame-shaped mature NFTs to massive filamentous aggregates were evident in the flies with relatively severe forms of tauopathies (Chanu and Sarkar 2017). Interestingly, in agreement to the fact that the morphological stages of NFTs signify the toxicity and severity level of the disease, it was observed that the size and frequency of the tangles progressively increase with the severity level and age of the fly, and the majority of the tangles adopt a flame-shaped morphology during the advanced stage of the disease (Chanu and

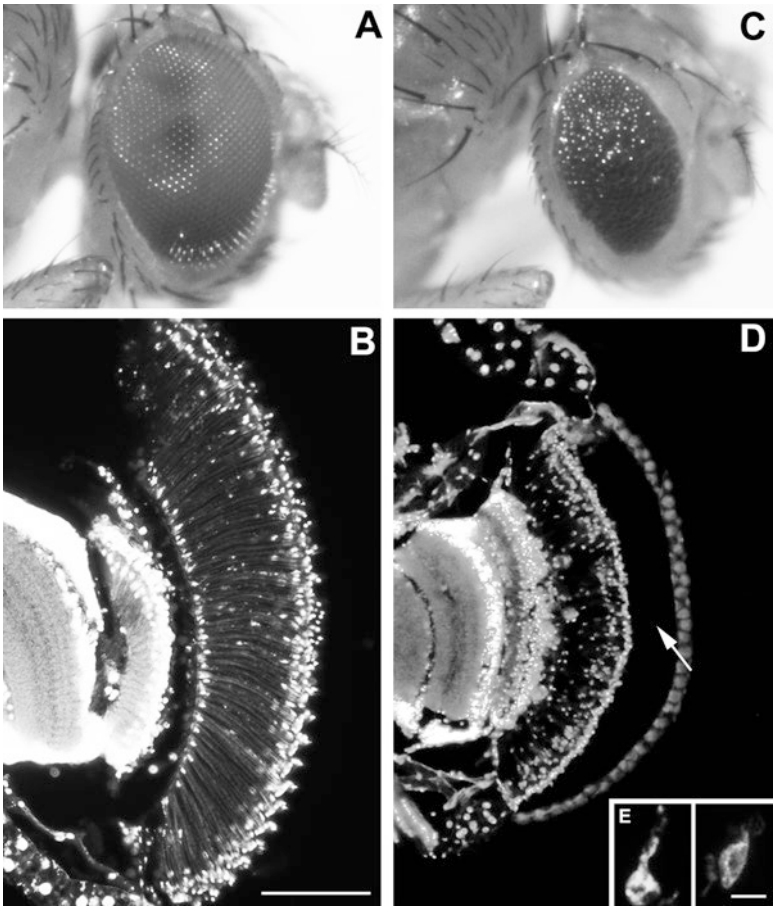

Fig. 3 Compared to the wild-type (**a** and **b**), eye-specific expression of human Tau^{WT}-transgene results in roughening of the eye surface (**c**) and widespread degeneration (arrow) of internal retinal tissues (**d**) as observed by DAPI staining. (**e**) Staining with total tau reveals the formation of typical flame-shaped neurofibrillary tangles (NFTs) in degenerating neuronal tissues. Scale: **b**, **d** = 100 μm; **e** = 10 μm

Sarkar 2017). It was also noted that the mature NFTs and phosphorylated tau filaments preferably localize around the brain vacuoles and degenerating/degenerated neuronal tissues, which clearly suggest that, in fly models, the aggregated tau-tangles make notable impact on the neuronal health and functioning. This also signifies a positive association between increased accumulation of insoluble neurofibrillary lesions and an enhanced level of neurotoxicity. Importantly, the presence of some of the disease-related phosphorylated tau epitopes, i.e. pT231, pT181, pS202/pT205, etc. in the pre- and matured NFTs indicates the fact that NFTs are composed of both the normal and disease-dependent hyperphosphorylated tau; however, NFTs in *Drosophila* could be best seen with the antibody that detects the total tau protein, regardless of their phosphorylation status.

We have recently demonstrated that targeted downregulation of dMyc, a *Drosophila* homolog of human c-myc proto-oncogene, dominantly suppresses tau-induced cellular and functional deficits by regulating abnormal tau hyperphosphorylation (Chanu and Sarkar 2017). Moreover, the reduced level of dMyc also restricts NFT formation in the neuronal tissues (Chanu and Sarkar 2017). Intriguingly, our findings have convincingly demonstrated that equivalent to human and mammalian model systems, accumulation of insoluble tau aggregates and their successive transformation into the forms of characteristic toxic NFTs lead to pathogenesis of human tauopathies in *Drosophila*.

Concluding Remarks

Interestingly, in spite of neuropathological heterogeneity of the tauopathies across human population and model organisms, there are several shared common features suggesting that components of the associated signalling cascades are arranged in an ordered manner. However, even after several pointers indicated towards an active involvement of NFTs in tau aetiology, it is still arguable if NFTs alone are a reason enough for tau pathogenesis and neurodegeneration. In view of the fact that tau aetiology is almost conserved in human and *Drosophila*, the fly models could be utilized to investigate the in-depth of the in-vivo biogenesis of NFTs, and to examine the order of cellular and biochemical events leading to neurodegeneration. Understanding these patho-cascades would not only provide means to establish the specific role(s) of NFTs in disease aetiology but also for designing the novel therapeutic strategies, which are desperately needed, considering that no effective treatment or disease-amending strategy is yet available for any of the tauopathies. In view of the fact that lowering the NFT load is emerging as a promising therapeutic approach (Bakota and Brandt 2016), the *Drosophila* system could be tremendously useful for the identification and characterization of the novel gene(s) and/or molecule(s) with disease-modifying capacity.

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Unraveling Alzheimer's Disease Using *Drosophila*

Catherine J. Yeates, Ankita Sarkar, Madhuri Kango-Singh, and Amit Singh

Abstract

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

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

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

Introduction

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

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

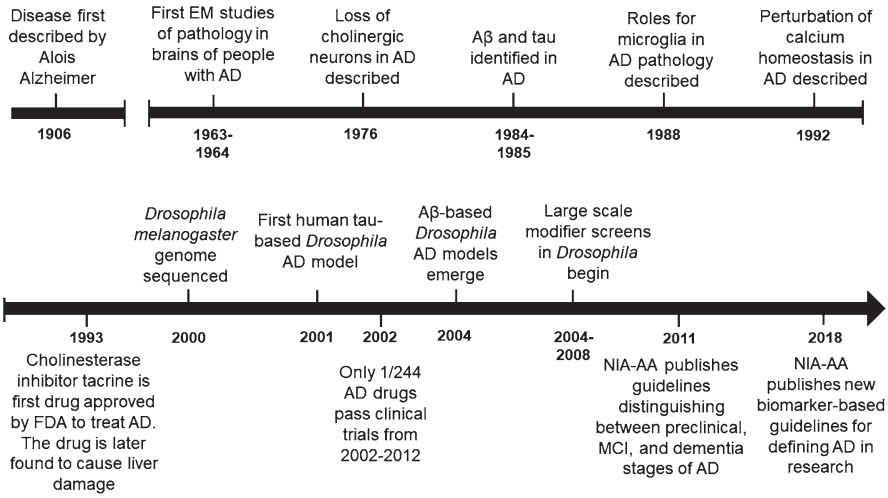

Fig. 1 Abbreviated timeline of AD research and its intersection with *Drosophila* research. *Drosophila* research has evolved rapidly, facilitating the use of large-scale modifier screens to search for new AD treatment targets. *NIA-AA* National Institute on Aging and Alzheimer's Association, *MCI* mild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

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

Pathology of AD

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

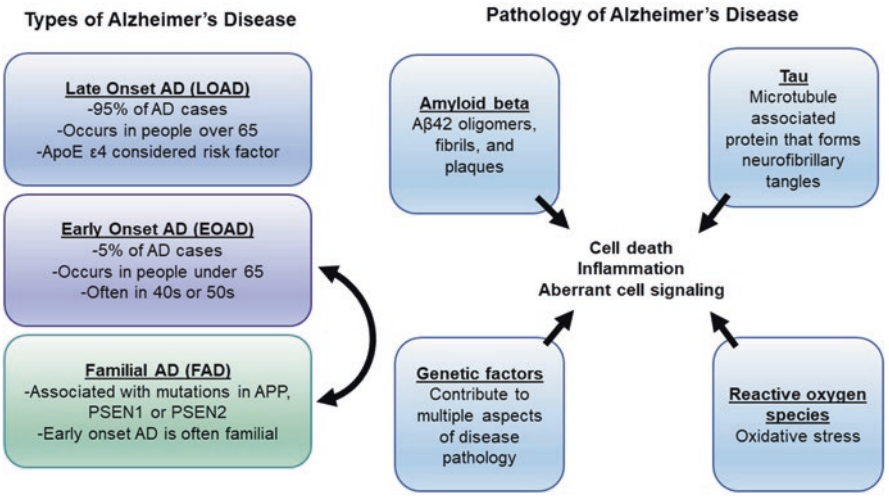

Fig. 2 Overview of the types of AD and pathology involved. AD can be categorized as late onset, early onset, or familial. Early-onset AD is frequently familial and may be called EOFAD. Many factors involved in AD pathology have been identified, and the best understood aspects of pathology are Aβ42, tau,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

Tau and Neurofibrillary Tangles

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

Amyloid Beta 42 (Aβ42)

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

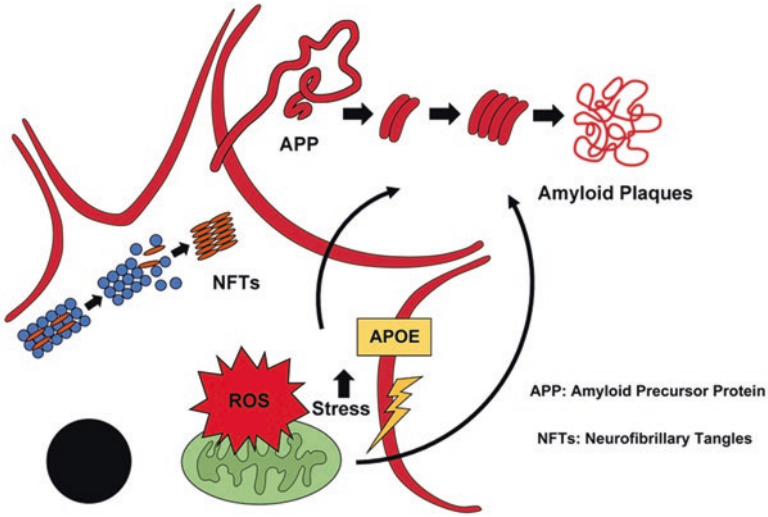

Fig. 3 Overview of the various mechanisms responsible for AD. A transmembrane protein, amyloid precursor protein (APP), is cleaved into Aβ42, which forms oligomers and eventually aggregates into amyloid plaques. Normally, microtubules (blue circles) are associated with tau (red), a microtubule-associated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

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

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

Genetic Risk Factors

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

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

AD Animal Models

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

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

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

Citations refer to the first publication of the models

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

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

Utility of *Drosophila* as a Model System

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

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

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

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

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

Modeling AD in *Drosophila*

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

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

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

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

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

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

Drosophila Eye Model

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

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

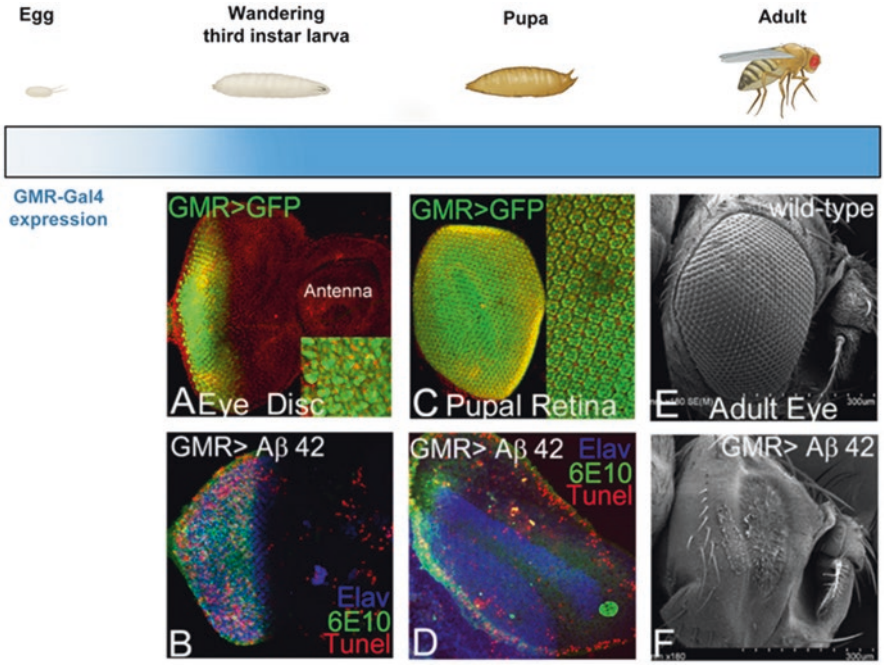

Fig. 4 Targeted misexpression of human Aβ42 in *Drosophila* eye triggers neurodegeneration as seen in AD. Using GMR-Gal4>Aβ42 to model AD in *Drosophila*. GMR-Gal4 expression turns on during the third instar larval stage. (**a**) Using GMR-Gal4 to drive UAS-GFP (GMR>GFP) triggers expression in the differentiating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

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

Suitability of *Drosophila* Model for Screens

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

Drug Screens

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

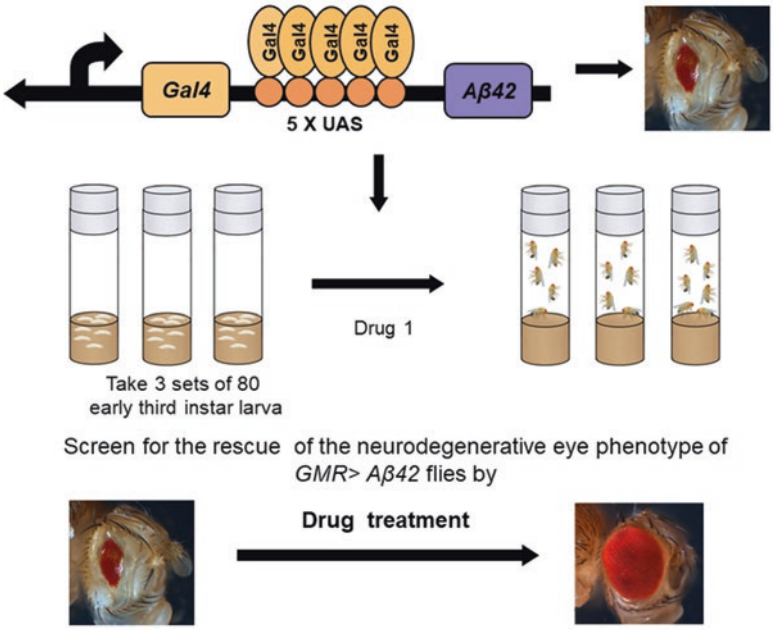

Fig. 5 Strategy for drug screen to identify modifiers of gain of function of Aβ42 (GMR-Gal4>Aβ42) in the *Drosophila* eye. First, 80 early third instar GMR-Gal4>Aβ42 larvae are collected in food vials. Larvae are subjected to drug treatment and observed as adults for rescue of the Aß42 neurodegenerative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

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

Genome-Wide Genetic Screens

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

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

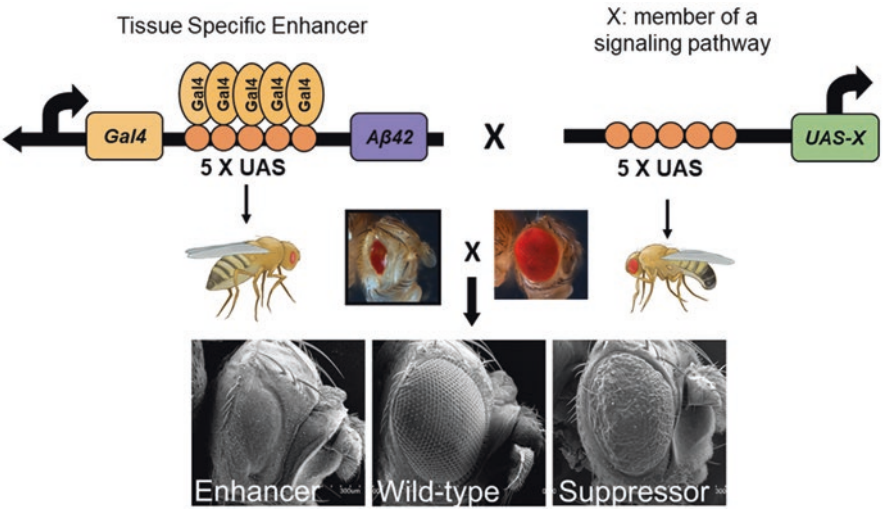

Fig. 6 Strategy for forward genetic screen to identify genetic modifiers of Aβ42 (GMR-Gal4>Aβ42) gain-of-function in *Drosophila* eye. Flies expressing human Aβ42 under the control of the GMR-Gal4 driver (small, rough eyes) are crossed to flies in which genes of interest (X) are expressed under UAS control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

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

Aberrant Activation of Cell Death Pathways

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

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

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

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

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

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

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

Current Treatments

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

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

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

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

Natural Products

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

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

Conclusions

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

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

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Conflict of Interest The authors declare no conflict of interest.

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Modeling of Human Parkinson's Disease in Fly

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Abstract

Years of in-depth research have contributed substantially to the understanding of the pathophysiology of Parkinson's diseases (PD). However, many crucial questions related to the etiology of the disease remain unanswered, which compelled the need for developing more realistic and genetically malleable model systems for modeling the precise neuropathology of the disease in vivo.

Ever-expanding genetic toolkit and conservation of implicated signaling pathways and neurological properties have prompted the use of *Drosophila melanogaster* (fly) as an instrumental model. Humanized fly models have aided in gaining insight into different cellular disturbances (protein aggregation and misfolding), mitochondrial deficits, and oxidative stress toward causation of Parkinson's disease. The transgenic and humanized *Drosophila* model provides a decisive platform to assess the pathogenic properties of rare variants and open a window to analyze the cellular processes and signaling pathways that have been disrupted, which is ultimately manifested by the death of dopaminergic neurons in the brain of Parkinson-affected subjects.

Apart from gaining molecular insight, toxin-induced models of *Drosophila* recapitulate multiple symptoms of environmental toxin-induced PD. Environmental toxin-induced models of *Drosophila* have proven to be an efficient means to study gene-environment interactions, which elevate susceptibility for Parkinsonism. Employment of *Drosophila* to scrutinize gene-environment interactions has led to the screening of many genetic risk factors.

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Additionally, the rapid development of genome manipulation technologies have paced up the development of more realistic models, which can precisely replicate all pathological features of the disease. This should be worthwhile to elucidate uncharted genetic and environmental risk factors, which are responsible for the complex pathogenesis associated with Parkinson's disease. The ease of genetic manipulations that mimic symptoms of PD in *Drosophila* makes it one of the most favorite model organisms for analyzing the underlying cause of PD, the second most prevalent neurological disorder after Alzheimer's disease.

Keywords
Parkinson's disease · *Drosophila* · Dopaminergic Neurodegeneration · α-synuclein · LRRK2 · PINK1 · Parkin · GBA · Paraquat · Rotenone · Environmental toxins

Introduction

Parkinson's disease was named after James Parkinson, and the medical description related to the disease was elaborated in his medical essay entitled "An Essay on the Shaking Palsy," in which he described the clinical attributes of six case reports in 1817 (Parkinson 1817). Later in the 1880s, French neurologist Jean-Martin Charcot more accurately attributed the clinical features of the disease, where he and his students described bradykinesia (slowness of movement) as one of the primary features of the disease. Based on the array of clinical symptoms and signs of the patients, they categorized the condition into two prototypes: tremors and rigid/akinetic form (akinesia). Charcot, through his studies on tremor over a large cohort of patients, contributed significantly to establish Parkinson's disease as a distinct neurological entity (Charcot 1879). Later he popularized the term "Parkinson's disease," coined by William Sanders in the year 1865. Friederich H. Lewy, an American neurologist in the year 1912, observed typical inclusion bodies in the nucleus basalis of Meynert and the dorsal vagal nucleus in the subjects affected with PD (Lewy 1912). Later Tretiakoff confirmed the presence of these inclusions in the neurons of the substantia nigra region among the PD patients and termed them as Lewy bodies (LB) (Trétiakoff 1919). Greenfield and Bosanquet in the year 1953 performed detailed pathological analysis and delineation of brain stem lesions in subjects affected with Parkinson's disease (Greenfield and Bosanquet 1953).

Introduction of a globally recognized rating scale by Hoehn and Yahr in 1967 proved to be a milestone to access the successive progression of the Parkinson's disease (PD) in the affected subjects (Hoehn and Yahr 1998). Involvement of striatal-nigral degeneration was first described in the pioneering works of Adams et al. (1964). Further researchers have shortlisted various symptoms which

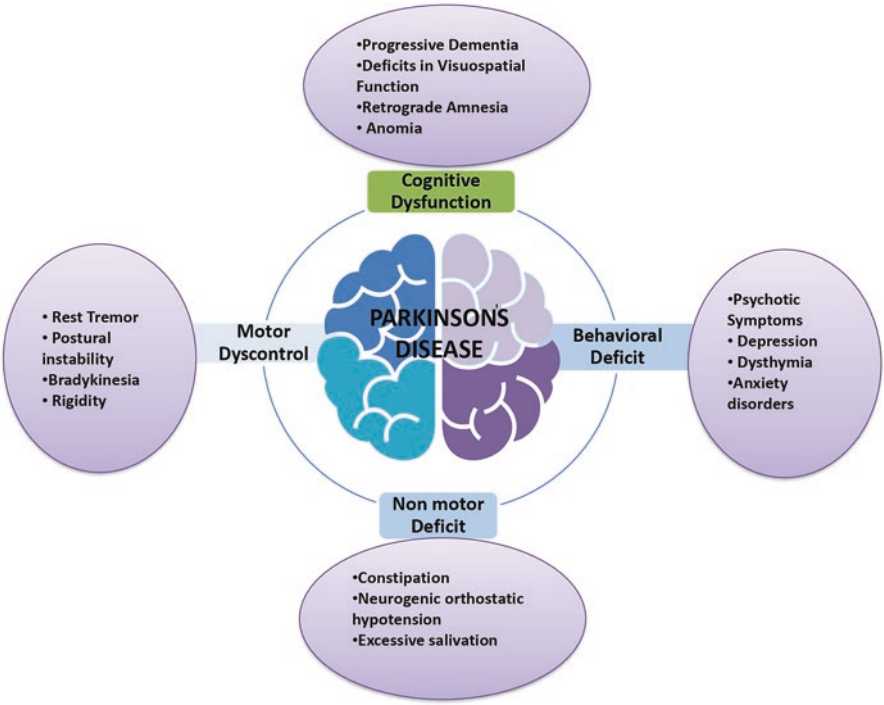

Fig. 1 The wide array of symptoms which frequently co-occur in subjects affected with Parkinson's disease (PD)

developed sequentially among the affected subjects; these include motor dysfunction, nonmotor deficit, behavioral deficit, and cognitive dysfunction (Fig. 1).

Further neuropathological assessments of Parkinson's subjects established the characteristic clinical attributes of Parkinson's disease. Neuronal loss in specific areas of the substantia nigra initiate from the ventrolateral area of substantia nigra in early-stage of the disease but become more widespread at the terminal stages of the disease (Damier et al. 1999). Pathogenesis of PD demonstrates three types of cellular defects that drive disease progression, abnormal protein aggregation, oxidative damage, and mitochondrial dysfunction (Schulz 2007). These discoveries and observations along with the advent of diagnostic technologies proved to be a turning point for delineation of the neuropathology of Parkinson's disease.

In order to answer a number of unresolved questions linked to the pathophysiology and the detailed molecular etiology of PD, researchers have utilized a variety of vertebrate and invertebrate model systems, which, with the aid of genetic and chemical tools, reproduce some pathological aspects of the disease condition. The PD animal models can be further grouped into two prototypes: toxin models, in which a neurotoxin has been administered for the degeneration of dopaminergic neurons,

and the genetic models, in which specific PD-related genes are mutated. Both these models not only successfully recapitulate the disease phenotypes but also aid in designing and accessing the efficiency of different therapeutic interventions in preclinical studies. Due to limitations of human-based genetic studies, researchers have utilized model organisms such as fruit flies, mice, and worms as well as neuronal cell lines such as the neuroblastoma cell line SH-SY5Y and the pheochromocytoma cell line (PC12). Utilization of cell culture for functional validation of different biochemical and molecular approaches are economical and limits the requisition of valuable clinical samples. However, there are many limitations associated with cell lines such as the difference in gene expression profiles of cell line versus that of primary tissues (Gillet et al. 2013). Over time, new mutations may be harbored in the cell culture, which may lead to change in the cell line characteristics (Rauch et al. 2011). Contrary to this, animal models are more reliable as they provide a chance to study fundamental cellular processes in the context of a whole organism.

To precisely mimic the features observed in PD patients, a wide array of models have been employed by researchers, which ranges from evolutionarily remote organisms such as yeast to nonhuman primates. However, none of the models mimic the cardinal features of the diseased conditions entirely (Jagmag et al. 2016). Among all these models, murine models have been widely accepted as being relatively cost-effective and involves limited ethical concerns compared to larger animals. Murine models gained preference due to the existence of a significant share of evolutionarily conserved genes and biological pathways associated with the clinical presentation of the disease (Zuberi and Lutz 2017). Further, murine models provide amenable scope to study variations in nonmotor symptoms such as depression, apathy, akathisia, dizziness, cognitive dysfunctions, and hallucination, which inevitably develops with disease progression among affected subjects (Bonnet and Czernecki 2013; Todorova et al. 2014). Murine models also face certain limitations, despite being the forefront model for undertaking molecular genetics research for addressing various aspects of PD. However, these models provide limited scope to inspect different aspects related to the gene-environment interaction (Chouliaras et al. 2010). Adding to this, most of the toxin-based murine models do not phenocopy the disease progression seen in PD (Dawson et al. 2018). *Drosophila* has proven to be a tractable model that has gained popularity among researchers in the form of critically acclaimed publications, which explore many unresolved queries related to the pathophysiology of PD. *Drosophila,* although an invertebrate model provides a simple platform along with an extensive genetic tools to model pathobiology associated with PD. Despite being evolutionarily distant from humans, *Drosophila* shares many fundamental cellular processes, along with conservation of primary signaling pathways and have orthologs of many genes implicated in PD (Ayajuddin et al. 2018).

Further, flies are capable of performing complex motor activities such as climbing and flight activities, which can mimic several motor symptoms associated with PD patients (Muñoz-Soriano and Paricio 2011). Maintenance of *Drosophila* stocks in the laboratory is relatively easy and inexpensive as compared to that of other

model organisms (Hales et al. 2015). Till date, various researchers have employed *Drosophila*, ranging from genetic models to toxin models. These models mimic different forms of PD, right from sporadic to familial forms of the disease. Development of toxin-based models of PD in flies is invaluable for the elucidation of development and progression of sporadic cases of PD, as the familial form of PD are rare. Several studies established that exposure of herbicide and environmental toxins such as rotenone, paraquet, 6-hydroxydopamine (6-OHDA), and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) may act as a risk factor in the development of sporadic PD (Whitworth et al. 2006; Botella et al. 2009; Lu and Vogel 2009; Ambegaokar et al. 2010; Hirth 2010).

Simulation of Sporadic Cases of PD Utilizing Toxin-Induced Models of *Drosophila*

Majority of the cases of Parkinson's are grouped as sporadic, and only about 10% of cases are accounted for having a positive familial history (Thomas and Beal 2011). Chronic exposure of agro toxins is considered as one of the prime causative factors implicated in the etiology of idiopathic cases of Parkinson's disease (Brown et al. 2005). Along with chronic exposure to toxins, other factors such as genetic profile, age, sex, diet, and smoking also plays a considerable role in causing the disease (Agim and Cannon 2015). Epidemiological and toxicological studies have produced conclusive evidence for the broad spectrum of chemical agents whose prolonged exposure may increase vulnerability to PD. Among them, particular emphasis has been laid on the evaluation of the potency of paraquat (1,1′-dimethyl-4,4′-bipyridinium), 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), and rotenone as a risk factor for PD. Various research groups, with the aid of different animal models, have prompted studies to expedite the deleterious cellular effects on the neurons in the substantia nigra pars compacta of the brain. Laboratory-based neurotoxicological studies with the help of animal models have proven that chronic exposure of certain neurotoxins leads to atrophy of dopaminergic neurons present in the substantia nigra, which ultimately leads to disruption of nigrostriatal pathway showing typical motor deficits (bradykinesia, postural instability, rigidity, and resting tremor) (Cannon and Greenamyre 2011). Majority of the toxin-based studies have utilized murine models. However, high genetic malleability along with distinct developmental stages and short life cycle designates *Drosophila* as a reliable model to undertake a study based on toxin-induced models of PD (Martin et al. 2014a, b). These fly models are marked by loss in dopaminergic neurons after administration of certain neurotoxin, accompanied by a prominent exhibition of behavioral and histological-pathological changes that relate with the classical hallmarks of PD. Several studies have been performed, which have established that pharmacological treatment could be used to model idiopathic cases of PD. *Drosophila* models of paraquat- and rotenone-induced Parkinsonism have been well established (Coulom and Birman 2004; Cassar et al. 2014).

Paraquat-Induced Neurotoxin Model of *Drosophila*

Paraquat exerts its toxic effects by rapid generation of superoxide radicals, a highly reactive oxygen species (ROS), which ultimately leads to cellular damage (Lascano et al. 2012). After ingestion, paraquat undergoes cyclic reduction-oxidation with the subsequent generation of superoxide radicals and singlet oxygen that later initiates lipid peroxidation (Bus et al. 1976). A large share of ROS generated due to paraquat ingestion arises from cellular sources, mitochondria being one of the chief contributors of ROS (Castello et al. 2007). After entry of paraquat in the mitochondrial matrix, membrane potential-dependent uptake across the mitochondrial inner membrane takes place, by rapid reduction of paraquat radical cations at Complex 1, that is associated with electron transport cycle (Castello et al. 2007). Patients suffering from acute toxicity of paraquat show lung, liver, and kidney malfunctions along with damage of the central nervous system (Conradi et al. 1983; Raina et al. 2008). Recently, a study has shown dynamic pathological changes in the human brain, particularly across extrapyramidal ganglia and hippocampus of paraquat-poisoned victims (Wu et al. 2012). Several experimental studies on animals provide evidence that systemic administration of paraquat leads to Parkinsonian-like syndrome.

Additionally, there is a considerable structural similarity between paraquat and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), well known for triggering Parkinsonian-like syndrome (Dauer and Przedborski 2003). The linking mechanism between paraquat exposure and Parkinson is further confirmed by results of multiple epidemiological studies, which suggests the elevation in risk for PD across subjects, after chronic paraquat exposure (Liou et al. 1997; Kamel et al. 2006; Costello et al. 2009). Many studies using in vitro cultures of neuronal tissues and cells further cemented the connection between paraquat exposure and PD. Injection of paraquat in mice is reported to induce acute motor deficits and nigral dopaminergic neuronal loss in a dose-dependent manner (Brooks et al. 1999; McCormack et al. 2002). The effect of paraquat is reported to be specifically limited across γ-amino butyric acid (GABA) neurons in the nigral and striatal regions of the murine brain (McCormack et al. 2002).

Paraquat treatment is also reported to increase α-synuclein aggregation in murine models (Manning-Bog et al. 2002; Fernagut et al. 2007). However, these toxin-based models show a lack of significant effect of paraquat on striatal dopamine depletion. Similar inferences were attributed to studies in which a loss in nigral dopaminergic neurons is detected (McCormack et al. 2002). Deficiency of striatal dopamine, which is one of the cardinal features of Parkinson's, and the lack of this feature limits the validity of the paraquat-based murine model to elucidate neurophysiological perturbations associated with Parkinson's disease (McCormack et al. 2002).

Despite being evolutionarily distant, *Drosophila* has provided novel insights into the progression of Parkinson's, with the aid of a combination of genetics and physiology. Over the past years, flies have proven to be an efficient model to study the effect of novel therapeutic compounds and also for deciphering the pathways that they are associated with (Sanz et al. 2017). Chaudhuri et al. (2007) showed

that sublethal exposure of paraquat leads to selective loss of DA neuronal clusters. This study showed that administration of paraquat leads to an elevation in catalase activity accompanied by motor deficits (Chaudhuri et al. 2007). This study implicates that oxidative stress is one of the prime factor causing PD pathogenesis.

Researchers utilized the paraquat-induced environmental toxin model of *Drosophila* to investigate the protective role of Hsp70 protein. The study further revealed that overexpression of Hsp70 diminished dopaminergic neuron degeneration through the inhibition of c-Jun N-terminal kinase (JNK) and caspase-3-mediated cell death. Hsp70-overexpressed flies also showed rescue of locomotory performance along with an extended lifespan (Shukla et al. 2014). In a similar study, the paraquat-induced Parkinson's disease model of *Drosophila* was utilized to investigate the ameliorative effect of minocycline. Minocycline prolonged the survival of dopaminergic neurons (DA) and rescued locomotory deficits in flies (Inamdar et al. 2012). Recently, a study utilized metabolomics to reveal the altered metabolic profile in paraquat-exposed flies. This study reported an increase of certain metabolites such as myo-inositol in brain tissues of flies, which mimicked the metabolite level in PD patients (Shukla et al. 2016). An independent study evaluated the therapeutic potential of the SOD-mimetic compound M40403 using a paraquat-induced Parkinson's disease model in *Drosophila*. The study employed *Drosophila* as an in vivo model and demonstrated the protective role of M40403 against oxidative stress induced by paraquat treatment (Filograna et al. 2016). These studies advocate the applicability of *Drosophila*-based paraquat-induced Parkinson's disease model to decipher different molecular players and pathways related to PD. These models proved to be a useful for accessing the therapeutic potential of novel compounds against PD.

Rotenone-Induced Neurotoxin Model of *Drosophila*

Rotenone is a crystalline isoflavone formulated with other pesticides such as carbonyl pyrethrins and serves as a broad-spectrum pesticide. Rotenone inhibits the transfer of electrons from iron-sulfur centers in complex I to ubiquinone, which ultimately leads to blockade of oxidative phosphorylation. The lipophilic nature of rotenone enables it to cross the blood-brain barrier (Talpade et al. 2000). The toxicity of rotenone is chiefly attributed to its ability to impede the mitochondrial complex I activity, which consequently accelerates the reactive oxygen species (ROS) production (Chance and Hollunger 1963; Fato et al. 2009). Exposure of rotenone also accounts for microtubule depolymerization, which may also contribute to its toxicity (Marshall and Himes 1978). Similar to paraquat, rotenone also induces cytotoxicity through the generation of oxidative stress and induction of reactive oxygen species (ROS) production (Sherer et al. 2003). Rotenone confers atrophy of dopaminergic neurons in the nigrostriatal system through interfering multiple pathways including acidification and translocation of DJ-1, elevation of ROS production, proteasomal dysfunction, and iron accumulation around nigral regions (Betarbet et al. 2006).

A number of studies have utilized *Drosophila* to model PD. Oral administration of rotenone at a particular concentration leads to degeneration of dopaminergic neuronal bodies in the brain (Coulom and Birman 2004; Hosamani 2009; Lawal et al. 2010; St Laurent et al. 2013).

Rotenone-treated flies show concentration-dependent lethality. Further, geotaxis assays demonstrated dose-dependent motor dysfunctions among flies after rotenone exposure. A study has been performed to screen for altered nonmotor symptoms like altered circadian rhythm utilizing rotenone-treated flies harboring deficiency of circadian photoreceptor, *cry*. Comparative analysis of tissue-specific gene expression of DA neurons of rotenone-treated and control flies pointed out the activation of crucial signaling pathways, namely, TGF-β and MAPK/EGFR signaling pathways. Rotenone-treated flies show reduced expression of armadillo/β-catenin along with impaired locomotory functions. Subsequently, overexpression of armadillo in DA neurons allayed rotenone-induced locomotory defects, implicating the role of Wnt signaling in the etiology of PD (Stephano et al. 2018). Comparative analysis of the wild-type and rotenone-exposed flies show significant elevation of stress markers accompanied by decreased levels of antioxidants (superoxide dismutase, catalase, glutathione-S-transferase, and glutathione). Rotenone-induced Parkinson's disease model of *Drosophila* has been used to access the efficiency of different therapeutic agents. In the year 2013, Sudati et al. have analyzed the therapeutic potential of *Valeriana officinalis* treatment in alleviating Parkinson's disease (Sudati et al. 2013). Another group has evaluated the therapeutic potential of Tianma Gouteng Yin (TGY), a traditional Chinese medicine decoction utilizing rotenone-induced *Drosophila* model of Parkinson's disease. TGY treatment rescued the impaired motor defects accompanied by improvement in survival rate in rotenone-treated flies (Liu et al. 2015). Recently a study has been published which accessed the neuroprotective potential of PTUBP, a dual inhibitor of soluble epoxide hydrolase (sEH) and cyclooxygenase (COX-2) against rotenone-induced neurodegeneration in the *Drosophila* model of Parkinson's disease (PD). The authors have utilized liquid chromatography with tandem mass spectrometry (LC-MS/MS) methods for quantifying the level of dopamine and its metabolites (DOPAC and HVA) to evaluate anti-parkinsonian activity of PTUPB (4-(5-phenyl-3-3-3-(4-trifluoromethyl-phenyl)-ureido-propyl-pyrazol-1-yl)-benzenesulfonamide) (Lakkappa et al. 2018). An interesting piece of work has been published by Liao et al. (2014) demonstrating an accurate procedure for measuring both long-term spontaneous locomotion and short-term startle-induced locomotion in a rotenone-induced *Drosophila* model of Parkinson's disease utilizing the *Drosophila* activity monitor system (Liao et al. 2014). This will further aid in accelerating therapies against Parkinson's disease.

Although the toxin-induced models of *Drosophila* have been helpful for delineating the multiple genetic and environmental factors associated with Parkinsonism. These models represent certain ineluctable drawbacks; lack of reproducibility of results in many instances is frequent due to the difference in the administration protocols of toxins. Most of the environmental toxin-based models partly reproduce the clinical symptoms and pathology of PD seen in humans. In order to curb these shortcomings, it was necessary to develop a more realistic model that could be

created by exposing single or multiple chemicals to flies with different genetic backgrounds.

Recently, several studies have utilized the co-mixture of multiple compounds and genetic backgrounds to address different queries related to the evaluation of gene-environment interactions that increase or decrease PD risk. A study has demonstrated that the neuroprotective effect of *Drosophila* vesicular monoamine transporter (dVMAT) protein, which is a transporter protein, is associated with sequestration of dopamine (DA) from free cytoplasmic space into the synaptic vesicles. Overexpression of the *Drosophila* vesicular monoamine transporter (dVMAT) protein protects DA neurons against rotenone-induced cell death (Lawal et al. 2010). Although the detailed mechanism by which dVMAT confers neuroprotection against rotenone remains unclear. In an independent study, RNAi-mediated knockdown of the tyrosine hydroxylase (TH) gene, across different subclasses of DA neurons, increased the lifespan of RNAi flies accompanied by the decline of DA neuronal loss in rotenone-treated flies (Bayersdorfer et al. 2010). These results indicate that DA metabolism is a contributing factor in the selective vulnerability of substantia nigra pars compacta DA neurons in PD and cytosolic DA can interact with rotenone.

On the contrary, many studies have shown that a spike in DA production confers protection against toxin-induced neuronal death. An interesting experiment was done by Chaudhuri et al. (2007), in which catecholamines-up (*Catsup*) was overexpressed across paraquat-treated flies. Catecholamines-up (*Catsup*) encodes a negative regulator of DA production in *Drosophila* through post-translational regulation of GTP cyclohydrolase I (GTPCH) and tyrosinase hydroxylaze (TH). Paraquat-treated flies with loss of function of the *Catsup* gene showed delayed neural degeneration accompanied by reduced morbidity (Chaudhuri et al. 2007). Conclusively, this experiment supports that upregulation of the dopamine pathway leads to protection against neurotoxicity by paraquat.

Genetic Models of *Drosophila* for Simulating Sporadic and Familial Cases of PD

Intensive research over many years have revealed many causative genes responsible for rare monogenic forms of PD including *DJ-1*, *PINK1*, *LRRK2* (leucine-rich repeat kinase 2), *α-synuclein*, and *Parkin*. Introduction of powerful approaches for disease gene identification, such as genome-wide association studies (GWAS) and advancement in linkage analysis, have contributed immensely to the identification of novel loci and genes associated with sporadic PD risk. The first GWAS report for disease gene identification of sporadic Parkinson's disease was published in 2005. Since then, this approach has identified multiple alleles, as well as led to the development of better computation-based models along with dedicated statistical tools. This has also improved the power and accuracy of genetic association, thereby allowing identification of ~21 susceptibility loci (Labbé and Ross 2014). Association of *LRRK2*, *SNCA* (Synuclein Alpha), *MAPT* (Microtubule-associated protein tau), and some of these loci have been also replicated in multiple studies (Mata et al.

2011; Trotta et al. 2012; Labbé and Ross 2014). Most of the genes implicated in causation of sporadic cases of PD disrupt either mitochondrial homeostasis or leads to aberrant protein folding and aggregation, ultimately leading to elevation of oxidative stress when mutated (Chai and Lim 2013).

Parkinson's disease is a complex disease due to the admixture of environmental and genetic factors. In 1993, researchers identified a pathogenic variant of α-synuclein in Italian families affected with an inherited form of Parkinson's disease (Polymeropoulos et al. 1997). Later, a number of studies have implicated that the mutant forms of genes such as *LRRK2*, *PARK7*, *PINK1*, and *Parkin* are some of the causative factors for familial PD. Familial cases of Parkinson's are comparatively rare, although majority of the neuropathological features displayed in these familial cases are relatively indistinguishable. Adding to this, the sporadic and familial forms of the disease have overlapping contributions of genetic factors and biochemical pathways (Chai and Lim 2013).

In order to get detailed insight into the underlying novel mechanisms of pathogenesis linked to the sporadic and familial forms of PD etiology, a variety of invertebrate and vertebrate models have been developed (Nagoshi 2018). These models have proven to be highly tractable for dissecting out the molecular mechanisms of PD. Invertebrate models like *Drosophila* has been instrumental due to the availability of a vast array of genetic manipulation tools and assays, which aided scientists in addressing the molecular mechanisms that increase risk of PD. In flies, inherited forms of PD are modeled by introduction of causal genetic variations from patients to their corresponding orthologs in flies or by insertion of human-based genes in wild-type or mutant forms.

Transgenic flies expressing wild-type and mutant forms of human α-synuclein showed a significant decline in their climbing abilities compared to that of wild-type flies and were accompanied with the formation of fibrillary inclusions containing α-synuclein and age-dependent loss of dopaminergic neurons, which paralleles the symptoms observed in PD patients, like locomotor deficits and progressive dopaminergic neuronal loss (Feany and Bender 2000). Further, transgenic flies replicated many cardinal features of Parkinson's disease marked by behavioral impairment and other pathological features of PD. Researchers have developed models to extend the findings from patient's neuronal tissue and replicated it in the flies in order to gain an in-depth knowledge about the elusive molecular pathways controlling the progressive dopaminergic neuronal loss in diseased condition.

LRRK2 *Drosophila* Models

LRRK2 encodes a large multidomain protein kinase of the ROCO protein family, which is involved in synaptic vesicle trafficking and orchestration of autophagy through a calcium-dependent activation of the CaMKK/AMPK signaling pathway (Marín 2006; Gomez-Suaga et al. 2011; Cirnaru et al. 2014). Mutation in leucine-rich repeat kinase (LRRK2) is associated with the late-onset, autosomal dominant form of PD (Gandhi et al. 2009). Studies implicate mutations in LRRK2 as one of

the most prevalent genetic causes of PD. Mutation spectrum in LRRK2 accounts for 4% of familial PD and 1% of sporadic PD across all populations (Xiong and Yu 2018). Most of the disease-causing variants in LRRK2 are centered on LRRK2 enzymatic domains (Islam and Moore 2017). Genotype-phenotype correlation revealed by molecular genetic studies have revealed close clinical and pathologic resemblance between sporadic forms of PD and LRRK2-mediated PD (Wallings et al. 2015). Different vertebrate and invertebrate models have proven to be instrumental in identifying and validating the molecular and cellular mechanisms underlying genetically linked disease. Among these models, fruit flies have turned out to be valuable, enabling dissecting out of molecular events causing pathogenesis by mutant LRRK2. Flies have indeed proven to be a reliable model for studying LRRK2-linked molecular etiology. The orthologue of human LRRK2, *CG5483,* is conserved in flies (Liu et al. 2008). The mutant flies display locomotor impairment, along with progressive loss of tyrosine hydroxylase-positive neurons and recapitulates the pathogenesis of the disease (Liu et al. 2008; Li et al. 2011). *Drosophila* models have contributed immensely to the understanding of disease pathogenesis caused by LRRK2 variants that affect signaling cascade; this provides us an insight into the fundamental molecular mechanisms of PD.

LRRK2 Knockout Models

Drosophila LRRK2 knockout lines have been generated to gain insight into the physiological function performed by the endogenous LRRK2. Notably, some groups have reported that knockout flies show a variable degree of sensitivity toward oxidative stress inducers, namely, hydrogen peroxide and paraquat (Imai et al. 2008; Wang et al. 2008). Detailed molecular and behavioral analysis of knockout lines by several groups declaimed the presence of neuronal atrophy or atypical patterning of DA neurons across mutant lines (Imai et al. 2008; Wang et al. 2008; Tain et al. 2009). The occurrence of withered DA neuronal bodies was marked by a severe reduction in tyrosine hydroxylase immunostaining along with locomotory deficiencies across *LRRK2* loss-of-function mutants. dLRRK knockout flies have been utilized to examine the response to oxidative stress (Lee et al. 2007). Conclusively, majority of knockout-based studies using *Drosophila* and murine-based models do not exhibit the characteristic DA-associated neural degeneration, which foretells that mutated forms of LRRK2, most likely contribute to PD pathogenesis through gain-of-function mechanism instead of loss-of-function mechanism (Yue 2009; Xiong and Yu 2018).

LRRK2 Transgenic *Drosophila* Models

Transgenic *Drosophila* models have been developed by the incorporation of human *LRRK2* and *dLRRK2* transgene. Transgenic models harboring human *LRRK2* (hLRRK2) or *dLRRK* pathogenic mutations showed an age-dependent decline of

DA neuronal bodies and DA-responsive motor deficit. Notably, functional characterization and gene expression profiling of various pathogenic mutations are characterized through overexpression of *hLRRK2* or *dLRRK* in *Drosophila.* A study focused on dominant G2019S mutation in LRRK2 kinase, as this mutation is the most frequent pathogenic mutation linked to Parkinson's disease (Bouhouche et al. 2017). The GAL4/UAS system was utilized to generate transgenic *Drosophila* expressing either wild-type human LRRK2 or LRRK2-G2019S, although neural expression of both LRRK2 and LRRK2-G2019S led to selective loss of dopaminergic neurons, locomotory dysfunction, and early mortality. However, the expression of a mutant form of LRRK2 caused a more severe Parkinsonism-like phenotype than that of wild-type LRRK2 (Liu et al. 2008). Another study reported dopaminergic expression of LRRK2 G2019S led to nonautonomous dysfunction and degeneration of photoreceptor neurons with the kinase-dead mutants (dLRRK-3KD and G2019S-K1906M) or the GTP-binding domain mutant like R1441C (Hindle et al. 2013). A study demonstrated that LRRK2-containing pathogenic Roc-COR domain mutation (R1441C or Y1699C) led to defective microtubule-based axonal transport in primary neurons leading to locomotory deficits in *Drosophila* (Godena et al. 2014). This indicates the potential role of LRRK2 GTPase activity as one of the causes of disease pathogenesis of PD. Recently, a study by Cording et al. reported that ectopic expression of either G2019S or I2020T mutant shows behavioral hallmarks of DA-based neuronal degeneration that is marked by delay in proboscis extension response and tremors, whereas R1441C or kinase-dead LRRK2 do not demonstrate such symptoms in flies (Cording et al. 2017). Conjointly, these studies support that various LRRK2 mutations cause DA-associated degeneration by distinct gain-of-function mechanisms across Parkinson-affected subjects. Recently, the components of the RNA-induced silencing complex (RISC) Argonaute 1 (Ago1) and Dicer1 as well as the miRNAs let-7 and miR-184 were shown to interact and enhance the pathogenicity of the mutant LRRK2 (Gehrke et al. 2010).

Insights into the Role of LRRK2 Functions

Years of extensive research have been devoted to investigating the potential role played by LRRK2 in neurite development. Although murine models, *C. elegans*, and in vitro studies have been used extensively (Li et al. 2011), *Drosophila* models have contributed immensely to our understanding of the molecular mechanism of PD. *Drosophila* models accompanied with extensively developed molecular genetic assays have been used to unveil the underlying physiological role played by *LRRK2* in the neuronal maintenance. Thus, *Drosophila* models have fueled pivotal discoveries related to the function of the *LRRK2* gene like vesicular trafficking, regulation of protein translation machinery as well as dendritic and synaptic dysfunction.

Vesicular Trafficking

Defective vesicular trafficking has been implicated as one of the prime causes of Parkinson's etiology. LRRK2 *Drosophila* models have been instrumental in underpinning the roles for LRRK2 in vesicle trafficking processes. *Drosophila* model have demonstrated the role of LRRK2 in regulating phosphorylation of Endophilin A (EndoA), a central component of synaptic endocytosis, and Synaptojanin 1 (SJ1), a synaptic vesicle protein. An independent study has reported the localization of dLRRK to the membranes of late endosomes and lysosomes. This study further confirmed the physical and functional interaction of dLRRK with Rab7L1, which has a direct role in lysosomal biogenesis and late endosomal transport (MacLeod et al. 2013). Further, dLRRK has been shown to regulate the dynamics of Golgi outposts (GOP), a prominent component in the dendritic secretory pathway. dLRRK has also been reported to interact with golgi Lava lamp (Lva), thus inhibiting the recruitment of dynein to Golgi membranes (Lin et al. 2015).

Protein Translation Machinery

A study by Imai et al. (2008) reported that LRRK and human LRRK2 could phosphorylate eukaryotic initiation factor 4E-binding protein (4E-BP), a negative regulator of eukaryotic initiation factor 4E-mediated protein translation, and these in turn affected the maintenance of dopaminergic neurons in *Drosophila (*Imai et al. 2008*)*. A report using postsynaptic knockdown and overexpression of fly homolog of LRRK2 and human LRRK2 transgene demonstrated the role of LRRK2 in the regulation of cap-dependent translation through targeting Furin 1 which is crucial for LRKK2 synaptic function (Penney et al. 2016). It has also been reported that phosphorylation of the ribosome protein S15 by LRRK2 regulates protein translation and mediates LRRK2-induced neurodegeneration. The outcome of these studies supports that LRRK2 regulates protein translation machinery (Martin et al. 2014a, b).

Dendritic Degeneration and α-Synaptic Dysfunction

Lin et al. (2010) first reported the role of LRRK2 in dendritic degeneration. They demonstrated that the mutant form of LRRK2 (G2019S) leads to dendritic degeneration through mislocalization of the tau protein (Lin et al. 2010). Recently, another group have shown that overexpression of either dLRRK or hLRRK induced retrograde enhancement of presynaptic release, while the loss of dLRRK led to deregulation in retrograde synaptic compensation (Penney et al. 2016). Collectively, these studies indicate that LRRK2 might play a pivotal role in the regulation of synaptic function.

α-Synuclein

α-Synuclein is a small presynaptic neuronal protein with three domains, namely, an N-terminal domain, a non-amyloid-β component of plaques (NAC) domain, and a C-terminal domain. α-Synuclein forms the core component of Lewy bodies, which is one of the cardinal features of Parkinson's (Spillantini et al. 1998). Under diseased conditions, α-synuclein forms an aggregate of high-molecular mass in the midbrain regions of the patients. α-Synuclein is encoded by the SNCA gene. Till date, the exact function of the protein in the human brain is unknown. Several autosomal dominant mutations have been screened in the coding region of the gene. The first case of A53T missense mutation was reported in an Italian family suffering from Parkinson's disease. Subsequently, duplications, triplications, and point mutations have been reported in the SNCA gene in the case of PD. Recently a number of genome-wide association studies (GWAS) have linked variants associated with the risk of developing a sporadic case of PD (Krüger et al. 1998; Singleton et al. 2003; Chartier-Harlin et al. 2004; Miller et al. 2004; Zarranz et al. 2004; Fuchs et al. 2007; Labbé and Ross 2014). Recent studies suggest that in Lewy bodies of PD-affected subjects, α-synuclein displays a misfolded conformation, which is phosphorylated explicitly at serine 129 residue (Fujiwara et al. 2002). To gain detailed insight into the physiological role and pathological implications of α-synuclein in PD etiology, animal models of synucleinopathies have been developed. Till date, some of the crucial roles played by α-synuclein in protein folding, synaptic plasticity, and dopamine release have been demonstrated utilizing different animal models (Abeliovich et al. 2000; Burré 2015). Sophisticated genetic tools, which extend a great platform to study behavioral or cognitive dysfunctions have led *Drosophila* to gain an edge over other in vivo models. Feany and Bender (2000) developed the first humanized fly model expressing either wild-type or familial PD-linked mutants (A53T and A30P) of human α-synuclein (Feany and Bender 2000). This transgenic model recapitulated several hallmarks of the PD.

α-Synuclein Transgenic *Drosophila* Models

In 2000, the first transgenic fly model expressing either wild-type or familial PD-linked mutants (A53T and A30P) of human α-synuclein was generated. These flies exhibited Parkinson's like feature like inclusion bodies, prominent locomotory deficits, adult-onset loss of DA neurons (Feany and Bender 2000). There upon, this model has been successfully used by several groups to model Parkinson's linked synucleinopathies. The same group further improvised the previously generated α-synuclein transgenic lines capable of replicating PD pathology by incorporation of the more efficient binary expression system, the Q system, that provide higher levels of effector transgene expression than the classical UAS-GAL4-based binary expression system (Ordonez et al. 2018). Chen and coworkers (2014) have generated a transgenic fly line expressing α-synuclein mutant, A30P. One of the central features of Parkinson's is marked by the presence of nonmotor disorders, including

depression, cognitive dysfunction, and hallucination, which become complicated in advanced phases of the PD. Akin to the patient's nonmotor symptoms, α-syn mutant flies also showed abnormal sleep-like behavior, abnormal circadian periodicity when the mutant α-synuclein transgene is expressed in a subset of serotonergic and DA neurons (Balija et al. 2011). α-Synuclein-based transgenic models have been widely used to uncover the role of α-synuclein in PD etiology. Further, these models also aided in identifying novel interacting partners of mutant α-syn-mediated toxicity.

Insights Gained from Fly α-Synuclein Models

Regulation of Vesicular Trafficking

Premier evidence of the involvement of α-synuclein was provided by Luc Maroteaux in year 1988, which indicated the co-localization of α-synuclein with synaptic vesicles (Maroteaux et al. 1988). Later, Jensen et al. demonstrated the binding of α-synuclein with vesicles from rat brain. In addition, they also reported that α-synuclein binds to mutant forms of the protein (A30P and A53T) devoid of vesicle-binding ability. In another study, neurodegenerative phenotype of the α-synuclein transgenic *Drosophila* model were rescued by expression of Rab-1 (Cooper et al. 2006). Overexpression of Rab7 is known to govern early-to-late endosomal maturation and endosome-lysosome transport, suppressed locomotory dysfunction in α-synuclein flies (Dinter et al. 2016). Overexpression of Rab-8, which is known to be involved in post-Golgi vesicular trafficking, also rescued the motor deficit in α-syn flies (Yin et al. 2014).

Oxidative Stress

There is significant evidence of the involvement of oxidative stress and mitochondrial dysfunction for causing clinical pathogenesis of Parkinson's disease. Transgenic fly models of α-synuclein have been reported to be sensitive toward hypoxia-induced oxidative stress. These transgenic flies showed prominent degeneration of dopaminergic neurons upon oxidative stress. On subsequent coexpression of Cu/Zn superoxide dismutase (SOD1), the enzyme that detoxifies superoxide radicals, the DA-associated neuronal loss was attenuated. Treatment with nicotinamide that suppresses reactive oxygen species generation also relieved the signs of locomotory impairments in α-syn transgenic flies (Botella et al. 2008).

Trinh et al. (2008) took a closer look at the implications of the phase detoxification pathway, especially glutathione metabolism, in α-synuclein-associated PD. Transgenic lines with loss-of-function gene mutations affecting glutathione metabolism pathways were used. It has been shown that α-synuclein flies with loss-of-function mutations showed higher dopaminergic neuron loss. The signs of DA-associated neuropathy were rescued by genetic or pharmacological

interventions that raised the glutathione biosynthesis or glutathione conjugation activity (Trinh et al. 2008).

Suppression of α-Synuclein-Induced Neurotoxicity by Molecular Chaperones

Chaperones are a specialized class of proteins that facilitate the refolding or degradation of misfolded polypeptides. Cells employ this special class of proteins to cope up with the misfolded proteins that progressively form distinctive protein aggregates, which appear in the form of inclusion bodies like Lewy bodies. Lewy bodies are the pathological hallmark of PD composed of misfolded proteinaceous aggregates. Aggregates of α-synuclein contribute significantly to the formation of Lewy bodies followed by synphilin-1 (Wakabayashi et al. 2000) and ubiquitin (Kuzuhara et al. 1988). The presence of a substantial portion of ubiquitinated α-synuclein in Lewy bodies suggests the activation of the cellular degradation machinery to cope with α-synuclein aggregate (Dimant et al. 2012).

Overexpression of a molecular chaperone, heat shock protein 70 (HSP70), relieved the α-synuclein-mediated toxicity marked by suppression of DA neuronal degeneration. It was further noted that elevation in the expression of HSP70 did not change the number of inclusions. These results were further confirmed by reduction in the levels of HSP70, through coexpression of Hsc4.K71S, a dominant negative form of *Drosophila* HSP70 that enhanced α-syn-induced DA neuronal degradation. Subsequent treatment with geldanamycin, an Hsp90 inhibitor, and heat shock transcription factor 1-activator compound, rescued the α-synuclein-induced neuronal death. This confirmed that in *Drosophila*, the protective effect is exhibited by HSP70 against α-synuclein-mediated neurotoxicity (Auluck et al. 2002). Another independent study demonstrated that reduction in the levels of tumor necrosis factor receptor-associated protein-1(TRAP1) in ectopically expressed α-synuclein in flies was associated with DA neuronal death marked by a decrease in the levels of dopamine. Overexpression of TRAP1 rescued DA neuronal death. The proof of principle was confirmed through in vitro studies in rat primary cortical neuron culture, which reconfirmed the protective effect of hTRAP against α-synuclein-mediated neurotoxicity (Butler et al. 2012). These studies suggest that systems that control protein quality, mitochondrial function, oxidative stress, and DA biosynthesis pathways are potential targets for developing therapeutic agents for α-synuclein toxicity.

Modeling GBA-Associated PD in *Drosophila*

The glucocerebrosidase (GBA) gene encodes a lysosomal enzyme. Mutations in the GBA gene are commonly associated with Gaucher disease (Hruska et al. 2006). Clinical studies reported case reports of patients suffering from Gaucher disease

along with the presence of atypical and rare phenotypes including dementia, fatigue, tremors, and poor balance akin to Parkinsonian features (Hruska et al. 2006). This finding prompted several scientists worldwide to undertake research to uncover the link between Gaucher and Parkinson's disease. A direct approach was undertaken in which the GBA gene was sequenced in a group of 17 patients with Gaucher's disease and parkinsonism. Sequencing of GBA revealed 12 different genotypes, with a prevalence of the N370S allele identified in 14 patients (82%), including five N370S homozygotes (Tayebi et al. 2003). Importantly, another study reported heterozygous GBA mutations in subjects affected with sporadic Parkinson's disease.

In addition, biochemical analysis of neuropathological specimens of the above patients revealed decreased glucocerebrosidase activity. Mutations in GBA are associated with a significant decline in the levels of the glucocerebrosidase protein (Neumann et al. 2009). The carrier of the GBA-associated mutations faces a higher propensity of developing PD, which suggests the presence of an unexplored yet a direct mechanistic link with the pathogenesis of PD (Mazzulli et al. 2016). A significant aggregation of oligomeric forms of α-synuclein was reported in a study conducted on patient brain samples. The presence of higher levels of α-synuclein was confirmed by the proteomic analysis of cerebral cortex samples of patients in the same study.

The utilization of transgenic and knockout models of *Drosophila* have thus revealed novel insights related to the GBA-associated PD.

Knockout Models of GBA-Associated PD

In *Drosophila,* two GBA orthologs, namely, *CG31148* (*dGBA1a*) and *CG31414* (*dGBA1b*), are conserved. Single-gene knockouts *dGBA1a* and *dGBA1*b (*dGBA1*a−/−, *dGBA1*b−/) or double-gene knockouts of GBA1a and GBA1b (dGBA1a,b−/−) were generated. Single-gene knockouts (*dGBA1a*−/−, *dGBA1b*−/−) showed some of the hallmarks of PD, marked by motor dysfunction and decreased lifespan. Conversely, dGBA1a−/− neither showed locomotory deficiency nor decreased lifespan. The GBA knockout flies showed significant alteration in lipid metabolism along with mitochondrial deregulation. Systemic administration of rapamycin (inhibitor of mTOR) rescued the locomotory deficits and widened the lifespan of GBA-deficient flies. Results indicate the implications of mTOR in GBA-associated PD (Kinghorn et al. 2016). Applications of the transposon-mediated knockout model were generated by Davis et al. (2016). The homozygous knockout flies displayed a shortened lifespan, along with behavioral deficits, and a significant increase in the aggregation of a ubiquitinated protein, especially α-synuclein, was distinctly noted, but the dopaminergic neuronal loss was not marked in the knockout flies (Davis et al. 2016).

Transgenic Lines of GBA-Associated PD

Transgenic flies are crucial for studying the effect of mutant alleles in the pathogenesis of the disease. L444P and N370S are the most prevalent mutant forms of GBA in the population. The transgenic fly models incorporating the hGBA forms N370S and L444P were generated (Suzuki et al. 2015; Maor et al. 2016). The mutant flies replicated many features alike GBA-associated PD. The mutants showed a significant decline in the glucocerebrosidase (GCase) level along with shortened lifespan, prominent motor dysfunctions, increased ER stress, and DA-associated neurodegeneration.

RNAi Lines of GBA-Associated PD

Suzuki et al. (2015) generated the RNAi knockdown models by specifically silencing dGBA1a and dGBA1b transcripts. The knockdown flies also showed decreased GCase activity. GBA-RNAi lines displayed climbing disability along with DA-associated neural degeneration and significant aggregation of α-synuclein. One of the interesting features of dGBA-RNAi lines is the retinal degeneration (Suzuki et al. 2015).

Both knockout and knockdown models have displayed the potential of efficiently modeling the diseased conditions marked by consistent disease symptoms such as shortened lifespan and DA neurodegeneration.

Insights into the Functions of GB

Detailed molecular and biochemical analyses of knockout/knockdown and transgenic models have provided conclusive directions related to the functions of GBA.

GBA as a Regulator of ER Stress

GBA plays a central role in regulating the unfolded protein response (UPR) in the ER (Maor et al. 2013). Immunostaining of mutant GBA flies (L444P andN370S) showed prominent aggregation of the mutant GBA protein in ER. UPR was intensified in mutant flies relative to that of the wild-type flies. This points out toward an increased level of ER stress due to misfolding of GBA (Sanchez-Martinez et al. 2016).

Another independent study reported abnormally large lysosomes in the brain tissues of GBA-knockout flies. Additionally, aggregation of Autophagy-related protein (*Atg8*), the fly light chain 3 (LC3) homolog, was observed in the lysosomal bodies. Further, the lysosomal-autophagic degradation marker, p62, showed significant aggregation in lysosomes, which signify the deregulation of the lysosomal-autophagic pathway in mutant flies (Kinghorn et al. 2016).

Parkin

Genetic depletion and loss-of-function analysis of a gene is easily feasible in *Drosophila* and has been readily used to explore precise genetic pathways operational in PD. Parkin is a 465-amino acid protein encoded by the *PARK2* gene. Parkin is a cytosolic protein containing an N-terminal ubiquitin-like domain linked to a C-terminal RING domain. Mutations in the *Parkin* gene have been implicated with the causation of autosomal recessive juvenile Parkinson's disease (Cesari et al. 2003). Several studies have linked this gene with familial and sporadic forms of PD. Immunoblotting and immunohistochemistry studies in patients with autosomal recessive juvenile Parkinson's disease (AR-JP) demonstrated the absence of Parkin in all regions of the brain in affected subjects; instead localization of the protein in Lewy bodies was seen (Shimura et al. 1999). The same group, uncovered the involvement of Parkin in protein degradation. It has been shown to function as an ubiquitin-protein ligase that interacts with the ubiquitin-conjugating enzyme UbcH7 (Shimura et al. 2000). The role of *Parkin* in maintaining mitochondrial function and integrity has been well established by employing in vivo models (Abou-Sleiman et al. 2006; Hardy et al. 2006). Various in vivo and in vitro models have been developed by different groups to undertake mechanistic studies for unraveling the role of Parkin in the pathogenesis of Parkinson's disease.

Insights Gained

Parkin null mutants of *Drosophila* exhibit clinical phenotypes such as the loss of dopaminergic neurons, degeneration of flight muscles, and mitochondrial abnormalities. In another study, Pesah et al. (2004) generated Parkin null lines using P-element mutagenesis. A detailed study of Parkin null flies revealed reduced lifespan and resistance to oxidative and cold stress. Mutant flies induced progressive, age-dependent degeneration of DA neurons as well as motor dysfunction. Mutants also showed degeneration of flight muscles and mitochondrial dysfunction, coinciding with the phenotypes reported in the previous work (Pesah et al. 2004). In an independent study by Haywood and Stavaley in the year 2006, double transgenic lines (UAS-α-Synuclein; UAS-Parkin) were generated, and it was reported that coexpression of Parkin with α-synuclein in the dopaminergic neurons of flies protects against the α-synuclein-induced neurodegeneration (Haywood and Staveley 2006).

Sang et al. (2007) developed *Drosophila* models with human wild-type and mutant human *Parkin*. They reported that expression of mutant (Q311X, T240R) but not wild-type human Parkin in *Drosophila* induces progressive, age-dependent degeneration of DA neurons as well as motor dysfunction (Sang et al. 2007).

Transgenic *Drosophila* models have been used for quantitative proteomic analysis. *Parkin* was ectopically expressed using the UAS-GAL4 system in neural cells. The study revealed mitochondrial proteins and several endosomal trafficking regulators such as v-ATPase subunits, Syntaxin 5 (Syx5/STX5), ALG2-interacting

protein-X (ALiX/PDCD6IP), and vascular sorting protein 4 and vascular sorting protein 35 (Vps4 and Vps35, respectively, PD-associated genes) that showed increased ubiquitination with over expression of Parkin (Martinez et al. 2017). In a separate study, utilizing transgenic flies and RNAi lines, interaction of Sloppy paired 2 (*SLP2*) with Parkin has been demonstrated in the mitochondria. It was further shown that overexpression of SLP2 rescues Parkin mutant phenotypes. The admixture of recent and previous findings uncovered the crucial role of *Parkin* in the maintenance of mitochondrial functions and modulation of DA-associated neuropathy (Zanon et al. 2017).

PINK1

PTEN-induced putative kinase 1 (PINK1) is a 581-amino acid serine/threonine kinase localized in mitochondria. PINK1, along with other molecular partners such as Parkin, it governs maintenance of mitochondrial quality control through regulation of mitophagy (Kawajiri et al. 2011). Mutations in PINK1 is recognized as the second most common cause of autosomal recessive Parkinson's disease (Gandhi et al. 2006). The PINK1 protein consists of the evolutionarily conserved kinase domain, which mediates different cellular functions of the protein, whereas the N terminus consists of mitochondrial targeting motif and a transmembrane domain. Till date, approximately 50 pathogenic mutations have been reported by different groups across various populations around the globe. Majority of the reported mutations cluster in the serine/threonine kinase domain of the protein, suggesting that it is essential for the normal functioning of the PINK1 (Kawajiri et al. 2011). Heterozygous mutations in PINK1 have been implicated as a susceptibility factor in the development of a sporadic form of PD.

Intensive research has been undertaken globally to address the role of PINK1 in the pathogenesis of autosomal recessive form of Parkinson's disease, facilitated by the employment of various in vivo models, ranging from vertebrates to invertebrates. Due to low genetic redundancy, reduced complexity, and versatile genetic tools, *Drosophila* has been preferred by many groups to model the PINK1 dysfunction and to understand it's implication in Parkinson's disease.

Knockout Models

The first PINK-1 model in *Drosophila* was generated by the deletion of *Drosophila* PINK1 ortholog (CG4523) by a P element-mediated precise gene knockout. PINK1 mutant showed impaired mitochondrial function and morphological aberrations marked by fragmented mitochondrial cristae and a significant decline in levels of ATP, along with severe male sterility, prominent degeneration of indirect flight muscles, and increased stress sensitivity. Notably, the phenotypes of PINK1 knockout flies shared significant phenotypic similarity with Parkin mutants.

Further, to explore whether PINK1 and Parkin function in a common genetic pathway, Parkin was ectopically expressed in the testes and indirect flight muscles of PINK1 mutants. This rescued male sterility and mitochondrial deficits in respective tissues of PINK1 mutants. This suggests PINK1 and Parkin function in a common pathway and PINK1 functions upstream of Parkin (Clark et al. 2006). In the same year Park et al. (2006) generated loss-of-function lines of PINK1 utilizing P element mutagenesis. The PINK1 mutant flies exhibited prominent degeneration of dopaminergic neurons and indirect flight muscles and locomotory dysfunctions along with prominent mitochondrial dysfunctions in degenerated tissues. Overexpression of Parkin compensated for the dopaminergic neuronal degeneration and restored the mitochondrial dysfunctions in PINK1 mutants marked by the restoration in the levels of mitochondrial DNA and ATP. This finding is in agreement with the study reported by Clark et al. (2006), which advocates Parkin and PINK1 function in the same pathway and that PINK1 acts upstream of Parkin (Park et al. 2006). Genetic interactions have shown loss of mitochondrial integrity in PINK1 and Parkin mutants. The results of the study illustrates the pivotal role played by the PINK1/Parkin pathway in mitochondrial fission (Poole et al. 2008).

In an independent study, utilizing transgenic *Drosophila* lines, PINK1 was reported as one of the critical regulators needed for mitochondrial morphogenesis and function. It has been demonstrated that PINK1 genetically interacts with the mitochondrial fission/fusion machinery and modulates mitochondrial dynamics through regulation of the fission/fusion pathway in dopaminergic neurons and indirect flight muscles (Yang et al. 2008). Utilizing transgenic fly lines and in vitro gene transfection studies, Kim et al. 2008 reported the physical interaction between PINK1 and Parkin, leading to PINK1-directed phosphorylation of Parkin. It has been further confirmed that this modification of Parkin is needed for mitochondrial localization. Further, multiple mutant lines with defects in various domains have been generated. The results suggest that the kinase activity of the protein is vital for the regulation of mitochondrial function and integrity. It has been assessed that PINK1 is crucial for the translocation of Parkin into the mitochondria, and the RING domain of Parkin is crucial for PINK1-mediated translocation of Parkin (Kim et al. 2008). It has also been confirmed that Parkin along with PINK1 actively regulates mitochondrial trafficking by delivering competent mitochondria to the lysosome-rich perinuclear area and facilitating mitophagy (Vives-Bauza et al. 2010). In an elegant study, it has been demonstrated that mitochondrial deubiquitinase, ubiquitin C-terminal hydrolase 30 (USP30), counteracts PINK1/Parkin-mediated mitophagy. Further, downregulation of USP30 in flies rescued mitochondrial defects and aberrations.

USP30 RNAi conferred protection to dopaminergic neurons in paraquat-fed flies (Bingol et al. 2014). Recently, a study utilizing transgenic flies expressing the mitophagy probe mito-Kiema demonstrated that PINK1 and Parkin are indispensable for age-dependent mitophagy in *Drosophila* (Cornelissen et al. 2018).

Concluding Remarks

In this chapter, we have highlighted reports that have utilized *Drosophila*, as a model to gain mechanistic insights into the disease pathogenesis (Fig. 2). Many genes, cellular processes and signaling pathways implicated in sporadic and familial forms of Parkinson's disease are well conserved in *Drosophila*. Analytical and genetic tools available in *Drosophila* have provided adequate opportunities to uncover the role of different candidate genes and environmental toxins in the etiology of Parkinson's disease. *Drosophila* is a competent model to dissect genetic interactions and identify key players that link mitochondrial dysfunction, impaired vesicular trafficking, along with aberration in regular translation machinery to progressive dopaminergic neurodegeneration. With access to more efficient and cost-friendly sequencing technologies, it has become easier to discover new mutations in familial as well as sporadic cases of Parkinson's disease. In parallel, the evolution of precise approaches to generate humanized mutations in endogenous *Drosophila* genes and easy manipulation of the fly genome assures that the amalgamation of human and fly genetics will provide an exquisite understanding of the underlying molecular pathways involved in the pathogenesis of Parkinson's disease as illustrated in Fig. 3. The wealth of knowledge gained by these findings can be utilized to resolve many unanswered questions regarding wide phenotypic variability and genetic risk factors related to Parkinson's disease.

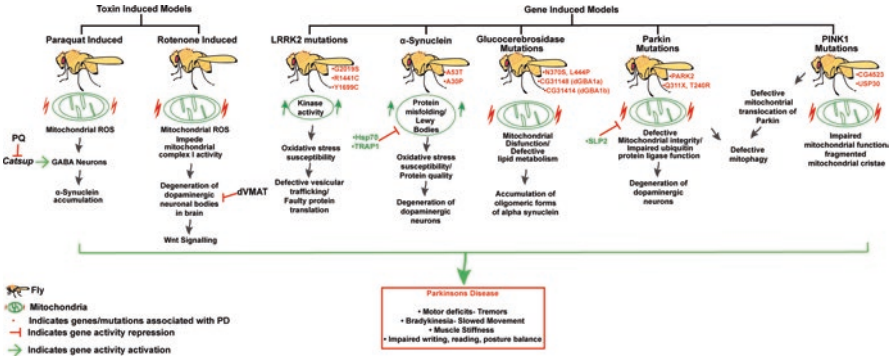

Fig. 2 Modeling Parkinson's disease in flies: The *Drosophila* models of Parkinson's disease can be divided into two classes: (**a**) toxin-induced model and (**b**) gene-induced models. Environmental effects, as well as genetic pathways, contribute to the progression of PD. These models provide probable causation and genes modulated in PD and provide information to work toward developing treatments against PD

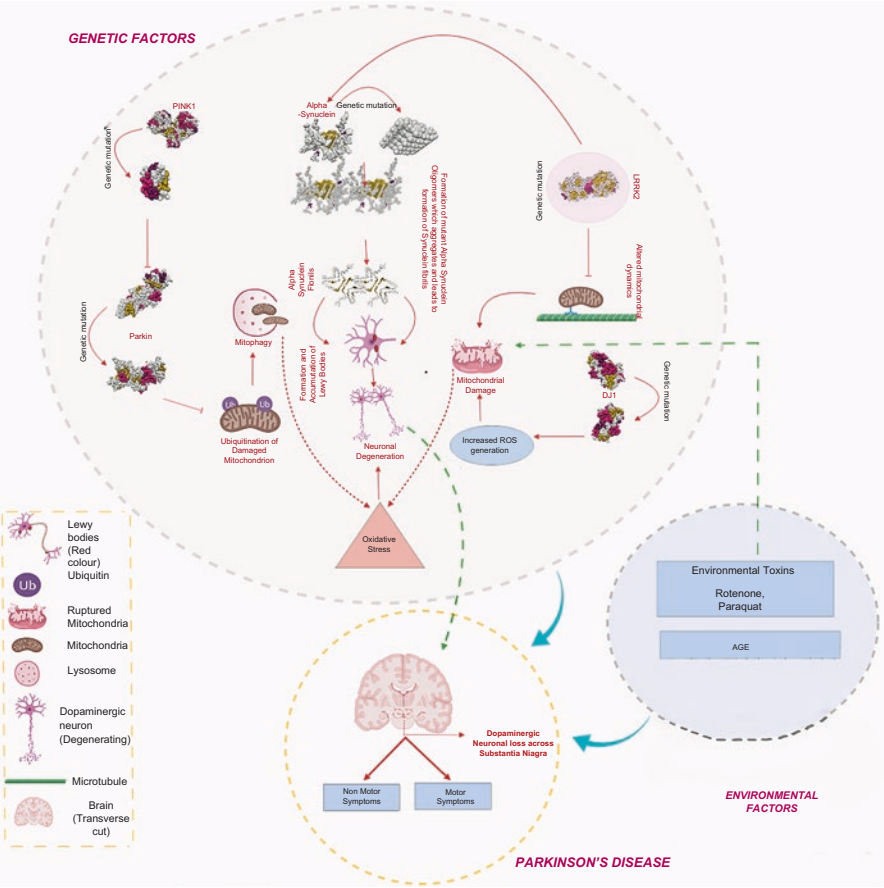

Fig. 3 Schematic summary of genes involved in the molecular genetics of Parkinson's disease (PD). Mutation in the causal PD genes *Parkin*, *PINK1*, and *LRRK2* may confer risk to the onset of Parkinson's disease by alteration in mitophagy and typical mitochondrial dynamics, which ultimately elevates the level of reactive oxygen species (ROS) with subsequent increase in oxidative stress, which finally leads to dopaminergic neuronal loss. Mutation in α-synuclein contributes to the etiology of PD by the formation of α-synuclein fibrils, which ultimately are accumulated and form Lewy bodies. Lewy bodies are accumulated in the neurons and lead to dopaminergic neuronal loss. Along with genetic factors, other factors such as age and exposure to environmental toxins like paraquat and rotenone also increase the risk of PD causation, mainly by the rise of oxidative stress. (Structure and backbone of mutated and wild-type forms of Parkin, PINK1, LRRK2, DJ1, and α-*synuclein* are derived from Protein Data Bank, https://www.rcsb.org/)

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Post-translational Modifications: A Mystery to Unravel Huntington's Disease Prognosis

Megha Chatterjee and Namita Agrawal

Abstract

Huntington's disease (HD) is a dominant debilitating neurodegenerative disorder caused by CAG trinucleotide repeat expansion in the huntingtin gene (*HTT*) on chromosome 4 of the human genome. The CAG repeat is highly polymorphic and varies from 6 to 35 in unaffected individuals and more than 35 repeats in HD patients. The encoded polyglutamine (polyQ) expansion in the exon 1 of the *HTT* gene that confers altered property to the protein leading to HD toxicity. However, in addition to the expansion of the polyQ stretch as a major cause of HD, recent reports suggest the involvement of post-translational modifications (PTMs) in metabolism, protein-protein interactions, and cellular toxicity. Various types of PTMs regulate protein stability, localization, function, and their interaction with other molecules and these have been reported in HD pathogenesis. Cleavage and clearance of mutant Htt (mHtt) and its interaction with other cellular processes are the key events leading to HD, and therefore, a better understanding of signaling pathways implicated in Htt protein modification is a mandate in the management of HD pathogenesis.

To address the complex processes involved in HD, various in vitro and in vivo HD models were considered. *Drosophila* proved to be one of the best model organisms due to the availability of powerful genetic tools and significant homology with fundamental cellular processes of humans. This chapter deals with the general effects of PTMs on wild type and mHtt and whether regulation of these PTMs can abrogate disease progression, particularly using *Drosophila* as a model organism.

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Huntington's disease (HD) · Post-translational modifications (PTMs) · *Drosophila* · Huntingtin protein · Neurodegeneration

Introduction

Huntington's disease (HD), a neurodegenerative disorder, is caused by the expansion of CAG trinucleotide repeat in the huntingtin (*Htt*) gene. Despite identification of the causative gene, due to the involvement of multiple complex pathways including a spectrum of PTMs, the complete molecular mechanism underlying HD is not yet clear. The neuropathology of HD includes dysfunction and preferential degeneration of specific neurons in the brain with variation in symptoms between patients but usually characterized by a triad of the motor, cognitive, and psychiatric symptoms. In addition to alterations in CNS, patients also suffer from metabolic and immune alterations, skeletal-muscle wasting, weight loss, cardiac failure, testicular atrophy, and osteoporosis (van der Burg et al. 2009). Patients usually die 20 years after onset, and in many cases, death results from fatal aspiration pneumonia.

Recent evidence also suggests that protein context and PTMs influence the neurotoxicity of the polyQ proteins. A myriad of post-translational modifications (PTMs) has been reported to occur in the Htt protein, namely, acetylation, phosphorylation, ubiquitination, SUMOylation, palmitoylation, and lipid modifications. In addition, one of the major PTMs, paramount to wt-Htt functioning and mHtt-induced toxicity, is the proteolytic cleavage (Ehrnhoefer et al. 2011) (Fig. 1). Interestingly, some PTMs of Htt have been shown to be protective against toxicity of mHtt, while others increase mHtt toxicity, suggesting a critical role of PTMs in HD pathogenesis. A significant number of reports related to HD toxicity by comparing various fragments of mHTT conclude that exon 1 is the most pathogenic fragment of mHtt. Intriguingly, the majority of well-characterized PTMs are also located on the N-terminal region of the Htt.

Usage of cell culture has been an initial and promising approach to decipher mechanistic insights into mHtt pathogenesis. However, to evaluate the function and the impact of individual PTMs on the disease phenotype, the in vivo system is often employed. Therefore, for many unanswered questions regarding the contribution of PTMs in mHtt-mediated toxicity, HD condition has been recreated in the fruit fly, *Drosophila,* by targeted insertion of the human *Htt* gene, and these transgenic flies mimicked symptoms of HD patients.

The present chapter is focused on the current interesting knowledge related to the role and impact of several PTMs involved in HD prognosis, with major emphasis on how *Drosophila* is used extensively as a model organism to identify pathways that regulate different PTMs, which is a promising avenue for therapeutic development.

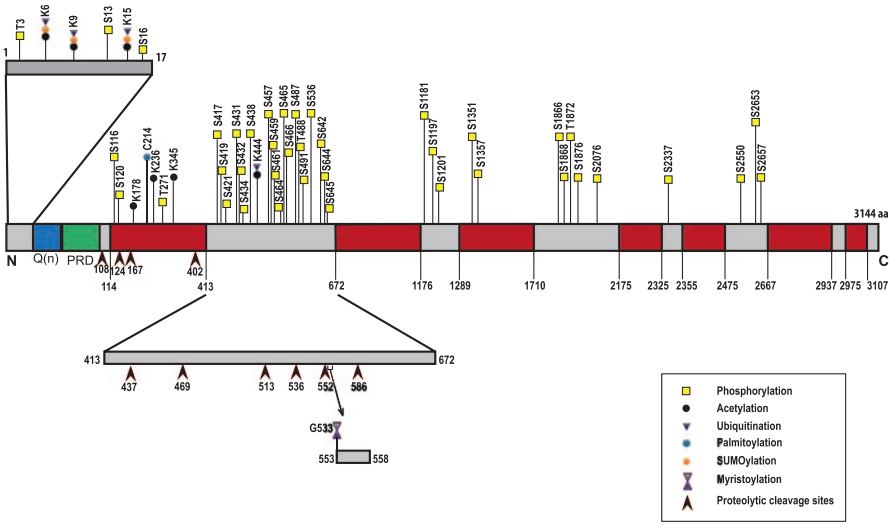

Fig. 1 Schematic representation of all the known post-translational modifications of the human huntingtin protein. All the known PTMs of human Htt and their putative sites on the protein are displayed in the figure. The different PTMs are represented by particular symbols mentioned in the inlet within the figure. Within the protein structure, the blue region indicates the polyglutamine (PolyQ) stretch; the green region indicates the proline-rich domain (PRD), and the red region indicates the HEAT repeat regions of the Htt protein

The Biology of Huntingtin

Etiology of Wild-Type Huntingtin Protein

The *Htt* gene (also called *IT15*) encodes a large 348-kDa protein located on the short arm of chromosome 4 in the human genome (4p16.3; Gusella et al. 1983) and has 67 exons. The postmortem brain tissue of the HD patients displayed the appearance of the amino-terminal fragments of Htt. The reports from fly models suggests that Htt exon 1 protein (Httex1p), an amino-terminal fragment, is sufficient to cause disease in HD models pointing toward the importance of the amino-terminal region of Htt in the disease progression. The first exon of Htt encodes 17 amino acids, followed by a polyQ repeat of variable length and a proline-rich domain (PRD) of 50 amino acids. The first 17 amino acids of Htt are highly conserved throughout mammalian evolution, and many of it's residues can be subjected to post-translational modification, suggesting an important function for these residues.

The NT17 domain forms an amphipathic α-helix. Due to it's flexible nature this domain can also adopt random coil and extended loop structures (Atwal et al. 2007; Kim et al. 2009). The PRD forms a proline–proline (PP) helix and provides stability to the polyQ structure. In addition, Htt protein contains several HEAT repeats forming antiparallel α-helices separated by non- helical regions.

These HEAT repeats are clustered into three to five alpha-rod domains, and these domains facilitate protein–protein interactions by providing a scaffold for protein complexes. Purified Htt is reported to adopt 100 structurally distinguishable conformations that can be attributed to these intramolecular interactions (Seong et al. 2010). Additionally, four domains in the Htt protein defined as PEST domains are enriched with proline, glutamic acid, serine, and threonine. Strikingly, these PEST domains are enriched with predictive proteolytic sites and various PTMs essential to the protein functions (DiFiglia et al. 1997; Wellington et al. 2000; Warby et al. 2008).

A characteristic feature of any protein is the presence of several functional motifs at particular locations in the protein. Htt has a cytoplasmic retention signal at the N-terminus and a nuclear export signal at the C-terminus (Xia et al. 2003; Rockabrand et al. 2007). A proline-tyrosine or PY-NLS is found between amino acids 174–207 has been predicted as a nuclear localization signal (NLS) of the protein (Desmond et al. 2013).

Htt is expressed ubiquitously, but the level of expression in the nervous system is higher than that in other tissues, which can be correlating to the preferential degeneration witnessed in the nervous system. Moreover, Htt has heterogeneous expression pattern within the same tissue. Mesenchymal cells have no or little Htt expression, whereas the epithelial cells of the same tissue have higher levels of Htt expression (Marques Sousa and Humbert 2013).

Intense research in the field of HD has led to the discovery of several critical cellular and physiological functions of wt-Htt. It is involved in various cellular processes such as trafficking, autophagic clearance, regulation of ciliogenesis, transcriptional regulation, embryonic development, maintenance of tissue, cell morphology, and cell survival (Saudou and Humbert 2016).

Mutant Huntingtin Protein

Expansion of CAG triplet repeats in the gene coding for the Htt results in an abnormal protein referred to as mutant Htt (mHtt) that gradually damages cells in the brain through varied mechanisms that are not yet fully understood. The increase in the CAG repeats occurs primarily due to the replication errors in the gene because CAG repeats are slippage-prone. The age of disease onset has an inverse correlation with the CAG repeat length; the longer the stretch of the CAG repeats, the earlier is the disease manifestation. HD is a familial and multifaceted disorder and therefore requires a holistic management plan taking into account clinical, environmental, and social factors.

One of the hallmark features of HD is the presence of mHtt ubiquitin-positive inclusion bodies (IBs) (DiFiglia et al. 1997; Becher et al. 1998). Initially, the mHtt aggregates were considered to be restricted within the nucleus, but a detailed observation suggested it's presence in the cytoplasm as well (Gutekunst et al. 1999). mHtt inclusion bodies contribute to the characteristic toxicity primarily by

sequestering essential proteins, mainly the components of ubiquitin-proteasome systems (UPS) (Cummings et al. 1998; Donaldson et al. 2003) and transcription factors (McCampbell et al. 2000; Steffan et al. 2000; Nucifora Jr et al. 2001) leading to the impairment of the UPS and transcriptional dysregulation, respectively. However, the sequestration of these essential cellular proteins by mHtt aggregates was also reported to be biologically insignificant (Yu et al. 2002; Schaffar et al. 2004; Bennett et al. 2005; Mitra et al. 2009).

In addition to the formation of inclusion bodies, mHtt is also reported to display dysregulation of transcription, autophagic dysregulation, proteasome inhibition, mitochondrial abnormalities, metabolic impairments, microtubule-based transport alterations, impaired calcium signaling, excitotoxicity, and dysfunction in neurotransmitter release (Ross and Tabrizi 2011; Saudou and Humbert 2016). Although the exact mechanistic basis of these cellular toxicity is yet to be established. A number of studies point towards the factors beyond the polyQ expansion of the Htt that critically determines the mHtt-induced toxicity in HD.

PTMs have been reported in most of the polyglutamine-containing proteins that interestingly often show similar effects on the disease phenotype. PTMs are complex biochemical modifications that can tightly regulate and control a variety of cellular processes. Several types of PTMs of Htt, like phosphorylation, acetylation, ubiquitination, SUMOylation, myristoylation, palmitoylation, and proteolytic cleavage have been reported till date (Ehrnhoefer et al. 2011).

Post-translational Modifications of mHtt

PTMs can occur on the amino acid side chains or at the protein's C- or N-terminus. PTMs of a protein extend the repertoire of the standard polypeptide chain translated from a gene. The various PTMs include the attachment of diverse biochemical functional groups to specific amino acids of a polypeptide such as acetate, phosphate, various lipids, and carbohydrates. Some PTMs also comprise attachment of proteins, such as ubiquitin and SUMO, to other target proteins. These modifications, alone or in combination change the chemical nature of an amino acid or extend the range of their probable molecular structures beyond the limits imposed by the 20 encoded amino acids. The molecular and functional consequences of PTMs include alterations in proteins' three-dimensional structure, protein interactome, subcellular localization, turnover, and activity. In addition, PTMs are often considered important pillars of cell signaling, thus enabling the cell to respond to a myriad of internal and external stimuli, as is the case during oxidative stress or during heat shock (Drazic and Winter 2014; Verdin and Ott 2015). Under normal conditions, most eukaryotic regulatory peptides, hormones, and neurotransmitters are synthesized in a biologically inactive form that undergoes a variety of post-translational processing steps to yield a functionally active protein (Harris 1989).

The first 17 amino acids of Htt, MATLEKLMKAFESLKSF, are highly conserved throughout mammalian evolution, suggesting an important function of these

residues (Arndta et al. 2015). Several PTMs, including phosphorylation, acetylation, ubiquitination, SUMOylation, lipid modifications, and proteolytic cleavage, have been witnessed to occur on the wt-Htt, which is altered in the mutant protein.

Impact of PTMs on HD Pathogenesis Using *Drosophila* as a Model

As discussed in many reports, HD pathogenesis is a complex set of events that occur simultaneously or sequentially. Therefore, to address mechanisms underlying this devastating disease, an appropriate model organism like *Drosophila melanogaster*, the common fruit fly, can be considered, as it helps tremendously to understand various aspects of disease prognosis and also uncover the cause–effect relationships between the causative gene and protein.

Essentially, the fundamental aspects of cell biology are conserved in human and flies including regulation of gene expression, membrane trafficking, cytoskeleton, neuronal connectivity, synaptogenesis, cell signaling, and cell death (Sang and Jackson 2005). Many genes and pathways that were originally studied in flies have subsequently been identified in mammals. Strengths of this simple model system also include a relatively short lifecycle of ~10 days, large number of progeny, simple and rapid genetic manipulation because of its smaller size of the genome (1.2×10^8 bp) than the human genome (3.3×10^9 bp). Moreover, to exemplify the neurological research, *Drosophila* neural circuitry has functional, developmental, and molecular similarities with those of human. In addition to all these similarities, the fly nervous system is less complicated than the mammalian nervous system, thus making it a reliable model to investigate human neuronal aspects (Sanes and Zipursky 2010).

The generation of transgenic flies stably harboring the human *Htt* gene with expanded and unexpanded CAG repeats, has been instrumental in the study of HD (Jackson et al. 1998). The HD condition was created by employing the commonly used bipartite UAS-GAL4 system that enables the spatial and temporal expression of human Htt in tissues of interest, in HD fly model, Htt are over-expressed in photoreceptor neurons (Brand and Perrimon 1993). The most frequent assay to assess neuronal dysfunction and degeneration in HD models of *Drosophila* includes the study of external eye morphology and internal eye sections of flies challenged with disease and normal conditions. Another assay for neuronal degeneration is pseudopupil analysis that provides a rapid quantitative readout of rhabdomere degeneration occurring due to mHtt expression in photoreceptor neurons. In addition, labeling of specific neuronal populations by using selective antibodies or by biomarker expression allows inspection of loss of a subset of neuronal cells. To evaluate behavioral symptoms, climbing and flight assays give insight into the extent of motor disabilities. Lifespan analysis is another crucial assay that readily recapitulates the toxic effect of the disease gene in terms of survival (Marsh et al. 2003; McGurk et al. 2015).

To monitor the effect of PTMs on the specific amino acid residue of Htt, the asystematic site-directed mutation SDM approach is employed. Site directed mutagenesis of serines to aspartate and alanine depict phospho-resistant and phosphomimic conditions, respectively (Warby et al. 2005; Gu et al. 2009). In similar manner, SDM of lysine to arginine and glutamate lead to acetylation resistance and phospho- mimic, respectively (Jeong et al. 2009; Chaibva et al. 2016).

PTMs play the role of key modulators of protein function in HD. The study of PTMs in Htt has been carried out extensively in various disease models and cell cultures. Several PTMs have thus been identified, and their impact on the functioning of wt-Htt ant mHtt-induced toxicity have been extensively studied. Besides the toxic fragments of mHtt, studies have strongly suggested the impact of altered PTM sites in the full-length Htt (Gu et al. 2009; Ratovitski et al. 2017).

Other than the addition of functional groups, another kind of PTM, i.e. proteolytic cleavage, has been suggested as a pivotal modification of Htt that has significant implications in HD pathogenicity (Cooper et al. 1998). As witnessed in both clinical settings and in disease models, several naturally occurring amino-terminal fragments of mHtt have been reported to confer differential toxic effect, particularly, exon 1 fragment. Interestingly, some of the proteolytic fragments are relatively harmless. Behavior of these naturally occurring fragments was monitored in many model organisms; however, a proper comparison can't be drawn due to differences between mouse strains, cell lines, transgene copy numbers, chromosomal location, polyQ tract length, and propensity of longer Htt peptides to be naturally processed into smaller fragments. *Drosophila* has an advantage over these model systems to compare the intrinsic toxicity of these fragments because all the transgenic flies with different fragments will have the same genetic background; Site specific insertions in the fly genome avoid additional toxicity and transgenic peptides undergo minimal proteolytic processing (Barbaro et al. 2015).

Some of the significant PTMs involved in HD pathogenesis are listed below, with major emphasis on published reports using the HD fly model (Fig. 2).

Phosphorylation

During the course of evolution, the interplay between phosphorylation and dephosphorylation of a biomolecule became one of the most crucial types of cellular processes (Hunter 2012). Biological phosphate esters (nucleic acids and phosphoproteins) and phosphate anhydrides (adenosine triphosphate and 3′-phosphoadenosine 5′-phosphosulfate) are stable at physiological temperature and aqueous conditions that are essential for the generation of many biological molecules and long-term storage of genetic information. Under physiological conditions, this reaction is readily catalyzed by protein kinases and can be reversed by protein phosphatases catalyzed through hydrolysis. Adenosine triphosphate (ATP), guanosine triphosphate (GTP), and phosphoenol pyruvate (PEP) are the known phosphate

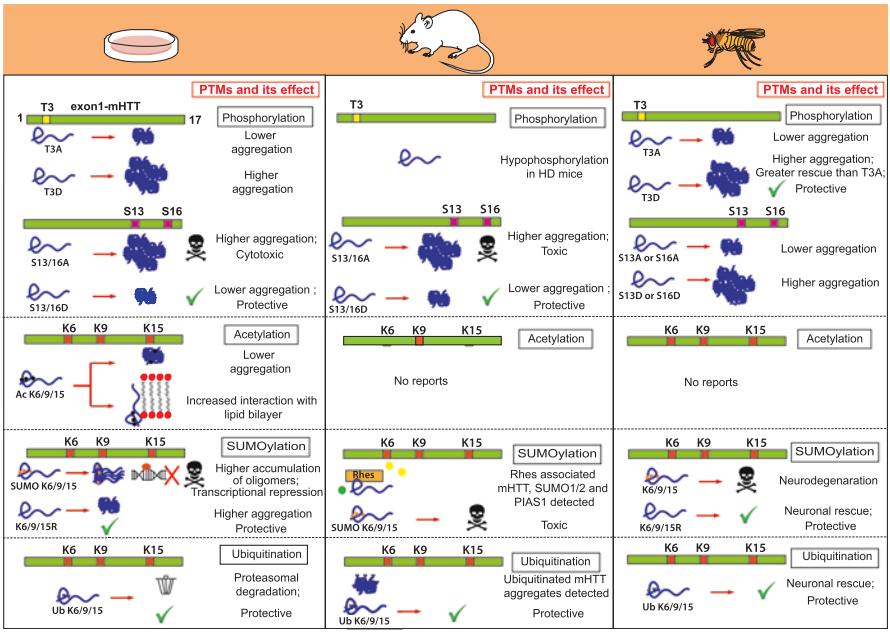

Fig. 2 Various PTMs that occur on exon 1 fragment of mHtt and their respective impacts on HD toxicity. The effects of these PTMs have been studied in vitro, in mouse and *Drosophila* models of HD. All the various reports of the PTMs of exon 1 mHtt along with their phenotypic effects studied in these model systems till date have been shown in the figure

donors under physiological conditions. In addition, nine out of 20 amino acids in protein provides putative sites for phosphorylation. Predominantly, in eukaryotes, three amino acids, namely serine (S), threonine (T), and tyrosine (Y) undergo phosphorylation. However, phosphorylation of six other amino acids, namely, lysine (K), arginine (R), histidine (H), aspartate (D), glutamine (E), and cysteine (C) are also chemically feasible substrates to the kinases. A striking feature of a phosphoamino acid is its greater than one negative charge, which in contrast to the only negatively charged amino acids aspartate and glutamate whose carboxyl side chains only have a single negative charge. A large hydrated shell and unique charge properties of the phosphate group ensure stable hydrogen bonds and salt bridges due to which inter- and intra-molecular interactions occur. Thus, protein phosphates may regulate conformational changes within protein monomers, an allosteric transition within a protein multimer, and may aid in the formation of biological polymers. In addition, phosphate groups covalently attached to the amino acids also regulate the subcellular localization. Additionally, it facilitates protein–protein interactions by aiding the recognition of the phospho-protein by a phospho-specific binding domain in other proteins (Pearlman et al. 2011; Wang et al. 2017).

In the context of HD, to date, a myriad of putative sites for phosphorylation, such as 307 serine, 170 threonine, and 62 tyrosine residues, has been reported to be distributed throughout the full-length Htt (Schilling et al. 2006). A detailed mass

spectrometric analysis of the phosphorylation sites in two cell populations, namely 293 T and PC12, was performed by Ellerby and coworkers, in the full-length Htt. They uncovered six novel putative phosphorylation sites: S536, S1181, S1201, S2076, S2653, and S2657 (Schilling et al. 2006). The location of these residues decides their molecular and functional consequences, as S536 is located in the proteolytic susceptibility domain, S1181 and S1201 are closer to an Htt nuclear localization signal, S2076 is within the HEAT domain 3, and S2653 and S2657 are in a C-terminal proteolytic susceptibility domain and a predicted calcineurin-binding motif (Aronin et al. 1999; Peters et al. 1999; Schilling et al. 2006). All these residues are displayed in Fig. 1, with yellow boxes indicating the phosphorylatable residues revealed to date (Humbert et al. 2002; Aiken et al. 2009; Thompson et al. 2009; Dong et al. 2012; Watkin et al. 2014; Huang et al. 2015; Ratovitski et al. 2017).

The three putative sites for phosphorylation in exon 1 of mHtt are T3, S13, and S16. Exon 1 is the most toxic fragment and reported to be significantly involved in HD pathogenesis; therefore, a consequence of phosphorylation on these three residues is of great interest. Phosphoresistant and phospho-mimicked mutations of T3 in cell culture and the *Drosophila* model revealed that phosphorylation increases mHtt aggregation; however, both the mutations reduce toxicity (Aiken et al. 2009). In a recent study, on the other hand, both the mutations at T3 led to a reduction in mHtt inclusions (Branco-Santos et al. 2017). In another study on HD mice and human HD samples, a reduction in the levels of T3 phosphorylation was witnessed. Phosphorylation of T3 in mHtt has also been observed to ameliorate the conformational rigidity imparted by the polyQ expansion (Cariulo et al. 2017). These reports indicate that therapeutic intervention of T3 phosphorylation in mHtt may prove to be beneficial.

In addition to T3, S13 and S16 are two serine residues that undergo phosphorylation by the IKK complex. Interestingly, the efficiency of S13 and S16 phosphorylation is found to be lower in mHtt than in wtHtt. It has been demonstrated that S13 is the direct substrate for this kinase and that the phosphorylation of S16 is facilitated by S13 phosphorylation. S13 and S16 phosphorylation has been reported to promote the proteasomal and lysosomal degradation of mHtt. Unfortunately, this mechanism becomes compromised in the mHtt-challenged cells, which might lead to the accumulation of mHtt (Thompson et al. 2009). Like T3, S13 and S16 phosphorylation of mHtt has been found to be neuroprotective in mice and cell culture models of HD (Gu et al. 2009). S13 and S16 have been reported as a critical determinant of Htt conformation. The phospho-mimicking mutations at S13/16 resulted in a loss of the alpha-helical conformation of the Nt17 domain of Htt (Atwal et al. 2011). S13/16 phosphorylation also plays a crucial role in determining the subcellular localization of mHtt and induces its nuclear accumulation (Atwal et al. 2011; Havel et al. 2011). Interestingly, the phospho-mimicked mutation of only S16 in exon 1 with expanded glutamine also increases the mHtt aggregate burden in *Drosophila* (Branco-Santos et al. 2017). A previous report suggests that the lysosomal clearance mechanism, which is present in mammals, is not well conserved in *Drosophila* due to lack of a component of chaperone-mediated autophagy (CMA), that is, LAMP 2A. Absence of LAMP 2A might be the factor responsible for

increased accumulation of exon 1 mHtt in phospho-mimicked S13/16D transgenic flies. This accumulation in transgenic flies can be well correlated with the disease condition, as the CMA pathway is compromised with age in HD patients (Thompson et al. 2009; Steffan 2010).

Functions of the putative residues located in the rest of the wt-Htt and aberrations in the mHtt have also been deciphered by several research groups. S536 is a putative site for calpain cleavage. However, in the case of mHtt, when this residue undergoes phosphorylation, the calpain-mediated cleavage is blocked and decreases cellular toxicity (Schilling et al. 2006). In mHtt, the phospho-resistant mutations on S116, S1201, and S2652 residues are reported to be protective suggesting that phosphorylation of these residues in mHtt may aggravate disease pathogenesis. However, some contradictory arguments have also been put forward, as S1201 and S1181 phosphorylation have protective effects in striatal neurons. Attributed to the discrepancies among model systems and cell populations, these effects of phosphomutations may be spatially and temporally specific and still need intense in vivo and in vitro research (Anne et al. 2007; Thompson et al. 2009; Watkin et al. 2014; Arbez et al. 2017).

Acetylation

The acetyl group (CH_3CO) has properties that can regulate various biological functions when attached to the polypeptide chains. In case of acetylation, the acetyl group donated by acetyl CoA is transferred to the targeted proteins. In a biological system, acetylation of protein occurs co-translationally and post-translationally and 80–90% of the protein is acetylated by the co-translational process at the N-terminus of the nascent polypeptide chains (Brown and Roberts 1976; Arnesen et al. 2009; Aksnes et al. 2015a, b). However, recent research suggests that the N-terminal acetylation of several proteins occurs post-translationally, indicating that acetylation is a complex system that can be catalyzed by N-terminal acetyltransferases (NATs) (Helbig et al. 2010; Helsens et al. 2011; Van Damme et al. 2011; Aksnes et al. 2015a, b). Earlier, the enzyme catalyzing the acetylation of histones was referred as histone acetyltransferases (HATs); however, after the discovery of non-histone proteins as substrates for lysine acetylation, the enzyme nomenclature was revised to lysine acetyltransferases (KATs). KATs are classified into two major groups: GNAT superfamily and the MYST family. Some other prominent KATs are p300 (E1A-associated protein 300 kDa), CBP (cAMP response element binding (CREB) protein), and TAFII 250 (TATA-binding protein-associated factor II 250) (Yang 2004). After lysine acetylation, the protein functions may be modified by both "loss-of-function" and "gain-of-function" mechanisms. In loss-of-function mechanism, when the acetyl group is added to the ε-amino group of a lysine residue, the positive charge of lysine is neutralized affecting its interaction with other biomolecules like DNA, RNA, or other proteins (Helbig et al. 2010; Van Damme et al. 2011; Aksnes et al. 2015a, b). In gain-of-function mechanism, the addition of an acetyl group creates new sites for protein interactions. Bromodomains are structural modules that

recognize and bind to such sites with acetylated lysine residues (Zeng and Zhou 2002; Ladurner et al. 2003; Matangkasombut and Buratowski 2003; Yang 2004). There are other proteins as well that interact with acetyl lysine residues without involving any bromodomain (Jeong et al. 2002).

In most of the prominent neurodegenerative diseases, imbalance in the acetylation level of the causative proteins shows significant relevance (Kim et al. 2006; Kilgore et al. 2010; Monti et al. 2010). As in HD, mHtt clearance is a beneficial step toward the prevention of cellular dysfunction and degeneration, and acetylation at lysine 444 (K444) of mHtt facilitates mHtt clearance in mouse and cell culture models. The stability of mHtt depends acetylation status of mHtt at residue K444, and in the primary neuronal cultures of the mouse, acetylation-resistant mutation led to the accumulation of mHtt. Interestingly, interaction of mHtt with the HAT domain of CBP suggest catalysis of lysine acetylation by CBP (Jeong et al. 2009). This is further supported by reports that CBP depletion enhances the toxicity, and overexpression of CBP suppresses mHtt-induced cellular toxicity (Nucifora Jr et al. 2001; Bates et al. 2006). In addition, lysine residues are also present within the caspase 6 fragments of mutant Htt (586 aa; described in the "proteolytic cleavage" section) (Graham et al. 2006). Three acetylation sites are also present in the NT17 domain of Htt through proteomic mapping by MS. These putative sites of acetylation in the NT17 domain are predicted to be important for interaction of this domain of Htt with the lipid membranes. It is reported that mimicking acetylation at lysine 9 and 15 (K9 and K15) results in a lower lipid membrane affinity than that of K6. In turn, the lower affinity of acetylated NT17 toward lipid membranes leads to a reduction in membrane damage associated with mHtt exposure (Chaibva et al. 2016). These reports suggest that altered acetylation levels of mHtt can be a crucial molecular switch that modulates mHtt-induced toxicity and thereby pathogenesis.

Palmitoylation and Myristoylation

Palmitoylation and myristoylation are the two major types of lipid modifications that occur in the cell besides prenylation. These modifications correspond to the addition of long-chain fatty acids to the amino acid residues of the polypeptide chains and make the protein more hydrophobic. This anchoring of lipid to the proteins facilitates the interaction of proteins with lipid bilayers, targeting the proteins to cell membranes and thus promoting subcellular protein trafficking. In palmitoylation, a saturated 16-carbon fatty acid chain forms a thioester bond post-translationally with the cysteine thiol side chain catalyzed by the enzyme palmitoyltransferases (PATs). It is a highly reversible modification attributed to the labile nature of the thioester bond, which plays an important function in shuttling-modified proteins between cellular compartments and allowing relocalization of the protein in the cell or within different regions of the membrane (Fukata and Fukata 2010). The recognition motifs for this PTM are not yet well defined, but some clear patterns have been uncovered by comparing different sites of palmitoylation. Palmitoylated proteins can be divided into four distinct types: (1) a single palmitoyl

modification frequently near the end of a protein; (2) palmitoylation proximal to a transmembrane domain; (3) dual palmitoylation and prenylation; and (4) dual palmitoylation and myristoylation (Bhattacharyya and Wedegaertner 2000; Tsutsumi et al. 2009; Guan and Fierke 2011). For dually lipid-modified proteins, the first modification, for example, prenylation or myristoylation, provides substrate proteins with weak membrane interaction, while the subsequent palmitoylation generates sufficient hydrophobicity for strong membrane affinity (Peitzsch and McLaughlin 1993; Shahinian and Silvius 1995).

Protein palmitoylation has been extensively studied in neurons and has been found to be involved in several aspects of neuronal functions. In addition, it signals for the proper folding and complex formation. Reduced palmitoylation often lead to protein aggregates are formed (Rakhilin et al. 1999; Drisdel et al. 2004).

N-myristoylation, on the other hand, is the addition of a 14-carbon fatty acid to glycine with an exposed –NH2 group after initiating methionine to cleave off the peptide chain. Predominantly, this process occurs co-translationally. However, post-translational myristoylation has also been reported following a caspase-mediated proteolytic cleavage at the preferred glycine (Zha et al. 2000; Dix et al. 2008; Mahrus et al. 2008; Martin and Hayden 2015). In contrast to the reversible palmitoylation, myristoylation is static in nature.

Palmitoylation of mammalian Htt at cysteine 214 is catalyzed by two palmitoyl acyl transferases (PATs), namely huntingtin-interacting protein 14 (HIP14) and huntingtin-interacting protein 14-like (HIP14L) (Huang et al. 2009). The interaction of mHtt with HIP14/HIP14L is reduced over that of wt-Htt, leading to reduced palmitoylation of the mutant protein. This reduced interaction is polyQ dependent, and with the disturbed interaction of mHtt and HIP14, a consequential reduction in palmitoylation leads to its altered cellular localisation. This strongly suggests that palmitoylation is essential for the trafficking of Htt (Yanai et al. 2006). Beyond the enzyme-substrate relationship, Htt also regulates the enzymatic activity of HIP14. Subsequently, the lower interaction of mHtt with these enzymes leads to reduced palmitoylation of their other substrates as well (Huang et al. 2011). Similar studies done in HIP14 and HIP14L deficient mice has strenthened the involvement of mHtt palmitoylation in HD pathogenesis. (Singaraja et al. 2011; Sutton et al. 2013).

Studies on the effect of palmitoylation of Htt on HD using *Drosophila* models are still ongoing. However, the description of *Drosophila* ortholog of HIP14, dHIP14 (CG6017), as a gene controlling synaptogenesis and embryonic motor axon guidance identified as the first mammalian huntingtin-interacting protein (HIP14), encodes a neuronal palmitoyltransferase (PAT) (Kraut et al. 2001).

Another lipid modification that has been reported in Htt is the post-translational myristoylation. However, unlike most of the other PTMs of Htt, myristoylation does not occur on the full-length Htt. The 14-carbon fatty acid, myristate, attaches to the Htt fragment that forms after caspase 3-cleavage. It is the N-terminal glycine residue, Gly553, of the $Htt_{553–588}$ fragment that is myristoylated. Like palmitoylation, in vitro reports indicate that the levels of this modification may be lower in mHtt than in wt-Htt (Martin et al. 2014). Interestingly, myristoylation of the $Htt_{553–588}$ fragment enhances the autophagic flux and increases autophagosome formation. However,

major insights to completely unravel the mechanistic basis of palmitoylation and myristoylation are still warranted.

Ubiquitination and SUMOylation

Ubiquitination is a PTM involves the transfer of a 76-amino acid protein ubiquitin, to lysine residues on a polypeptide chain. The effect of ubiquitination on its target is the degradation of protein by the proteasomal pathway, altering cellular location and promoting or preventing protein interaction (Rock et al. 1994). The binding of a single (monoubiquitination) or multiple ubiquitin molecules (polyubiquitination) to the same target protein, triggers the signal for proteasomal degredation (Wilkinson et al. 1995). The tagging process initiates with the generation of Ub-thiol ester, catalyzed by the ub-activating enzyme E1 by using ATP (Haas and Rose 1982).

The process starts with activation of ubiquitin by a ubiquitin-activating enzyme (E1) and generates a thioester-linked E1 ~ Ub conjugate. The activated Ub is then handed over to a ubiquitin-conjugating enzyme (E2) by a transthiolation reaction. Finally, ubiquitin ligase (E3), which can bind both the substrate and the E2 ~ Ub conjugate, mediates the transfer of Ub onto the ε-amino group of lysine in the target protein, forming an isopeptide bond. E3s have been classified into three families, namely, RING, HECT, and RING between RINGS (RBR). All E2s interact with an E1 enzyme and one or more E3s. In addition, E2s play an important role in the determination of where and how the target protein is modified (Callis 2014).

The ubiquitin-proteasomal pathway involves a cascade of three enzymes that associates the polyubiquitin chain with the proteins to mark them for degradation through the proteasome, a multicatalytic protease complex that degrades the ubiquitinated proteins to small peptides (Baumeister et al. 1998; Glickman and Ciechanover 2002; Pickart 2004). Ubiquitination sites have proven to be very useful in determining cellular states, as ubiquitin on nascent misfolded polypeptides mark disrupted protein synthesis and protein folding. These disruptions play critical roles in several neurodegenerative diseases such as HD, Alzheimer's disease, and Parkinson's disease; thus, these ubiquitination sites play an active role as biomarkers.

Balancing the rate of protein synthesis and degradation in the cells is an essential mechanism and must be maintained (Mitch and Goldberg 1996). To orchestrate this complex cellular balance, a contrasting PTM, SUMOylation, in concert with ubiquitination, occurs on the cellular proteins and balances the protein turnover rate. Over a decade post-discovery of ubiquitination, SUMOylation was discovered as a major PTM (Matunis et al. 1996; Mahajan et al. 1997). SUMO (small ubiquitin-like modifier) proteins are members of the UBL (ubiquitin-like proteins) family and ~11 kDa in size. UBLs share a similar three-dimensional structure with ubiquitin and also function as protein-based modifiers (Kerscher et al. 2006; Van der Veen and Ploegh 2012). Historically, SUMOylation, a lysine-targeted PTM, has been associated solely with the proteins involved in nuclear organization and function (Kamitani et al. 1997). However, subsequent reports strongly suggest it's involvement in several extranuclear cellular processes, modulating properties of the target protein and

thus having a myriad of functional and molecular consequences (Henley et al. 2014). Defective SUMOylation has emerged to as a significant modification in a growing number of neuronal disorders.

Like ubiquitination, SUMO conjugation requires cellular machinery involving activation, conjugation, and ligation. The three-step enzymatic cascade involves ATP-dependent activation of the SUMO precursor protein by the E1 enzyme, transfer of the activated SUMO to the E2 enzyme, and E3-catalyzed conjugation of SUMO to its target protein. Strikingly, a difference in the enzymatic cascade of ubiquitination and SUMOylation is the diversity of the E2 enzyme. Unlike a diverse population of the E2 enzyme in ubiquitination, a sole E2 serves the role in SUMOylation with no substrate specificity. SUMO precursor proteins are present in all the eukaryotes tested to date. Yeast, *Caenorhabditis elegans,* and *Drosophila* express only a single SUMO protein. However, more than one SUMO protein is expressed in other eukaryotes, including higher plants and vertebrates. In the mammalian system, three to four SUMO proteins are expressed, named as SUMO1–4 (Saitoh and Hinchey 2000; Bohren et al. 2004; Johnson 2004; Owerbach et al. 2005).

A number of PTM sites have been identified in the NT17 domain of Htt, and some of these sites are associated with multiple modifications. Ubiquitination and SUMOylation have common putative substrate residues in Htt. In addition, they target the same lysines, K6, 9, and 15 of the NT17 domain that are targeted for acetylation. In vitro reports suggest that SUMOylation facilitates the mHtt accumulation and reduce the levels of visible inclusions, possibly by raising the cellular levels of toxic oligomers (Steffan et al. 2004). On the other hand, ubiquitination of polyQ is reported to trigger the formation of mHtt aggregates (Donaldson et al. 2003). Paradoxically, SUMO modification may mask the cytoplasmic retention signal located in the NT17 domain, thus allowing the nuclear accumulation of mHtt, which is considered a characteristic attribute of mHtt-induced cellular toxicity (Muslin and Xing 2000; Alefantis et al. 2003). Evaluation of the influence of Htt SUMOylation and ubiquitination on HD toxicity using transgenic *Drosophila* revealed that modification of lysines reduces HD pathology, while unmodified lysines produces significant cytotoxicity. These results also suggested that modification of lysines also prohibits ubiquitination despite the fact that HD pathology was significantly reduced due to inhibition of SUMOylation, suggesting SUMOYlation supersedes ubiquitination (Steffan et al. 2004). Using *Drosophila* model the SUMO conjugation pathway have been implicated in other polyQ diseases as well (Chan et al. 2002).

Other studies using human brain samples and a mouse model of other polyQ diseases have also confirmed the SUMO-1's critical involvement in disease pathology (Ueda et al. 2002). Ubiquitination of mutant proteins has been implicated in both abrogation and aggravation of toxicity in several polyQ diseases (Warrick et al. 2005; Al-Ramahi et al. 2006; Adachi et al. 2007; Choi et al. 2007).

In HD, Htt ubiquitination has been reported to be mediated by the ubiquitin ligase E2-25K (Kalchman et al. 1996). E3 ligase, Rhes, on the other hand, mediates mHtt SUMOylation (Subramaniam et al. 2009). However, Rhes has a higher affinity

toward mHtt over wt-Htt. Selective neuronal pathology in the striatum can be attributed to the high expression levels of Rhes in the striatal neurons (Falk et al. 1999).

A recent study points to the importance of mHtt ubiquitination in protein turnover, as K48 and K63 ubiquitin linkages occur in mHtt, and association of p62 with ubiquitinated mHtt increases aiding to the autophagic degradation of the mutant protein. From the therapeutic standpoint, these reports indicate that increased K48/K63 ubiquitination may account for the autophagic clearance of mHtt (Ehrnhoefer et al. 2018). In addition, reports have indicated that the inclusion bodies are dynamic structures and sequester proteins including enzymatically active proteins that are involved in continuous de-ubiquitination and ubiquitination processes (Juenemann et al. 2018).

Proteolytic Cleavage

Proteolytic cleavage is a significant yet underappreciated. It is ubiquitous and irreversible and involves a myriad of proteolytic enzymes. These proteases catalyze the breakdown of proteins into smaller polypeptides or amino acids. Subsequently, neo-N and C termini emerge along with the newly formed fragments of the native protein. These proteolytic processing events often impart novel functions to the neoproteins by activation, inactivation, or excision of protein fragments from a parent molecule. These events, in turn, lead to the regulation of several cellular processes such as DNA replication, cell cycle progression, cell proliferation, and cell death. This also includes pathological processes such as inflammation, cancer, arthritis, cardiovascular disease, and neurodegenerative disorders. Degradomics and terminomics are two recently emerged fields under proteolytic cleavage determined to uncover the biology of proteolysis products and the natural protein N and C termini (Rogers and Overall 2013). To completely comprehend the functions of the proteases, one needs to identify their specific substrates, the cellular role of these substrates, and the processing events. However, various proteases remain to be characterized, as there are no known substrates for them. Proteases form one of the largest enzyme families in humans adding to the complexity of their complete comprehension (Puente et al. 2003).

It was in the 1990s when the concept of generation of smaller and diffusible fragments from a full-length polyQ protein emerged. These smaller fragments were then thought to be responsible for the characteristic aggregation witnessed in the polyQ protein cytotoxicity. This was referred to as the "toxic fragment hypothesis" and is strongly supported by evidence gathered from different murine and fly models since the inception of this idea. However, the corresponding wt-Htt protein also undergoes proteolysis, but the fragments generated are different than the polyQ proteins suggesting that the proteolytic cleavage of the mutant protein is strongly polyQ-dependent. In HD, the striatum and cortex of patients' brain bear intranuclear aggregates of amino-terminal mHtt fragments, and these truncated mHtt forms have been witnessed in the murine model as well (DiFiglia et al. 1997; Kim et al. 2001; Wellington et al. 2002; Landles and Bates 2004). Interestingly, mice

expressing the N-terminal fragment exhibits more severe HD phenotype than the one expressing full-length mHtt. Noticeably, mHtt is cleaved differentially by various proteolytic enzymes such as caspases, calpains, and aspartic endopeptidase. The caspases that cleave mHtt include caspase 2, 3, 6, and 7, and they have their cleavage sites located between amino acid residues 513 and 586 (Wellington et al. 2000; Kim et al. 2001; Hermel et al. 2004). Two types of calpains, that is, calpain 1 and 2, primarily cleave mHtt at sites located between amino acids 469 and 536 (Gafni and Ellerby 2002; Gafni et al. 2004). Some of the other proteolytic sites are at amino acid positions 124, 167, 402, and 437 (Martin et al. 2018). Besides the well-studied N-terminal fragments, C-terminal fragments of mHtt have also been found in postmortem HD brain samples; however, their involvement in HD pathogenesis still needs to be investigated (Mende-Mueller et al. 2001; Landles et al. 2010).

Transgenic *Drosophila* plays a vital role in strengthening the significance of mHtt proteolysis as a crucial determinant of HD toxicity. In a study using the *Drosophila* model, seven well-studied naturally occurring mHtt fragments were analyzed for their ability to confer toxicity, as transgenic *Drosophila* offers the same chromosomal location, controlled genetic background, and expression level. In addition, it eliminates the possibility of having the transgenic peptides get further cleaved in the experimental setting. These N-terminal fragments are 90, 108, 469, 513, 536, 552, and 586. These numbers indicate C-terminal amino acid residues with identical N-terminal of the Htt protein and the polyQ expansion region, where 108 is the product of aspartic endopeptidase cleavage; 469 and 536 fragments produced by calpain cleavage; 513, 552, and 586 are the products of caspase cleavage (Gafni et al. 2004; Graham et al. 2006; Landles et al. 2012). The fragment 90 represents exon 1 and is the product of aberrant splicing of the gene. Interestingly, inclusion bodies could only be detected in smaller fragments like exon 1 and 108 of mHtt in *Drosophila* eye neurons, with exon 1 being the highest aggregation and accumulation prone of all. The assessment of the pathogenic potential of these fragments revealed that the caspase and calpain fragments are relatively non-pathogenic in the *Drosophila* model. Fragment exon 1 and 108 displayed the highest level of toxicity in respective orders and considered a potential contributor of pathogenicity in the *Drosophila* model of HD (Barbaro et al. 2015). Noticeably, results of the *Drosophila* study regarding exon 1 being extremely pathogenic was also displayed in other model systems including mouse, worms, and yeast (Mangiarini et al. 1996; Marsh et al. 2000; Hughes et al. 2001; Steffan et al. 2001; Morley et al. 2002).

Therefore, it is evident that proteolytic processing of mHtt is an essential event in HD pathogenicity and progression, as it could produce a repertoire of smaller fragments out of the full-length protein, each having a distinct property, and contributes differentially to disease progression. However, the reason behind some of the fragments to have a greater pathogenic potential than others is still incomprehensible. In addition, the cascade of proteolytic events of full-length wt-Htt and mHtt and their precise consequences are obscure that further needs thorough investigations.

Conclusion

The nine known neurodegenerative polyQ disorders (HD; spinal and bulbar muscular atrophy (SMBA); dentatorubropallidoluysian atrophy (DRPLA); and spinocerebellar ataxias (SCA) 1, 2, 3, 6, 7, and 17) are all characterized by a polyQ expansion in the respective disease-causing protein (Pennuto et al. 2009). For most of the polyQ-containing proteins, PTMs have been involved that often show similar effects on the disease phenotype (Ehrnhoefer et al. 2011). Taken together, the similarities in PTMs among different polyQ proteins could account for resemblance in disease phenotype such as aggregate formation, symptoms, and transcriptional dysregulation. However, the interplay between different PTMs is likely different in each disorder reflecting the importance of protein context with differential effects on cell type toxicity of the polyQ tract in each disease. In HD, the causative protein Htt gains toxic properties owing to the expansion of the polyQ stretch in the protein. However, the cellular and physiological functions of wt-Htt may be regulated both by alternative splicing and through a multitude of PTMs. These include phosphorylation, acetylation, ubiquitination, SUMOylation, lipid modifications, and proteolysis. Some of these PTMs have been documented to go awry in mHtt which may be a strong contributor to the polyQ toxicity witnessed in the diseased condition. Many of the enzymes catalyzing the PTMs are known, and it can possibly be helpful to identify and test the respective agonists and antagonists in the appropriate in vitro and in vivo disease model system. Thus, the sites of PTMs on the protein and the enzymes involved in the catalysis may provide an excellent starting point for therapeutic intervention.

In view of limitations linked with human genetics, *Drosophila* has emerged as an excellent disease model for the human neurodegenerative disorders due to availability of flexible, yet powerful, genetic tools and owing to the similarity between some of the fundamental cellular processes with human. In addition, to gain mechanistic insights, *Drosophila* has been extensively utilized to screen several genetic and chemical modifiers. Therefore, *Drosophila* offers a great platform to disclose the PTM code of Htt and therefore can potentially pave the way toward designing a novel therapeutic strategy for disease treatment.

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Understanding the Pathogenicity of Noncoding RNA Expansion-Associated Neurodegenerative Disorders

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Abstract

Recent decades of cutting-edge research have unraveled abnormal nucleotide repeat expansions that manifest themselves in the form of neuronal or neuromuscular disorders. Depending on the location of the repeat expansion, which may be either in the coding or in the noncoding region, cells may succumb to death owing to protein toxicity or RNA toxicity or even both. This chapter highlights the anomalies in the noncoding regions, that is, in FXTAS, DM1, DM2, SCA8, and *C9orf72*ALS/FTD. Repeat expansion in the noncoding region poses multitudes of cellular pathogenicities. The repeat expanded transcript forms secondary structures, which may either confer neuroprotection or result in neurodegeneration. The expanded RNA can act as a molecular sink and titrate away RNA-binding proteins. The depleted pool of RNA-binding proteins hinders with important functions like splicing and RNA processing. Alternatively, some repeat expanded RNAs can form into small RNAs (sRNAs) that may result silencing of target-complementary sequences. In addition, expanded repeats may be aberrantly translated to produce short peptides despite lacking a start codon, by a phenomenon known as repeat associated non-ATG (RAN) translation. Faithful animal models and high-resolution molecular techniques have led to a paradigm shift in our understanding of repeat expansion disorders. Toxicity owing to RNA expansion has far more overwhelming implications than that previously perceived. It underlies even classical polyQ dis-

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eases like SCA3. For identifying the pathogenic involvement of coding as well as the non coding RNAs as the critical underlying mechanisms of expansion disorders, *Drosophila melanogaster* can be credited immensely. It has not only helped unravel the underlying molecular mechanisms of the disease pathogenicity but has also provided us with novel avenues for therapeutic interventions. In this chapter, we have highlighted knowledge obtained from the *Drosophila* model in understanding the complex noncoding repeat expansion-associated neurodegenerative disorders. Five major disorders caused by expansion in the non-coding region have been discussed elaborately in this chapter. The readers will be enlightened about the contribution of this tiny fly, not only as an unerring in vivo model but also as a robust tool and platform for therapeutic interventions.

Keywords
Fragile-X syndrome · Fragile-X-associated tremor/ataxia syndrome · Myotonic dystrophy 1 · Myotonic dystrophy 2 · Spinocerebellar ataxia 8 · *C9orf72*ALS/FTD · *Drosophila* models · RNA foci · RAN translation · Alternative splicing

Introduction

For several decades, the notion of RNA as just an inert intermediate persisted. Our knowledge was restricted to its role as a transitional messenger molecule of the central dogma. The discoveries of new classes of RNAs and their associated functions in recent years have brought a huge paradigm shift in our perception of RNAs in the cells (Holt and Schuman 2013). The functioning of a normal cell depends largely on the accurate localization and interpretation of a large repertoire of RNAs, all of which may not always code for protein. These nonprotein-coding or noncoding RNAs (ncRNAs) contain information critical for cellular functions (Mattick and Makunin 2006). A diverse class of noncoding RNAs plays momentous roles in the regulation of gene expression, thereby comprising the gene regulatory networks (Iyengar et al. 2014) that act in a *cis* or *trans* manner. The 5′ and 3′ untranslated regions (UTRs) of mRNA function like an address box for the mRNAs. These noncoding regions are the pivotal *cis*-acting units that regulate mRNA expression. These localization signals are recognized by RNA-binding proteins (RBPs) (Darnell 2013). The RBPs assemble the transcripts and regulate gene expression (Hentze et al. 2018). RBPs bind either directly to the regulatory sequences and coding region of mRNAs or indirectly to the structural motifs in RNA, thereby guiding the transport, localization, and regulation of target mRNAs in the form of ribonucleoproteins (RNPs) or RNA granules (Kiebler and Bassell 2006; Lee and Lykke-Andersen 2013; Chen and Shyu 2014). The broader picture is brilliantly discussed in some recent reviews (Harvey et al. 2018; Hentze et al. 2018). A fraction of ncRNA also acts in *trans* to regulate the stability and expression of mRNAs. This category is best exemplified by miRNAs (Doyle and Tenenbaum 2014). The repression of specific

targets at post-transcriptional levels by miRNAs regulates a plethora of developmentally critical functions (Picao-Osorio et al. 2017; O'Connell et al. 2012; Carrington and Ambros 2003).

Recent scientific progress in the field of neurobiology has established RNA, both coding and noncoding, as a critical player in relaying the extrinsic signal from the microenvironment and mounting a suitable compartmentalized functional response from the neuron. Spatially and temporally distinct signaling domains subdivide neurons into highly dynamic biological compartments (Holt and Schuman 2013). Tightly controlled temporal and spatial localization of RNAs in the subcellular compartments is pivotal for tightly synchronized development, differentiation, specialization, and highly polarized dynamics in metazoan neurons (Martin and Ephrussi 2009). RNA-based gene regulatory mechanisms, mainly directed by ncRNAs, are vividly discussed in some recent reviews (McNeill and Van Vactor 2012; Sharma et al. 2013; Holt and Schuman 2013; Li et al. 2019).

The use of microsatellite markers in linkage analyses for diseases has been a useful tool through the late twentieth century. However, the identification of microsatellite repeat as the causative factor for diseases was a surprise in disease biology. Identification of the increased size of a polymorphic tandem CAG repeat in the coding region of the androgen receptor as the cause of androgen insensitivity associated with Kennedy's Disease (La Spada et al. 1991) and identification of the CGG repeat expansion in the 5′ untranslated region (5′ UTRs) of the *FMR-1* gene as the cause of Fragile X syndrome (Fu et al. 1991) marked the beginning of shift in paradigm. This was followed by the identification of the expanded repeat in the 3′ UTR of myotonic dystrophy 1 gene (*DM1*) (Mahadevan et al. 1992; Brook et al. 1992; Fu et al. 1992). Subsequently, within the next two years, 5 neurodegenerative and neuromuscular diseases, namely, Huntington's disease (HD), spinal and bulbar muscular atrophy (SBMA), myotonic dystrophy (DM1), spinocerebellar ataxia type 1 (SCA1), and dentatorubrapallidoluysian atrophy (DRPLA), were established to be caused by unstable triplet expansions (Plassart and Fontaine 1994). Till date, nearly 40 disease-causing unstable tetra-, penta-, hexa-, and even dodecanucleotide repeat expansions have been identified, housed in both the coding or the noncoding regions of genes (Mirkin 2007; Paulson 2018).

Neurodegenerative disorders are characterized by progressive loss of neurons in the central nervous system and peripheral nervous system. Abnormalities in the RNA processes underlie many neurodegenerative diseases (Belzil et al. 2013). *Drosophila* has contributed immensely to our current knowledge of repeat expanded RNA disorder and has proved to be a robust platform to perform in vivo therapeutic interventions for targeting pathogenic RNA-associated neurodegenerative disorders. In this chapter, neurodegenerative disorders that are largely caused due to repeat expansion in the noncoding region of the gene (noncoding transcripts, 5′ UTR and 3′ UTR) have been addressed. The non-coding expansion mutation manifests itself in the form of depletion of wild-type protein and a gain of function of toxic RNAs (RNA GOF) (Koon and Chan 2017). Figure 1 briefly recapitulates some of these disorders, along with the groups that have generated *Drosophila* models in understanding this unconventional group of disorders. In this chapter, the lessons

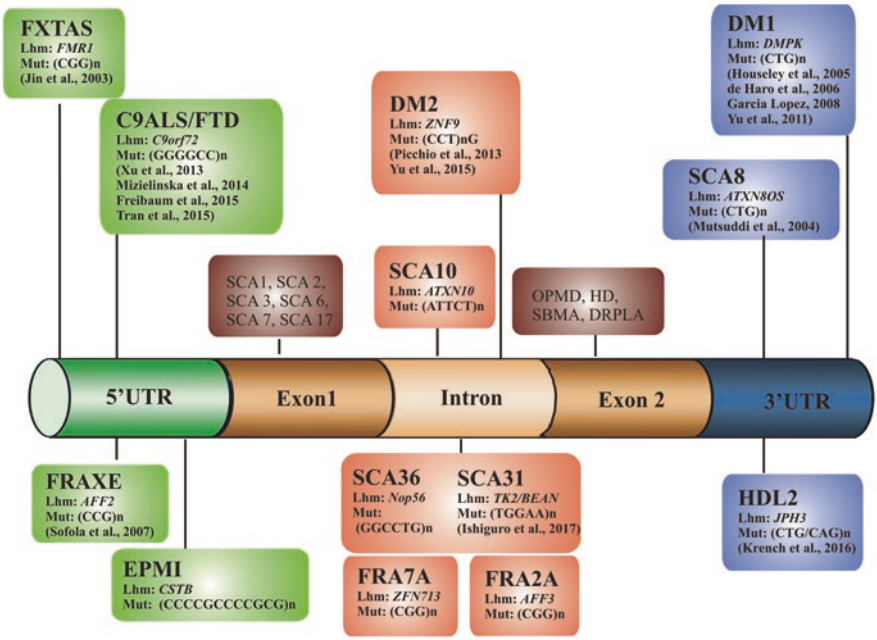

Fig. 1 Schematic representation of a gene with all its coding and noncoding components and associated neurodegenerative disorders. The balloons represent the expansion-associated neurodegenerative disorders placed according to the site of mutant locus within its host gene. Each balloon mentions the name of the disorder, the mutant repeat, the host gene, and publications that established *Drosophila* model for each disease. Abbreviations used – *Lhm* Locus harboring mutation, *Mut* Mutation, *UTR* Untranslated region, *FXTAS* Fragile-X-associated ataxia/tremor syndrome, *ALS/FTD* Amyotrophic lateral sclerosis/frontotemporal dementia, *DM* myotonic dystrophy (*Dystrophia myotonica*), *SCA* spinocerebellar ataxia, *OPMD* Oculopharyngeal muscular dystrophy, *HD* Huntington's disease, *SBMA* Spinal-bulbar muscular atrophy, *DRPLA* Dentatorubralpallidoluysian atrophy, *FRAXE* Fragile-X-E syndrome, *EPMI* Progressive myoclonus epilepsy 1 (*Unverricht–Lundborg Disease*), *FRA7A* CGG expansion at fragile site 7A, *FRA2A* FRA7ACGG expansion at fragile site 2A, *HDL2* Huntington's disease-like 2

learnt from *Drosophila* have been discussed elaborately for five RNA expansion disorders, namely, FXTAS, DM1, DM2, SCA8, and *C9ORF72* ALS/FTD.

FXS and FXTAS (Fragile X Syndrome and Fragile X-Associated Tremor/Ataxia Syndrome)

FMR1 is located on the long arm of the X chromosome. The mutation in the *FMR1* gene results in three clinically distinct disorders, namely fragile X syndrome (FXS), fragile X-associated tremor/ataxia syndrome (FXTAS), and fragile X-associated primary ovarian insufficiency (FXPOI). FXTAS, FXPOI, and almost all cases of

FXS result from the expansion of an unstable CGG repeat tract at the 5′ untranslated region of the *FMR1* transcript (Hayward et al. 2017).

Fragile-X syndrome (FXS), previously known as Martin-Bell syndrome, is the most common form of heritable X-linked intellectual disability, characterized by a spectrum of autistic features, with an incidence of 1 in 1250 males and 1 in 2500 females (Bakker et al. 1994). This X-linked mental retardation is associated with Xq27.3 and is named so because of its linkage to a metaphase chromosome with an isochromatid gap commonly called as a "fragile site" on X-chromosome in the position Xq27.3 (Sutherland 1979; Harrison et al. 1983; Mulligan et al. 1985). In 1991, mutant locus associated with FXS, *FMR-1,* was found to contain a CGG trinucleotide repeat (Verkerk et al. 1991), making it one of the first disorders to be identified that was caused by the expansion of trinucleotide repeats. *FMR1* is a 38 kb long gene encoding a 632-amino acid long Fragile X mental retardation protein (FMRP). The pathogenicity arises due to an abnormal trinucleotide repeat expansion in a noncoding region of the gene, affecting the expression of *FMR1* (Pieretti et al. 1991). The normal repeat range is 5–54 repeats, while more than 200 repeats have been reported in diseased individuals (recently reviewed by Hayward et al. 2017 and Mila et al. 2018). The repeat expansion leads to aberrant silencing of *FMR1* by hypermethylation of CGG repeats in the 5′-CpG island and flanking promoter sequences (Bell et al. 1991; Heitz et al. 1991 and Oberlé et al. 1991) through *FMR1* mRNA, which forms an RNA:DNA duplex in the genome (Colak et al. 2014).

Interestingly, after the identification of the mutation for FXS, the premutation allele of the fragile-X comprising of only 55–200 repeats became a subject of much speculation and research in the early twenty-first century. These premutations expand into full mutation, as they are transmitted through maternal germline. Initially, the carriers of these premutations were thought to be absolutely asymptomatic, just like in other disorders until research findings from Hagerman lab unraveled the curious case of fragile X premutation. The carriers are not asymptomatic. The population of carriers comprise of a subgroup that has some physical features of FXSlike mild cognitive problems, emotional glitches, and radiologically detectable lesions (Hagerman et al. 2001; Jacquemont et al. 2003). Prevalence of the carriers is approximately 1 per 700 men and 1 per 250 women. Unlike FXS, in which trinucleotide expansion leads to transcriptional silencing of *FMR1* and reduced levels of FMRP, the carriers with premutation allele show no abnormalities in *FMR1* expression (Pieretti et al. 1991). Hagerman's group observed that male carriers for the premutation (>100 repeats) had elevated *FMR1* mRNA, despite only mildly reduced FMRP protein (Tassone et al. 2000), suggesting that the symptoms of the carriers are not associated with lower production of FMRP (Hagerman et al. 2001). This increase in the level of mRNA possibly positively correlates with the size of CGG expansion (Greco et al. 2006). Studies like these suggested the involvement of an alternative mechanism at play, which was evidently associated with the *FMR1* transcript and not the protein FMRP.

To dissect out the association of the premutation with the symptoms of the carriers, a group led by Stephen T. Warren created a *Drosophila* model that expresses a portion of normal or premutation-length human *FMR1* 5′ UTR under the control of the

UAS-GAL4 system (Brand and Perrimon 1993). They placed the 90 ribo-CGG (90rCGG) repeats upstream to the EGFP in a *Drosophila* expression vector pUAST and studied its effect on various larval tissues. Interestingly, the model showed degenerative phenotypes, exclusively in the photoreceptors and neurons. The 90 rCGG repeat induced the formation of ubiquitin-positive inclusions, similar to what is seen in the postmortem brains of the male carriers (Greco 2002). The neurodegeneration was seen to be progressive with age, another feature that supports the faithfulness of the model generated. The most interesting outcome of this work was implication of 90 CGG repeats of RNA, which by itself could result in neurodegeneration in a dosage- and repeat-length-dependent manner without the involvement of any mutant FMRP protein (Jin et al. 2003). This work was not only remarkable for the insight that it provided into the pathophysiology of *FMR1* premutation carriers but also first of its kind for experimental demonstration of RNA-mediated neurodegeneration (Magleby 2004).

Thus, the underlying mechanism for disease pathogenesis for FXTAS is different than that of FXS. The latter is caused by a repeat length greater than 200, resulting in loss-of-function of the FMRP protein. On the other hand, FXTAS is mainly due to the untranslated CGG repeat in the range of 55–200 in the 5′ UTR of *FMR1* locus, resulting in RNA toxicity without altering the level or stability of the FMRP protein. It is a late-onset neurodegeneration affecting male carriers of the premutation in or beyond the fifth decade of their life and is characterized by progressive tremor, ataxia, parkinsonism, and cognitive decline.

By 2003, studies had implicated the premutation itself causes premature ovarian failure (POF) among female carriers and tremor/ataxia syndrome (FXTAS) among older male carriers (Hagerman and Hagerman 2002; Oostra and Willemsen 2003).

Using the same fly model of $rCGG_{90}$, Hagerman's group postulated a mechanism underlying the pathophysiology of FXTAS. They hypothesized that rCGG repeat-binding proteins become limiting to the cell, as these proteins are sequestered by the expanded rCGG repeats. In 2007, the group employed biochemical and genetic approaches to report the association of crucial RNA-binding proteins, Purα and hnRNP A2/B1 with premutation-length CGG repeats. Purα physically interacts with the rCGG repeats in a sequence-specific manner. Purα was also shown to be present in the rCGG inclusion bodies both in the fly brain and in the postmortem patient brain samples. Suppression of the pathogenic phenotype was shown by overexpression of Purα that further consolidated their hypothesis. In a nutshell, their findings suggested that Purα plays a crucial role in the pathogenesis of FXTAS (Jin et al. 2007). The role of Purα with FXTAS-associated neurodegeneration was consistent with a report published in 2003, in which Purα null mice showed tremor and gait abnormality, shortly after birth (Khalili et al. 2003).

Parallel to this report, the role of RBPs in the pathogenesis of FXTAS was examined using a *Drosophila* model. A genetic screen for modifiers using candidate genes encoding RNA-binding proteins in combination with *GMR-Gal4*-driven pathogenic *UAS-rCGG$_{90}$-EGFP* led to the identification of CUG-binding protein (CUGBP1) as a suppressor of *rCGG*-mediated neurodegeneration. Furthermore, CUGBP1 was shown to bind to *rCGG* repeat through hnRNP A2/B1 (Sofola et al. 2007a, b).

Using *Drosophila* as a model, the group also showed the premutation allele of *FMR-2,* the gene associated with FRAXE, the most common form of non-syndromic X-linked mental retardation, to result in RNA-mediated neurodegeneration (Sofola et al. 2007a, b).

Identification of miRNAs in the clearance of toxic proteins and their involvement in neuronal functioning further directed research towards the identification of regulatory miRNA for different neurodegenerative disorders, thereby providing therapeutic alternatives. In 2007, another report by Nelson's group demonstrated suppression of the rCGG phenotype by RNA interference in *Drosophila.* The argonaut-mediated RNA degradation pathway was shown to interfere with the pathophysiology of expanded rCGG. It was also proposed and reported that neurodegeneration caused by rCCG as an antisense transcript might also lead to neurodegeneration; however, no human mutations of rCCG expansions in FXTAS have been reported. Nevertheless, it might account for some unknown causes of ataxia (Sofola et al. 2007a, b). The concept of antisense transcription associated with neurodegenerative disease like spinocerebellar ataxia 8 (SCA8) has been discussed elaborately later in this chapter.

By the end of first decade of the twenty-first century, RNA interference strategies to knockdown mutant mRNA and proteins had already been explored for therapeutics of polyQ disorders using in vivo models. A screening for miRNA was performed by Peng Jin's group in 2012 using *elav-Gal4*-driven *UAS-CGG$_{60}$-EGFP* flies. Their screening unraveled nine miRNAs including *miR-277*, which were significantly altered. *miR-277* is known to shorten lifespan in *Drosophila* by metabolic alteration of TOR kinase activity (Esslinger et al. 2013). Using genetic interaction studies, *miR-277* was found to modulate *rCGG$_{90}$*-mediated neurodegeneration in *Drosophila*. Their study also identified downstream targets of *miR-277*, which, in turn, modulated the rCGG-mediated neurodegeneration (Tan et al. 2012). Given the crucial role miRNAs play in development, which includes neurogenesis as well, this study opened up a broader area for therapeutic interventions for these disorders.

Currently, there are two widely known hypotheses for molecular mechanisms underlying RNA repeat expansion-mediated neurodegeneration: RNA gain-of-function (GOF) toxicity and RAN translation producing toxic polyglycine peptide. The intranuclear RNA aggregates, seen in the patient brain as well as in *Drosophila* photoreceptor neurons, not only show sequestration of rCGG-binding proteins but also include proteins that are present in intranuclear inclusions of polyglutamine repeats like Hsp70 (Iwahashi et al. 2006; Greco et al. 2006). HL Paulson's group demonstrated repeat-associated non-AUG (RAN)-initiated translation triggered by CGG repeats, leading to polyglycine-containing peptides, FMRpolyG, in the *Drosophila* model *rCGG$_{90}$*, as well as in FXTAS patient postmortem brain samples. The toxicity of *rCGG$_{90}$* in *Drosophila* is largely due to FMRpolyG production. The group proposed that alternate use of a close match initiation codon at 5′ of the CGG repeat permitted by a stalled 43S ribosome initiation complex trigger the generation of polyG from at least two of the three reading frames available. However, each reading frame may have a different mechanism of translation (Todd et al. 2013; Reddy and Pearson 2013).

In addition to the above mechanisms, the pathophysiology of FXTAS has also been associated with antisense *FMR1* RNA (Ladd et al. 2007; Mila et al. 2018), mitochondrial dysfunction (Ross-Inta et al. 2010; Hukema et al. 2014; Alvarez-Mora et al. 2017), and R-loop-induced DNA damage response (Loomis et al. 2014). Unfortunately, despite so much of enriched knowledge on the molecular mechanism underlying the disease, so far there is no cure for this disorder (Kong et al. 2017). Hopefully, in the near future, we might get closer to therapeutic intervention.

Myotonic Dystrophy (Type I and Type II)

Myotonic dystrophy is a common autosomal dominant disorder with a worldwide incidence of 1 in 8000. The earliest cases of *Dystrophia myotonica* (DM) was reported more than a century ago, with the description of patients with severe myopathies, along with cataract and atrophy of the testicles. Dr. W. J. Aide recollects the earliest evidences of the DM to have appeared as early as 1886, which then was addressed as "atypical Thomsen's disease" by medical practitioners (Aide 1922). In 1909, Steinert and Batten-Gibbs independently reported pioneer cases of muscular atrophy, which they termed as *Myotonia atrophica* (Batten and Gibb 1909; Steinert 1909). DM is a multisystemic disorder, with seemingly unrelated symptoms, like myotonia and progressive muscle weakness, along with ocular, central nervous system, cardiovascular, and respiratory abnormalities. Among all the symptoms, respiratory abnormalities cause most of the lethality. DM exhibits genetic anticipation, that is, as the disorder is transmitted from one generation to the other, with increase in repeat length, decrease in the age of onset, and increase in the severity (Fleiseher 1918; Höweler et al. 1989). Contributions by clinical neurologists and geneticists not only determined the non-Mendelian pattern of inheritance of this disease but also showed variable penetrance and maternal transmission bias in the congenital form (Harley et al. 1993). The mutation underlying the disorder was identified in 1992 on the chromosome 19q13.3 (Jansen et al. 1992; Aslanidis et al. 1992) as a trinucleotide CTG repeat within the 3′ untranslated region of the DMPK gene (Mahadevan et al. 1992; Fu et al. 1992). This CTG repeat was found to be transcribed but not translated in the heart, muscles, and brain (Brook et al. 1992). Ribonuclear foci detected in DM patient fibroblasts and muscle biopsies included *DMPK* transcripts with expanded CUG repeats (Taneja et al. 1995). Interestingly, this CTG repeat region resides in the CpG island, which becomes hypermethylated when expanded. This hypermethylation results in the spread of heterochromatin region and reduction in expression of the *SIX5* gene located downstream to the CpG island (Cho et al. 2005; Filippova et al. 2001; Klesert et al. 1997; Klesert et al. 2000; Yanovsky-Dagan et al. 2015).

Around 1994, a new form of DM was reported. This alternate form shared many symptoms of the classical DM but still had certain distinct characteristics that were unique and different from myotonia congenita, paramyotonia congenita, and DM. Initially, the report described the unique clinical condition of weakness in the pelvic girdle of the patients as proximal myotonic myopathy or PROMM. The patients had nonspecific, mild myopathies; however, unlike classical DM, the

patients did not report problems in mastication, swallowing, or respiration, nor apparent muscle wasting or cardiac abnormality were observed. Strikingly, in these patients, the length of the CTG region in the only DM locus known at that time was comparable to that of the normal population. This report laid the foundation of a genetically novel disorder associated with the development of late-onset myotonia, with mild proximal leg weakness and occasional cataract (Ricker et al. 1994; Ricker et al. 1995), that is presently known as myotonic dystrophy type 2 (DM2). Shortly following the clinical characterization, the second myotonic dystrophy locus was identified as a tetra-nucleotide CCTG expansion in the intronic region of the *ZNF9* gene on chromosome 3q21 (Ranum et al. 1998; Day et al. 1999) and was found to be associated with PROMM pathophysiology (Ricker et al. 1999).

Although the pathophysiology of the PROMM or DM2 is more benign than that of DM1, the length of expansion is much larger, the average repeat number being 5000 (Liquori et al. 2001). The similarity of molecular and clinical features between DM1 and DM2 raised the possibility of involvement of the toxic RNA itself in manifesting the two multisystemic defects, since the muations causing DM1 and DM2 did not have any functional correlation with each other and the only common thread between them was repeat expanded transcripts.

The mutations associated with DM1 are located at the 3′ end of the DMPK gene on chromosome 19. However, the loss-of-function or gain-of-function studies of DMPK in mice were not conclusive of its implication in the pathogenesis of the disorder (Reddy et al. 1996; Jansen et al. 1996; Hamshere and Brook 1996). However, the mutation in *warts,* the *Drosophila* homolog of DMPK, could be correlated with epithelial and other tumors like neuroendocrine, neurofibromatosis, and endocrine adenomas reported in DM1 patients and their asymptomatic relatives, thereby advocating the role of heterozygous mutation in the DM kinase gene in the development of these tumors (Justice et al. 1995). Such inconclusive association of the DMPK protein further raised the possibility that the mutant RNA toxicity may underlie the disease pathogenesis.

Swanson's group identified triplet repeat expansion RNA-binding factors, which are titrated away by the secondary structure formed by $(CUG)_n$ and $(CCUG)_n$ expansion that aggregates in the form of RNA foci (Mankodi et al. 2001). Among all of these mediator proteins, some of them are very important for normal development. The most notable RNA-binding proteins recruited in the toxic ribonuclear foci include those of the muscleblind-like (MBNL) family and CUG RNA-binding protein (CUGBP) family with ETR-3-like factor (also known as CELF), which play a vital role in normal RNA function like splicing, polyadenylation, stabilization, and translation (Mateos-Aierdi et al. 2015). Specifically, the abnormal sequestration of MBNL and stabilization of CELF ultimately leads to alternative splicing of many different premessenger RNAs, which results in multisystemic abnormalities. For example, splicing deregulation of Dystrobrevin 1, Dystrophin, CLCN1, BIN1, and RYR1 contributes to the development of muscle weakness and myotonia, whereas aberrant splicing of troponin T and Tau may lead to the cardiac and CNS manifestations, respectively (Gourdon and Meola 2017; Sansone 2016; Nakamori et al. 2007; Nakamori et al. 2013).

MBNL1 plays a role in stabilizing the RNA aggregates; however, it is not absolutely indispensable for the formation of foci (Querido et al. 2011). The role of the MBNL protein, as a major modulator of DM1 pathogenesis, was further supported by a number of reports highlighting the importance of this protein in maintenance of muscle homeostasis and functions like splicing (Ho et al. 2004; Machuca-Tzili et al. 2006; Wang et al. 2012; Llamusi et al. 2013). In the past two decades, a number of therapeutic alternatives have been reported in DM1, but blocking the interaction between the toxic CUG expansion RNA and MBNL is the hotbed for therapeutic intervention. *Drosophila* muscleblind (Mbl), an ortholog of the human muscleblind-like proteins (MBNLs), is critical for the differentiation of photoreceptor and muscle cells, thereby implicating sequestration of muscleblind as one of the leading causes of DM1 pathogenesis (Miller 2000; Mankodi et al. 2001).

Drosophila mutants of many RNA binding proteins (RBPs) like *warts, Bruno,* and *SIX* families of genes show phenotypic similarity to the clinical symptoms of DM patients. These studies further strengthened the importance of *Drosophila* as a reliable and accurate model for studying *Dystrophia myotonica*. The human homologs of these genes are important RNA-binding proteins that are found to be involved in the DM1 pathogenesis (Winchester et al. 1999; Good et al. 2000; Harris et al. 2000; Kirby et al. 2001). More information of the role played by RBPs in neurodegenerative disorders will be discussed in the next chapter.

To explore the role of toxic RNA in DM, a *Drosophila* model was created by Monckton's group, which had CTG expansion in the 3′ UTR of a reporter gene. $(CUG)_{162}$ forms RNA foci that colocalizes with muscleblind, a paralleling phenotype seen in DM1 patients (Houseley et al. 2005). Another *Drosophila* model, UAS-i $(CUG)_{480}$, expressing noncoding mRNA containing 480 interrupted CUG repeats, demonstrated several aspects of DM1 pathology when expressed in the developing photoreceptor neurons or muscles like the nuclear accumulation of CUG transcripts, muscle wasting, degeneration, splicing misregulation, and diminished muscleblind function in vivo. The pathology in the UAS-i$(CUG)_{480}$ fly model was found to be associated with a decreased level of Muscleblind (Mbl). Their data demonstrated the titration of Mbl from the soluble fraction of the cells with CUG expanded repeats (de Haro et al. 2006; Vicente-Crespo et al. 2008). The degenerative phenotype of DM flies could be rescued by overexpression of Mbl. This model caught the spotlight of the therapeutic platform owing to its faithful reflection of a number of clinical features associated with the disease (see Fig. 2).

Since then, a number of path-breaking findings have been unraveled using this model. The disorganized photoreceptor phenotype and semi-lethality due to a targeted expression of $i(CUG)_{480}$ served as a background for screening genetic and chemical modifiers. A study led to the identification of 15 genetic and 10 chemical modifiers of CUG-induced toxicity (Garcia-Lopez et al. 2008). Using the same model, a large-scale peptide library screening was carried out, which led to the identification of a D-amino acid hexapeptide (ABP1). ABP1 suppressed the formation of RNA foci and splicing defects due to MBNL sequestration and also rescued CUG-induced degeneration in brain and eye, as well as muscle wasting. The rescue

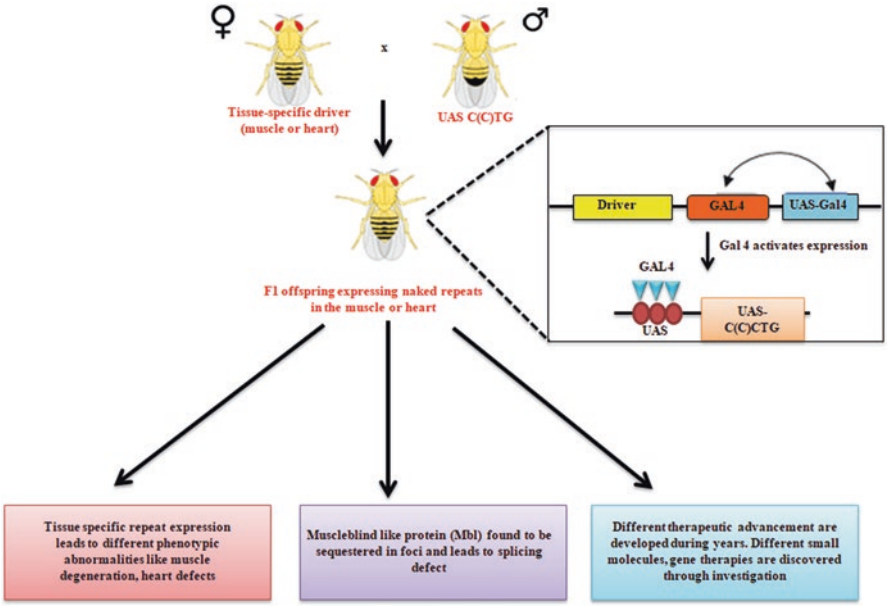

Fig. 2 Gal4-UAS system used to express transgenes in the *Drosophila* tissue. Different Gal4 flies are crossed with UAS strains carrying different lengths of repeats to drive the repeat expression in the F1 offspring. The F1 offspring flies showed disease-related important phenotypes upon repeat expression. Muscle degeneration, heart defects, Muscleblind (Mbl) sequestration splicing defect

could be achieved by both oral administration and transgenic expression in the fly and DM1 mice model. Both in vitro and in vivo studies suggested that the hexapeptide causes a shift in the conformation of the secondary structure of the expanded CUG RNA, thereby restoring the homeostasis of the cells in spite of expression of pathogenetic RNA (García-lópez et al. 2011).

One of the several attributes of using *Drosophila* as a model for the disorder is the highly amenable bipartite gene activation systems. Using the $i(CUG)_{480}$ *Drosophila* model, heat shock-induced expression of expanded RNA facilitated the study of adult muscle atrophy eliminating the interference of the developmental role of this expanded RNA. This inducible model revealed apoptosis and autophagy-associated genes that were upregulated in DM1 flies. Overexpression of mTOR and DIAP1, as well as miRNA-mediated silencing of autophagy-associated genes rescued the muscle wastage in the fly model. The heat shock inducible model of *Drosophila* helps in distinctly studying the adult-onset DM1 toxicity. The results were validated using skeletal muscle biopsies from DM1 patients (Bargiela et al. 2015).

The $i(CUG)_{480}$ model of *Drosophila* has also provided a robust platform for in vivo drug testing for DM1. In order to deal with a disorder like myotonic

dystrophy that operates through multiple molecular pathways, small-ligand molecules have been developed that can target the disorder at multiple levels (Gonzalez et al. 2017). Using in vitro approach, a multi-target ligand was designed that could combat the disorder at three levels, inhibiting transcription of CUG_{exp} and MBNL1 sequestration and hydrolytically degrade the $CUG_{exp.}$ One of the ligands was also tested for its potency in the $(iCUG)_{480}$ DM1 model of *Drosophila* and was shown to rescue the degenerating photoreceptor neurons in adult flies as well as motor function of crawling larvae (Nguyen et al. 2015). Similarly, another potent inhibitor of protein sequestration, developed recently by the same group, was also tested on the model (Luu et al. 2016).

The cardiac abnormalities, which are one of the most critical features of DM1, have also been studied in *Drosophila* (Chakraborty et al. 2015; Cerro-Herreros et al. 2017). GMH5-Gal4 was used to drive pure expanded 250 CUG repeats in cardiomyocytes that mimicked pathogenetic features of DM1, like, ribonuclear foci formation, muscleblind sequestration, and several other cardiac abnormalities associated with DM1. This model was used for in vivo drug discovery, by rescuing of the defective cardiac phenotypes by pentamidine, a potent suppressor of DM1 repeats (Warf et al. 2009), thus releasing MBNL1 bound to CUG and reduce ribonuclear foci formation in *Drosophila* heart (Chakraborty et al. 2015). Recently, drug daunorubicin was also demonstrated to rescue the cardiac phenotype by the same group (Chakraborty et al. 2018). Mbl overexpression in the fly heart improved lifespan and cardiac dysfunctions in DM1 model flies (Chakraborty et al. 2018). These results have shown that overexpression of muscleblind by compounds without affecting the CUG repeats can be an alternative therapeutic strategy, where the degree of titration of muscleblind-like proteins can affect the dynamics of the CUG-MBNL complex.

Several *Drosophila* models for DM1 have been developed by different groups working in this area; however, *Drosophila* model for DM2 is a relatively new addition; Bonini's group in 2014 generated transformant expressing 700 pure, uninterrupted tetranculeotide CCUG-repeat expansion, recapitulating the mutations underlying DM2 (Yu et al. 2015). When driven with *GMR,* Gal4 shows mild reduction in MBNL levels. Another model expressing $(CCUG)_{106}$ indicated the involvement of apoptosis in DM2. This model developed by Bergmann's group was also shown to be a potent in vivo model for drug screening (Yenigun et al. 2017). The DM2 model expressing pure uninterrupted $CCUG_{1100}$ repeats revealed in vivo toxicity similar to that of the DM1 model expressing CUG_{250} uninterrupted repeats including toxicity in muscle and cardiac functions (Cerro-Herreros et al. 2017), and this was quite surprising, as the severity of DM2 is less than that of DM1. It was shown that the effect of expression of 1100 uninterrupted repeats was like that of DM1. A genetic modifier screen for factors that quenched the toxicity in DM2 was carried out using the same fly model. A recent publication has shed additional insight into the molecular pathogenesis of DM2. It has been depicted that an RNA-binding protein, rbFOX1, partly release the sequestered MBNL1 from CCUG expansions, as it competes with MBNL1 to bind to the expanded repeats, consequently ectopic expression of rbFOX1 corrects the alternative splicing defects and

muscle atrophy in the DM2 [*UAS-CCTG(1100)x)*] model of *Drosophila* but not in the DM1 (*UAS-CTG(250)*x_x) model (Sellier et al. 2018), suggesting some distinct mechanisms underlying these two disorders.

Membrane dSERCA, which is also an Mbl splice target, was demonstrated to rescue DM1 induced hypercontractility in a larval model of DM1 (Picchio et al. 2013). Similarly, genetic screening of RNA-binding proteins in the i$(CUG)_{480}$ fly model, unraveled Smaug to be a strong suppressor of toxic phenotype by restoring the impaired function of CUGBP1 (de Haro et al. 2013). Both of these modulators have close homologs in humans, making these potent candidates for therapeutic intervention in DM1. Elevated expression of a member of the CUGBP1/CELF1 family of proteins, Bruno3, has been recently associated with reduced myofiber length and myoblast fusion in a $DM1_{960}$ model of *Drosophila* (Picchio et al. 2018).

In order to facilitate research on therapeutic interventions, luciferase reporter-based spliceosensor flies were generated that exploited the splicing defects that are well established for this disorder. It has been shown before that alternative splicing of cardiac troponin T (cTNT) and insulin receptor (IR) is regulated by the MBNL protein (Ho et al. 2004). Based on this knowledge, transgenic lines of *Drosophila* expressing luciferase-coupled MBNL1 splicing targets, called *INSR* minigene, were generated to quantify splicing dysregulation seen in DM1. This immensely robust, sensitive, and high-throughput technology was utilized for large-scale screening of chemical modulators of DM1 pathogenesis, challenging the, usually practiced "brute force" in vitro drug testing platforms. Thus, powerful *Drosophila*-based screening tools led to the identification of several splicing modulators, which were also found to ameliorate other pathogenic features of DM1 like foci formation and reduced lifespan (García-Alcover et al. 2014). This highly versatile yet simple tool has taken *Drosophila* model to an astounding new level of in vivo drug discovery platform, which could be useful, not only for DM but also potentially expandable for other disorders associated with splicing defects.

In the past decade, the therapeutic role of MBNL1 and CUGBP1 and their associated targets in DM1 pathogenesis have been well established and worked out; nevertheless, CUG repeat toxicity, independent of Mbl titration, has been reported sparsely in fly models (Picchio et al. 2013). In the past decade, as the concept of titration of critical factors like MBNL by toxic RNA expansion was gaining a stronghold, the implications of RNA interference, and bidirectional transcription was also getting unraveled. The role of the antisense CAG repeat transcript of the DM1 locus in restricting the H3-K9 methylation of the CTG repeats in wild-type DM1 locus was previously reported by Tapscott's group (Cho et al. 2005). In order to understand the role of antisense RNA interference in DM1 pathology, Nancy Bonini's group generated a fly model that co-expressed CAG repeat transcripts along with CTG repeat transcript. Strikingly, not only did they observe the generation of triplet-derived siRNA, but they also observed elevated toxicity as compared to the pure uninterrupted CTG expansion induced toxicity. Their observation was completely in contradiction to the observations reported for rCGG/rCCG repeats in

the FXTAS model of *Drosophila,* discussed at the beginning of this chapter. The group proposed that the bidirectional transcription of the repeat region adds additional complexities to the pathogenesis of DM1 (Yu et al. 2011). Role of RNA interference in the pathology of DM1 was further consolidated with the identification of miRNAs that are altered in the muscles of the DM1 fly model, thereby implicating the role of CTG expansion in miRNA dysregulation associated with the disease. Remarkably, defects in some of these miRNAs, *miR-1, miR-7* and *miR-10*, were found to be conserved, even in muscle biopsies from DM1 patients. The DM1 fly model was not only used to establish a direct link between CTG toxicity and miRNA dysregulation but also demonstrated the therapeutic potential of miRNA (Fernandez-Costa et al. 2013).

The role of miRNA in therapeutics was further explored by Ruben Artero's group, as they developed micro RNA sponge, *miR-'X'SP* (Fulga et al. 2015).*UAS-miR-'X'SP* driven by *Mhc-Gal4* sequesters the micro RNAs, *dme-miR-277*, and *dme-miR-304*, which are involved in the regulation of *muscleblind* RNA isoforms. This elevates the level of Mbl expression in the control and DM1 *Drosophila* model. Enhancing muscleblind expression through miRNA sponges, direct a novel approach to rescuing pathogenic phenotypes and molecular defects associated with DM1 that is effective right at the level of gene expression (Cerro-Herreros et al. 2016).

The line of distinction between the role of repeat expansion in the coding region leading to protein gain-of-function versus that in the noncoding region leading to RNA gain-of-function started to blur with the identification of a novel molecular mechanism called RAN translation, repeat-associated non-ATG-initiated translation. Laura Ranum's group demonstrated RAN translation in DM1 expansion transcripts in a DM1 mouse model and in human tissues, that leads accumulation of polyglutamine expansion proteins. Similar observations were also made for SCA8, which will be discussed in the next section (Zu et al. 2011). Poly-LPAC and poly-QAGR tetrapeptide were also reported to be found in association with DM2 expanded CCTG·CAGG transcripts in a patient's brain biopsies. It was hypothesized that, possibly, at a certain threshold, RNA expansion exceeds the sequestration capacity by RNA-binding proteins; consequently, the expanded transcripts are exported to the cytoplasm, where these undergo RAN translation (Zu et al. 2017). RAN translation has also been demonstrated in *Drosophila* SCA31 disease model (Ishiguro et al. 2017).

After decades of research, *Drosophila* has not only provided insight into the molecular mechanisms of DM1 and DM2 disorders but also proven to be a robust platform for drug testing and discovery. Most of the therapeutic interventions have been centered around releasing the sequestered MBNL1 from the expanded CUG transcripts by depleting the CUG expansion transcripts, interfering with the interaction of toxic transcripts with MBNL1, where viral vector-mediated hU7-snRNA-CAG expression, hammerhead RNA ribozyme, antisense oligonucleotides, or small ligands have been used. However, as of now, there is no treatment available for this disorder (López-Morató et al. 2018).

Spinocerebellar Ataxia 8

Ataxias are characterized by slowly progressive neurological symptoms associated with lack of voluntary coordination of muscle movements like maintaining gait, walking, speech, and eye movements. However, shortening of life is not usually witnessed (Ayhan et al. 2014). They can be dominantly or recessively inherited. Commonly seen spinocerebellar ataxias are the dominantly inherited ataxias, resulting from atrophy of the cerebellum, affecting about 0.3–2 people per million globally. Till date, about 31 Spinocerebellar Ataxias (SCAs) have been identified (Wang et al. 2018; Sullivan et al. 2019). Spinocerebellar ataxia 8 (SCA8) was the first SCA identified to be caused by a locus encoding a noncoding RNA mapped at 13q21 (Koob et al. 1999). The clinical feature that distinguishes SCA8 from other SCAs is the extreme speech problem and word production at an early stage and a severe progressive truncal titubation. Severe atrophy is seen in the vermis and hemispheres of the cerebellum. SCA8 is further characterized by extreme maternal bias and variable penetrance (Koob et al. 1999; Day et al. 1999; Silveira et al. 2000). Initially, pathogenic SCA8 was found to be ranging from 107 to 127 CTG repeats as against 74–94 CTG repeats found in unaffected individuals; however, the pathogenic expansions vary among different affected families (Koob et al. 1999; Day et al. 1999; Juvonen et al. 2000). Strikingly, the length of the CTG repeat expansion can be correlated with neither the disease severity nor the age of onset (Day et al. 1999). At times, very long expanded CTG repeats (>250) have been found to be nonpathogenic, whereas some affected individuals were found to have relatively shorter expansions (Juvonen et al. 2000; Silveira et al. 2000). The distinction between a pathogenic and nonpathogenic stretch of repeats is blur for this disorder. Recently, a pathogenic CTA/CTG expansion of just 51 repeats was reported in China, which showed a strong linkage with the SCA8 locus (Wang et al. 2018); hence, the length of the shortest full-penetrant allele remains elusive and an inconclusive basis for diagnosis (Sobrido et al. 2001; Roda et al. 2017).

The molecular aspects of the disorder were first described by Laura Ranum's group. The intergenerational instability of the expansion and variable penetrance of the disorder clouded the strong linkage between the expanded locus and the disorder. To implicate the expansion as the underlying mutation, the technique of RAPID cloning (repeat analysis pooled isolation and detection) of individual clones containing the expanded trinucleotide repeats was carried out (Koob et al. 1998). Clones of novel CTG expansion mutation from the genomic DNA of a single affected individual were isolated. Interestingly, a complete absence of any ORF in the locus was observed; thus making SCA8 the first SCA identified to be caused by a nontranslated stretch of repeats (Koob et al. 1999). To prove the pathogenicity of the expanded SCA8 allele, a mouse model expressing a BAC clone harboring the human mutant SCA8 expansion was generated. Further characterization revealed the presence of exons but no ORFs in any of the splice variants, suggesting similarities with the DM1 pathogenic CTG expansions (Koob et al. 1999). It was the second example of a pathogenic CTG expansion disorder to be reported after DM1. But unlike DM, its symptoms are not multisystemic. SCA8 is usually a late-onset,

gradually progressive neurodegenerative disorder, characterized by ataxia, which is not demonstrated in DM, whereas the CNS abnormalities in DM, like cognitive impairment and sleep disorders, are not manifested in SCA8 (Day et al. 1999). The genetic studies were further complicated by the occurrence of polymorphic CTA repeat interruptions in the CTG expansions, which, upon replication, gives rise to alleles that are variable not only in their lengths but also in their sequence configurations (Day et al. 1999). This possibly plays a role in the somatic mosaicism and meiotic instability of the locus in both expanded and normal alleles (Silveira et al. 2000). However, upon identification of the SCA8 locus, it was hard to pin down the underlying molecular cause of the difference between the clinical manifestations of the two noncoding CTG expansion disorders. Whether the pathophysiological difference seen in the two disorders owes partly to the widespread expression of the mutant DM allele as against restricted expression of the mutant SCA8 allele, remains an elusive question.

Noncoding SCA8 transcripts overlap with the transcription and translation start site of a sense strand gene, which was found to have putative actin-organizing domain named *Kelch-like-1 (KLHL1)*. Strikingly the gene was found to be localized primarily in the cerebellar region, the region most susceptible to SCA8 expansion toxicity (Nemes et al. 2000). This finding suggested the possibility of interference by the expanded SCA8 locus with the regulation and translation of *KLHL1*. However, the involvement of this gene in cerebellar function remains uncertain (Mandrile et al. 2016).

Laura Ranum's group developed a mouse model with CTG_{116} repeats that demonstrated progressive neurological phenotypes. Their work established the formation of intranuclear polyglutamine inclusion bodies in the cerebellar Purkinje and brainstem neurons in the mice model and SCA8 patient tissue. The polyQ was found to be encoded by CAG repeats in a novel antiparallel transcript, Ataxin 8 (*ATXN8*), complementary to the CTG repeat on the opposite strand. Thereafter, the SCA8 pathogenic repeat allele has been called ATXN8 opposite strand (*ATXN8OS*). The SCA8 pathogenesis occurs because of toxic gain-of-function at the protein as well as the RNA levels, involving *ATXN8* encoding a nearly pure polyglutamine protein and *ATXN8OS* encoding a noncoding transcript (Moseley et al. 2006). This report highlighted the importance of mutation on both strands in microsatellite disorders. The mechanism of bidirectional transcription was also reported for myotonic dystrophy (Cho et al. 2005), thereby aligning the two disorders on similar lines of underlying pathomechanisms. Bi-directional transcription can be a plausible mechanism to explain the somatic instability associated with SCA8-associated neurodegeneration (Nakamori et al. 2011).

With the emerging role of SCA8 as the antisense strand, the mechanism underlying SCA8-mediated pathogenesis became an area of intense research and debate. To probe into the subject, we developed a *Drosophila* model expressing the human SCA8 noncoding RNA. Four exons were placed under the UAS element, generating transgenic flies expressing non-pathogenic SCA8 $(CTG)_9$ and pathogenic SCA8 $(CTG)_{112}$. The pathogenic CTG repeat configuration used for generating the flies is $CTA_3CTG_5CCG_3CTG_{112}$ from a patient whose ataxia was demonstrated to be

genetically linked to SCA8 locus. Expression of pathogenic transcripts with *GMR*-GAL4 led to disorganized ommatidia and mechanosensory bristles in the adult fly. Age-related progressive neurodegeneration was observed in the photoreceptor neurons. The flies expressing pathogenic constructs faithfully exhibited some of the characteristic features associated with the disorder like adult onset of the neurodegeneration, variable penetrance, and age-dependent-progression (Mutsuddi et al. 2004). Utilizing the model, we also demonstrated that the localization of the SCA8 transcript does not alter with the expansion of the repeats. This model served as a sensitized background for screening genetic modifiers of SCA8-associated neurodegeneration, which lead to the identification of *staufen, muscleblind, split ends,* and *yu/spoonbill.* Interestingly, all of these are neuronally expressed RNA-binding proteins. The pathogenic SCA8 expansion phenotype deteriorated with the loss of function of *mbl*, suggesting a correlation between the molecular mechanisms underlying the two CTG expansion neurodegenerations known at that time, myotonic dystrophy, and spinocerebellar ataxia 8. The proof of principle for our *Drosophila* model was demonstrated as mbl, which was identified from our screen, later reported to be co-localized with pathogenic SCA8 transcripts in histological sections obtained from postmortem SCA8 brain samples (Daughters et al. 2009). We demonstrated that SCA8-sequestered Staufen is an RNA-binding protein required for transport of neuronal RNA. As expected, polyQ inclusion bodies were not observed in our fly model, as the expression of bidirectional transcripts was absent; however, RNA foci formation was observed both in the nucleus and in the cytoplasm, which paralleled RNA foci localization in postmortem brain sample of SCA8 patients. This SCA8 model demonstrated the disease phenotype faithfully and thus could be used for identification of genetic modifiers of the disease. In an attempt to understand the *modus operandi* of these modulators of SCA8, Spoonbill was expressed in wing muscle and photoreceptors by direct physical interaction with the expanded allele through its KH domain using the same *Drosophila* SCA8 model (Tripathi et al. 2016). Spoonbill is a putative PKAAP protein involved in neuronal cell fate determination and oocyte development and has been implicated in a multitude of neuronal functions in large-scale screens (Hadad et al. 2011; Tripathi et al. 2017). KH RNA-binding domain could deplete the pathogenic SCA8 ribonuclear foci and thus suppress the neurodegenerative phenotype in flies.

We hypothesized that pathogenicity of the expanded repeats owes to the accumulation of the transcript, instead of the length of the transcripts. These transcripts titrate away critical RNA-binding proteins or factors that are required for maintaining cell homeostasis (Mutsuddi et al. 2004; Mutsuddi and Rebay 2005). The hypothesis was further substantiated by Laura Ranum's group who generated the mice model of SCA8, which demonstrated co-localization of MBNL with polyglutamine inclusion bodies (Daughters et al. 2009).

RAN translation from the bidirectionally transcribed strands *ATXN8S* and *ATXN8OS* RNA has been reported to produce short stretches of amino acids like polyglycine, polyserine, and polyalanine in the absence of a start codon; however, their biological function remains elusive (Zu et al. 2011; Chen et al. 2013). Recently, Laura Ranum's group reported the presence of directly translated polyGln and a

RAN-translated polyAla to be present in the RNA foci of SCA8. In addition, poly-Ser aggregates in the white matter, leading to age-progressive demyelination and axonal degeneration in SCA8 (Ayhan et al. 2018).

The therapeutic intervention has not been yet achieved for the disorder; however, the KH domain obtained in our study has strong potential in ameliorating the pathogenicity. Either by the decay of the transcripts or by altering the secondary structure of the transcripts, the KH domain mediates suppression of pathogenic SCA8.

ALS/FTD *C9orf72*

Frontotemporal dementia (FTD) represents a multitude of neurodegenerative disorders that are associated with frontotemporal lobar degeneration. It is characterized by early-onset dementia, similar to Alzheimer's disease. The earliest reports of the clinical cases date back to the late nineteenth century. In 1911, Pick's Bodies (named after Arnold Pick, who described one of the earliest cases of FTD) were identified by Alois Alzheimer, as distinct spherical inclusions that are TAU protein positive. This feature marked the histopathological hallmark of this non-Alzheimer's dementia, also called as Pick's disease or dementia of frontal lobe type. Since then, a multitude of FTD cases without Pick's Bodies have been identified, making Pick's disease just a subgroup of a frontotemporal dementia in the present day.

ALS is the third most common neuronal disorder (described in detail in chapter "Understanding Motor Disorders Using Flies") and possibly the most dreaded one. Progressive paralysis leading to death from respiratory malfunctioning within two to three years of the onset of the symptoms has been reported. Amyotrophic lateral sclerosis (ALS) is largely associated with loss of muscle mass and degeneration of the neural track running down both sides of the spinal cord. This disease is characterized by the presence of scar tissue-like appearance of the spinal cord after degeneration. ALS was previously called Charcot's Disease, after the French neurologist Jean-Martin Charcot, who first described the disorder in 1869. The demise of the great Stephen Hawkins recently reverberated the horror of this disorder in our minds. He succumbed to death after fighting with a rare form of slow-progressing ALS for more than four decades.

In 1981, reports started surfacing that described the ALS patients showing symptoms of FTD, ALS-associated neuropathology along with frontal and temporal degeneration, and degeneration of sub-substantia nigra. Neuropathologically, ubiquitin-positive and tau- and alpha-synuclein-negative inclusions became a combined hallmark for ALS as well as FTD (Ferrari et al. 2011). Similarly, in 1987, FTD patients also demonstrated some morphological aspects of dementia-ALS. Currently, it is well reported that the patients of both the disorders share some of the symptoms (Ferrari et al. 2011).

Finally, in 2006, TDP-43 (TAR DNA-binding protein) was shown to be the component protein in the ubiquitinated neuritic inclusions, hallmarks of both FTD and ALS. In the affected central nervous system, the pathological TDP-43 was found not only to be ubiquitinated but also to be highly phosphorylated. Accumulation of

TDP-43 in the inclusions specific to sporadic and familial FTD and sporadic ALS suggested the possibility of the diseases to be collectively considered as proteinopathies of TDP-43 (Neumann et al. 2008; Arai et al. 2006).

By the end of the first decade of the twenty-first century, a number of genetic mutations had been identified for ALS and FTD, like *SOD1*, *TARDBP* (TDP-43), optineurin (*OPTN*), valosin-containing protein (*VCP*), and fused in sarcoma *(FUS)*. However, the treatments effective in *SOD1* mouse models have not been successful with ALS clinical trials, suggesting the presence of alternate mechanisms at play for TDP-43 proteinopathies (DeJesus-Hernandez et al. 2011). The association of the two disorders with noncoding RNA came into light in the year 2011. The October edition of the journal *Neuron* in the year 2011 resonated with exciting reports implicating an expanded hexanucleotide repeat in 9p21-associated ALS and FTD. Bryan J. Traynor's group reported the role of a locus *p21* on the short arm of chromosome 9, identified from a genome-wide association study of ALS in Finland (Laaksovirta et al. 2010), reverberating another report implicating the locus in sporadic cases of ALS (Van Es et al. 2009). The group studied the genetic lesion that accounts for 9p21-associated ALS and FTD. Their study led to the identification of a hexanucleotide repeat GGGGCC expansion in the first intron of the transcripts of *C9orf72,* with pathogenic lesions spanning more than 30 repeats (Renton et al. 2011). Parallel to this, another group lead by Ian Mackenzie and Rosa Rademakers reported the presence of the hexanucleotide in the noncoding region that leads to missplicing of *C9orf72* transcript along with the formation of RNA foci in TDP-43 proteinopathies (DeJesus-Hernandez et al. 2011).

Identification of this mutation highlights the fact that the functionality and stability of noncoding RNAs are stringently regulated by *cis* sequences. These mutations are the major loci responsible for the disorders, that is, in ~40% familial and ~8% sporadic ALS cases, highlighting the role of mutant RNA in the pathogenesis of neurodegenerative disorders (Orr 2011).

The exact biochemical function of the protein coded by *C9orf72* is largely unknown. The possible mechanisms that were suspected for the pathogenicity of this disorder included loss of function of the C9ORF72 protein or gain of function of expanded *C9orf72* RNA. On the one hand, *C9orf72* null mice was shown to develop age-related inflammatory sensitivity; on the other hand, *C9ORF72* was not absolutely critical for motor functions. These observations made the pathogenic contribution of loss-of-function of C9ORF72 protein, obscure (Freibaum and Taylor 2017). Identification of the ability of *C9ORF72* to be able to form a G-quadruplex structure supports the hypothesis of foci formation and sequestration of crucial RNA-binding proteins, thereby advocating the RNA gain-of-function hypothesis (Fratta et al. 2012).

The expanded *C9orf72* RNA gain-of-function hypothesis gained pace with the identification of various RNA-binding proteins that were critical in the ALS-FTD pathogenesis. In a *Drosophila* model developed by Peng Jin's group, $(rGGGGCC)_{30}$ repeats were shown to be sufficient to cause neurodegeneration. The forward genetics approach helped in unraveling purα as the major RNA-binding protein to get sequestered by the expanded RNA, also forming inclusions in fly eye as well as

patient cerebellum. The protein interacts with the RNA in a sequence-specific manner. The overexpression of purα mitigates $(rGGGGCC)_{30}$ repeats-associated neurodegeneration not only in *Drosophila* but also in mammalian neuronal cells (Xu et al. 2013).

Another pathomechanism underlying *C9orf72* was identified as the unusual peptide formation with the help of RAN translation of the sense and antisense strands of the *C9orf72*. Dipeptide repeats (DPRs) were shown to undergo translation from the sense as well as the antisense strands of the expanded repeat at six reading frames giving rise to five distinct DPRs GA, GR, PA, PR, and GP (Freibaum and Taylor 2017). However, the mechanisms of RBP sequestration by expanded RNA remains nonexclusive to RAN translation explanation.

To understand the role of DPRs and RAN translation in disease pathogenesis, Paul Taylor's group generated flies expressing 8, 28, or 58 GGGGCC repeat-containing transcripts using phiC31 integrase-based site-specific insertion into the *Drosophila* genome. In order to detect the repeat-associated RAN translation, their model had the UAS sequence preceding the repeat sequences and a GFP in the reading frame; however, the repeats as well as GFP did not have a start codon. This RAN translation *Drosophila* model demonstrated dosage- and repeat length-dependent degeneration in neuronal tissues when driven using *GMR-Gal4*. Expression of $(GGGGCC)_{58}$ in the motor neuron using *OK371-Gal4* resulted in significant impairment of locomotor activity. Similar defects were also seen when pathogenic GGGGCC repeats were expressed in the neuromuscular junctions, muscles, and neuronal tissues. An unbiased thorough screening revealed 18 genetic modifiers that were involved in nuclear export of RNA and nucleocytoplasmic transport of proteins, thereby implicating nuclear RNA retention and compromised nucleocytoplasmic transport as pathogenic processes involved in ALS-FTD. In this model, RAN translations were observed for longer pathogenic stretches of repeats. In an attempt to understand the role of DPRs in driving neurodegeneration, flies were generated that directly expressed AUG-poly(GA), poly(GR), or poly(GP) with an N-terminal GFP. Poly$(GR)_{50}$-GFP was shown to be highly lethal for *Drosophila,* and poly$(GA)_{50}$-GFP elicited a degenerative eye phenotype, thereby providing another plausible mechanism by which $(GGGGCC)_{58}$ poses neuronal toxicity in addition to the toxic RNA produced (Freibaum et al. 2015). Similar results were also obtained by another group that created a *Drosophila* overexpression model with pure repeats that could translate into DPRs compared to their flies with "RNA-only" repeats that had stopped codon interruptions in all the frames without interfering with the tertiary structure of G-quadruplex (Mizielinska et al. 2014). A *Drosophila* model expressing dipeptide $(GR)_{80}$-FLAG has also been shown to downregulate Notch signaling in *Drosophila* (Yang et al. 2015).

With deeper understanding of pathogenic disorders, a neuroprotective role of RNA foci is surfacing up. The expanded G_4C_2 repeats form nuclear foci prevent toxic RNA to migrate into the cytoplasm and translate into DPRs. This hypothesis got a strong foothold with the study in *Drosophila.* Fen-Biao's group generated fly models expressing 160 G_4C_2 repeats flanked by intronic sequences. This long spliced intronic repeat transcript formed nuclear foci abundantly. Interestingly, it

failed to elicit any neuronal toxicity or RNA processing defect. However, a much shorter 36 G_4C_2 repeat was transported to the cytoplasm and translated to produce DPR abundantly, which proved to be highly toxic. The levels of DPR produced by the shorter repeat was almost 100-fold higher than that produced by intronic 160 G_4C_2 repeats (Tran et al. 2015). Unlike initial speculations, now it is well understood that long expanded sense and antisense RNAs of *c9orf72* are by themselves not toxic to *Drosophila* neurons; rather possibly these RNAs reduce DPR production, thereby conferring the cells with some extent of neuroprotection (Moens et al. 2018). However, the tertiary structures formed by these expanded RNAs can still prove to be a critical therapeutic endpoint. Recently, a screening of previously described chemotypes revealed some small molecules that interfered with pathogenic interactions of the expanded RNA with RBPs and/or RAN translation. Small molecules binding to the G-quadruplex structure ameliorate C9ORF72 pathology in the *Drosophila* model and iPS neurons from patients, and their lack of an off-target effect makes them a promising solution to this fatal disease (Simone et al. 2017; Schludi and Edbauer 2017).

The mechanism of DPR-mediated toxicity in the affected and the nearby cells has become a topic of intense research (Westergard et al. 2016). The ALS fly models were extensively utilized to understand the mechanism of DPR pathogenicity. Another group generated expression constructs with ATG-mediated translation of single DPRs in a way that the transcripts generated did not form the secondary structure. This approach helped to study the pathogenic contribution of the DPRs without the confounding factors of RNA toxicity and RAN translation. Utilizing this faithful model for DPRs that reverberates the previous observations of DPR-mediated toxicity in adult flies and motor neurons, a screening was performed using previously described 55 nucleocytoplasmic transport genes (Jovičič et al. 2015). This screening revealed 15 enhancers and four suppressors of $(PR)_{25}$ toxicity (Boeynaems et al. 2016). Another elegant observation about DPRs came into light with transcriptome analysis of GFP conjugated 47 or 50 DPR proteins expressed in HEK293T cells. The screening revealed that arginine-rich DPRs interact with RNA-binding proteins and low-complexity sequence domains that mediate assembly or themselves are components of membraneless organelles such as nucleoli, Cajal bodies, P-bodies, and stress granules, which exist like phased-out condensed liquid cellular matrix (Gomes and Shorter 2018). A total of 126 interacting partners were screened in the in vivo orthologous approach followed in the GFP-GR_{50} expressing flies. A total of 84.9% of the interactors were found to be functionally significant, as these genetically modulated the DPR-mediated toxicity. The robustness of *Drosophila* for such screen can be assessed by the sheer proportion of modulators identified. Thirty-five of the total suppressors (28%) identified in the human cell lines proved to increase viability to greater than 70% of GFP-GR_{50}-expressing flies. Additionally, it has been demonstrated that DPR, GRs, and PRs modulate physical dynamics of the membraneless organelles, and phase transition of TDP-43, FUS, and hnRNP that contribute to the DPR-mediated toxicity, leading to widespread cellular abnormality in ALS/FTD (Lee et al. 2016). The toxicity of arginine-rich DPRs was recently found to exclusively affect the glutamatergic neurons, including

the motor neurons in *Drosophila*. An interesting study of DPRs with different toxicities tested in different populations of neurons revealed that GR/PR repeats as short as 36 repeats were sufficient to result in motor defects in *Drosophila.* This study provided a critical site for pharmacological interventions, as inhibition of vesicular glutamate transporter effectively rescued the degenerated motor functions in *Drosophila,* by restoring the elevated extracellular glutamate levels (Xu and Xu 2018).

To shed more light on the relationship between TDP-43 and C9orf72 repeat mediated pathogenicity, a recent publication reported that accumulation of GGGGCC-derived repeat peptides, but not the expanded RNA, resulted in TDP-43 dysfunction and karyopherin-α2/4 pathology (Solomon et al. 2018). Modulation by the other RNA-binding proteins FMRP and Orb2 were also seen using the *Drosophila* model for C9orf72 neurodegeneration. The model also implicated the transport granule dysfunction as an additional mechanism for pathogenicity (Burguete et al. 2015). (For more information on TDP43 and ALS see Chap. 13)

ALS *c9orf72* fly models showed insight into not only the disease mechanisms but also RNA metabolism (Zhang et al. 2018a). The amenable genetics of this versatile model made the study of the complex mechanism easier to unravel. *C9orf72*-associated ALS-FTD is possibly a consequence of a number of parallel process. Kudos to this fly, cause now we can implicate the relative contribution of different mechanisms without the confounding effect of the other.

Conclusion

Microsatellite expansion neurodegenerations are a unique set of disorders that may have common or different pathogenic mechanisms. A high degree of clinical overlap has been witnessed despite different proteins or polypeptides being implicated in each case. Recently, a study identified *ATXN8OS* to harbor a mutation in patients diagnosed with ALS in Japanese population. These patients did not have mutation in *C9orf72*. This report brought into light another occasional symptomatic manifestation of *ATXN8OS* mutation associated with upper and lower motor neurons (Hirano et al. 2018). In this scenario, *Drosophila* models have proved to be the Pandora's box, that help us understand the relative contributions of overlapping complex mechanisms. Pathogenicity for several disorders like HD and some SCAs had been classically contributed to polypeptide expansion. However, with a deeper understanding of mechanisms underlying such diseases, aberrant RNA intermediates have also been accounted for some level of pathogenicity. SCA3-associated neurodegeneration is caused by CAG repeat expansion in the *Ataxin-3* gene. Classically, the pathogenicity was attributed to polyQ alone (Paulson et al. 1997). However, in 2008, the *Drosophila* model of SCA3 showed that both translated and untranslated CAG repeats resulted in neurodegeneration (Li et al. 2008).

Drosophila genetics has also helped us in understanding the crosstalk between pathological cellular processes for several disorders. Spinocerebellar ataxias make up a group of around 30 disorders that have overlapping clinical features but are

caused by distinct genetic loci (Wang et al. 2011b). SCA12 is a relatively rare neurodegeneration. The trinucleotide repeat expansion in the 5′ UTR region of *ppp2R2B*, a gene enriched in the neurons, has been reported (Holmes et al. 1999); however, the CAG trinucleotide repeat expansion does not code for polyQ. The encoded protein Bβ2 is located on the outer membrane of mitochondria, and elevated levels are associated with mitochondrial fission (Wang et al. 2011b). The role of the mammalian *ppp2r2b* during development is unclear; however, *Twins,* the *Drosophila* homologue of *ppp2r2b*, is associated with numerous cellular functions like mitosis, cell fate determination, and circadian rhythms (Uemura et al. 1993; Sathyanarayanan et al. 2004; Bajpai 2004; Wang et al. 2011a). To get a clearer understanding of the contribution of the mutation in disease pathogenesis, a *Drosophila* model ectopically overexpressing the gene was created by Ting Chou's group. Neuronal apoptosis and shortened lifespan, as seen in the patients, was faithfully reflected in the animal model. Using this model, it was reported that elevated dSOD2 levels by antioxidant treatment not just alleviated the degenerative phenotypes but also improved the lifespan of the flies (Wang et al. 2011b). This report brought an interesting involvement of mitochondrial disintegration as one of the critical causes of SCA12 pathogenesis. Recently, another *Drosophila* model, spinocerebellar ataxia, was developed by Nagai and Ishikawa's group. Ectopic expression of the pathogenic repeat expansion of UGGAA in *Drosophila*, the repeat expansion that leads to SCA31, revealed interesting findings. The fly model not only demonstrated neurodegeneration but also showed accumulation of RNA foci and RAN translated pentapeptide repeat proteins. Strikingly, motor neuron disease-associated RNA-binding proteins, FUS, hnRNPA2B1, and TDP-43, resulted in the proper folding of the expanded RNA and regulated RAN translation through some structural alterations of the $UGGAA_{exp}$ RNA. Their work not only brought insight into the pathophysiology of SCA31 but also established "crosstalk" of underlying mechanisms between RNA expansion disorders and RNA-binding proteins-associated proteinopathies (Ishiguro et al. 2017; Jackson 2017).

The expanded RNA can form a wide array of secondary and tertiary structures (Fig. 3) ranging from sRNA, ds hairpin, and G-quadruplex structures, thereby interfering with numerous nonexclusive cellular processes as mentioned in previous sections. The ability to form ribonuclear foci has been witnessed in all dominantly inherited repeat expansion-associated neuronal disorders. However, their instability dynamics or neuroprotective role has always been a topic of intense debate (Wojciechowska and Krzyzosiak 2011; Zhang and Ashizawa 2017). CAG repeats like CUG repeats are fully capable of forming ribonuclear foci and sequestering the critical RNA-binding proteins like MBNL1 and thus affects alternate splicing; however, some difference in the preference of substrate pre mRNA suggests distinct *modus operandi*. With deeper understanding of molecular toxicity of classic polyQ disorders, it is now clear that the RNA intermediates play a crucial role in the pathogenesis of these neurodegenerative disorders by nucleolar stress, RNA processing, and RNA export mechanisms (Tsoi and Chan 2013; Koon and Chan 2017; Zhang et al. 2018b); trigger RAN translation (Banez-Coronel et al. 2015); or even induce innate immune response (Samaraweera et al. 2013; Richards et al. 2013).

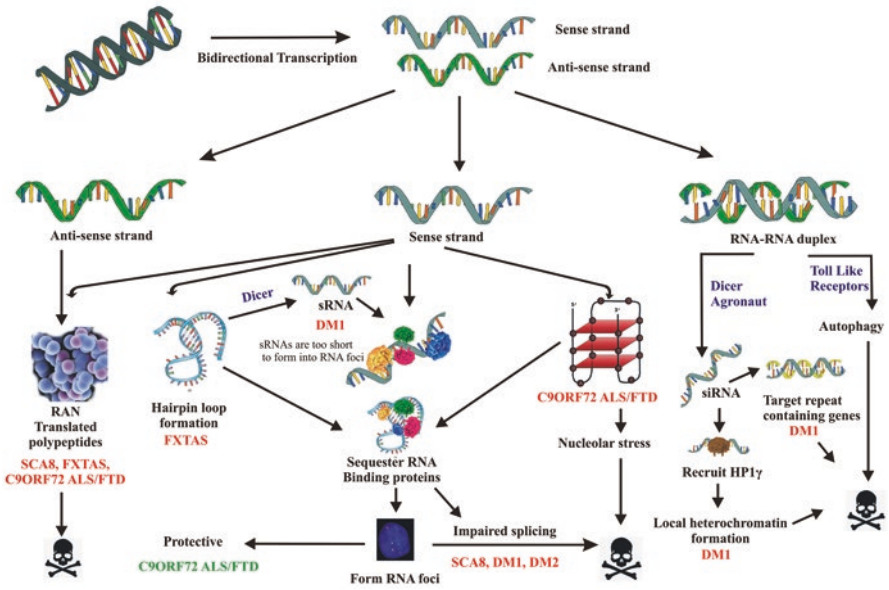

Fig. 3 Possible RNA toxicity mechanisms underlying repeat expansion-associated neurodegenerative disorders caused primarily by RNA expansion. Repeat expansion mutations occur at the genome level. The affected locus may undergo sense and antisense transcription. The sense or the antisense strand of the transcript, may give rise to small RAN-translated peptides as seen in the case of SCA8, FXTAS, DM, and *C9orf72*ALS/FTD. RAN-translated products are detrimental for the cell, as they result in nucleolar stress and destabilize the dynamics of membraneless organelles. The sense strand of transcript is versatile in it's mode of toxicity. It has the potentiality to sequester RNA-binding proteins in its native form, in its hairpin structure, or in its tertiary form of G-quadruplex. RBPs along with other interacting partners result in RNA foci formation, which are usually toxic to the cells during later stages of neurodegeneration as seen in most of the repeat expansion disorders. Hairpin loop of single-stranded RNA can give rise to short RNAs (sRNAs) by Dicer. These short RNAs are too short to form RNA foci; however, these can sequester the RNA-binding proteins and impair the splicing machinery as seen in DM1. G-quadruplex structures are seen in C9orf72ALS/FTD, which may either directly recruit nucleolin (NCL) and induce nucleolar stress or sequester RNA-binding proteins. However, in this case, some reports suggest a neuroprotective role of these RNA foci. The bidirectional transcription products may also form RNA:RNA duplex. These duplexes gives rise to short siRNAs that may target CAG-containing genes like *Atx2* and *TATA-Binding Protein* in case of DM1. Another alternative fate of these siRNAs is also seen in DM1 pathogenesis in which the siRNA recruits the heterochromatin protein 1 (HP1γ), resulting in local silencing of a gene. In DM1, autophagy is also seen. In a recent report, TLR genes have been shown to get altered and result in autophagy due to expression of double-stranded CTG repeat transcripts, suggesting a possible mechanism underlying DM1-associated autophagy

Steady understanding of the widespread contribution of RNA toxicity in neurodegenerations and a certain level of overlapping mechanisms between repeat expansion disorders have led to the development of therapeutic strategies targeting expanded RNA. Recently, a short 13-amino acid peptide P3 and polyQ-binding protein were shown to effectively mitigate both RNA and protein toxicities in the *Drosophila* model of HD (Zhang et al. 2016). Currently, three basic approaches are

followed, namely, oligonucleotide-based therapeutics, peptide-based therapeutics, and small-molecule-based therapeutics (Koon and Chan 2017). In the current scenario, *pan*-acting drugs are better alternatives. *Drosophila* provides a versatile platform for therapeutic interventions. It would not be an overstatement to say that this tiny fruit fly can prove to be a torchbearer for future therapeutic interventions. The research involving therapeutic breakthroughs depends largely on mice models, patient samples, or transformed cell lines; however, crucial knowledge like the role of miRNA sponges in alleviating DM pathophysiology has been unraveled using the *Drosophila* model of the disease (Cerro-Herreros et al. 2016). For widely studied disorders like myotonic dystrophy, a multitude of RNA-based therapeutic alternatives have been worked out. Antisense oligonucleotides that act like molecular cutters or blockers, RNAi-based approach, or antago-miRs that mainly culminate into the release of sequestered RNA-binding proteins hold promising therapeutic alternatives (Overby et al. 2018). One such antisense oligonucleotide-based drug has found its way to clinical trials (ClinicalTrials.gov identifier: NCT02312011). This success should be a source of motivation for researchers all over the world to utilize RNA-associated interventions coupled with robust drug delivery technologies to alleviate neurodegenerative disorders.

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The Expanding Role of RNA-Binding Proteins in Neurodegeneration

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Abstract

In eukaryotic cells, gene expression is regulated at various levels after generation of a primary RNA transcript, including mRNA processing, transport, stability, and co-and post-transcriptional regulation. These processes are tightly controlled by the action of a multitude of RNA-binding proteins (RBPs). As soon as an RNA is transcribed, RBPs regulate the RNA at every step, starting from processing up to its final degradation. RNA processing plays a fundamental role in regulating multiple events during nervous system development. So far, RBPs have been shown to be important for neurogenesis, neurite outgrowth, maintaining neural stem cells, synapse formation, and plasticity. In addition, studies have depicted that several neurological diseases are associated with deregulated genes involved in RNA metabolism. Moreover, alterations in RNA-binding proteins are associated with many neurodegenerative disorders such as amyotrophic lateral sclerosis (ALS), fragile X syndrome (FXS), spinal muscular atrophy (SMA), and many others. *Drosophila* has been one of the best model organisms to understand neurodegeneration at the molecular level. In this chapter, we report the use of *Drosophila* in comprehending recent advances that link RBPs with neurodegenerative processes. This will help in advancing our knowledge as to how RBP dysfunction contributes to neurological diseases.

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Ribonucleoproteins · RNA toxicity · Proteinopathies · Transport granules · Processing bodies

Introduction

RNA-binding proteins (RBPs) are involved in all aspects of the RNA life cycle and have diverse roles, starting from transcription, post-transcription editing, splicing, ribosome biogenesis, export, translation, and processing, to finally its degradation. An important component of all these regulations is exerted through interaction with RNA-binding proteins that recognize RNA and form a ribonucleoprotein complex (RNP). However, some RBPs show transient interaction with RNA, whereas others bind to RNA until its degradation. These RBPs are further classified according to domains that include a wide variety of RNA-binding motifs that recognize specific RNA sequences. On the basis of RNA-binding domains (RBDs), RBPs are grouped into four families: RNA recognition motif (RRM), zinc-finger domain (ZnF), K-homology domain (KH), and double-stranded RNA-binding motif (dsRBM) (Lukong et al. 2008; Lunde et al. 2007; Glisovic et al. 2008). A number of studies from the last decade show that RBPs may contain more than one kind of RNA-binding domain, suggesting its diverse function in RNA metabolism. In addition, auxiliary domains and flanking regions associated with the core RNA-binding domains alter the binding and assembly of RBP with RNA (Rudolph and Klostermeier 2015). The term "RNA-binding protein" can be misleading, as these proteins can sometimes bind to RNA as well as DNA and affect a large number of biological processes. Many of these RBPs form extensive protein-RNA or protein-protein interactions, providing a number of permutations and combinations that affect different cellular processes spatially and temporally. RBPs are expressed during nervous system development; further mutation studies have shown that several RBPs play a role in the asymmetric division of neuroblasts, cell fate determination, and neurogenesis (Broadus et al. 1998; Sakakibara et al. 2002). In addition, defects in the functioning of RBPs have been often linked to a broad spectrum of neurodegenerative disease (Lunde et al. 2007).

Neurodegenerative diseases as diverse as Parkinson's disease, Alzheimer's disease, Huntington's disease, spinal muscular atrophy, amyotrophic lateral sclerosis, and spinocerebellar ataxias are characterized by neuronal damage, which progresses over time. The majority of these diseases share a common mechanism involving accumulation and deposition of the misfolded protein that forms aggregates, which is a hallmark of neurodegenerative diseases as discussed in earlier chapters; for example, α-synuclein in Parkinson's disease (PD), huntingtin protein in Huntington's disease (HD), amyloid-β (Aβ) in Alzheimer's disease (AD), transactive response DNA-binding protein 43 (TDP-43) in frontotemporal dementia (FTD) and amyotrophic lateral sclerosis (ALS), and ataxin in spinocerebellar ataxia (Skovronsky et al. 2006). Recent findings have shown the role of abnormal RNA processing and misregulation of RNA-binding proteins, which leads to neurodegenerative diseases.

There are numerous RBPs that have been found to be aggregated in different neurodegenerative diseases (Maziuk et al. 2017). In this chapter, we shall discuss how these RBPs are linked with neurodegenerative diseases. It has been reported that mutation in genes encoding RBPs leads to the abnormal production of a protein that affects RNA metabolism and leads to neurodegeneration. The discovery of TDP-43 (TAR DNA-binding protein) that has been classified as an RNA-binding protein highlighted the significance of RNA metabolism in diseases. TDP-43 regulates different processes like transcriptional repression of the HIV-1 genome by binding to the double-stranded TAR DNA sequence motif through its RRM domain, pre-mRNA splicing of cystic fibrosis transmembrane conductance regulator (CFTR), microRNA processing through interaction with Drosha, and transport of mRNA to dendrites and its translation (Ou et al. 1995; Freibaum et al. 2010; Buratti and Baralle 2001; Gregory et al. 2004). In addition, RBP TDP-43 plays an important role in ubiquitination and hyperphosphorylation, and its mislocalization from the nucleus to cytoplasm is associated with cytoplasmic inclusions, a common characteristic associated with amyotrophic lateral sclerosis (ALS) and frontotemporal lobar degeneration (FTLD) (Liu-Yesucevitz et al. 2014; Neumann et al. 2006). Mutations in RBPs are associated with a variety of neurodegenerative diseases, and this is discussed in detail in this chapter. An RNA-binding protein, ELAV4, a Hu family of protein, is associated with the age-at-onset (AAO) trait in Parkinson's disease (Noureddine et al. 2005). Alzheimer's is associated with abnormal accumulation of the tau protein, which promotes stress granules. TIA1, an RNA-binding protein that co-localizes with these tau proteins, enhances neurodegeneration (Vanderweyde et al. 2016). In Huntington, FUS/TLS, an RNA-binding protein, physically interacts with mutant Htt aggregates and enhances the pathogenesis of the disease (Doi et al. 2010).

In earlier chapters, we have seen how *Drosophila* has aided in understanding of the molecular basis of neurodegenerative diseases. In this chapter too, we shall discuss how fruit fly genetics has provided us an insight into the involvement of various RBPs and its molecular pathways in causing neurodegeneration. Specifically, we will discuss the role of RBPs in RNA biogenesis and its contribution to neurodegeneration. This chapter deals with examples of RBPs, where alteration in the genes encoding for RNA-binding proteins is associated with neurodegeneration (Table 1).

RNA-Binding Proteins (RBPs)

RNA assembles with proteins forming dynamic complexes, so-called ribonucleoproteins (RNPs). These RNA-binding proteins are involved in each and every step of RNA metabolism. Most of these RBPs were discovered over the last three decades and are composed of small RNA-binding domains (RBDs), which specifically bind to RNA targets. These RBPs belong to different RBD families, namely, RNA recognition motifs (RRMs), zinc-fingers, KH domains, DEAD-Box, Pumillio, and double-stranded RNA-binding motifs (dsRBMs) (Cléry and Allain 2012).

The RNA recognition motifs (RRMs) family, also known as RNA-binding domain (RBD)-containing proteins or ribonucleoprotein (RNP) domain, is the most abundant

Table 1 Selected RBPs associated with neurological diseases

Human RNA-binding protein	Neurological disease	Pathological alterations	Comments	*Drosophila* ortholog	Reference
Nova1-2	Paraneoplastic opsoclonus myoclonus ataxia (POMA)	Ectopic expression; autoantibody production	Synaptic proteins; neuronal inhibition	*Pasilla* (*ps*)	Buckanovich et al. (1993), Ule et al. (2003), Seshaiah et al. (2001)
Elav2-4/Hu	Paraneoplastic subacute sensory neuropathy syndrome	Ectopic expression; autoantibody production	Nuclear mRNA splicing; translation repression; amino acid biosynthesis; synaptic cytoskeletal dynamics; putatively involved in neurogenesis	*Elav* (embryonic lethal, abnormal vision)	Dalmau et al. (1992), Ince-Dunn et al. (2012), Myer et al. (1997), Gamberi et al. (2006)
Rbfox3 (NeuN)	Mental retardation; epilepsy; autism	Mutation	Transmission; membrane excitability alternative splicing; polyadenylation	*Rbfox1* RNA-binding Fox protein-1	Jin et al. (2003), Barnby et al. (2005), Yeo et al. (2009)
Ptbp2		Mutation	Expression in subsets of glial cells and in mitotic neuronal progenitors; translation repressor activity	*dmPTB/hephaestus*	Licatalosi et al. (2012), Heimiller et al. (2014)
QKI quaking	Psychiatric diseases; schizophrenia; ataxia	Reduction of expression	Myelination in CNS; vascular development; stability; translation; alternative splicing; localization	*QKI*	Hardy (1998), Lauriat et al. (2008), Hafner et al. (2010)

FMRP	Fragile X syndrome	Repeat expansion mutation	Presynaptic proteins; postsynaptic proteins; translation; transport; stability	*FMRP*	Darnell (2010), Yu et al. (1992), Brown et al. (2001)
TDP-43	Amyotrophic lateral sclerosis; frontotemporal lobar dementia	Mutation; cytoplasmic aggregate	Neuronal development; neuron survival; synaptic transmission; alternative splicing; microRNA biogenesis	*TBPH*	Neumann et al. (2006), Mackenzie et al. (2010), Lerga et al. (2001)
Fus	Amyotrophic lateral sclerosis; frontotemporal lobar dementia	Mutation; cytoplasmic aggregate	Neuronal impulse; neuronal projection; transcription; alternative splicing; transport; gene silencing	*Cabeza (Caz)/Fus*	Mackenzie et al. (2010), Vance et al. (2009)
MBNL1–3	Myotonic dystrophy	Repeat expansion	Neural differentiation; alternative splicing	*Muscleblind (mbl)*	Kanadia et al. (2003), Charizanis et al. (2012), Irion (2012)
DDX59	Orofaciodigital syndrome; microcephaly; intellectual disability; epilepsy	Loss-of-function mutation	Enriched in oligodendrocytes	*mahe (maheshvara)*	Surabhi et al. (2015), Salpietro et al. (2018)

RBD in higher vertebrates. A significant number of proteins house RRM domains and are involved in RNA processing and transport. Interestingly, RRM domains are capable of physically interacting with DNA and protein. Proteins containing RRM domain include hnRNP proteins (A1, A2/B1, and C1/C2), spliceosomal proteins (U1A, U1, 70k, and U2B″), nucleolin, U2 auxiliary factor (U2AF), ELAV, and poly(A)-binding proteins (Maris et al. 2005). The zinc finger domain (ZnF) family, although known to primarily interact with DNA, has been now shown to interact with RNA as well. Examples of proteins having ZnF domains are TFIIIA, a transcription factor involved in the transcription of eukaryotic ribosomal 5S RNA, and MBNL1 (Muscleblind-like 1), a tissue-specific alternative splicing regulator that promotes muscle differentiation (Cléry and Allain 2012). The hnRNP K homology (KH) domain is present in a number of proteins such as DDX53, FMR1, NOVA1, NOVA2, PCBP1-4, QKI, and SF1. The dsRBM's main function is to bind double-stranded RNA (dsRNA). This family of proteins is involved in a variety of biological processes implicated in nuclear import and export, as well as cytoplasmic and nuclear retention. Staufen, Dicer, and Drosha are examples of proteins having dsRBD. The DEAD-box family is involved in pre-mRNA splicing, translation initiation, and miRNA biogenesis. A majority of them that belong to the DEAD box family are RNA helicases, eIF4A, Ded1, p68/p72, and DDX59. Proteins in the RGG box domains bind to G-quartet motif and regulate mRNA and rRNA biogenesis. FMRP, nucleolin, and EWS are the examples of RNA-binding proteins having RGG box domain. PUM-H (Pumilio-homology) domain plays a role in mRNA stability and translation, and examples are Pum1 and Pum2 (Albert and Darnell 2004; Cléry and Allain 2012).

RBPs affect the life of RNA from its genesis until its degradation (Glisovic et al. 2008). Moreover, RBPs regulate the post-transcriptional fate of mRNA and affect splicing, editing, localization, and translation. Extensive studies have revealed the delicate regulatory network of neurogenesis, among which neurons have their own system for regulating RNAs. Several RNA-binding proteins are expressed in neurons, which perform a variety of functions like RNA metabolism, processing, localization, and expression as described earlier. Alternative splicing allows new proteins from pre-mRNA with different binding partners and functions, which are further regulated by RBPs (Keene 2007). RBP quantitatively regulates protein by stabilizing or destabilizing mRNA transcripts. Finally, RBPs transport mRNA along axons and dendrites and regulate subcellular localization and local translation. Thus, deregulated RBP leads to impaired cellular function, triggering the development of disease (Darnell et al. 2013; Doxakis 2014). This chapter focuses on the multifunctional role of RBPs associated with neuronal function and dysfunction. In the next section, we shall discuss the emerging role of RBPs in neurodegenerative diseases.

Role of RNA-Binding Proteins in Neurodegeneration

RNA metabolism is a fundamental part of basal processes of molecular biology like transcription and translation, among which RBPs comprise 3–11% of the total proteome of bacteria, archaea, and eukaryotes. RBPs act as a mediator to regulate gene expression at different levels. They are highly conserved from bacteria to human

and are known to influence the structure and interaction of the RNA, and also plays a critical role in various kinds of RNA processing like transcription editing, splicing, ribosome biogenesis, export, translation, and degradation ultimately affecting cell growth and viability (Beckmann et al. 2016).

Neurodegeneration is an irreversible progressive loss of neuronal structure and function. Protein misfolding and mis-conformation leading to aggregate formation have often been linked to neurodegenerative disease. The current understanding of the protein aggregate formation relies on studies based on misfolding and conformational loss of the protein in neurodegenerative diseases like Alzheimer's, Parkinson's, and Huntington's. The misfolded protein form of beta sheet structure tends to dimerize and form oligomers, which subsequently aggregate to form insoluble fibrils. The possible cause of the protein aggregate formation seems to be multiple including genetic mutation leading to misfolding of protein.

One of the characteristics of neuronal and glial cells are their remarkable morphological and functional diversity, which is achieved through post-transcription gene regulation such as alternative splicing and RNA editing. Since RNA-binding proteins are involved in alternative splicing and RNA editing in all tissues, specifically neurons are highly vulnerable to RBP dysregulation. Investigation of the involvement of RNA-binding proteins in neurological defects and understanding their activity in CNS can give an insight into their role in disease pathology.

Mutations in RBPs are known to be central in many neurodegenerative diseases like Alzheimer's (AD), Parkinson's (PD), frontotemporal lobar dementia (FTLD), and amyotrophic lateral sclerosis (ALS).

Over the past decade, several RBPs are known to be associated with neuronal functioning and play an important role in neuronal development. Alterations in these RBPs contribute to disease mechanism in neurodegenerative disorders. At the molecular level, it is desirable to understand how a mutation in a gene induces degeneration later in the life cycle of a neuron. One of the best possible explanations seems to be alteration in post-transcriptional gene regulation of genes expressed in the neurons. For example, in the case of ALS and FTD, mislocalization and aggregate formation of a common RNA-binding protein, TDP-43, in the cytoplasm mark the last stage of the disease. TDP-43 is known to be involved in the stability, translation, and transport of its target RNA. Taking into consideration that RBPs are the major player in post-transcriptional gene regulation, it is desirable to have an insight into the role of RBPs in neurodegeneration (Baloh 2011). Interestingly, some RBPs can even cause disease if expressed in different tissues other than neuronal tissues. In some neurological syndromes, ectopic expression of RBPs restricted to neurons is triggered by a tumor outside the nervous system, which is recognized by the immune system and produces an immune response by generating autoantibodies against these proteins (Albert and Darnell 2004; Buckanovich et al. 1993). There is increasing evidence that mutations in RBPs are associated with neurodegeneration. However, very little is known about the mechanism that links the mutation in RBPs to neurodegeneration. In the previous chapters, we have seen that repeat expansion is a common mechanism in neurodegenerative diseases. These expansions of repeats create a sink of RNA-binding sites, which sequester RBPs to the pathogenic transcripts, thus generating a gain of transcript toxicity. In addition to this, mutation and

alteration in RBPs, which help in transport, storage, and degradation of mRNAs in the form of RNA granules, transport granules, stress granules, and processing bodies, are associated with numerous neurological diseases (Thomas et al. 2014; Kiebler and Bassell 2006). Here, we present human RBPs and its homolog in *Drosophila* with the focus on different mechanisms related to RBP deregulation, which causes neurodegeneration (Fig. 1) (De Conti et al. 2017; Wolozin and Apicco 2015; Zhou et al. 2014).

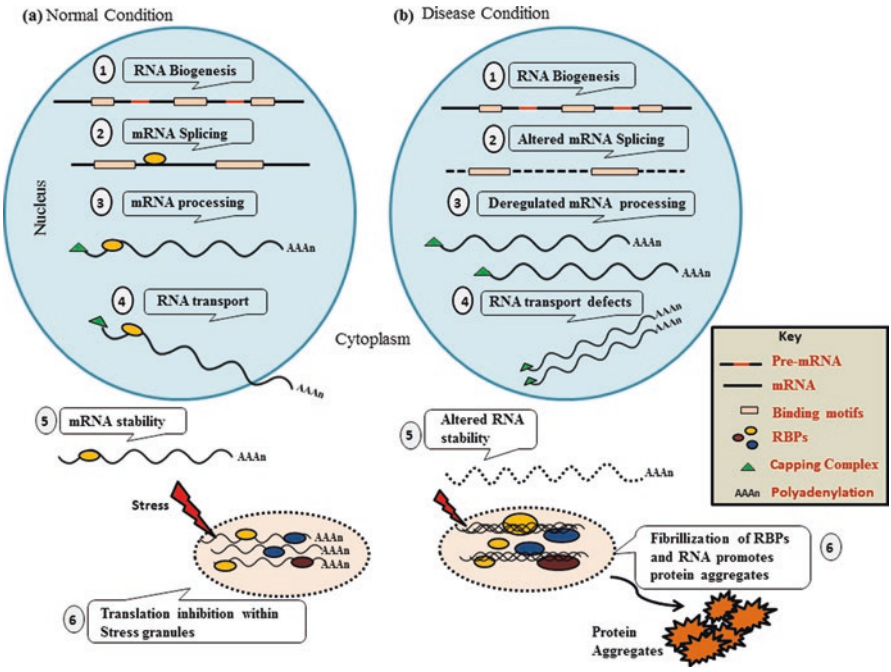

Fig. 1 RBPs deregulation in neurodegeneration: (**a**) In normal neurons, RBPs bind to mRNA to regulate its splicing and processing. RBPs help in the transport of RNAs into the cytoplasm and maintain their stability by binding to the 3′ UTR. Under cellular stress, RBPs localize into RNPs and are strongly associated with RNA and other RBPs and halt translation. (**b**) In the case of diseased neurons, mutation or loss of RBP leads to altered mRNA splicing. RBP loss results in transcript accumulation in the nucleus due to defects in transport from the nucleus to cytoplasm as well as alters mRNA stability. Altered RBPs in stress granule promote protein aggregate formation by trapping translation machinery and other RBPs, thus inhibiting their normal function. **(c)** Repeat expansion can be present in exons, introns, and UTRs of a gene. These expanded repeats form RNA aggregates or RNA foci, which lead to sequestration of RBPs. Lack of RBPs results in altered splicing and mRNA processing, ultimately leading to protein dysfunction and RNA toxicity. **(d)** Processing of micro-RNA is regulated at multiple steps: Drosha and Pasha complex cleaves pri-miRNA to pre-miRNA. The resulting pre-miRNA is exported from the nucleus to cytoplasm with the help of the exportin complex. Finally, Dicer cleaves pre-miRNA in the cytoplasm into miRNA duplex, which is further coupled with an RNA-induced silencing complex (RISC). This complex facilitates the binding of miRNA to their target mRNA and finally leads to its degradation. (e) In a neurodegenerative disease like ALS/FTD, miRNA processing is dysregulated. RBPs mislocalize to the cytoplasm, resulting in an increase in the level of pri-miRNA and reduction in the level of pre-miRNA. As a result of disrupted miRNA processing, normal duplex formation is altered and results in increased mRNA targets

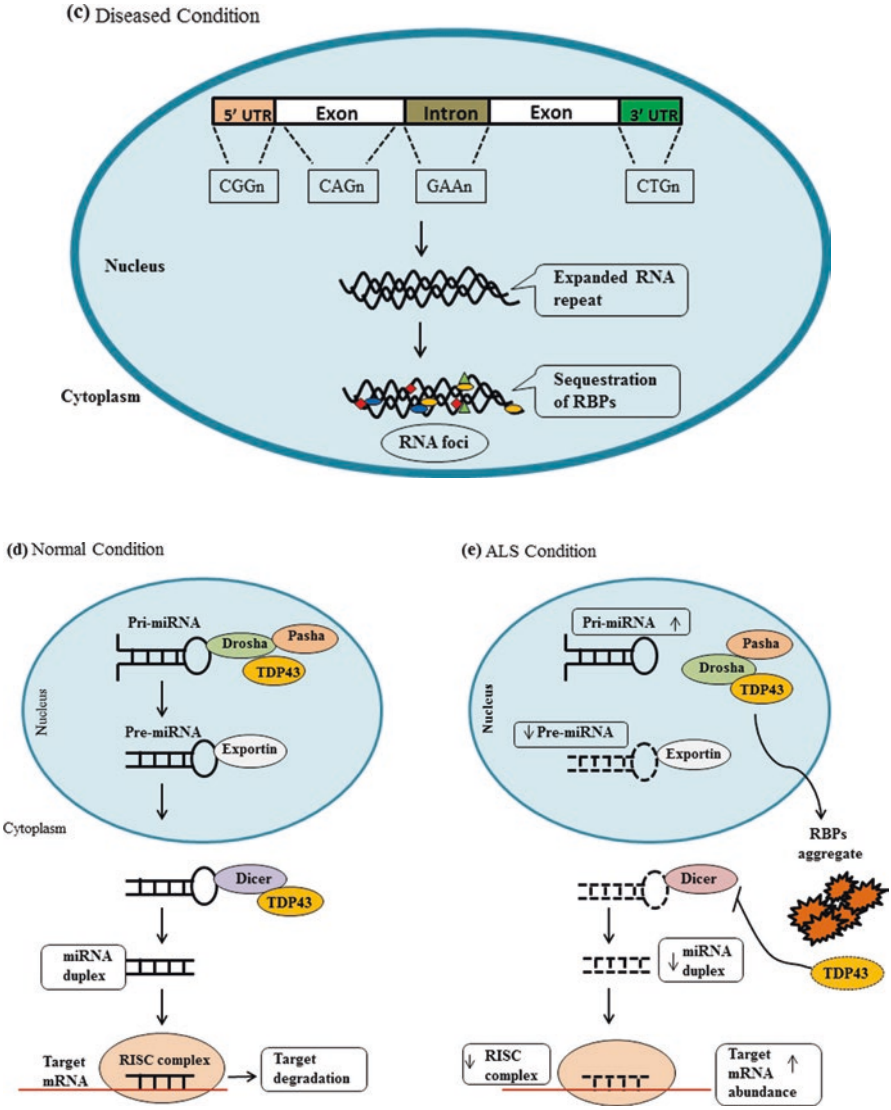

Fig. 1 (continued)

Autoimmunity-Induced Neurodegeneration

Paraneoplastic neurological syndromes (PNS) are rare disorders affecting the central, peripheral, and autonomic nervous systems in patients with cancer. PNS are a group of degenerative conditions resulting from autoimmunity initiated by an immune response against both cancer and the nervous system. The Hu family of

proteins was identified as the target antigen in PNS or Hu syndrome. The sera of patients contain antibodies that recognize antigens in neurons and in small-cell lung tumors. In mammals, the Hu protein family includes four members, namely, HuR, HuB, HuC, and HuD, among which HuR is ubiquitously expressed, while HuB, HuC, and HuD expression are restricted to neurons. Tumor outside the nervous system will induce an ectopic expression of the Hu family of proteins, RBPs that are recognized by autoantibodies and results in PNS. Interestingly, this Hu family of proteins is extremely homologous to the *Drosophila* proteins Elav and Sex-lethal (Szabo et al. 1991; Albert and Darnell 2004). In *Drosophila*, Elav was identified in mutants with an embryonic lethal abnormal vision phenotype. Elav family proteins (Elav, Fne, and Rbp9) belong to RBPs with well-characterized RNA recognition motifs: an N-terminal domain, a hinge region, and RNA recognition motifs. Elav is expressed at all stages of development and present exclusively in neurons, suggesting that Hu proteins play a similar role in the control of the development of the human nervous system. In vitro experiments have shown that Elav, Fne, RBP9, and HuR bind Elav target RNA with similar affinity. Even in non-neuronal tissues such as wing disc, all of these proteins can regulate the alternative splicing of Elav target genes (Zaharieva et al. 2015; Koushika et al. 1996; Good 1995; Okano and Darnell 1997). Similarly, ectopic expression of Nova1 and Nova2, neuron-specific KH-type RBPs associated with breast cancer and fallopian cancer, causes the production of autoantibody against these proteins. This results in dysfunction of the motor nervous system along with neuronal cell death, causing paraneoplastic opsoclonus-myoclonus ataxia (POMA). It has been reported that the *Drosophila* gene *pasilla* (*ps*) has homology to the Nova family of RBPs which is based on conserved motifs and high sequence similarity. Pasilla shows expression in the salivary gland and other tissues with no neuronal expression like that of the Nova proteins, which shows a high level of expression in neuronal tissues. Further, *pasilla* mutants are associated with the secretory defects in the salivary gland, suggesting that the defects in the secretory mechanism could be one of the causes linked to autoimmune disease in POMA patients (Seshaiah et al. 2001).

RNA Toxicity-Induced Neurodegeneration

The abnormal expansion of a nucleotide repeat sequence in coding or noncoding RNA of a gene leads to aggregate or RNA foci formation, which sequesters RNA-binding proteins by reducing the available pool in the cell, as a result, altering its normal function.

One of the examples of RNA toxicity is myotonic dystrophy (DM). DM is a neurological disorder and has effects throughout the body characterized by muscle weakness and wasting associated with cardiac, hormonal, respiratory, digestive, and mental effects. DM is an autosomal dominant disease caused by repeat expansion in the noncoding region of the *DMPK* gene. Expansion of a CTG triplet repeat in the 3′ UTR of the *myotonic dystrophy protein kinase* (*DMPK*) gene encoding a

cytosolic protein kinase leads to DM. CTG repeat ranges from 5 to 30 under normal conditions, whereas in DM1 patients, these repeat varies from 50 to 5000 copies. DM type 2 (DM2) results from CCTG repeat expansion in gene *ZNF9*, encoding a nucleic acid-binding protein. CTG and CCTG repeat expansion is transcribed into CUGs and CCUGs, respectively, which induces the formation of aggregates called RNA foci. Expanded CUG and CCUG repeats form double-stranded RNA hairpin secondary structures, which recruit RBPs and result in the alteration of their normal function (Timchenko et al. 1996; Cho and Tapscott 2007; Wang et al. 2007). RNA toxicity results from sequestration of RNA-binding proteins like CELF proteins, which include CUG RNA-binding protein 1 (CUGBP1) and the muscleblind-like (MBNL) proteins. These RBPs regulate different aspects of mRNA biogenesis such as alternative splicing, stability, and transport. In vitro studies have shown that a member of the CELF family of protein CUGBP1 was first identified for binding to the $(CUG)_8$ transcript and is involved in splicing (Timchenko et al. 1996). MBNL 1–3 in vertebrates and Muscleblind (Mbl) in *Drosophila* are key regulators of alternative splicing. MBNL is sequestered in nuclear foci and regulates alternative splicing by binding to intronic sequences in the pre-mRNA. Depending on the specific binding site on pre-mRNA, it results into mis-spliced mRNA, including or excluding specific exons, which prevent it from performing its normal function and is a major cause of disease (Fardaei et al. 2002; Jiang et al. 2004; Miller et al. 2000).

To understand the muscleblind function and pathogenesis of myotonic dystrophy, transgenic fly was generated with a CTG repeat flanked by 100 bp of human *DMPK-3′ UTR* incorporated into the 3′ UTR of GFP reporter gene. In situ hybridization showed ribonuclear foci formation in larval and adult muscle cells, as well as in salivary gland nuclei at different developmental stages. Despite ubiquitous expression of the transgene RNA, foci formation was not observed in any other organ, indicating the need for other cell type-specific factors. In mammalian tissue, MBNLs are ubiquitously distributed throughout, with its expression in the cytoplasm and nucleus in the muscle cell. Unlike mammalian MBNLs, muscleblind in *Drosophila* is restricted only to few tissues like the muscle, imaginal discs, and salivary gland. In case of DM patient cells, these MBNLs are recruited to ribonuclear foci. Similarly, flies expressing CTG repeat show colocalization of Muscleblind with ribonuclear foci. Further, it was seen that ectopic expression of *muscleblind* in neuronal cells promotes formation of ribonuclear foci, which was not observed earlier, indicating that *muscleblind* is sufficient to promote RNA foci formation (Houseley et al. 2005; Fardaei et al. 2002).

Another example of RNA toxicity-related neurodegenerative condition is Fragile X syndrome. Fragile X syndrome is the most common form of hereditary mental retardation. It is caused by mutation in the fragile X mental retardation (*FMR1)* gene on X chromosome, by the expansion of CGG triplet repeat in the 5′ untranslated region of the *FMR1* gene. Normal individuals have 5–40 repeats, while patients with fragile X syndrome have more than 200 repeats. Those individuals who carry CGG repeat expansion between 60 and 200 are referred to as permutation carriers (Ciaccio et al. 2017). Recently, it has been found that these permutation carriers are normal with respect to fragile X syndrome, while they show age-dependent fragile

X-associated tremor/ataxia syndrome (FXTAS). FXTAS is an inherited late-onset neurodegenerative disease with progressive tremor, ataxia syndrome, peripheral neuropathy, and cognitive decline. Two molecular mechanisms are associated with FXTAS: first one is toxic polyglycine peptide generation by CGG repeat-associated non-AUG-initiated (RAN) translation and the second one is through RNA toxicity (Glineburg et al. 2018). Here, we have discussed about FXTAS caused by the elevation FMR1-mRNA, which exerts a toxic RNA gain-of-function effect leading to sequestration of various RNA-binding proteins to the expanded CGG sequence. Sequestration of proteins is seen for hnRNP A2/B1, MBNL1, lamin A/C, α-internexin, CUGBP1, Sam68, Rm62, TDP-43, and DGCR8, which affects mRNA splicing and transport leading to neuronal toxicity and cell death (Iwahashi et al. 2005; Sofola et al. 2007; Sellier et al. 2010; Qurashi et al. 2011; Tan et al. 2011). The transgenic *Drosophila* model of FXTAS expressing permutation length repeat (90 CGG repeats) from 5′ UTR of the human *FMR1* gene was generated to study RNA-mediated neurodegeneration. The fly model for FXTAS exhibits retinal degeneration and locomotor difficulty (Jin et al. 2003). Interestingly, the *Drosophila* model effectively mimics the molecular and cellular alterations of FXTAS. Overexpression of CUGBP1 and hnRNP A2/B1 suppresses the phenotype of CGG transgenic fly. hnRNP A2/B1 directly interacts with CGG repeats, whereas CUGBP1 indirectly interacts with the repeat through hnRNP A2/B1 (Sofola et al. 2007). Similarly, overexpression of the RNA-binding protein Purα and Rm62 results in the suppression of FXTAS-mediated neurodegeneration. Rm62, the *Drosophila* ortholog of the p68 RNA helicase, is involved in transcription, pre-mRNA processing, and RNA export. In FXTAS fly model, permutation of CGG repeats leads to the sequestration of Rm62, which results in decreased expression of *rm62* post-transcriptionally. Interestingly, this altered expression of rm62 affects the RNA export of its target *Hsp70* mRNA and, in turn, results in nuclear accumulation of transcripts involved in stress and immune response. These nuclear-enriched transcripts build up stress, further triggering neuronal cell death and neurodegeneration. Additionally, Rm62 overexpression was found to suppress the neuronal toxicity caused by permutation CGG repeats (Qurashi et al. 2011). Similarly, TDP-43 fly ortholog TBPH suppresses neurodegeneration in FXTAS fly model mediated through hnRNP A2/B1 (He et al. 2014). The DiGeorge syndrome critical region 8 (DGCR8) and DROSHA play a role in microRNA biogenesis. Sequestration of both of these proteins by extended CGG repeats leads to neuronal dysfunction. In summary, RNA-binding proteins sequestered by expanded RNA repeats or RNA toxicity play a role in the pathogenesis of neurodegenerative diseases (Sellier et al. 2013).

Neurodegeneration Induced by Altered Granule Dynamics

Transport RNP Granules

Neurons are among the most structurally complex cells having diverse morphological, molecular, and functional properties. Neurons are dynamic structure with axons and dendrites having high degree of spatial compartmentalization. Neurons are

clearly compartmentalized into pre- and post-synaptic regions, soma, dendrites, synapse, and axon, which are essential for their functions. Axons and dendrites reaches long distance, and further communication between neurons involves synapse formation. Neurons undergo very tightly regulated development for their normal function. Signaling within and between neuronal compartments requires sophisticated mechanisms for information processing, sometimes in a spatially restricted manner. Many large and complex cells such as neurons compartmentalize information to specific domains by targeting mRNAs or proteins. However, to concentrate the protein or mRNA locally, a cell uses different strategies like degradation of mRNA in regions where they are not required, make it everywhere and transport it to a particular region, or make it only where it is needed. Neurons use these strategies to maintain their local proteome and to concentrate the proteins in a particular location in a tightly regulated manner. In particular, RBP forms organized ribonucleoprotein particles (mRNPs) that can have a different role from the transfer of specific mRNA to regulate local translation (Krichevsky and Kosik 2001; Zeitelhofer et al. 2008).

Among these ribonucleoproteins, neuronal transport granules in the axons and dendrites of neurons perform diverse functions like transport, storage, or degradation of RNAs. To satisfy the rapid change of environment driven by neuronal inputs or metabolic state, transport of mRNA in neurons is highly regulated. These motile granules help in the transport of mRNA and contain translational components. In neurons, the mRNA has to reach the dendrites before entering into the translation pathway. Transport RNPs move as a discrete unit together to control localized translation of mRNA and regulate protein expression in specific subdomains of cell upon reaching their final destination. In order to prevent premature translation and degradation of mRNA, these granules are translationally arrested during transport by the action of silent mRNAs, RBPs, clusters of ribosome, and miRNA (Kiebler and Bassell 2006). Several RBPs are involved in the formation of transport granules. These include Smaug, Nanos, Pumilio, TDP-43, FUS/TLS, FMRP, SMN, and hnRNPA2 (Thomas et al. 2014). Mutation and alteration in the expression of these RBPs associated with tRNP granules affect translation of mRNA transcripts in neurons. In spinocerebellar ataxia type 1 (SCA1), mutation in *Ataxin1* causes protein accumulation in neurons and causes toxicity leading to neurodegeneration. Human Pumilio protein, Pum1 and Pum2, plays an important role in the nervous system. Pumilio is a member of the PUF family of RBP involved in a diverse range of processes including regulation of mRNA stability. The RNA-binding protein Pum1 directly regulates the Ataxin level by binding to its highly conserved Pumilio-binding element (PBE) on 3′ UTR and regulates its mRNA stability (Gennarino et al. 2015). The *Pumilio* gene is highly variable among species. *Drosophila* contains one variant of *Pumilio.* In *Drosophila,* Pumilio controls dendritic morphogenesis and affects synapse morphology, its function, and translation control. Pumilio contributes to synapse formation by repressing the translation of *eIF4E* post-synaptically in both flies and mammals. Second, it inhibits neuronal excitability by regulating the translation of paralytic (*para*) and vertebrate *Scn1a* voltage-gated sodium channel, which is needed for proper synapse development (Mee et al. 2004; Vessey et al. 2010). Another report shows that *disc large 1* (*dlg1*), an ortholog of

vertebrate *PSD95*/*Dlg4* contains Pumilio-binding element (PBE) to which Pumilio binds and regulates synapse formation. Additionally, transcripts associated with RNA regulation contain neuron-specific 3′ UTR with specific PBE sites. In one of the studies, it was found that Pumilio regulates transcripts associated with Parkinson's disease (Galgano et al. 2008; Turrigiano 1999). Both mammalian and *Drosophila* Pumilio regulate the ERK and p38 pathway, thus regulating both neuronal and muscle cells (Kim et al. 2012; Lee et al. 2007). Interestingly, similar neuronal defects were observed in *nanos* mutant flies in combination with the *pumilio* mutant. Furthermore, Nanos is repressed by Pumilio in the peripheral nervous system in *Drosophila*. In flies, *nanos* transcripts are transported along dendrites through motile granules in the peripheral nervous system. Smaug, another RBP associated with transport granules, regulates RNA at different levels. Smaug interacts with different RNA molecules through Smaug recognition elements (SREs). Smaug1/Smad4A, mammalian homologs of Smaug, show expression in hippocampal neurons and regulate synaptogenesis. Further mutation in these proteins leads to abnormal synaptic response. In addition, Smaug regulates repression of mRNA at different levels. Smaug helps in deadenylation of *nanos* transcript, inhibits translation initiation by blocking 40S recruitment, blocks both cap-dependent and cap-independent translations, and thus affects neuronal development. All these data suggest that Pumilio-Nanos-Smaug shows a conserved role in neuronal development in both *Drosophila* and mammals. Alteration in these proteins involved in transport granules affects proper translation of mRNA, leading to altered neuronal development, ultimately resulting in neurodegeneration (Pinder and Smibert 2013; Nelson et al. 2004; Zaessinger et al. 2006; Jeske et al. 2011).

Processing Bodies

Translational regulation and mRNA degradation play an important role in the regulation of gene expression. The processes of translation and mRNA decay are coupled in eukaryotic cells. Processing bodies (P-bodies) play a fundamental role in mRNA storage and decay, nonsense-mediated mRNA decay, adenylate-uridylate-rich element-mediated mRNA decay, RNA-mediated silencing components, and RNA decapping machinery. These P-bodies are highly dynamic structures associated with transport of RNPs. Studies have shown that FMRP, Staufen, and TDP-43, neurologically associated disease proteins, are recruited to P-bodies (Barbee et al. 2006; Sephton and Yu 2015). However, the mechanism of involvement of P-bodies in neurological disease is poorly known, and further studies are needed to better understand their role in neurological diseases.

Stress Granules

Stress response such as hypoxia, heat shock, chemical exposure, and aging in eukaryotic cells often inhibits translation initiation and forms cytoplasmic stress

granules. Under stress condition, a cell tries to conserve its energy as much as possible. To maintain cells' energy, the translation process is reduced and only a few essential proteins are translated, which is needed for survival. As a result, stress granules are formed, which are nonmembranous assemblies of mRNA, RBPs, and many other proteins affecting mRNA function together with stalled ribosomes and translation initiation factors. These granules are reversible in nature, disassemble upon release of stress, and result in translation initiation of repressed mRNA. Stress granules also interact with P-bodies and other cytoplasmic RNP granules forming a dynamic structure in the cytosol for controlling mRNA function. If the stress is prolonged, stress granules rapidly increase their size by recruiting additional RBPs followed by the formation of protein–protein associated aggregation (Morimoto 2011; Heck et al. 2014). Several RBPs are found to be associated with stress granules, namely, SMN, FUS/TLS, TDP-43, ATXN2 TAF15, hnRNPA1, dFMR1, RIN, and many others (Sephton and Yu 2015). Different environmental conditions, chronic stress, or aging enhance the formation of stress granules, which forms a stable and long-lived protein aggregate, further leading to aggregation of proteins, a characteristic feature of neurodegenerative disorders.

In amyotrophic lateral sclerosis (ALS) and frontotemporal lobar degeneration (FTLD), neuronal and glial cells form cytoplasmic inclusions having the RNA-binding protein TAR DNA-binding protein of 43 kDa (TDP-43) and fused in sarcoma (FUS). Interestingly, stress granule marker proteins were found to be additional components of TDP-43 and FUS cytoplasmic inclusions (Bentmann et al. 2013). In *Drosophila*, Fragile X mental retardation protein 1 (dFMR1), an RNA-binding protein that negatively regulates translation, localizes with stress granules in the embryonic muscles under hypoxia condition. Similarly, rasputin (RIN), a homolog of G3BP, was shown to be present in stress granules formed due to low oxygen levels (Irvine et al. 2004; Van Der Laan et al. 2012).

RNA-Binding Proteins and Spreading of Disease

A hallmark of neurodegenerative disease proteinopathies is aberrant aggregation, accumulation, and misfolding of proteins in the brain. In each neurodegenerative disease, the nature of protein aggregates and its distribution are different and unique for specific disorders. In AD, there are β-amyloid protein deposits, α-synuclein in PD, a polyglutamine-rich version of the Huntingtin protein in Huntington disease, and many more. However, the mechanism through which these aggregates spread during disease is still unclear.

Recent studies have suggested that protein aggregate contributes to the propagation of neurodegenerative disease by crossing the cell membrane. Interestingly, specific proteins have been found to misfold and accumulate, which can act as seeds of aggregation having the ability to form pathogenic assemblies ranging from small oligomers to large aggregates (Jucker and Walker 2013). These aggregates increase in size followed by fragmentation resulting in multiple seeds, inducing further formation of aggregates and leads to enhancement of proteinopathies that spread in a

prion-like manner. Transformation of a protein into prion is accompanied by an increase in beta-sheet structure and its ability to form aggregates. Prions are proteins that acquire amyloid conformations and become self-propagating, which help in the progression of the disease.

Recent reports suggest that a prion-like mechanism can also be applied to RBP aggregates. The glycine-rich domain of RBPs, which has similarity with yeast prion domains, is often termed as prion domains. In vitro evidence has shown that misfolded and mutant forms of TDP43, SOD1, and FUS/TLS have the ability to induce aggregation/misfolding, suggesting a prion-like propagation (Jucker and Walker 2013; Chia et al. 2010; Furukawa et al. 2011; Nomura et al. 2014). Taken together, different studies suggest that a neurodegenerative disorder associated with RBP dysfunction can propagate through a prion-like manner.

Drosophila Models of Neurological Diseases

Drosophila Model of Amyotrophic Lateral Sclerosis (ALS)

Amyotrophic lateral sclerosis (ALS) is a common adult-onset neurodegenerative disorder characterized by degeneration of motor neurons in the brain and spinal cord. In ALS, both the lower and upper motor neurons degenerate and die, leading to gradual muscle weakness, stiffness, twitching, and atrophy. This disease is progressive and affects voluntary muscle movements like chewing, walking, breathing, and talking. Typically, death occurs within 3–5 years due to respiratory failure when the first symptom appears. The majority (~90%) of ALS cases are sporadic, while only 10% of cases are familial. Interestingly, many of the genes associated with ALS encode RNA-binding proteins, which include transactive response DNA-binding protein 43 (TDP-43), fused in sarcoma/translocated in liposarcoma (FUS/TLS or FUS), ataxin-2 (ATXN2), TATA-box-binding protein-associated factor 15 (TAF15), Ewing's sarcoma breakpoint region 1 (EWSR1), heterogeneous nuclear ribonucleoprotein A1 (hnRNPA1), heterogeneous nuclear ribonucleoprotein A2/B1 (hnRNPA2/B1), matrin 3 (MATR3), and T-cell-restricted intracellular antigen-1 (TIA1). The *Drosophila* model has been established for many ALS-causing alleles (Kapeli et al. 2017; Zhao et al. 2018).

Modeling TAR DNA-Binding Protein 43 (TDP-43) Mutations in *Drosophila*

A large number of disease-associated alleles are involved in neurological disorders. *Drosophila* has proven to be an invaluable model system to understand the molecular mechanism of these alleles. Nonetheless, in cases like ALS, which can be caused by a number of mutations, *Drosophila* helps in deciphering the role of various disease-associated alleles to the disease phenotype.

The human TAR DNA-binding protein 43 (TDP-43) gene is a common disease-causing factor implicated in ALS, frontotemporal dementia, and Alzheimer's disease. TDP-43 plays a role in various cellular processes such as apoptosis, axonal

transport, transcription, alternative splicing, and mRNA stability (Buratti and Baralle 2008). TDP-43 contains a nuclear localization sequence (NLS), a nuclear export signal, and a glycine-rich C-terminus to mediate protein–protein interaction. TDP-43 mainly resides in the nucleus and is capable of undergoing nucleocytoplasmic shuttling. In both sporadic and familial cases, deposition of TDP-43 forms cytoplasmic inclusion or "TDP-43 proteinopathies" with continuous loss from the nucleus. The *Drosophila* ortholog TBPH has been used to understand the molecular dysfunction associated with TDP-43 mutations in ALS (Olesnicky and Wright 2018). Researchers have performed rescue experiments with disease-associated TARDBP variants in TBPH loss-of-function flies by assessing phenotype like the death of adult bursicon neurons. Further, it was found that ALS-associated mutations failed to rescue the reduced life span and neuronal death phenotype. In addition, mutant alleles promoted mislocalization of TDP-43 from the nucleus to cytoplasm in comparison to that of wild-type TDP-43 (Broeck et al. 2015). Other studies have also provided evidence that gain of function of TDP-43 can cause neurodegenerative phenotypes. TDP-43 expression is strictly regulated through an autoregulatory feedback loop, which maintains its alternative isoform, subcellular localization, and stability; therefore, loss of this regulation leads to neuronal toxicity (Ayala et al. 2011). Interestingly, upon mimicking the autoregulatory feedback loop in the transgenic *Drosophila* model, six RBPs were identified, namely, Rsf1, B52, x16, SC35, Rbp1, and SF2, as regulators of TDP-43. All these reports have helped us in unraveling new targets for therapeutics in neurodegenerative disease (Pons et al. 2017).

In some studies, it was found that TDP-43 is associated with *futsch* mRNA in *Drosophila* neurons (Coyne et al. 2017; Romano et al. 2016). TDP-43 maintains stability and regulates translation and expression of *futsch* mRNA at the neuromuscular junction (NMJ) in *Drosophila*. TDP-43 induced proteinopathy at NMJ and leads to the reduction of *futsch* transcripts compared with the transcript level of controls. Interestingly, genetic interaction studies have shown that overexpression of *futsch* is neuroprotective by reducing TDP-43 proteinopathy, extending life span, and rescuing microtubule-associated NMJ abnormalities as well as locomotor dysfunction. MAP 1B, the mammalian homolog of Futsch, was also found to accumulate in motoneuron cell bodies in ALS patients in a similar way to the observation in *Drosophila* motor neurons. TDP-43 helps in understanding how it regulates the function of its target mRNA, which further affects neurodegeneration (Coyne et al. 2017). Interestingly, other groups have identified that mitochondrial dysfunction is also associated with ALS, among which the *Parkin* and *PINK1* genes are important for mitochondrial function. Earlier studies have shown *Parkin* as RNA target of TDP-43, which require RNA-binding function of TDP-43. In contrast, TDP-43 indirectly regulates PINK1 at the level of proteasomal degradation resulting in the accumulation of PINK1 in the cytoplasm, causing cytotoxicity. Using knockin flies, it was further revealed that TDP-43 overexpression misregulates Parkin and PINK1 (Sun et al. 2018).

TDP-43 associates with many of the stress granules as a normal physiological response during cellular stress. The heat shock cognate protein 70 (Hsc70-4) is a

ubiquitous molecular chaperone member of heat shock protein 70 (HSP70) family. Hsc70-4 is involved in different functions like protein folding, stress response, and chaperone-mediated autophagy. Importantly, TDP-43 overexpression in motor neuron causes a reduction in *hsc 70-4* mRNA levels at neuromuscular junctions, which is involved in synaptic vesicle cycling. Impaired interaction between Hsc70-4 and TDP-43 leads to impaired synaptic vesicle cycling. However, overexpression of both Hsp70-4 and TDP-43 ameliorates defects in locomotion and lifespan-related overexpression of TDP-43. All these findings emphasize over TDP-43 dysfunction and its effect on target RNAs in the context of ALS (Van Der Laan et al. 2012, Coyne et al. 2017; Khalfallah et al. 2018).

Modeling Fused in Sarcoma (FUS) Mutations in *Drosophila*

ALS encompasses different subtypes, among which mutations in TDP-43 account for a major proportion of ALS cases. Fused in Sarcoma/translated in liposarcoma (FUS/TLS), an RNA-binding protein, is the second major cause for disease etiology. It has been implicated in different cellular functions, including regulation of gene expression, RNA localization, splicing, and translation. Like TDP-43, mutation in FUS results in its nuclear clearance and cytoplasmic accumulation, causing FUS toxicity in motor neurons (Guerrero et al. 2016). In order to understand the neurodegeneration caused by FUS, the *Drosophila* model has been generated. Human FUS/TLS mutations causing ALS were ectopically expressed in eyes, resulting in loss of mechanosensory bristle and disorganized ommatidia. Expression of FUS/TLS mutant in the nervous system caused pupal lethality. Further, a conditional model for ALS was developed; upon expressing the mutant FUS/TLS in neurons, it leads to larval crawling and locomotor defects and shortens lifespan. Abnormal nuclear to cytoplasmic localization of FUS has been shown in human ALS patients. Deletion of nuclear export signal rescued the mutation-dependent FUS/TLS toxicity, indicating that its cytoplasmic localization is responsible for neurodegeneration (Lanson et al. 2011; Daigle et al. 2012). In contrast, other groups have done in vivo studies to find out whether the neurodegeneration in ALS is caused by FUS cytoplasmic toxicity or by loss of FUS nuclear function. Cabeza (Caz) is the *Drosophila* ortholog of human FUS. Caz knockout flies were generated, which showed locomotion and neuromuscular junction defects. In contrast to earlier studies, it was found that a decrease in the level of nuclear Caz is enough for causing neurodegeneration even in the absence of cytoplasmic aggregates (Machamer et al. 2018). Recently, *Drosophila* cholinergic neurons were identified as a model for studying FUS protein aggregate formation and its cytoplasmic mislocalization. A mutation in NLS of Caz caused its mislocalization to cytoplasm, resulting in disruption of dendrites and axonal transport. These findings suggest that disrupted transport of synaptic machinery in axons upon Caz overexpression leads to neuronal hyperexcitability, similar to what has been previously been reported in ALS patients (Chesnut et al. 2018).

Modeling C9orf72 Aberrations in *Drosophila*

Repeat expansions GGGGCC in C9orf72 locus are currently the most common genetic cause of ALS in 40% of familial cases and 7% of sporadic cases. These repeat expansions form pathogenic RNA aggregates, which sequester RNA-binding proteins, resulting in altered RNA metabolism. Human Znf106, an RBP residing on chromosome 15, has a strong association with ALS. Znf106 ortholog in Zpf 106-knockout mice developed neurodegenerative phenotypes (Guerrero et al. 2016; Celona et al. 2017). In *Drosophila,* the UAS-GAL4 system was used to overexpress the GGGGCC repeat sequence in a tissue-specific manner, and this exhibited 50% pupal lethality and locomotor defects in flies that successfully enclosed from the pupal case. Moreover, expression of the hexanucleotide repeat resulted in a reduced number of active zones within the larval neuromuscular junction. In addition, when both Zpf 106 and GGGGCC repeats were coexpressed, it suppressed locomotor defects and other neurotoxic phenotypes. This suggests that Zpf 106, an RNA-binding protein, protects neurons from neurotoxicity caused by C9orf72 in ALS (Celona et al. 2017).

One of the proposed hypotheses by which C9orf72 mutation occurs in ALS is through repeat-associated non-ATG-initiated (RAN) translation of sense and antisense RNA into toxic dipeptide repeat proteins (DPRs). Overexpression studies done in *Drosophila* have shown that DPRs are indeed toxic and causes neurodegeneration (Wen et al. 2014; Freibaum and Taylor 2017). Boeynaems' group thus performed targeted RNAi screen in adult *Drosophila* eyes to identify modifiers of DPR-induced toxicity. A large number of genes involved in transport across nuclear pore component, importins, exportins, and arginine methylase were found to be modulating DPR toxicity (Boeynaems et al. 2016). In contrast, other groups have shown that overexpression of these hexanucleotide repeats can be recognized by mRNA localization machinery and can form cytoplasmic granules localized to neurites. Additionally, overexpression of an expanded repeat in *Drosophila* da neurons results in degeneration of dendritic branches (Peters et al. 2015). Thus, *Drosophila* genetics provides a powerful tool for studying different factors that is known to cause ALS.

Modeling Fragile X syndrome in *Drosophila*

Fragile X syndrome (FXS), earlier known as Martin-Bell syndrome or marker X syndrome or FRAXA, is the first X-linked, heritable intellectual impairment syndrome that results from mutation in the *FMR1* gene, a ubiquitously expressed RNA-binding protein. An FXS patient suffers from different symptoms such as mental retardation, anxiety, aggression, hyperactivity, cardiac disorders, sleep disorder, and autistic-like behaviors. FXS is caused by the expansion of a CGG triplet repeat within the fragile X mental retardation gene (*FMR1*) located on the X chromosome. Normally, the repeat ranges from 5 to 40, whereas individuals with 55–200 repeats have *FMR1* gene premutation. Individuals with CGG repeat greater than 200 have a full mutation for FXS. Repeat expansion silences the expression of the *FMR1* gene,

leading to low expression of the fragile X mental retardation protein (FMRP). FMRP functions mainly as a translation regulator that binds to different RNAs and is associated with neural tissues as well as synaptic transmission (Darnell et al. 2011). *FXR1* and *FXR2* are paralogs of *FMR1* in humans, while flies have only one *dfmr1* gene. The *Drosophila* homolog of *FMR1* was first identified in 2000 by Wan and colleagues and was named *dfmr1*. To investigate *FMR1* function at the genetic and molecular levels, *dfmr1* mutant flies have been used. Neurons in the central and peripheral nervous systems of mutant *dfmr1* flies exhibit excessive synaptic growths, while expression of human *FMR1* rescued these synaptic defects (Tessier and Broadie 2008). Interestingly, mutant for *dfmr1* exhibits a similar type of defects that were observed in human FXS patients. Loss of function of *dfmr1* mutants shows a variety of phenotypes such as flight and climbing defects, which resembles delayed motor development. Further, olfactory and learning defects were observed, which parallels learning and memory impairment in patients. In addition, using the *UAS-GAL4* system, cardiac-specific *dfmr1* RNAi knockout flies were examined, and it was found that it regulates heart rate during development similar to that in FXS patients having cardiac defects with altered *FMR1* levels (Novak et al. 2015). Further, tissue-specific RNAi studies unrevealed the role of *dfmr1* at different developmental stages. A study based on *Torsin* RNAi knockout flies revealed that *Torsin* works together with *dmf1* to regulate synaptic plasticity and is involved in locomotion (Nguyen et al. 2016). *dfmr1* knockdown by RNAi causes defect in the regulation of immune cell phagocytosis of bacteria as a result of sensitivity toward bacterial infection which was significantly enhanced, indicating the role of *dfmr1* in phagocytosis (O'connor et al. 2017). Among the different targets of FMRP, some RNA targets include autism candidate genes. In FXS, increase in PI3K signaling is associated with neuronal dysfunction including inherited intellectual disability, schizophrenia, and autism. PIKE, the PI3K enhancer, binds to metabotropic glutamate receptor subunit and activates PI3K signaling. *CenG1A, a Drosophila* ortholog of PIKE, is translationally repressed by FMRP. To determine whether the reduction in levels of PI3K signaling rescues the neuronal phenotype, dosage of *CenG1A* was reduced in *dfmr1* mutant flies, which rescued mushroom body defects, short-term memory defects, and fusion of axonal projections (Gross et al. 2015).

Zeynep's group has identified a novel FXS-causing frameshift mutation in the *FMR1* gene. Frameshift mutation leads to a premature stop codon, resulting in a truncated protein with a novel nuclear localization sequence (NLS) to be formed at its C terminus. This NLS sequence can target the FMRP protein into the nucleus. Finally, using a *Drosophila* model, the authors overexpressed patient mimetic constructs into the nervous system to understand the molecular nature of a mutant allele. Interestingly, the mimetic protein localized to the nucleolus and showed some defects in neurons. This finding has shown that an allele has a neomorphic function other than that of translation regulation (Okray et al. 2015).

Drosophila has been a favored organism for genetic research, as it provides an array of diverse tools for manipulating gene expression. Earlier studies have shown that FMRP is needed during brain development. Thus, it has been hypothesized that FMRP may function as an activity sensor and can directly regulate the translation of

proteins required for synapse stabilization during late brain development. Interestingly, in order to better understand FMRP function, the neuronal remodeling optogenetic technique has been used to see neuronal activity. Optogenetics is a biological technique that enables one to target specific neurons with light-sensitive proteins, for example, ion channels, ion pumps, and enzymes, to manipulate neuronal activity through illumination. The dendrite of neurons undergoes dendritic refinement, a key step in neuronal circuit formation during brain development. In addition, it has been predicted that FXS and autism result from defects in synaptic connectivity and activity-dependent circuit formation. Researchers have used Flylight lines to manipulate specific subsets of neurons in the brain. They have identified *dfmr1*, which act as a regulator of dendritic refinement in extrinsic neurons of the mushroom body. Loss of *dfmr1* function results in elaborated dendritic arborization in mushroom body neurons. The *dfmr1* null mutant does not respond to optogenetic stimulation. Even dendritic arbors fail to undergo a critical period of refinement, indicating that this refinement process is dependent on the function of FMRP to regulate neurite remodeling (Doll and Broadie 2015; Doll et al. 2017).

Modeling Fragile X-Associated Tremor Ataxia Syndrome (FXTAS)

Fragile X-associated tremor/ataxia syndrome (FXTAS) is a neurodegenerative disorder with core features of tremor, dementia, and ataxia. In contrast to FXS, FXTAS results from intermediate CGG repeat expansions (50–200 repeats) in the *FMR1* locus discussed earlier in this chapter. Those with a permutation allele leads to the upregulation of *FMR1* mRNA resulting in mRNA toxicity. However, recent studies have predicted that repeat-associated non-AUG-(RAN) translation produces FMRP with polyglycine residues (FMRPpolyG), which mediates toxicity. Whether CGG repeat is pathogenic through RNA gain-of-function or by translation into a toxic protein is unclear. In *Drosophila*, an altered ubiquitin proteasome system (UPS) results in CGG repeat-induced degeneration. Researchers have used the line in which CGG repeats are preceded by a stop codon prior to 5′ of repeats, which inhibits the generation of FMRPpolyG. In addition, for overproduction of FMRPpolyG, an AUG start codon was placed just upstream of the CGG repeat sequence, which results in enhanced expression of the RAN-translated product FMRPpolyG. However, concomitant alteration of the ubiquitin-proteasome system thus resulted in enhanced necrosis in photoreceptor neurons in *Drosophila*. All these data suggest that toxicity in neurons is been mediated by RAN translation of FMRPpolyG and plays a strong role in disease pathogenesis in comparison to CGG repeat-containing mRNA alone (Glineburg et al. 2018; Oh et al. 2015).

Spinal Muscular Atrophy

Spinal muscular atrophy (SMA) is a neurodegenerative disease characterized by degeneration of motor neurons in anterior horn cells of the spinal cord, which results

in muscle weakness and atrophy. It is one of the most common autosomal recessive disorders associated with mutation/deletion within the survival of the motor neuron 1 (*SMN1*) that leads to reduction in the SMN protein level. SMN is found in the cytoplasm and nucleus of all cells and is a part of the multiprotein complex. This SMN complex facilitates the assembly of spliceosomal small nuclear ribonucleoproteins (SnRNP). SMN has many interacting partners, including mRNA-binding proteins and actin regulators. It also functions as a molecular chaperone involved in mRNA metabolism. Moreover, it plays an important role in diverse cellular process, which regulates neuronal growth, differentiation, maturation, and axonal guidance. SMA patients are classified into four categories depending on the severity of motor dysfunction and age of the person. Among the different types, SMA type I is the most severe form with severe motor dysfunction, and patients were predicted to die before the age of 2 years. However, SMA type IV is characterized by an age of onset beyond 30 years with mild muscle weakness (Burghes and Beattie 2009; Edens et al. 2015).

Modeling SMN in *Drosophila*

Drosophila contains a single copy of *SMN* ortholog having 41% homology with human *SMN1*. To investigate the function of *SMN*, transgenic flies with several *SMN* constructs were generated by P-element-mediated transformation. Interestingly, ectopic expression of human *SMN* led to pupal lethality with a number of morphogenetic and differentiation defects (Miguel-Aliaga et al. 2000). Hypomorphic SMN mutant displayed flightlessness with acute muscle atrophy due to defective axonal arborization in motor neurons and a failure to form muscle filament (Rajendra et al. 2007). Howard and colleagues have shown that *SMN* expression and concentration are needed for the normal function of neuromuscular junction (NMJ). Additionally, 27 different genetic modifiers of the *SMN* phenotype were identified, among which *wit*, *mad,* and *dad* are members of the BMP signaling pathway. Thus, with an increase in BMP signaling, the NMJ defects caused by loss of *SMN* function were rescued by the *SMN* phenotype (Chang et al. 2008). Another group used *Drosophila* as a model to examine 12 different *SMN* missense mutations originally identified in SMA patients. Moreover, *SMN* patient-mimetic alleles in *Drosophila* are biochemically similar to those of humans and display a wide range of phenotypic defects. Intragenic complementation assay indicated that some mimetic alleles affect the YG box self-oligimerization domain of SMN, which displays a stronger phenotype in a dominant negative manner in comparison to null allele. Interestingly, the wild-type protein suppresses the YG box mutant phenotype and heterodimerizes with it. Understanding the molecular basis of the mutant alleles will help in future targeted therapeutic approaches to treat SMA (Praveen et al. 2014).

RNAi knockdown of *SMN* allows researchers to partially downregulate the gene of interest allowing to examine adult phenotypes, as the null allele of *SMN* leads to pupal lethality. This approach was used to identify 340 genes as modifiers of *SMN* function. Among these modifiers, 20 genes were associated with modifying the pupal lethality phenotype and 11 genes were modifiers of the SMN NMJ phenotype, with seven affecting SMN expression. Moreover, from the RNAi screen, 322

modifiers conserved to human genes were identified. Thus, such modifier screens provide insight into human SMN modifiers that may contribute to disease pathology and aid in identification of therapeutic targets (Bowerman et al. 2017).

Conclusion

It is now apparent that RNA-binding proteins play a fundamental role in neuronal development including neurogenesis, differentiation, and synaptic plasticity. Most of the RBPs are involved in post-transcriptional gene regulation and regulate multiple RNA targets. However, deficits in RBP expression and disruption in mRNA metabolism result in the pathogenesis of neurodegenerative disorders. Despite the fundamental role of RBPs in RNA processing and development of the nervous system, only a few RBPs have been studied in details with regard to neuronal function. Therefore, a major goal in this field is to understand the role of RBPs in neuronal functioning and to unravel its neural-specific targets. Another major challenge is to understand whether RNA species affected by altered RBPs are responsible for neuronal cell death or global RNA processing events, that eventually triggers neurodegeneration. Further question is to understand how these RBPs fine-tune between RBP–mRNA and RBP–RBP interaction in different RNP processing granules, which further regulates neuronal proteins. Due to the high level of conservation of RBPs, genetic techniques available, *Drosophila* model provides us an unique opportunity to study the role of RBPs within the nervous system in vivo. Ease of generating transgenic flies along with a wide variety of tissue-specific genetic manipulations, makes *Drosophila* an unparalleled model organism to study RBP function in the nervous system. Altogether, these findings will help us in developing a number of therapeutic strategies to target specific RBPs associated with multiple neurodegenerative diseases.

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Modeling Hereditary Spastic Paraplegias in Fruit Flies: Potential of Its Genetic Paraphernalia

Sajad Ahmad Bhat and Vimlesh Kumar

Abstract

Hereditary spastic paraplegias (HSPs) are a large group of heterogeneous inherited neurological diseases characterized by spasticity or progressive stiffness in the lower extremities. The genetic basis of HSPs is very diverse, and the number of candidate genes being identified has been increasing due to better diagnostic approaches, including the advance and access of next-generation sequencing. Currently, the number of genomic loci associated with HSPs in humans are more than 80 and the corresponding number of identifiable genes are greater than 70. *Drosophila* has evolved as a powerful genetic model to explore these disease-causing genes in vivo. A comprehensive review of the previously studied HSP causative genes in flies revealed high similarity and potential of these organisms to provide novel insights into the underlying cellular pathways. We also found high conservation of HSP-related genes in flies with more than 60% of the human genes having corresponding sequences in *Drosophila*. Therefore, the study of HSP genes in flies can unravel valuable information for designing future therapeutic strategies.

Keywords

Hereditary spastic paraplegias · Neurological disorders · Spastin · Kinesin family members · Endoplasmic reticulum · Cytoskeleton · Neuronal signaling

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Introduction

Adolf Strümpell was the first to describe hereditary forms of spastic paraplegia in 1883, which were further explained in detail by a French neurologist Maurice Lorrain. This disease is often referred to as Strümpell-Lorrain disease or familial spastic paraparesis (Faber et al. 2017). Hereditary spastic paraplegias (HSPs) have been reported across the globe with a pooled average of 1.8 per 100,000 (Ruano et al. 2014). Symptoms may arise from early childhood to late adulthood. In humans, the disease affects the upper motor neurons of the corticospinal tract, a long multi-synaptic neuronal track emanating from the cerebral cortex and extending all the way to the skeletal muscles. The axons of these neurons span over long distances and help in rapid neurotransmission for voluntary movements. This lengthy course of axons requires efficient and complex intracellular machinery to ferry the cargo to their destinations, which also makes them vulnerable to neurological disorders (Blackstone 2012).

The disease may segregate as autosomal dominant, autosomal recessive, or, less frequently, X-linked or mitochondrial trait (Finsterer et al. 2012). HSPs are classically divided into two groups, pure and complex. In the pure form, the spasticity and weakness in lower extremities represent the main symptoms along with frequent urinary disturbances, whereas complex forms are often accompanied by other neurological or non-neurological symptoms. These include distal amyotrophy, cognitive impairment, thin corpus callosum, Parkinsonism, retinopathy, seizures, and various other symptoms (Harding 1983, 1993; Fink 2013).

The complexity of the human brain and the technological limitations to investigate these pathologies in patients necessitate the employment of model organisms to unfold the mechanistic underpinnings of these diseases. Murine models offer high genomic similarity and, in most cases, the data faithfully extrapolate to humans. However, long life cycle and economic requirements make them unsuitable for large-scale genetic and pharmacological screens. With a sharp increase in the number of human disease genes, as in the case of HSP, a suitable substitute is required to accelerate the research. At first glance, the fruit fly, whose brain is barely the size of a grain of sand, seems a very unlikely choice to study these complex diseases. However, this airborne insect is a workhorse of genetics and the reason behind six Nobel prizes from the year 1933 to 2017.

Drosophila melanogaster has contributed immensely in understanding the basis of a broad spectrum of diseases (Lu and Vogel 2009; Jaiswal et al. 2012; McGurk et al. 2015). More than 75% of known human disease genes were found to have related sequences in *Drosophila* (Bier 2005; Fortini et al. 2000; Reiter et al. 2001; Inlow and Restifo 2004). Despite the great similarity that spans almost the entire array of human genes, the *Drosophila* genome is comparatively compact with less redundancy in the gene families simplifying their study. *Drosophila* toolbox is equipped with a vast repertoire of genetic tools that enables various genetic manipulations within a short time span. These comprehensive tools are available in the form of loss-of-function null mutants, hypomorphs, and almost a complete range of RNAi lines. The Galactose responsive transcription factor GAL4-Upstream

Activation Sequence (Gal4-UAS) system can be employed to modulate the gene expression in a spatiotemporal manner. The gamut of Gal4 drivers offers control over gene expression in almost any tissue with precision. Also, the temperature dependence of Gal4 transcription factor and, in many cases, the availability of multiple Gal4-drivers for the same tissue offer the choice to vary the strength of gene expression (Brand and Perrimon 1993; Duffy 2002). Further, many genetic lesions like nucleotide repeat disorders as well as studying the effect of point mutations are also possible, which is becoming increasingly easier with the advance of CRISPR-Cas9 genome editing technology. So, the bargain of the time factor, the luxury of genetic tools, and the versatility of flies against high genome similarity and complex behavioral analysis in murine models put *Drosophila* at the forefront to study HSP causative genes, which are relentlessly increasing in number.

For a general review of spastic paraplegia, we recommend the readers to go through these articles (Blackstone 2012, 2018) and the Spastic Paraplegia Foundation website (https://sp-foundation-org.presencehost.net/). *Drosophila* as a model for HSPs has been previously reviewed (Wang and O'Kane 2008; Ozdowski et al. 2015). This chapter provides an update on the recent literature and a large number of genetic loci and genes that have been since discovered. Table 1 lists all the genes that have been modeled in flies. Orthologs exist for more than 60% of HSP genes. Table 2 lists all the known or putative *Drosophila* orthologs of the genes which are yet to be explored.

The enormous numbers of HSP causative genes seem a formidable task to handle, but fortunately, most of the genes that are central to HSP condense into a limited number of common pathogenic themes. These include axonal transport, endosomal trafficking, mitochondrial function, lipid metabolism, endoplasmic reticulum morphogenesis, and axon pathfinding (Fig. 1). However, some genes may

Table 1 List of HSP causative genes studied in *Drosophila*

	Type	MOI	Human gene	Type	*Drosophila* ortholog
HSP genes modeled in *Drosophila melanogaster*					
1	SPG4	AD	*SPAST*	P(mostly) or C	*Spastin (CG5977)*
2	SPG3A	AD	*ATL1*	P(mostly) or C	*Atlastin (CG6668)*
3	SPG12	AD	*RTN2*	P(mostly) or C	*Reticulon-like 1 (CG33113)*
4	SPG6	AD	*NIPA1*	P or C	*Spichthyin (CG12292)*
5	SPG20	AR	13q13	C	*Spartin (12001)*
6	SPG10	AD	*KIF5A*	P or C	*Kinesin heavy chain (CG7765)*
7	SPG39	AR	*PNPLA6*	C	*Swiss cheese (CG2212)*
8	SPG31	AD	*REEP1*	P or C	*ReepA (CG42678)*
9	SPG72	AD AR	*REEP2*	P	*ReepA (CG42678)*
10	SPG30	AR	*KIF1A*	P or C	*Unc-104 (CG8566)*
11	SPG7	AR	*SPG7*	P or C	*Spg7 (CG2658)*

MOI mode of inheritance, *AD* autosomal dominant, *AR* autosomal recessive, *P* pure, *C* complicated

Table 2 List of HSP causative orthologs in *Drosophila*

	Type	MOI	Human gene	Type	*Drosophila* ortholog
HSP genes with putative *Drosophila* orthologs					
1	SPG8	AD	WASHC5	C	Strumpellin
2	SPG9A	AD	ALDH18A1	P or C	CG7470
3	SPG11	AR	Spatacsin	C	CG13531
4	SPG13	AD	HSPD1	P or C	Hsp60A
5	SPG15	AR	Spastizin/ZFYVE26	C	CG5270
6	SPG35	AR	FA2H	P or C	fa2h
7	SPG42	AD	SLC33A1	P	CG9706
8	SPG45 & 65	AR	NT5C2	C	CG32549
9	SPG46	AR	GBA2	C	CG33090
10	SPG49	AR	TECPR2	C	CG11141
11	SPG54	AR	DDHD2	C	PAPLA1
12	SPG56	AR	CYP2U1	C	Cyp18a1
13	SPG59	AR	USP8	C	Usp8
14	SPG60	AR	WDR48	C	CG9062
15	SPG61	AR	ARL6IP1	C	Arl6IP1
16	SPG63	AR	AMPD2	C	AMPdeam
17	SPG66	AR	ARSI	C	CG8646
18	SPG67	AR	PGAP1	C	PGAP1
19	SPG69	AR	RAB3GAP2	C	Rab3-GAP
20	SPG70	AR	MARS	C	MetRS
21	SPG71	AR	ZFR	P	Zn72D
22	SPG74	AR	IBA57	C	CG8043
23	SPG77	AR	FARS2	P	PheRS-m
24	–	AD/AR	BICD2	C	BicD
25	–	AR	LYST	C	mv
26	–	AR	CCT5	C	Cct5
27	–	AR	Alsin	P	Als2
28	–	AR	EXOSC3	C	Rrp40
29	–	Mitochondrial	MTATP6	P or C	mt:ATPase6
***Drosophila* genes with moderate similarity scores**					
30	SPG2	XR	PLP1	P or C	M6
31	SPG22	XR	SLC16A2	P or C	Kar
32	SPG53	AR	VPS37A	C	Vsp37A
33	SPG73	AD	CPT1C	P	whd
34	SPG76	AR	CAPN1	C	CalpA
35	SPG78	AR	ATP13A2	C	anne
36	SPG79	AR	UCHL1	C	Uch

MOI mode of inheritance, *AD* autosomal dominant, *AR* autosomal recessive, *XR* X-linked recessive, *P* pure, *C* complicated

These data were compiled from DRSC integrative ortholog prediction tool (DIOPT) and Ensemble

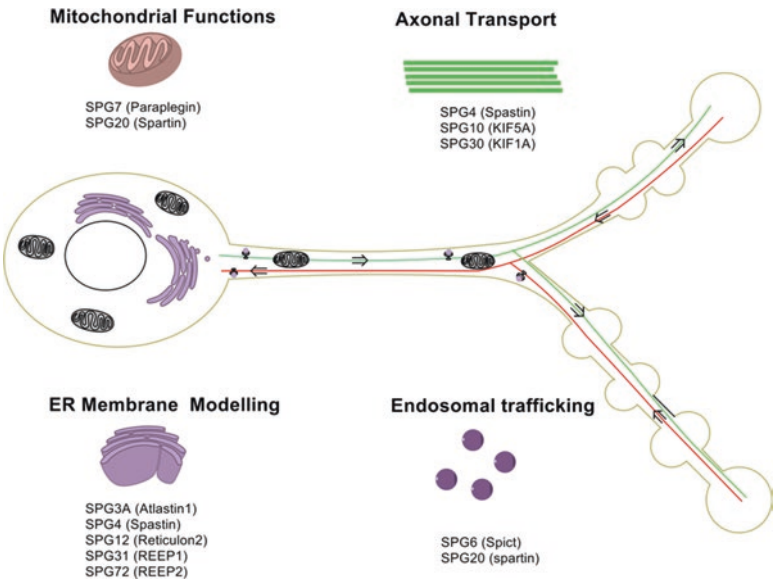

Fig. 1 HSP orthologs in *Drosophila* grouped into major functional modules: A simplified depiction of a *Drosophila* motor neuron emphasizing the subcellular compartments and the site of action of HSP causative genes modeled in flies. Spastin, atlastin, reticulon, and REEP proteins help in shaping the ER; spartin and paraplegin regulate mitochondrial function; Spastin and KIF proteins are involved in axonal transport, while spict and spartin mediate endosomal trafficking

often work in a number of pathways and cannot be restricted to one domain (Blackstone 2012; Klebe et al. 2015).

Here, we discuss the HSP causative genes, which have been modeled in flies.

Spastin (SPG4)

SPAST gene encodes spastin, a member of AAA (ATPase associated with various cellular activities) protein family. The presence of two initiation codons in *spastin* open reading frame gives rise to two isoforms M1 and M87 of 68 kDa and 60 kDa molecular weight, respectively (Claudiani et al. 2005; Kozak 2002). The shorter isoform is the predominant isoform in all tissues at all stages of development. M1 isoform, on the other hand, is detectably present in the adult spinal cord only (Solowska et al. 2008, 2010). N-terminal region of M1 isoform bears a hydrophobic hairpin that helps in membrane localization and binding to broad classes of endoplasmic reticulum proteins like Receptor Expression-Enhancing Proteins (REEPs), reticulons, and atlastins (Park et al. 2010). The C-terminal of spastin, common to both isoforms, possesses domains required for microtubule binding and severing including the AAA ATPase domain. Spastin assembles in the form of hexamers and breaks microtubules by severing long microtubules into shorter polymers by

hydrolyzing ATP (Roll-Mecak and Vale 2005; Baas et al. 2006). SPG4 mutations are the most common cause of autosomal dominant, pure HSP accounting for nearly 15–40% cases. More than 200 causative mutations including missense, non-sense, and deletions have been identified in the gene (Hazan et al. 1999; Fonknechten et al. 2000; Depienne et al. 2007; Shoukier et al. 2009).

The *Drosophila* spastin shows high similarity with the human counterpart, with all the major domains conserved between the two. RNAi-mediated knockdown of *Drosophila spastin* resulted in synaptic undergrowth in larvae, while synaptic strength was increased. Consistent with the microtubule-severing role of spastin, levels of acetylated microtubules were robustly elevated at Neuromuscular Junctions (NMJs). The synaptic function was restored by pharmacologically restoring the microtubule dynamics (Trotta et al. 2004). However, contrary to this, *spastin* loss-of-function mutants displayed synaptic overgrowth forming grape-like clusters of boutons, particularly at the NMJ terminals. Neurotransmission was impaired with a reduction in evoked responses or excitatory junction potentials (EJPs). The mutants also showed severe locomotor and other behavioral defects. The eclosion rate was nearly 20%, with escapers showing severely compromised jumping and flight behaviors. They also showed a reduction in the levels of α-tubulin and futsch (*Drosophila* ortholog of microtubule-associated protein MAP 1B) in the overall and stable microtubule populations, especially at the synaptic terminals. The defects were rescued by the neuronal overexpression of spastin. Overexpression of spastin in larval and embryonic muscles dramatically reduced the microtubule network. However, neuronal overexpression of spastin collapsed the embryonic CNS, implying that it may play a role in the breakdown of microtubules during the axonal growth phase (Sherwood et al. 2004). Together, these studies infer that spastin controls the level of stable microtubules.

In a following study, Orso *et al.* found that overexpression of spastin, bearing a pathogenic mutation homologous to a human mutation in spastin, elicits the same phenotypes as that of spastin RNAi knockdown, reducing the size of NMJs and stabilizing the microtubules indicating a dominant negative behavior of these mutations. Microtubule destabilizing drug vinblastine was able to ameliorate these phenotypic defects substantiating the role of spastin mutants in stabilizing the microtubule network. This study implies a dominant negative nature of spastin mutation in addition to the more diverse set of mutations, which reiterate the haploinsufficiency model of spastin mutations (Orso et al. 2005).

Drosophila null mutants overexpressing human spastin constructs showed significant rescue of eclosion rates, synaptic morphology, microtubule network, and behavior comparable with the results of fly constructs revealing a high conservation between the proteins. Interestingly, expression of pathogenic mutations located in the catalytic domain of spastin improved the eclosion rate. It indicates a role of spastin outside microtubule severing function, which does not require ATPase domain. The heterozygous mutation for S44L, which are typically asymptomatic in humans, still exhibited some cellular defects in flies demonstrating that this mutation may still be causing some defects to neurons without any outward disease symptoms (Du et al. 2010). In an unbiased genetic screen, p-21 activated kinase 3

(pak3) was found to be a genetic interactor of spastin. Reduction of pak3 suppresses the abnormal synaptic morphology, neurotransmission, and the microtubule defects in the spastin mutants (Ozdowski et al. 2011). Cold temperature also alleviates the eclosion rate, survival, and synaptic defects in spastin mutants. The effect is, however, not restricted to spastin and was seen in other mutants like *few* and kat-60L1, indicating a broad role of temperature on neuronal phenotypes (Baxter et al. 2014).

Haploinsufficiency is the predominant genetic model for HSP because most of the pathogenic mutations in spastin are truncations leading to translation of a meager amount of the shortened protein. Also, non-sense-mediated decay (NMD) clears the mRNAs bearing premature termination codons (Lykke-Andersen and Jensen 2015; Popp and Maquat 2016). Recently, cell culture-based work has proposed a novel mechanism involving the role of truncated proteins in the toxicity of HSP. Truncated M1 isoform showed more toxicity toward neurite outgrowth than truncated M87. Since M1 isoform still retains the N-terminal part, it is possible that it might retain its ability to insert into ER, but the interaction with REEP1 and atlastin-1 may be lost, hence affecting its functions. Interestingly, N184X mutation fosters the translation at a novel, third start codon in *SPAST*, producing a novel 48 kDa M187 isoform, which retains the microtubule severing activity. It appears that presence of the N184X mutation was essential for the identification of this start codon by the translational machinery because this isoform was not detectable in cells transfected with wild-type spastin cDNA (Solowska et al. 2017). Thus, these data imply that truncated proteins can exacerbate the pathogenesis of HSP.

Spastin has also been related to axon regeneration as the loss of a single copy of spastin significantly reduced axon regeneration. It was found that spastin is a key regulator that controls axon regeneration of laser-transected sensory axons or dendrites without affecting the normal developmental axon outgrowth or pruning in flies. These observations are interesting because HSP can remain asymptomatic for a long period before its onset. Most of the phenotypes observed across various model organisms reveal neurodevelopmental defects, which are not a general phenomenon in HSPs. Thus, this study offers a probable postdevelopmental mechanism to explain the disease discourse (Stone et al. 2012; Trotta et al. 2004; Sherwood et al. 2004; Wood et al. 2006; Jinushi-Nakao et al. 2007; Yu et al. 2008). Rao *et al.* found that the endoplasmic reticulum was concentrated at the tip of growing axons. The ER concentration was observed in axon regeneration and not in case of dendrites. This was, however, defective in case of spastin mutants and atlastin RNAi knockdown animals. Thus, it seems that some HSP proteins may work together with underlying microtubules in controlling the axon regeneration. These observations are in line with the ability of spastin to interact with ER proteins and also its role in microtubule severing (Rao et al. 2016). However, it remains to be seen whether the reason for the late onset of degeneration is a postdevelopmental defect or a result of early assaults, which snowballs over a period resulting in an observable phenotype.

Ubiquitous overexpression of *spastin* increased the size of lipid droplets (LDs) in fat bodies, while decreasing their overall number. Spastin knockdown, on the other

hand, showed a decrease in the number of lipid droplets. Ubiquitous expression of pathogenic mutant spastin^{K467R}, known to abolish ATP binding ability and act as a dominant negative form, also reduced the number of LDs. In line with the role of spastin as a positive regulator of LD formation at the tubular ER, overexpression of wild-type spastin in neurons and muscles increased the LD number. The M1 spastin isoform contains an N-terminal LD targeting signal comprising a hydrophobic domain interrupted by an arginine residue. LDs have been proposed to act as sequestration or degradation platforms for proteins and can have a possible role in intermembrane lipid trafficking. However, given the limited role of neurons in this foray, the function of spastin in LD metabolism in the context of neurons needs further investigation. Nonetheless, this opens up yet another possible mechanism underlying HSP (Papadopoulos et al. 2015).

Kinesin Family Member 5A (SPG10)

Kinesin Family Member 5A (*KIF5A*) encodes the heavy chain of kinesin-1, which is a heterotetramer consisting of two identical kinesin heavy chains and two identical light chains (Goldstein 2001). Mutations in *KIF5A* causes autosomal dominant, pure, or complicated forms of HSP (Reid et al. 2002). Protein complexes, organelles, and mRNAs need to be transported inside polarized neurons along the roadways laid down by cytoskeletal proteins, and microtubule-associated protein family of kinesins mainly mediate the anterograde traffic along these tracks (Hirokawa 1998; Goldstein and Yang 2000). Kinesins are plus-end-directed molecular motors which carry the cargo toward axon terminals, while minus-end-directed dyneins mediate the retrograde transport toward the cell body mainly carrying endocytic material, old and damaged components for degradation and/or recycling. Kinesin superfamily proteins (KIFs) possess a conserved ~360 residue globular head domain (also referred to as the catalytic core) which contains a catalytic pocket for ATP hydrolysis and a binding site for microtubules. In analogy with the structural features of kinesin-1, the catalytic core, also called as head, is followed by stalk region and a tail domain at the other end (Miki et al. 2005).

The *Drosophila Kinesin heavy chain* (CG7765) is the only ortholog of three of the vertebrate KIF5 proteins. KIF5A and KIF5C express mainly in the neuronal cells, and KIF5B has a broader expression and is expressed in the nervous system as well as glia of mouse (Kanai et al. 2000). Kinesin is ubiquitously expressed and *Drosophila Khc* mutants suffer severe morphological and physiological consequences; null mutants are morphologically smaller and mostly die at the second instar larval stage. The larvae exhibit compromised tactile behavior and become paralyzed mostly in the posterior segments (Saxton et al. 1991). Electrophysiological analysis of hypomorphic *Khc* alleles revealed that compound action potential amplitude wanes by a factor of four due to impaired sodium channel activity. EJC amplitude also emulates the paralytic behavior with amplitudes dropping by almost a factor of three in the anterior segments and by a factor of five in the posterior segments. The posterior segments show the most dramatic reduction in the

neurosecretion (Gho et al. 1992). The number of boutons also reduced by a factor of nearly four in the A2 segment and by a factor of five in the A6 segment with respect to the controls. The axons showed unusual swellings that contained cargoes of fast axonal transport like synaptic vesicles, synaptic membrane proteins, lysosomes, and mitochondria (Hurd and Saxton 1996).

Pan-neuronal knockdown of *Khc* reduces the viability, but the simultaneous reduction of *Khc* in both the neurons and glia causes lethality. The rescue experiments with Khc^{8} null allele established the role of glial cells. Ubiquitous expression of *Khc* successfully rescued the lethality, while pan-neuronal expression could only rescue 6% flies, which were very sluggish and died within six days. Ubiquitous expression with glial-specific repressor dropped the viability to 60%. Glial-specific knockdown reduced and delayed the evoked responses at the muscles. The larvae also had axonal swellings, the majority of which had mislocalization of the septate junction protein Neurexin IV. These observations indicate the role of Khc in cargo transport in glia and its contribution to neuronal function (Schmidt et al. 2012). In vitro studies showed that pathogenic mutations of *KIF5A* could slow down the movement of motor proteins along microtubules or may interfere with their interaction with microtubules (Ebbing et al. 2008). Wild-type larvae or larvae lacking one copy of *Khc* show similar locomotion, but the larvae ectopically expressing pathogenic mutation Khc^{N262S} (corresponding to human Khc^{N256S}) have severe defects exhibiting the classic distal degeneration. The impairments are strikingly similar to those observed in *Khc* null larvae. Thus, the mutation acts as an antimorph or dominant negative, which is further validated by amelioration of phenotypic defects by the overexpression of wild-type Khc, which dilutes its antimorphic activity (Füger et al. 2012). It is interesting to note that Schmidt *et al.* found Khc^{6} allele as antimorphic and were not able to rescue it with a wild-type copy of Khc. However, Saxton et al. were able to rescue lethality by a genomic rescue construct probably because of very high levels of endogenous *Khc* expression, which dilutes the antimorphic Khc^{6} (Füger et al. 2012; Saxton et al. 1991). These observations provide a strong case for dominant-negative effects of some mutations as opposed to haploinsufficiency. While flies lack neurofilaments, it recapitulates most of the phenotypic defects in *Khc* mutants and undermines the role of neurofilament transport defects in HSP pathogenesis (Wang and Brown 2010). Synapse and axon loss thus seems to be a cardinal feature of HSP as limited studies on human tissues suggested a dying-back axonopathy (Wharton et al. 2003). Therefore, understanding the mechanism of distal degeneration and axonal transport may take us one step closer for better therapeutics.

Interestingly, mutations in *KIF5A* have been identified in other neurodegenerative diseases like amyotrophic lateral sclerosis (ALS). However, mutations causing SPG10 are almost exclusively missense and are located in the N-terminal motor domain. On the other hand, ALS-causing mutations are predominantly in the C-terminal cargo-binding region, with many being loss-of-function alleles of *KIF5A*. HSP-related mutations in KIF5A mainly affect the microtubule binding, ATP hydrolysis, and movement of molecular motors, and hence anterograde transport of cargo along axons and dendrites (Ebbing et al. 2008; Henthorn et al. 2011).

Primarily, motor neurons seem to be the targets of ALS-related mutations, which frequently accumulate cytoplasmic aggregates. It is reinforced by the fact that KIF transports granules containing RNA and RNA-binding proteins including ALS-related FUS and hnRNPA1 (Kanai et al. 2004; Nicolas et al. 2018). This inability of cargo binding might lead to their accumulation in cell bodies. The nature of *KIF5A* mutation, therefore, defines the course of neurodegeneration.

Kinesin Family Member 1A (SPG30)

Kinesin Family Member 1A (Kif1A) belongs to the kinesin-3 family and mutations in this gene cause pure or complex HSP (Erlich et al. 2011; Klebe et al. 2012; Lee et al. 2015; Citterio et al. 2015; Ylikallio et al. 2015; Hotchkiss et al. 2016). *Kif1A*-null mice die shortly after birth, show significant neuronal death, and have prominent motor and sensory disturbances. The transport of synaptic vesicle precursors exhibits specific and significant decrease (Yonekawa et al. 1998).

Drosophila unc-104/Imac is the ortholog of *KIF1A* and also bears similarity to *KIF1B* and *KIF1C*. *Kinesin family member 1C* (SPG58) is also implicated in HSP (Dor et al. 2014; Tesson et al. 2015). *Unc-104* null mutants are embryonic lethal, and motor neuron growth cones fail to form synaptic boutons. The embryonic nerve endings have very few active zones and rarely have synaptic vesicles (Pack-Chung et al. 2007). Despite its role in axonal transport, these mutants behave differently from Kinesin-1 family; *Drosophila unc-104* mutants do not form axonal swellings. Non-lethal Khc mutations also lead to axonal clogs or jams; hence lack of focal swellings is unlikely due to differences in allele severity and represents functional differences. So, the *KIF5A* and *KIF1A* seem to mediate specific functions, and focal swellings do not reflect a general outcome of defective axonal transport (Pack-Chung et al. 2007; Füger et al. 2012). A hypomorphic mutation *unc-104^{bris}*, caused by a point mutation in the forkhead-associated (FHA) domain, survives till larval stage and exhibits severely reduced anterograde transport of synaptic and dense core vesicles along with a reduction in the number of active zones. Null and hypomorphic alleles of *unc-104* also have bouton formation defect (Pack-Chung et al. 2007; Barkus et al. 2007; Kern et al. 2013). *Unc-104^{bris}* induces a severe decrease in Rab3 immunoreactivity, a synaptic vesicle-associated protein, at NMJs and ectopic overexpression of Rab3 transgene partially rescues the defect in active zone numbers (Zhang et al. 2016). *Unc-104^{bris}* mutants show severely compromised synaptic functions with dramatically reduced EJP and mEJP amplitudes. The mutant also shows a 20-fold decrease in the mEJP frequency (Zhang et al. 2017). These data suggest the different mechanistic underpinnings of seemingly similar proteins in HSP. The nature of mutation, however, can dictate the phenotypic defects and might explain some differences in these studies. Deletion and point mutation-based defects, as used in these studies, might not precisely mimic the human conditions. Hence, a detailed and comparative study of disease-related mutations can demystify this case.

Atlastin 1 (SPG3A)

SPG3A accounts for nearly 10% of cases of autosomal dominant pure HSP (Zhao et al. 2001; Reid 2003). It is the second most common cause of HSP and the most common early-onset form (Wilkinson et al. 2003; Abel et al. 2004; Dürr et al. 2004; Namekawa et al. 2006). Atlastin-1 is a large oligomeric membrane-associated GTPase belonging to the dynamin superfamily of proteins and is highly enriched in the central nervous system (CNS). It typically comprises N-terminal GTPase domain with three conserved GTP binding motifs and two C-terminal transmembrane domains. It localizes mostly in tubular ER and the ER-Golgi intermediate compartment and, in particular cases, at the *cis*-Golgi apparatus (Zhu et al. 2003; Rismanchi et al. 2008; Blackstone 2012). Atlastin-1 is required for ER network formation involving the homotypic fusion, that is, merging of ER membranes. Depleting atlastins or overexpression of its dominant-negative forms in cells leads to the formation of unbranched ER (Hu et al. 2009; Orso et al. 2009). The neuronal rough ER is mainly concentrated in the soma and the surrounding somatodendritic volume, while the tubular smooth ER (SER) mainly localizes in the distal dendrites and the axon (González and Couve 2014).

There are three atlastins (named atlastin-1, -2, and -3) in humans, which likely represent the functional paralogs, but atlastin-1 is the only member which is highly enriched in CNS (Zhao et al. 2001; Zhu et al. 2003; Praefcke and McMahon 2004; Rismanchi et al. 2008). Some HSP genes have a role in endoplasmic reticulum morphogenesis, including spastin, atlastin-1, receptor accessory protein 1, REEP-1, and reticulons, which collectively may account for nearly 60% of HSP cases in North America and North Europe (Blackstone et al. 2011; Montenegro et al. 2012). *Drosophila* has a single but highly conserved ortholog of atlastin and has a very similar structural domain organization as that of mammalian isoforms. Lee et al. fortuitously identified an insertion mutant of *Drosophila atlastin* (named atl^1) having a P-element insertion in the first intron of CG6668. Transcript levels in the mutants were abolished and protein levels were also not detectable. It is a bang-sensitive mutant paralyzed by mechanical shock. The mutant surprisingly is viable but sterile, has a short lifespan and reduced climbing ability, which worsens with age. The defects were attributed to a great degree to dopaminergic neurons because transgenic expression of wild-type atlastin in the dopaminergic neurons displayed significant rescue. Administration of dopamine precursor L-DOPA or dopamine D_1 receptor agonists could significantly alleviate the paralysis and mobility defects in mutants (Lee et al. 2008). However, contrary to these findings, methylphenidate, an amphetamine that influences neurotransmitters like glutamate, serotonin, and dopamine and acts at dopamine D1 receptor and noradrenergic α2 receptors, did not show beneficial effects on human HSP patients (22 patients, with 6 recognized as SPG4) (Klebe et al. 2006). Also, two of the patients with SPG3A type HSP showed normal nigrostriatal integrity reiterating that dopamine may not be effective in treating HSP. These disagreements can arise from various reasons like the nature of atl^1allele, which may not represent a clean mutant because another deletion mutant atl^2 is hemizygous and homozygous lethal. It may also be attributed to

species-specific differences in selective neuron vulnerability, and finally, these two proteins might vary in some molecular functions (Albin et al. 2008).

Downregulation of *atlastin* in larval muscles causes ER fragmentation, while enlarged ER profile is observed by its overexpression, likely due to membrane fusion involving GTPase-dependent mechanism (Orso et al. 2009). A deletion mutant in *Drosophila* Atlastin atl^2 survives till pupal stage, with very few escapers. These mutants showed frequent clustering of boutons together with a reduction in muscle surface area. The bouton number was significantly increased indicating the role of atlastin during development. Expression of wild-type *atlastin* in muscles, but not in neurons, rescued the defects suggesting a role of atlastin primarily in muscles. The integrity of the subsynaptic reticulum and postsynaptic scaffolding proteins like disc-large (dlg) and α-spectrin was also compromised. Alteration in the levels of atlastin had deleterious effects on the morphogenesis of Golgi and ER, with loss-of-function mutants showing a reduction in the levels of Lys-GFP-KDEL and GalTase-GFP expression indicating the poor formation of these organelles. These phenotypes were partially restored by the postsynaptic expression of *atlastin* in the muscles. Further, *Drosophila* spastin was shown to physically interact with atlastin and functionally work together to disassemble microtubules in muscles. Spastin null mutation suppressed the atlastin overexpression defects in the muscles. Most of the abnormalities were dramatically rescued by administration of the microtubule-destabilizing drug vinblastine (Lee et al. 2009).

Loss of atlastin had no major effects on the ER organization in the cell bodies; however, initial axonal segments displayed aberrant ER morphology, and the nerve terminals revealed a diffused pattern of the organelle as compared to the basket like structure in wild-type larvae representing the fragmentation of ER. Targeted knockdown of atlastin and expression of a dominant-negative atl^{K51A} allele in motor neurons recapitulated similar phenotypes. The atl^2 mutants showed compromised neurotransmission with a twofold decrease in the evoked potentials at 0.6 mM extracellular Ca^{2+} concentration. Increasing the bath $[Ca^{2+}]$ partially restored this defect probably because nerve terminal ER can influence cytoplasmic $[Ca^{2+}]$ and at high concentrations, it may be less limiting for the neurotransmitter release. Muscle-specific knockdown of *atl* does not affect neurotransmitter release. Expression of UAS-atl^+ in motor neurons caused accumulation of large ER puncta in motor neuron cell bodies as well as axons and decreased EJP amplitude. Loss of atlastin in either neurons or muscles leads to progressive locomotor deficits accompanied by degeneration of specific muscles within the adult thorax preceded by the accumulation of aggregates containing poly-ubiquitin and escalation of ROS production. It leads to the activation of JNK/Foxo stress pathway. The administration of TOR-Kinase inhibitor rapamycin pharmacologically rescued locomotor defects and poly-UB aggregate accumulation caused by either muscle or neuronal atl knockdown. These results show that *atl* loss can potentially trigger cell degeneration through cell-autonomous and non-autonomous mechanisms, possibly through synaptic means and rapamycin might hold therapeutic potential for at least some forms of HSP (Xu et al. 2016).

Downregulation or overexpression of atlastin, specifically in the motor neurons, was sufficient to cause locomotor defects in both larvae and adults. However, these defects were rescued only by expression of atlastin in broader neural circuits and not specifically in the motor neurons. Axons displayed the abnormal distribution of secretory organelles and accumulation of some presynaptic components in the distal axons. The number of vesicles in the periphery of active zones was decreased and membranous organelles with small vesicle-like structures reminiscent of multivesicular bodies (MVBs) accumulated in the atlastin knockdown larvae. This implies the role of atlastin in biogenesis and mobilization of synaptic vesicles non-adjacent to the active zones. Knockdown of atlastin also impaired the synaptic transmission with compromised readily-releasable and reserve pools of synaptic vesicles (Gregorio et al. 2017). In contrast to a previous study (Summerville et al. 2016), EJPs were found to be unaffected. The discrepancies may root from the use of voltage-clamp versus current-clamp or differences in $[Mg^{2+}]$ (Gregorio et al. 2017). Due to its similar structure and highly conserved functions concerning ER morphogenesis, *Drosophila* ortholog of atlastin may reveal some key insights into the molecular basis of HSP.

Reticulon 2 (SPG12)

Reticulons and REEP/DP1/yop1p family of proteins are essential for the generation of endoplasmic reticulum sheets and tubules. These proteins share a common feature, termed as reticulon homology domain (RHD), comprising of two long hydrophobic stretches. These are thought to occupy more space in the outer leaflet than the inner leaflet of the ER lipid bilayer. The aforementioned double-hairpin hydrophobic domains lead to the ER-membrane curvature, a process termed as hydrophobic wedging (Voeltz et al. 2006; Hu et al. 2008; English et al. 2009). These proteins are largely restricted to the tubular ER and not seen on the continuous nuclear envelope sheets and peripheral ER. Mutations in *reticulon-2* cause autosomal dominant spastic paraplegia (Montenegro et al. 2012).

The *Drosophila* genome encodes two reticulons *Rtnl1* and *Rtnl2*, orthologous to the four reticulons found in mammals. Only *Rtnl1* is widely expressed, while *Rtnl2* shows restricted patterns of expression confined mainly to testis and fat bodies. Deletion mutant of $Rtnl1^{1}$ (in which all RHD-encoding exons are deleted) is viable and fertile with no obvious signs of developmental abnormalities. The flies, however, exhibit a short lifespan, with nearly 39% decrease in lifespan at 29 °C (Wakefield and Tear 2006). Another hypomorphic mutant generated by EMS-induced mutagenesis of a GFP-exon-trapped *Rtnl1* was also viable, establishing the non-essential but an important role of Rtnl1 (Röper 2007). Rtnl1 displayed a reticular distribution in epidermal cells overlapping with the ER retention signal KDEL. In CNS, however, it was expressed in motor neuron axons. Neuropil, which is rich in synapses, axons, and dendrites, exhibited higher levels of Rtnl1 in contrast to its low expression in cell bodies where KDEL is mostly located. At the neuromuscular junctions, it co-localized with the microtubule marker Futsch. This distribution

pattern indicates that it might be localized on the smooth ER which resides preferentially in axons rather than the cell bodies. Flies with ubiquitous RNAi knockdown of *Rtnl1*, leading to almost complete depletion of the protein, were viable, consistent with the mutant phenotype. The flies exhibited normal locomotor activity in early life, which, however, deteriorated with age, indicative of the progressive degeneration caused by *rtnl1* loss. Depletion of *rtnl1* leads to a loss of reticular pattern in epidermal cells to a more diffused pattern and the ultrastructural analysis revealed a structural reorganization of ER with a threefold surge in the average length of the ER sheets. It also leads to an ER-stress response indicating a crucial role of Rtnl1 in the maintenance of tubular ER structure. Importantly, loss of *rtnl1* caused pronounced defects in the distal abdominal segments of the larvae, with NMJs showing fewer but enlarged mitochondria indicating that it might affect the localization or the fission/fusion balance of mitochondria in the neuron terminals of longer axons (O'Sullivan et al. 2012).

Expressing Acsl::myc to label axonal ER revealed partial loss of ER staining in posterior axons of *rtnl1* depleted animals sparing the anterior part along with more irregular staining in the posterior region. These defects were partially rescued by a $rtnl1^{Pacman}$ genomic clone. Loss of *rtnl1* causes mild irregular organization of ER in the axons, which were aggravated by loss of another ER protein REEPB. *Rtnl1* mutants also displayed large accumulations of the synaptic vesicle protein CSP (cysteine string protein) in many peripheral nerves, which was rescued by two copies of $Rtnl1^{Pacman}$genomic clone (Yalçın et al. 2017). Like atlastin, loss of $rtnl^{1}$causes increased arborization and leads to a reduction in the EJPs. Overexpression of $rtnl^{1}$ also reduces the transmitter release. Knockdown of $rtnl^{1}$ in other cells of the tripartite synapse-like muscle and glia also conferred similar defects. Ubiquitous expression of wild-type $Rtnl^{1}$ was sufficient to rescue these defects, but its expression in motor neurons, muscle, or glia alone could not rescue these abnormalities. These phenotypes were, however, rescued by expression of $rtnl^{1+}$ simultaneously in all the three tissues, indicating that Reticulon1 might regulate intercellular signaling among these cell types. Increased bath [Ca^{2+}] also rescued the transmitter release defect as in the case of atlastin. Both these proteins mutually suppressed the reduction in EJP raising the possibility of their antagonizing effect on ER (Summerville et al. 2016). Thus, Rtnl1 reproduces the cardinal features of the reticulons in the regulation of ER architecture and importantly the axon length-dependent defects involving the role of the ER.

Receptor Expression Enhancing Protein-1 (SPG31)/Receptor Expression Enhancing Protein-2 (SPG72)

SPG31 is the third most common form of HSP. REEP and Reticulon family of proteins contain reticulon homology domain (RHD) and play an essential role in the formation and maintenance of peripheral ER tubules (Züchner et al. 2006; Park et al. 2010; Esteves et al. 2014; Blackstone 2018). *Drosophila* and humans both possess six REEP proteins, and sequence comparisons reveal *CG42678 or ReepA*

(previously designated as *Reep1*) as a single ortholog of mammalian REEP1-REEP4. *CG8331* (designated as *REEPB*) is orthologous to *REEP5* and *REEP6* because of its high sequence similarity and widespread expression. The remaining *REEP* genes in flies have restricted expression in testes and larval fat body. REEPB is strongly expressed in the epidermal cells and larval CNS, but was also detectable in segmental nerves and the NMJ. However, ReepA::GFP fusion constructs, corresponding to particular isoforms, exhibited weak expression in epidermal cells and third instar larval CNS, but were not detected in axons and presynaptic terminals. *ReepA*$^{-}$*ReepB*$^{-}$ double mutants displayed longer ribosome-studded ER sheets and increased ER stress in epidermal cells. Rtnl1 and REEP proteins, therefore, seem to be crucial for maintaining the integrity of the ER network. Loss of ReepB disturbs the distribution of ER in the posterior axons, while ReepA is likely having a subtle or no role in these phenotypes. Loss of Rtnl1 leads to ER fragmentation in axons, which is worsened by the loss of ReepB, while this was not observed in the *Reep* mutants independently. Live imaging and photobleaching experiments further confirmed the continuous distribution of ER in axons and loss of reticulon and Reep proteins caused occasional gaps, which represented physical breaks in the ER continuity. Therefore, ReepA, which is orthologous to the REEP proteins implicated in HSP, is not detectable in peripheral nerves and has meager contributions in ER disruption defects; while ReepB is enriched in these structures and together with Rtnl1 leads to ER-related defects in the peripheral nerves. However, even in case of *ReepA*$^{-}$*ReepB*$^{-}$*Rtnl1*$^{-}$ triple mutants ER distribution was not entirely abolished from the axons implying the redundancy of these proteins and the likelihood of unidentified proteins that may be part of this ER structuring team (Yalçın et al. 2017).

Non-imprinted in Prader-Willi/Angelman Syndrome 1 (SPG6)

NIPA1 is a widely expressed transmembrane protein enriched in brains and mutations in this gene lead to a dominant, pure form of HSP (Rainier et al. 2003). *Drosophila CG12292* is the ortholog of mammalian NIPA1 and has been designated as *spichthyin* (*spict*) since it appears to be orthologous to SPG6 as well as *ichthyin*. While *Drosophila spict* mRNA is broadly distributed, it shows enriched expression in CNS and muscles during embryogenesis. In *Drosophila*, S2 cells spict displayed significant overlap with the early endosome marker Rab5, but not substantially with the late endosomal-multivesicular body marker hook or Rab11, which marks recycling endosomes or the lysosomes. Similar patterns were also observed at larval NMJ. Rab5 expression was less intense in boutons of spict mutant NMJs, but was not affected in neuronal cell bodies and axons of larvae or in spict-depleted S2 cells; therefore, it seems to be spatially important at these locations. *Spict* mutants showed a significant increase in NMJ arborization, with bouton numbers reaching twice that of normal and these defects were rescued by neuronal expression of wild-type spict and not by expressing the protein in the muscle. It has, therefore, an important function in restricting the growth of NMJs

(Wang et al. 2007). Bone Morphogenetic Protein (BMP) pathway plays a key role in the regulation of synaptic size at the *Drosophila* NMJ (Aberle et al. 2002; Marqués et al. 2002; McCabe et al. 2003, 2004). Double mutants of *spict* and key BMP receptor subunits like *tkv (thickveins)* and *saxophone (sax)* belonging to type-I and type-II receptor subunit *wit (wishful thinking)* and type-II ligands *Gbb (Glass bottom boat)* or *medea (med)* were all indistinguishable from homozygous BMP mutants alone. Heterozygous combinations of BMP mutants also partially suppressed the overgrowth. Levels of phospho-Mothers against decapentaplegic (pMad), Tkv, and Wit were all upregulated in the NMJ boutons. Therefore, spict checks the levels of BMP receptors at NMJ. Spict was shown to physically interact with Wit and partially colocalize with Tkv and Wit at NMJ boutons. Also, in S2 cells it tips the relocalization of Wit from the surface to the early endosomal compartment. Thus, spict works antagonistically to BMP signaling acting likely through receptor trafficking. *Spict*-overexpressing neurons display decreased levels of α-tubulin and acetylated α-tubulin. The decreased level of tubulin and microtubules was suppressed by constitutively active Tkv implying that spict may be regulating microtubules through BMP signaling. Synaptic vesicle protein accumulated in the axons of BMP mutants like *tkv*, *sax, gbb, wit* and in spict-overexpressing axons of larvae, however, axons of spict mutants seemed unaffected. Tkv expression suppressed the defect in spict-overexpressing animals (Wang et al. 2007). It remains to be seen whether spict-mediated receptor trafficking affects axonal transport and whether it has some role in HSP. Spichthyin, along with other cytoskeleton-regulating proteins like spastin and atlastin, also plays a role in axon regeneration revealing yet another postdevelopmental role of this protein (Rao et al. 2016). The relation between these processes and HSP, however, is far from clear.

Spartin (SPG20)

Spartin bears an N-terminal MIT (microtubule interacting and trafficking) domain, a central Eps15 interacting domain, and a C-terminal senescence domain (Ciccarelli et al. 2003; Bakowska et al. 2005). Mutations in the gene coding for Spartin (SPG20) cause an autosomal-recessive and complicated form of HSP called Troyer syndrome. In addition to spasticity in lower extremities, it is clinically characterized by dysarthria, short stature, cognitive impairment, and distal muscle wasting (Cross and McKusick 1967; Patel et al. 2002; Proukakis et al. 2004; Manzini et al. 2010). *Drosophila* Spartin (*CG12001)* is the single ortholog of SPG20 and its null allele (*spartin1)* is viable and fertile. Spartin localizes in larval brain and the ventral nerve cord and is also enriched at the NMJ exclusive to presynapse, with its punctate pattern partially overlapping with an early endosomal marker Rab5. Spartin loss-of-function induced the overgrowth of NMJs and led to a significant increase in the satellite boutons. The EJP in mutants was significantly reduced. These synaptic abnormalities were restored to the wild-type levels by the expression of a Spartin transgene in neurons, but not in muscles. Interestingly, expression of human Spartin also exhibited a

similar rescue in NMJ morphology, implying high functional conservation among these genes. Overexpression of Spartin had inverse effects on NMJ morphology establishing its role as a repressor of synaptic growth (Nahm et al. 2013).

Human Spartin is known to interact with endocytic and trafficking protein Eps15 (Bakowska et al. 2005) and a similar physical association was observed in *Drosophila*. Spartin and Eps15 colocalized at the *Drosophila* NMJs (Nahm et al. 2013). *Spartin* and *eps15* mutants show similar phenotypic defects (Koh et al. 2007) and double mutants of these genes worsened the defects suggesting that they might belong to the same pathway. Similar to *Eps15* loss-of-function mutants, $Spartin^{1}$ also shows endocytic defects. Moreover, Spartin-Eps15 interaction is essential for its function since Spartin does not rescue NMJ defects in the absence of Eps interacting domain. Levels of presynaptic P-Mad levels are upregulated in $spartin^{1}$ and removing one or both copies of BMP receptor Wit suppresses the overgrowth, strongly linking the gene to BMP pathway (Nahm et al. 2013). Spartin promotes endosomal trafficking of Wit to lysosomal degradation (Nahm et al. 2013). Interestingly, mammalian Spartin also exhibits similar internalization and endosomal sorting of EGFR suggesting conserved functions of two genes (Bakowska et al. 2007). Stable microtubules and Futsch levels are increased in *Spartin* mutants. Mutating *futsch* or treatment with microtubule-destabilizing drug vinblastine strongly suppresses synaptic growth defects mediated by elevated BMP signaling (Nahm et al. 2013). There is an intimate relationship between BMP signaling and HSP. For instance, NIPA1 and its *Drosophila* ortholog regulate BMP receptor trafficking and other HSP proteins like Atlastin, Spastin, and Spartin act as BMP inhibitors (Wang et al. 2007; Tsang et al. 2009). Indeed, *Spartin* mutants show progressive, age-dependent neurodegeneration, which is ameliorated by BMP inhibitor Dad in neurons (Nahm et al. 2013). Infact, heterologous expression of Spartin in yeast cells displayed pro-survival effects, decreased ROS production, and improved lifespan likely through regulation of mitochondria-assisted metabolism (Ring et al. 2017).

Paraplegin (SPG7)

Mutations in *Paraplegin* gene cause pure or complicated autosomal recessive HSP. It is an m-AAA metalloprotease of the inner mitochondrial membrane where it carries out protein quality control and also has a role in ribosomal assembly (Casari et al. 1998; Rugarli and Langer 2006). *Drosophila CG2658* encodes the fly ortholog of *Paraplegin* with 58% identity. It contains a predicted AAA domain, an M41 metallopeptidase domain, and an N-terminal mitochondrial targeting sequence. A CRISPR/Cas9-mediated deletion of the gene is viable and fertile at a young age. The flies are, however, short-lived, display behavioral defects, and show progressive muscle and neuronal degeneration. The retrograde mitochondrial transport in segmental nerves is increased and the activity of respiratory chain complexes I and II is reduced. The degenerating tissues have electron-dense aggregates and dysmorphic mitochondria implying the role of mitochondrial dysfunction. In photoreceptor neurons, the neurodegeneration seems to initiate at the synaptic terminals, which

corroborates with the dying-back axonopathy observed in HSP. So, the study provides key insights into the mechanism that may underlie this form of HSP (Pareek et al. 2018).

Patatin-Like Phospholipase Domain Containing-6 (SPG39)

Patatin-Like Phospholipase Domain Containing-6 (PNPLA6), also referred to as neuropathy target esterase (NTE), is a conserved lysophospholipase residing on the cytoplasmic face of endoplasmic reticulum and converts lysophosphatidylcholine to glycerophosphocholine (Zaccheo et al. 2004; Glynn 2005). Mutations in PNPLA6 lead to autosomal-recessive spastic paraplegia causing progressive weakness of lower and upper limbs (Rainier et al. 2008, 2011). The protein was identified as a molecular target of toxic organophosphorus compounds that cause axonal degeneration in large neurons and lead to organophosphorus-induced delayed neuropathy (OPIDN), very similar to spastic paraplegia (Smith 1930; Johnson 1990; Lotti 1991; Moretto 2000; Atkins and Glynn 2000). Recently, mutations in PNPLA6 have also been linked to a host of other syndromes, including Boucher–Neuhäuser syndrome, Gordon Holmes syndrome, and in patients with childhood blindness caused by photoreceptor death and clinical features of Leber congenital amaurosis and Oliver McFarlane syndrome (Deik et al. 2014; Synofzik et al. 2014, 2015; Topaloglu et al. 2014; Kmoch et al. 2015).

Drosophila swiss cheese (sws) is the ortholog of PNPLA6 (Lush et al. 1998; Moser et al. 2000) and *sws* mutant flies exhibit adult nervous system degeneration by around day 5 of adulthood detectable by neuronal apoptosis and the formation of spongiform lesions within the CNS, hence the name. Multilayered wrappings of glia are also observed around neurons (Heisenberg and Bohl 1979; Kretzschmar et al. 1997). *Drosophila sws* is expressed in the cortex of adult CNS within most or all neurons, which restricts to specific subsets of neurons with aging. It shares the esterase domain with PNPLA6 that mediates the phospholipase activity. In addition, both SWS and PNPLA6 share a domain that can specifically bind to and inhibit the PKA-C3 catalytic subunit of Protein kinase A mimicking the inhibitory function of the canonical regulatory subunits (Bettencourt da Cruz et al. 2008). Treatment of flies with organophosphorus compounds like vertebrates induces behavioral defects and neurodegeneration (Wentzell et al. 2014). The SWS protein functions cell autonomously in glia and neurons as the expression of wild-type *sws* in each cell type only rescues the respective defects in the mutant (Mühlig-Versen et al. 2005). Knockdown of *sws* specifically in the glia induces the formation of large membranous glial structures in the lamina, which are also observed in the mutants and likely originates from the defective pseudocatridge glia. The defects were reversed by the expression of *Drosophila* or mouse wild-type gene. SWS is also essential for glial wrapping of the neurons by ensheathing glia. Loss of *sws* in glia leads to locomotion defects and the reduction in the reliability with which the neurons respond to increasing frequencies in giant fiber system implying the role of glia in neuronal functions. Hyperwrapping was, however, not observed by the knockdown of *sws* in

the neurons and glia individually indicating that the defects perhaps arise due to lack of neuron–glia communication. The glial functions, unlike neurons, were observed to be dependent only upon the phospholipase function and independent of its role as a non-canonical PKA regulatory subunit role (Dutta et al. 2016).

Genes Not Yet Studied in the Context of HSP

Besides the above-mentioned genes, there are some *Drosophila* mutants which are available but not necessarily studied broadly in the context of HSP. It includes *neuroglian (nrg)*, an ortholog of L1-CAM (SPG1) mutations in which cause an X-linked complicated spastic paraplegia, associated with MASA syndrome, X-linked hydrocephalus, and X-linked agenesis of the corpus callosum (Bianchine and Lewis 1974; Rosenthal et al. 1992; Jouet et al. 1994), now considered to be part of a single clinical syndrome called CRASH (Fransen et al. 1995). Although *nrg* has been well studied in *Drosophila* with mutations causing embryonic lethality and established roles in processes like neurite growth, axon guidance, and dendrite morphogenesis (Hall and Bieber 1997; Islam et al. 2004; Yamamoto et al. 2006), they have not been directly related to HSP. *Nrg* mutants with a missense mutation in an extracellular domain alters the function of central synapse by regulating the microtubule cytoskeleton, a potential link to HSP (Godenschwege et al. 2006). Since L1-CAM mutations cause complex phenotypes, therefore, it becomes difficult to model *nrg* for HSP and derive inferences, which are specific to HSP.

Likewise, Seipin/BSCL2, an integral membrane protein of endoplasmic reticulum is implicated in SPG17, a complicated form of HSP associated with amyotrophy of hand muscles (Patel et al. 2001; Windpassinger et al. 2004). It is attributed to mutations affecting a glycosylation site in seipin (Windpassinger et al. 2004; Ito et al. 2008). *CG9904* is the closest ortholog of BSCL2 and loss of this gene leads to reduced lipid storage in the fat body and ectopic accumulation of lipid droplets in the non-adipose tissue (Tian et al. 2011). Seipin interacts with ER Ca^{2+}-ATPase SERCA and regulates intracellular calcium homeostasis (Bi et al. 2014). Reduction of seipin was shown to hamper axon regeneration and the protein has been proposed to work along with atlastin and spastin and may help in the concentration of ER at the growing axon tips to regulate axon regeneration. However, the role of seipin in context of HSP is obscure (Rao et al. 2016).

Conclusion

Drosophila genome may not be comprehensive enough to model all HSP-causing genes, but it encodes orthologs of crucial genes implicated in the majority of the HSP cases. The genes with key roles in endoplasmic reticulum morphogenesis, including spastin, atlastin-1, receptor accessory protein 1, REEP-1, and reticulons, which may account for nearly 60% of HSP cases in North America and North Europe, are well conserved in flies. The genetic data from model organisms,

however, should be carefully reviewed because one gene can lead to distinct diseases depending upon the type and location of mutation within the gene. The observations should be substantiated by independent approaches like employing pathogenic mutations relevant to the disease and using alternative reagents wherever possible. The upwelling in the pathogenic HSP genes over the past few years has added to the complexity of the disease spectrum, but some cellular mechanisms seem to be at the heart of many genetic lesions. These include axonal transport, microtubule stability, endoplasmic reticulum membrane modeling and shaping, mitochondrial function, BMP signaling, lipid metabolism, and endocytic trafficking. Targeting these modules may prove beneficial to tackle multiple genetic lesions at a time. HSP is still treated symptomatically. HSP research in flies, though cannot be a panacea for the entire HSP spectrum, can help us to delve into the underlying mechanisms which can lay the foundation for potential targeted treatments.

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Drosophila as a Model System for the Identification of Pharmacological Therapies in Neurodegenerative Diseases

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Abstract

Neurodegenerative diseases (NDs) constitute a very important problem in our current society, as they are usually associated with the aging process. NDs are devastating disorders that lead to severe disabilities and ultimately to death and have a considerable impact on human health. Although intense efforts are being made to shed light on the pathophysiology underlying these diseases, an important concern is that NDs are incurable and existing therapies are only directed to

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relieve their symptoms or delay the progression of the disease. Therefore, the development of new therapeutic approaches against NDs is urgent and challenging. In such a scenario, *Drosophila* is a very valuable model organism to study the pathophysiology underlying a wide range of NDs. Besides, *Drosophila* models of NDs have also become a very important tool for therapeutic discovery to treat these diseases. Here, we review the different experimental approaches used for the identification of therapeutic compounds in fly models of NDs, including the methods used for drug administration and the assays carried out to evaluate the efficacy of the candidate compounds. We also provide information about a number of studies performed in different *Drosophila* models of human NDs aimed to discover new potential therapies for these disorders.

Keywords
Drosophila · Neurodegenerative diseases · Compounds · Chemical libraries · Drug screens · Phenotypic assays

Introduction

Neurodegenerative diseases (NDs) constitute a very important problem in our current society since they are usually associated with the aging process. Although some are idiopathic, many of them appear as a consequence of mutations in specific genes or the presence of genetic variants that alter risk (Mitsui and Tsuji 2014). NDs are histologically characterized by the progressive loss of neurons from specific regions of the brain, which determine the pathological hallmarks and the phenotypic manifestation of a particular disease. Clinically, NDs are characterized by specific neurological symptoms such as cognitive decline, ataxia, Parkinsonism, or motor weakness. Thus, they are devastating disorders that lead to severe disabilities and ultimately to death, and hence have a considerable impact on human health. In the last few years, intense efforts have been made to shed light on the pathophysiology underlying these diseases, sometimes complicated by their multifactorial and complex nature. However, one of the most important concerns is that NDs are incurable, and existing therapies are only directed to relieve their symptoms or delay the progression of the disease. For all these reasons, identification of new drugs and the development of new therapeutic approaches against NDs are urgent and challenging (Durães et al. 2018; Singh et al. 2018).

Several strategies can be used in the drug discovery process to treat human disease. The chosen strategy depends not only on the molecular understanding of its pathophysiology but also on the availability of cellular or animal models able to reproduce relevant aspects of the disease. A possible strategy is to look for a drug or compound aimed to modify the activity of a signaling pathway or a specific protein that is altered in the corresponding disease. This target-based approach requires previous knowledge of that protein and of its role in the development of the disease and is mainly based on in vitro cell cultures or biochemical assays (Strange 2016;

Pandey and Nichols 2011). Numerous cell models of human NDs have been used for this purpose; however, they are limited in terms of complexity and, thus, compounds identified in such target-based screens often fail to exert the expected effect when administered to human patients (Strange 2016; Pandey and Nichols 2011; Dawson et al. 2018). An alternative approach is based on the identification of compounds able to modify an observable characteristic of an animal, tissue, or cell model of human disease (Strange 2016). This phenotype-based approach is mainly performed in whole animals and may provide information on critical parameters in drug development like absorption, distribution, metabolism, excretion, or toxicity (Strange 2016; Pandey and Nichols 2011), which are impossible to obtain in traditional in vitro assays. Thus, the development of animal models of NDs that consistently reproduce pathological features linked to these disorders is essential for preclinical therapeutic development. The phenotype-based search of new therapies is an increasingly used approach, in which the ability of different compounds to modify or reverse a disease-related phenotype in a model organism is evaluated, even in the absence of a known target (Swinney and Anthony 2011). Relevant models of NDs have been generated not only in rodents but also in lower vertebrate and invertebrate animals, such as the fruit fly *Drosophila melanogaster* (Dawson et al. 2018; Lambrechts et al. 2017). Several key factors make *Drosophila* a uniquely powerful animal model for neuroscience research. First, its relatively short life span allows addressing questions of brain function more rapidly than in other animal model organisms. Besides, flies have a complex central nervous system (CNS) with neurons and glia, which is protected by a blood-brain barrier and is similarly organized, but less complicated than that of the mammalian brain (McGurk et al. 2015). Indeed, *Drosophila* has proven to be a very valuable model organism in the field of neurodegeneration, contributing to a better understanding of a wide range of NDs (Lambrechts et al. 2017). More importantly, fly models of human NDs are also being used in primary chemical screens and validation of compounds for therapeutic discovery to treat these diseases (Strange 2016; Pandey and Nichols 2011; Fernández-Hernández et al. 2016).

In this chapter, we will discuss the advantages of using *Drosophila* in the drug discovery process. First, we will describe the different experimental approaches commonly used for the identification of therapeutic compounds in fly models of human diseases, including the methods used for drug administration and the different assays designed to evaluate the efficacy of the candidate compounds. Subsequently, we will introduce a number of studies performed in different *Drosophila* models of human NDs to discover new potential therapies for these disorders.

Drosophila and Its Potential in the Drug Discovery Process

In the discovery process for therapeutics for human diseases in *Drosophila*, different considerations must be taken into account. The foremost important issue is to generate an appropriate fly model of the disease that could be able to reproduce its

key physiological aspects; thus, fly models should exhibit phenotypes (neuronal, behavioral, molecular, etc.) reminiscent to those observed in patients. In order to identify potential therapeutic compounds that could be beneficial in the *Drosophila* model, they must be efficiently administered using different strategies that will depend on the developmental stage in which modified phenotype will be observed. Another important consideration in drug discovery is to decide whether to test candidate compounds with a specific mode of action or to perform high-throughput chemical screens with large collections of chemical compounds, either synthetic or of natural origin. In this section, we will describe the different techniques and approaches that are used in *Drosophila* to efficiently manage these issues.

Generation of Fly Models of Neurodegenerative Diseases

Once a human gene involved in an ND is identified, the way to obtain a fly model of the ND depends on how the disease-causing mutations affect gene expression. For gain-of-function mutations, these models typically involve transgenic individuals expressing the human gene carrying a disease-causing dominant mutation. In addition, the wild-type allele of the human gene or even the fly ortholog can be overexpressed in flies. If the disease is caused by loss-of-function mutations, models express a targeted loss-of-function mutation generated in the own fly ortholog. In all these cases, it is expected that models show phenotypes that resemble some features of the human conditions.

The most common method of expressing human-disease genes in *Drosophila* makes use of the GAL4/UAS binary system (reviewed in Caygill and Brand 2016). The gene of interest is subcloned under the control of the yeast Upstream Activating Sequences (UAS), which in turn are activated by the yeast transcriptional factor GAL4, whose expression is controlled by an endogenous fly promoter. The system retains its activity when expressed in *Drosophila* cells. Regarding the methods of disrupting gene function in *Drosophila*, they traditionally involve the use of transposable elements (Cooley et al. 1988). Transposable elements became powerful tools when several genome-wide projects generated thousands of fly stocks, each one harboring a single transposon construct, mainly a P-element, inserted at a known site in the genome. Some of these insertions were used to generate specific mutations of a gene of interest by inducing mobilization or imprecise excision (Hummel and Klämbt 2008). In RNA-mediated interference (RNAi), double-stranded RNA (dsRNA) is cleaved in vivo into short fragments ultimately degrading the homologous mRNA (Kavi et al. 2008). This method was combined with the GAL4/UAS system to knockdown the expression of a specific endogenous gene in a ubiquitous way or in specific tissues. More recently, a variety of powerful genome engineering techniques have been developed that allow modifying genomic DNA at the nucleotide level. These included new transposon-based methods and the recent introduction of the Clustered Regularly Interspaced Short Palindromic Repeat/CRISPR associated (CRISPR/Cas) system. This technique has been used in *Drosophila* to delete, interrupt,

substitute, tag, and edit multiple genes (Gratz et al. 2015), and can be used to create any desired change in the genome sequence. A complete description of these potent technologies is included in Chapter 2 of this book.

Although most *Drosophila* models of human NDs are based on genetic manipulation of the fly genome, there are also examples of chemically induced models in which flies are treated with different agents (either environmental or synthetic) that are able to damage neurons. The best-known toxin-induced models are those generated to study Parkinson's disease (PD), and they represent a useful tool to mimic sporadic forms of the disease. Several toxins are able to produce PD-related pathology and symptoms, and therefore they have been used to establish PD models in animals. In these neurotoxic models, compounds that produce irreversible effects like 6-hydroxydopamine (6-OHDA), 1-methyl-1, 2,3,6-tetrahydropyridine (MPTP), rotenone, and paraquat are used (Dawson et al. 2018; Blesa et al. 2012). All of them can induce oxidative damage and mitochondrial dysfunction, thus causing cell death in dopaminergic (DA) neuronal populations and reflecting phenotype similar to what is seen in PD. In *Drosophila*, toxin-based PD models are mainly generated by administration of MPTP or rotenone to adult flies, while paraquat and rotenone are also used to investigate the susceptibility of genetic PD models and their role in neuronal cell death (Aryal and Lee 2018). Pharmacological models have also been generated in animals to study specific aspects of other NDs. For example, mitochondrial impairment and excitotoxicity-induced cell death are mechanisms of neurodegeneration in Huntington's disease (HD), which can be mimicked by 3-nitropropionic acid and quinolinic acid treatment, respectively (Ramaswamy et al. 2007). However, toxin-induced models have not been used to study NDs other than PD in *Drosophila*.

Routes of Drug Administration

Drosophila is a holometabolous insect whose life cycle is divided into four developmental stages: embryo, larva, pupa, and imago or adult. The larva goes through three instars in which it grows and reaches the final size, and at the end, it undergoes metamorphosis into a pupa. This developmental event comprises of profound change and reorganization of larval tissues to form the adult structures. The fly biological cycle takes 10 to 12 days at 25°C and adult flies can live around 40–50 days. Drugs can be administered at embryonic, larval, or adult stages using different ways of delivery (see Fig. 1).

Although drugs can reach embryos through maternal feeding (Fig. 1a), exposition of embryos to chemicals is usually performed by *in vitro* incubation or injection (Rand 2010). In an in vitro assay, the chorion that covers the fly embryo must be removed or permeabilized to facilitate the drug entering into the embryo (Fig. 1b). Embryo microinjection has largely been used for gene transgenesis and initially was done by hand using drawn glass capillaries. Microinjection systems were later optimized for high-throughput protocols (Zappe et al. 2006) and novel microdevices have been developed for precise delivery of biological reagents, including drugs, into specific locations of fly embryos (Fig. 1c) (Ghaemi et al. 2017). Injection strategies

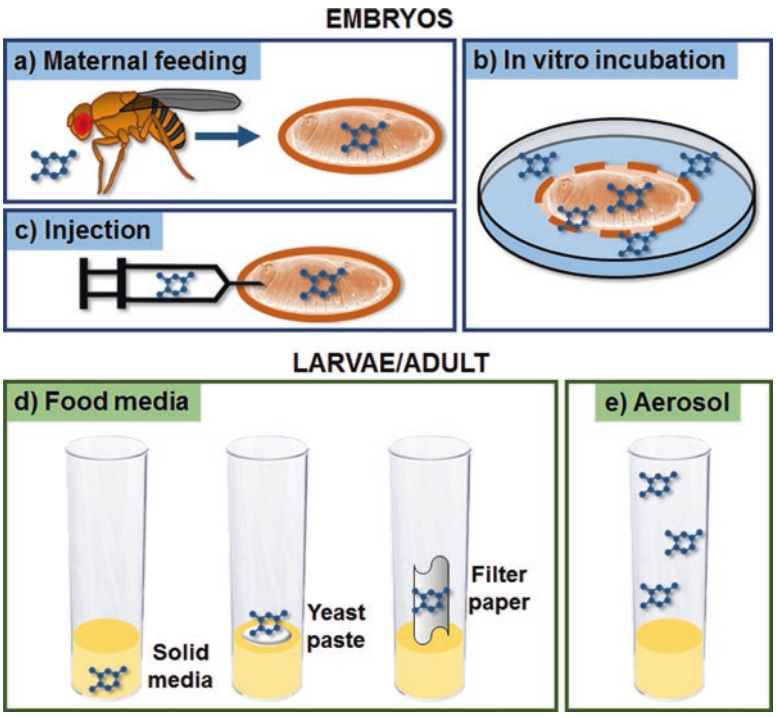

Fig. 1 *Drug administration*. *Drosophila*'s life cycle is divided into different stages of development, from embryos to adults, in which drugs can be administered. In embryos, drugs can be delivered by maternal feeding (**a**), by in vitro incubation (**b**), or by microinjection (**c**) after the chorion has been eliminated or permeabilized. In the case of larvae and adults, drugs can be administered by diluting them in the solid media, in a yeast paste, or in a filter paper with a sucrose/drug mixture (**d**). Another possibility is to deliver the drug via aerosol saturating the atmosphere of the culture vials (**e**)

can be also applied for larva and adults of *Drosophila* (Bijelic et al. 2005). Specifically, the adult flies are subjected to intra-abdominal injection (not shown), a procedure previously applied in insects with bigger size than *Drosophila,* such as the honeybee (Manev et al. 2003). This technique allows the drug to diffuse throughout the organism (Dzitoyeva et al. 2003) and is chosen when other methods for drug delivery do not work in adults. The easiest mode to expose adult flies and larvae to chemical compounds is by mixing such compounds in the solid food medium (Fig. 1d) (Manev et al. 2003). It is usually composed of cornmeal, yeast, glucose, agar, water, and a fungicide. Some authors include a coloring agent, which allows to verify whether larvae and adults have ingested the food, and thus the drug previously dissolved in it (Soriano et al. 2013). The colorant stains the digestive tract and it could be traced along the adult fly abdomen and the larva. It is also possible to administer a drug in a liquid medium containing yeast paste to improve feeding of larvae (not shown). This method is selected for short exposure regimens of drugs. For adult flies, the drug could be mixed with sucrose and deposited on a filter paper (Fig. 1d); alternatively,

flies can be exposed to an atmosphere containing vaporized compounds in aerosol particles (Fig. 1e) (Li et al. 2000). A limitation of this latter method is the need for the active compound to reach a volatile state.

There are different strategies to deliver drugs depending on whether the pharmacological treatment is acute or chronic. In the first case, drugs are administered to the individuals for a brief period of time, but after an interval of starvation (around 18 h). In this case, flies would eat more food and thus more drug than in a normal regimen without starvation would be ingested. The effect of drugs could be monitored by scoring different phenotypes that are described below. It would allow the researcher to determine the peak of maximum effect and the decay of the drug action based on the changes of such phenotypes. In the chronic treatment, individuals are reared in the food, previously mixed with the drug, for more than 24 h. This ensures that the drugs reach their steady-state level. To ensure a prolonged action of drugs, treated individuals must be moved to new fresh vials containing the medium supplemented with compounds, since effectivity of drugs will reduce with time as they are metabolized in the fly organism.

Throughput of Chemical Screens and Selection of Drugs

Drosophila models of NDs can be used in the validation process of drugs identified in massive analysis performed in mammalian cell culture. However, they are increasingly being used in primary chemical screens because they allow to directly examine the effects of compounds in living organisms (Pandey and Nichols 2011). An important aspect to be considered in the initial drug discovery process using *Drosophila* models of NDs is to decide which and how many compounds will be tested for their efficacy to suppress disease-related phenotypes or molecular/cellular dysfunctions exhibited by embryos, larvae, or flies due to neuronal dysfunction and degeneration. In some cases, a set of candidate compounds based on their specific properties or mechanisms of action are selected, which will depend on the pathway, protein, or cellular defect to be targeted. The throughput of this primary chemical screen will vary from low to medium and may be limited in the number of candidate compounds that will be tested in the *Drosophila* model. A similar strategy will be followed when the assay used to evaluate the effect of the compound in the neurodegenerative defect involves a detailed examination of model individuals (see the following section). In addition, *Drosophila* models of NDs are also being used in high-throughput drug screening (HTS) in which large libraries comprising hundreds or thousands of different drugs, even with disparate mechanisms of action, are tested in model animals. This approach facilitates the identification of chemical modifiers of degeneration on a large scale and in a fully unbiased manner. Although these assays are not usually automated, as it happens with traditional HTS approaches performed in cell culture, they still allow us to screen tens/hundreds of compounds per week and enable us to identify high-quality leads by scoring visible and easily quantifiable phenotypes (see the following section). Currently, several libraries of compounds for HTS are available from different pharmaceutical

companies (Volochnyuk et al. 2018), each containing sets of compounds with chemical and pharmacological diversity like small molecules, drugs approved by different agencies, natural products, or kinase inhibitors, among others. Some of these libraries have already been used in *Drosophila* models of NDs for drug discovery. For example, the Prestwick Chemical Library (Prestwick Company, Illkirch, France) has a collection of 1280 molecules comprising mostly approved drugs, which have recently been used to screen a *Drosophila* model of Friedrich's ataxia (FRDA) for compounds able to rescue cardiac dysfunction in adult flies (Palandri et al. 2018). A total of 11 compounds that prevented cardiac dysfunction were identified in this study; paclitaxel was identified as one that has the strongest protective effect (Palandri et al. 2018). In addition, methylene blue, a compound previously proven to be beneficial for FRDA (Seguin et al. 2015; Tricoire et al. 2014), was also identified in the screen, thus supporting the utility of this approach in drug discovery using *Drosophila* (Palandri et al. 2018).

New therapies for NDs can come from three main sources: existing drugs, and synthetic or natural products (Durães et al. 2018). As mentioned above, several libraries of compounds contain drugs already approved by the Food and Drug Administration (FDA), the European Medicine Agency (EMA), or molecules that have reached the clinical trials, but show low efficacy for a specific disease. Drug repurposing is a new trend in drug discovery, where new therapeutic applications for an existing drug are identified (Durães et al. 2018; Langedijk et al. 2015). Therefore, the use of libraries of compounds in *Drosophila* models of NDs may have an important impact in drug repurposing. Besides, *Drosophila* models can be used to validate already existing drugs that have been computationally predicted to have an ameliorative effect on a particular ND. For example, a recent study performed in a *Drosophila* model of PD has revealed the potential of old drugs as new therapeutics for this disease (Styczyńska-Soczka et al. 2017). PD model flies expressing human α-synuclein (α-syn) in the brain were used to validate the effect of two FDA-approved drugs, astemizole (an antihistamine drug) and ketoconazole (an antifungal medication). Both compounds were able to rescue the dysfunction of PD model flies and ketoconazole also suppressed DA neuron death, a hallmark of this disease (Styczyńska-Soczka et al. 2017). These results support the use of *Drosophila* models of NDs as important in vivo tools for drug repurposing, and to validate candidate hits identified by in silico techniques.

Finally, it should be mentioned that although synthetic drugs are commonly used for the management of NDs, they often have side effects that affect patients after chronic treatments (Ansari 2010). Therefore, many researchers are trying to determine the pharmacological properties of plants/plant-derived compounds in different model organisms. Among them, *Drosophila* is one of the most commonly used animal models in medicinal plant research (Panchal and Tiwari 2017). Several studies have already shown that different metabolites of medicinal plants represent promising therapeutic agents for various diseases like chronic and progressive NDs due to their anti-inflammatory and antioxidant properties, among others (Rasool et al. 2014). As an example, it has been shown that curcumin, a polyphenol derived from turmeric herb (*Curcuma longa*), was able to decrease amyloid β (Aβ) peptide

toxicity in a *Drosophila* model of Alzheimer's disease (AD) as well as other AD-like symptoms such as locomotor defects or reduced longevity (Caesar et al. 2012). Indeed, curcumin has been shown to prevent and treat AD in several in vitro and in vivo models, due to its pleiotropic effects. However, the bioavailability of curcumin should be improved in order to translate the success of pre-clinical studies to clinical outcomes (Tang and Taghibiglou 2017).

Assays Used in Drosophila to Evaluate the Therapeutic Potential of Compounds

The use of *Drosophila* in genetic research started at the beginning of the twentieth century, and currently, it is one of the most popular model organisms used in biological and medical research (Stephenson and Metcalfe 2013; Feala et al. 2008). Indeed, *Drosophila* is one of the best-known organisms utilized for genetic, developmental, behavioral, and molecular/biochemical studies. A variety of different assays can be carried out in this organism to score and quantify a neurodegenerative defect (Lambrechts et al. 2017; McGurk et al. 2015; Panchal and Tiwari 2017). In this section, we will highlight the biological assays that are most frequently used in *Drosophila* models of NDs for testing the therapeutic potential of candidate compounds. Thus, changes in neuronal degeneration/dysfunction as well as in other disease-related defects can be evaluated by different means after the administration of specific drugs or collections of compounds.

Developmental Assays

These assays allow evaluation of the in vivo effect of drugs on *Drosophila* development, which is often altered in ND models. One of the most frequently used developmental assays is lifespan analysis in adult flies. The average life span or life expectancy of wild-type adult flies in optimal temperatures are about seven weeks and is easily measured in the laboratory using large cohorts of age-matched individuals. Flies are transferred every two–three days to fresh culture vials and the numbers of dead individuals are recorded till the last fly dies; thus, the life span of the corresponding fly strain can be calculated using the recorded data (Linford et al. 2013; Bauer et al. 2004). As mentioned above, fly models of NDs, in which disease-related genes can either be overexpressed or inactivated, typically have a shortened life span compared to control or wild-type flies (Lambrechts et al. 2017; McGurk et al. 2015). In the drug discovery process for NDs in *Drosophila*, the life span assay can be performed by feeding the model flies on a drug-containing diet in the search of life extension activity associated with the tested compound (Fig. 2a). These results should be always compared to those obtained in flies fed with normal diet without any addition of drug (control). Therefore, different values of life span obtained in drug-containing and control vials will allow us to learn about the possible beneficial effect of the drug in disease model flies (Linford et al. 2013; Bauer et al. 2004). This assay has been used to test the therapeutic potential of several compounds in *Drosophila* models of PD and FRDA (Soriano et al. 2013;

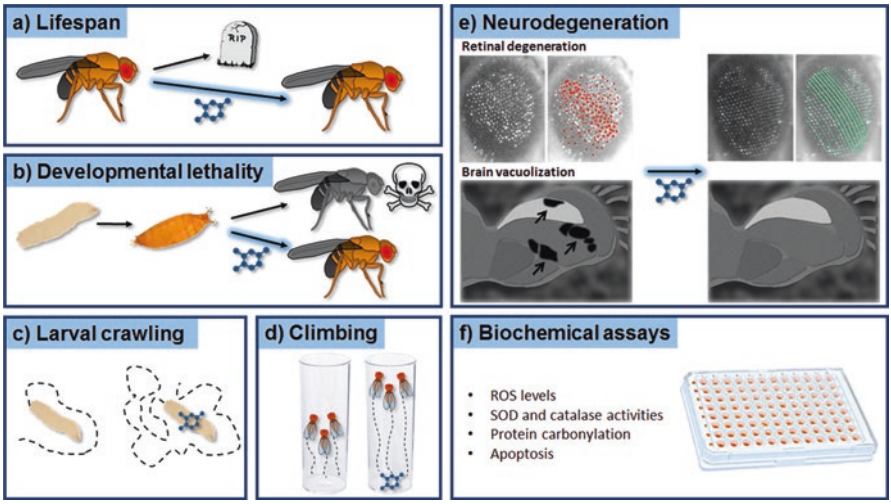

Fig. 2 *Assays to identify candidate therapeutic compounds in Drosophila*. To evaluate the effect of different drugs in the neurodegenerative defects of the *Drosophila* ND model, several kinds of assays can be carried out. The one used for a particular ND will depend on the phenotype exhibited by the *Drosophila* model. Beneficial drugs can cause an extension of life span in fly ND models (**a**) or suppress a developmental arrest phenotype at any stage of the *Drosophila* life cycle (**b**). Changes in locomotor abilities in the *Drosophila* model due to compound administration can be studied by different techniques like the crawling assay in larvae (**c**) or the climbing assay in adult flies (**d**). Neurodegenerative defects in the *Drosophila* model can be manifested by retinal degeneration, thus producing a rough eye phenotype in which ommatidia are disorganized (depicted in red). Besides, brain degeneration in ND flies also develop vacuoles (pointed by arrows). Both phenotypes can be suppressed by therapeutic compounds resulting in either regular ommatidial organization (depicted in green) or suppression of vacuole formation in the brain (**e**). Finally, a whole plethora of biochemical assays can also be performed to evaluate the effect of a specific drug, whether related to oxidative stress or autophagy/apoptosis in *Drosophila* models of NDs (**f**)

Calap-Quintana et al. 2015; Lavara-Culebras et al. 2010). Besides, similar studies in other *Drosophila* models of NDs have also been reported (see below).

Neuronal dysfunction in *Drosophila* may also cause lethality at any developmental stage of this organism. As previously mentioned, the life cycle of *Drosophila* has four stages: embryonic, larval, pupal, and adult. There are several examples in the literature showing that *Drosophila* models of human diseases often suffer a developmental arrest, usually dying at any stage before adult eclosion. This is observed in a transgenic *Drosophila* model of HD expressing a 588 amino acid N-terminal fragment of the human Huntingtin (Htt) protein with a pathogenic polyglutamine tract of 138 repeats (HttQ138) (Akbergenova and Littleton 2017). Pan-neuronal expression of pathogenic Htt led to overgrowth of synaptic connections due to defective endosomal trafficking, among other neuronal defects. In addition, it caused robust pupal lethality, with only a few rare adults able to emerge from pupal cases (Akbergenova and Littleton 2017). This pupal lethality phenotype could be used for screening compounds with therapeutic potential for HD. In this assay, model flies were cultured in

vials with regular (control) or drug-containing medium in which the embryos transitioned into larvae and subsequently developed into pupae. Using this assay, one could identify compounds that were able to suppress or reduce pupal lethality, therefore increasing adult viability (Fig. 2b). A similar approach was used in a *Drosophila* model of myotonic dystrophy 1 (DM1) (Garcia-Lopez et al. 2008). In this model, targeted expression of a 480 interrupted CUG repeat RNA in the *Drosophila* mushroom bodies (brain structures involved in learning, sleep, and memory) developed into a female-specific semilethal pupal phenotype. This phenotype was used to screen chemical suppressors of neuronal toxicity caused by CUG RNA in which drugs that were able to increase the viability of flies were identified (Garcia-Lopez et al. 2008).

Behavioral Assays

Drosophila is a very versatile model organism that shows both innate and higher-order behaviors. They are tightly regulated by genetic and environmental factors and can be easily assayed, therefore allowing to determine whether the animal is physiologically compromised in a specific scenario (Neckameyer and Bhatt 2016; Nichols et al. 2012). Behavioral assays are especially useful in the study of *Drosophila* models of NDs. Indeed, one of the most common symptoms associated with human NDs such as PD or AD is the progressive loss of locomotor abilities, which can be easily evaluated in vivo in the *Drosophila* models (Lambrechts et al. 2017; McGurk et al. 2015; Panchal and Tiwari 2017). There are several assays to study the locomotor behavior in *Drosophila*, which can be carried out either in larval or adult stages (Neckameyer and Bhatt 2016; Nichols et al. 2012).

The *Drosophila* larva undergoes two stages before pupation and metamorphosis: foraging and wandering. The foraging stage spans from the beginning of first instar to late third instar, in which larvae are photophobic and will actively move away from bright light (Sawin-McCormack et al. 1995). Approaching late third instar, larvae enter the wandering stage where they leave the food to find an appropriate pupation site. At the onset of wandering, their repulsion to light decreases until larvae behave indifferently toward bright light stimuli (Sawin-McCormack et al. 1995). *Drosophila* larvae present a complex collection of coordinated locomotor behaviors including crawling, turning, rolling, and burrowing (Heckscher et al. 2012); these larval movements can be defective in *Drosophila* models of NDs. The most commonly used assay to study locomotor activity in third instar larvae is the larval crawling assay, which can be performed with model larvae in the absence or presence of a candidate drug (Nichols et al. 2012). In this assay, larvae are incubated into drug or control solutions for 15 min. Subsequently, they are placed on a petri dish with agarose over a graph paper with a 0.2 cm^2 grid and the number of grid lines crossed by each larva in 1 min is counted. Thus the results obtained will allow one to quantify locomotion in treated and untreated larvae and this will help us in identifying potential therapeutics for the corresponding ND. A suppression of locomotion dysfunction in model larvae by a specific drug will be reflected in the crawling assay by an increase of larval movements (Fig. 2c). This assay is specifically used when locomotor behavior can't be evaluated in model flies since overexpression or inactivation of disease-related genes causes lethality in pupal or adult stages (Nichols et al. 2012).

Locomotor activity in adult flies can be easily studied by means of the climbing assay, also known as negative geotaxis assay, which is based on the natural tendency of flies to move against gravity when tapped to the bottom of an empty vial (Nichols et al. 2012; Ali et al. 2011). Wild-type flies will immediately start climbing up the walls, while *Drosophila* models of NDs often present disease-related motor deficits and, as a consequence, exhibit a reduced climbing ability (Lambrechts et al. 2017; McGurk et al. 2015; Panchal and Tiwari 2017). In the traditional climbing assay, groups of ten individuals of the same genotype or treatment are tapped to the bottom of the vial and the number of flies that can climb above a certain height or in a fixed period of time is recorded (Nichols et al. 2012; Ali et al. 2011). This assay can be used in the drug discovery process using fly models of NDs in which candidate therapeutic compounds will be able to improve their climbing ability (Fig. 2d). This assay has been successfully used in the search for new therapies against PD and FRDA (Soriano et al. 2013; Calap-Quintana et al. 2015; Sanz et al. 2017; Soriano et al. 2016). Numerous examples of similar experiments performed with model flies for other NDs have also been published (see below). An alternative to the standard climbing assay is the rapid iterative negative geotaxis (RING) assay, which provides a reproducible, sensitive, and high-throughput approach to quantify locomotor ability in adult flies (Gargano et al. 2005). In this assay, several groups of flies with different genotypes or treated with different drugs can be tested simultaneously using large number of animals, therefore making it a suitable assay for drug screening (Nichols et al. 2012).

Assays to Measure Neuronal Degeneration

Drosophila models of NDs often exhibit either neuronal/brain defects or degeneration of specific neuronal populations in adult flies, which can be scored and quantified; therefore, these neurodegenerative defects can be used in the drug discovery process to identify candidate compounds that are able to modify these disease-specific phenotypes (Lambrechts et al. 2017; McGurk et al. 2015; Panchal and Tiwari 2017). In some cases, neurodegeneration can be easily examined by simply observing model flies under the dissecting microscope. Indeed, one of the most commonly used phenotypes for this purpose in fly models of NDs is retinal degeneration, which occurs when a disease-related gene is specifically overexpressed or inactivated in the *Drosophila* adult eye causing toxicity and photoreceptor degeneration (Ambegaokar and Jackson 2010; Wang et al. 2014). Compared with the normal eye, the degenerative eye often shows disruption of ommatidial structure and is externally rough (Lambrechts et al. 2017; McGurk et al. 2015; Panchal and Tiwari 2017). Since the eye is a non-essential tissue, retinal degeneration does not affect viability. This is the reason why external eye roughness can be used in large-scale chemical screens in which potentially therapeutic compounds that are able to suppress neurodegeneration will also reduce the level of eye roughness and will be easily identified (Fig. 2e). However, a more detailed analysis of the fly brain could be required to evaluate neurodegenerative defects in several *Drosophila* models of NDs (Lambrechts et al. 2017; McGurk et al. 2015; Panchal and Tiwari 2017). Sometimes, neurodegeneration in *Drosophila* is accompanied by formation of vacuoles in the brain, which can be visualized and quantified in sections of brains

embedded in plastic or paraffin using histological approaches (Sunderhaus and Kretzschmar 2016). In this particular case, a potentially therapeutic compound would be able to reduce the number of vacuoles in brains of model flies (Fig. 2e). Besides, there are several NDs in which specific populations of neurons in the brain are affected. Therefore, selective antibodies or expression of specific markers are used to assess the integrity/loss of such neurons in *Drosophila* models (McGurk et al. 2015), where immunostainings can be performed either in brain sections or in whole-mount preparation of adult brains (not shown). For example, DA neurodegeneration can be evaluated with an anti-tyrosine hydroxylase antibody in sections of brains from PD model flies (Muñoz-Soriano and Paricio 2007) in which these neurons are specifically lost. Immunostainings of adult brains can also be carried out in *Drosophila* models of AD, which will allow to evaluate the presence of Aβ plaques in fly models (Panchal and Tiwari 2017). Since protocols involving brain mounting and immunostaining are laborious and time-consuming, they are often used as secondary assays to validate candidate compounds identified in HTS or large-scale screens (as an example see Lin et al. 2016). In all these assays in which the effect of candidate compound on a neurodegenerative defect is evaluated in brains of model flies, the compounds are fed from the first instar larval stage. When the treated individuals reach adulthood, brains are dissected, whole-mounted or sectioned, and the corresponding immunostainings are performed.

Biochemical Assays

As previously mentioned, NDs are characterized by damage and loss of neurons in specific regions of the nervous system. It is considered that multiple mechanisms contribute to neuronal degeneration, including inflammation, mitochondrial dysfunction, and oxidative stress (OS) (Bourgognon and Steinert 2019; Neal and Richardson 2018). As ND advances, OS increases, which provides a clear target for therapeutic intervention. OS results from increments of formation of free radicals as reactive oxygen species (ROS), reduction in antioxidant defense, or a combination of both processes. These processes provide a battery of biochemical markers to test the redox status of cells and, therefore, to examine the antioxidant properties of compounds selected for treatment. The most commonly used assays to analyze OS markers are based on determination of the levels of ROS, H_2O_2, malondyaldehide (as an indicator of lipid peroxidation), and of protein carbonyl content (as a marker of protein oxidation). All of them can easily be measured in *Drosophila* using different commercial kits. In addition, the activity of antioxidant enzymes is quantified to know the defense capacity of these cells (Fig. 2f). The enzymes that constitute the first line of cellular antioxidant defense are superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx). The first line of defense antioxidants also comprises metal ion-binding proteins that chelate iron as in transferrin, as this metal participates in the generation of free radicals. In addition, abnormal metabolic function, reduction of ATP production, altered morphology, and impaired fission-fusion balance have also been observed in mitochondria of different NDs (Tan et al. 2019; Elfawy and Das 2018; Panchal and Tiwari 2018). All these processes as well as cellular death can also be examined in flies to evaluate the efficiency of

pharmacological treatments (Fig. 2f). Numerous examples of biochemical assays performed in several *Drosophila* models of NDs to test the therapeutic potential of candidate compounds have been published (see the following section).

Identification of Pharmacological Therapies in Fly Models of Neurodegenerative Diseases

In the last decades, several *Drosophila* models of NDs have been developed and successfully used in the drug discovery process. Potential therapeutic compounds for these diseases have been discovered in *Drosophila* using different approaches. In some cases, studies were performed with candidate compounds with known mechanisms of action; however, several reports of high-throughput chemical screens, in which collections of drugs were used, can also be found in the literature. Despite the throughput of the assay, compounds are currently tested for their capability to suppress a given phenotype exhibited by model flies. In this section, we will describe the results obtained in studies performed to discover new treatments for several NDs and discuss the relevance of their findings in the pathophysiology of the corresponding disease.

Parkinson's Disease

Parkinson's disease (PD) is a neurodegenerative disorder caused by the selective loss of DA neurons in the substantia nigra pars compacta (SNc). The reduction of dopamine levels leads to the classical motor symptoms of this disease such as resting tremor, rigidity, bradykinesia, and postural instability; however, non-motor symptoms such as mood alterations, sleep disturbances, or even dementia are also observed in PD patients (Farlow et al. 2014; Samii et al. 2004). Although most PD cases are sporadic, 5–10% of all patients suffer from familial forms of PD caused by mutations in specific genes (Lill 2016). Interestingly, most causative genes to PD are evolutionary conserved and some are misregulated in sporadic forms of the disease (Vanhauwaert and Verstreken 2015). Current therapies to treat PD are based on a dopamine replacement therapy, in which dopamine precursors (L-DOPA), dopamine agonists (i.e., bromocriptine, lisuride, pergolide), or inhibitors of dopamine metabolism (i.e., selegiline, entacapone) are employed (Athauda and Foltynie 2015). However, these treatments fail in stopping or delaying the progression of the disease. In order to discover more effective treatments for PD, new approaches are being carried out (Pandey and Nichols 2011; Abdel-Salam 2008). Recent studies have revealed multiple pathological mechanisms underlying PD like α-syn aggregation, mitochondrial dysfunction, impaired autophagy, endoplasmic reticulum (ER) stress, microglial activation, metabolic alterations, and disruption of calcium (Ca^{2+}) homeostasis (Błaszczyk 2018; Charvin et al. 2018). Therefore, compounds targeting these pathogenic mechanisms are now being considered to develop new therapies for PD and have been tested in fly models of the disease (see Table 1).

Table 1 Compounds identified using *Drosophila* models of Parkinson's disease

Parkinson's disease			
Drosophila model	Compound	Phenotype	Reference
α-syn	Rifampicin	Eye degeneration	Yedlapudi et al. (2016)
	Nortriptyline	Eye degeneration	Collier et al. (2017)
	AGK2	Dopaminergic neuronal loss	Outeiro et al. (2007)
	Fendiline	Locomotor defects	Hillman et al. (2012)
	Astemizole	Locomotor defects and dopaminergic neuron survival	Styczyńska-Soczka et al. (2017)
	Ketoconazole		
	Opuntia ficus-indica (prickly pear) extract	Life span	Briffa et al. (2017)
	Padina pavonica (peacock's tail) extract		
LRRK2	Pyrroloquinoline quinone	Dopaminergic neuronal loss and mitochondrial morphological defects	Ng et al. (2017a)
	Lovastatine	Locomotor defects and neurodegeneration	Lin et al. (2016)
PINK1	Grape skin extract	Life span, mitochondrial aggregation, mitophagy, and autophagy	Wu et al. (2018)
DJ-1β	Dexrazoxane	ROS levels and locomotor defects	Sanz et al. (2017)
	Pterostilbene		
	Minocycline		
	Sodium phenylbutyrate		
	Dalfampridine		
	Methylene blue		
	α-tocopherol	ROS levels, superoxide dismutase and catalase activities, and protein carbonylation Life span Locomotor defects	Casani et al. (2013), Lavara-Culebras et al. (2010), and Sanz et al. (2017)
	Melatonin	Life span	Lavara-Culebras et al. (2010)
	Vitamin C	ROS levels, superoxide dismutase and catalase activities, and protein carbonylation	Casani et al. (2013)
UCH	Curcumin	ROS levels, locomotor defects, neurodegeneration, and dopamine levels	Nguyen et al. (2018)

(continued)

Table 1 (continued)

Parkinson's disease			
Drosophila model	Compound	Phenotype	Reference
Paraquat-induced	Minocycline	ROS levels, locomotor defects, neurodegeneration, and life span	Inamdar et al. (2012)
	Bacopa monnieri (herb of grace) extract	ROS levels, ATP levels, and life span	Srivastav et al. (2018)
MPTP-induced	Resveratrol	ROS levels, locomotor defects, and brain lesions	Abolaji et al. (2018)
Iron-induced	Hesperidin	Locomotor defects, life span, and dopamine levels	Poetini et al. (2018)

ROS reactive oxygen species, *MPTP* 1-methyl-4-phenyl-1, 2,3,6-tetrahydropyridine

As mentioned above, α-syn aggregation leads to DA neuron death. Hence, molecules able to inhibit or block this aggregation represent a promising therapeutic strategy to treat PD (Yedlapudi et al. 2016). Several compounds that inhibit α-syn aggregation have been tested in *Drosophila* PD models. For instance, rifampicin or nortriptyline, currently employed as antidepressants, have effectively suppressed α-syn aggregation and neurotoxicity in a *Drosophila* model expressing a human pathogenic A30P α-syn protein (Yedlapudi et al. 2016; Collier et al. 2017).

Mitochondrial dysfunction plays a central role in PD, which leads to an increase of ROS levels. ROS may affect biomolecules such as proteins or DNA causing irreparable damages (Salazar et al. 2018). Therefore, a number of antioxidant compounds such as curcumin, tocopherol, or resveratrol have been tested in *Drosophila* models of PD as candidate therapies for the disease. In these experiments, it was found that these compounds are not only able to reduce ROS levels, but also to suppress other phenotypes in model flies like locomotor defects and reduced life span (Sanz et al. 2017; Abolaji et al. 2018; Nguyen et al. 2018). In addition, a deficiency in complex I of electron transport chain (ETC) has been also reported in PD, which leads to a leak of electrons from ETC and, in consequence, to an increase of ROS levels and a reduction of ATP synthesis (Bose and Beal 2016; Lenaz et al. 2006). In this context, methylene blue has emerged as a potential PD treatment by counteracting these effects because it is a redox-active alternative electron acceptor/donor that bypasses complex I/II (Biju et al. 2018; Lee and Boelsterli 2014). Indeed, this compound has been able to rescue locomotor defects and to decrease protein carbonylation levels (a consequence of high ROS levels) in PD model flies deficient of the *DJ-1β* gene (Sanz et al. 2017). In addition, alterations in mitochondrial morphology, as well as deregulation of fusion/fission dynamics, have also been detected in PD. Under high OS levels, there is excessive mitochondrial fission driven by Drp1, which leads to mitophagy (Ng et al. 2017a). In such a scenario, it was demonstrated that pyrroloquinoline quinone, a compound that stimulates mitochondrial biogenesis through increasing PGC-1α expression, ameliorated DA neuron loss in PD model flies mutant for *LRRK2* (Ng et al. 2017b).

In the CNS, the innate immune response is mainly mediated by microglia and astrocytes. Microglia exerts a protective role under healthy conditions. However, in injured brains, microglia releases mediators that promote neuroinflammation and increase ROS levels, thus damaging surrounding tissues (Joe et al. 2018; Subramaniam and Federoff 2017). Indeed, activation of microglia has been detected in brains of PD patients (Perry 2012) and is considered as a new target for therapeutic intervention in PD. Regarding this, Outeiro and collaborators performed a chemical screen to identify compounds that could inhibit SIRT2 (Outeiro et al. 2007), which plays an important role in lipopolysaccharide-induced microglial activation (Chen et al. 2015). One of the compounds, AGK2, was validated in a *Drosophila* PD model based on α-syn overexpression, being able to suppress DA neurodegeneration exhibited by model flies (Outeiro et al. 2007). Accordingly, it was shown that minocycline, which also reduces microglial activation (Fan et al. 2007), was found to be beneficial in a paraquat-induced *Drosophila* model and in *DJ-1β* mutant flies (Sanz et al. 2017; Inamdar et al. 2012).

Deregulation of Ca^{2+} homeostasis is also associated with the pathogenesis of several NDs, including PD. There are evidences showing that high intracellular Ca^{2+}levels might be due to an increased Ca^{2+}influx across the plasma membrane and due to the dysfunction of intracellular Ca^{2+}stores (ER and mitochondria) (Zaichick et al. 2017). Currently, L-type channel blockers are being widely used as antihypertensives to treat high blood pressure and other cardiovascular diseases. Interestingly, it has been shown that the drug fendiline, a Ca^{2+}receptor blocker, is able to suppress motor defects in a *Drosophila* PD model expressing α-syn (Hillman et al. 2012). Therefore, it could be possible to repurpose these drugs to treat PD (Zaichick et al. 2017; Ortner and Striessnig 2016).

Potential therapeutic compounds with heterogeneous mechanism of actions have also been identified and tested in different *Drosophila* PD models like lovastatin, which was found to be neuroprotective in LRRK2-G2019S flies by activating antiapoptotic Akt/Nrf signaling and decreasing caspase 3 levels (Lin et al. 2016). Besides, other candidate therapeutic compounds for PD have also been identified using bioinformatic approaches, looking for existing drugs predicted to target deregulated pathways in PD (Sun et al. 2016). Using this methodology, astemizole and ketoconazole were selected as potential therapeutic agents, which were subsequently validated in vivo using a *Drosophila* PD model expressing wild-type *α-syn*. Indeed, both compounds improved locomotor ability and DA neuron survival in PD model flies (Styczyńska-Soczka et al. 2017). In addition, it has been already demonstrated that nature is an invaluable resource of promising new treatments; therefore, extracts obtained from diverse plants should be taken into consideration as potential therapies. Indeed, extracts obtained from *Bacopa monnieri*, grape skin, *Opuntia ficus-indica*, or *Padina pavonica* have been shown to be effective in suppressing phenotypes exhibited by several PD model flies (Srivastav et al. 2018; Wu et al. 2018; Briffa et al. 2017). Also, hesperidin, a flavonoid isolated from citrus fruits was able to improve locomotor ability as well as to increase dopamine levels and the activity of antioxidant enzymes such as superoxide dismutase, catalase, or glutathione S-transferase (GST) in an iron-induced *Drosophila* model of PD (Poetini et al. 2018).

Alzheimer's Disease

Alzheimer's disease (AD) is the most common ND. The cause of this disease is yet unknown, but it is thought that intracellular neurofibrillary tangles (mainly composed by hyperphosphorylated tau protein) and extracellular deposition of amyloid plaques (primarily composed by Aβ peptides) in the brain lead to neuronal and synaptic loss and to a severe brain atrophy (Shankar and Walsh 2009). To date, treatments for AD are symptomatic and based on counterbalancing the neurotransmitter disturbance of the disease (Yiannopoulou and Papageorgiou 2013); however, their efficacy is still in question (Liu et al. 2015). Currently, the search for new treatments is based on either reducing Aβ plaque formation or improving its clearance as well as reducing intracellular neurofibrillary tangles (Yiannopoulou and Papageorgiou 2013), but other approaches are being used (see below). In Table 2, several candidate therapeutic compounds for AD tested in *Drosophila* models of the disease are shown.

A number of strategies have been developed to reproduce Aβ plaque formation or presence of intracellular neurofibrillary tangles in animal models of AD. In *Drosophila*, several models of AD based on overexpression of Aβ42, Tau as well as other related genes like APP (amyloid precursor protein) and BACE-1 (β-secretase 1) are available (Tan and Azzam 2017). Model flies exhibit several phenotypes that can be tested to evaluate the efficacy of different compounds like defects in synaptic activity as well as locomotor defects or impaired learning and memory (Bonner and Boulianne 2011). Furthermore, several biochemical assays can be performed in *Drosophila* models to quantify Aβ aggregation in brains (Chakraborty et al. 2011). So far, different studies have been published in which *Drosophila* models of AD are used to identify potential therapeutic compounds for this disease. In some cases, plant extracts are tested in fly models. Indeed, extracts from 23 medicinal plants used in Chinese traditional medicine were tested in a *Drosophila* model of AD based on Aβ42 overexpression and five of them showed protective activity against Aβ42 neurotoxicity and were able to increase survival rates and reduce photoreceptor neuron degeneration (Liu et al. 2015). In a separate study, flies co-expressing human APP and BACE-1 were treated with acacetin, a compound obtained from the plant *Agastache rugosa*. This compound improved locomotor ability and life span in AD flies and also reduced Aβ levels and protected them against photoreceptor degeneration (Wang et al. 2015). Another example is salidroside, a compound obtained from the plant *Rhodiola rosea*, which was able to improve locomotor ability, to increase life span and to reduce the amount of Aβ plaques in brains of AD model flies (Chen et al. 2016a). However, parallel to natural compounds, synthetic compounds have been tested in *Drosophila* models of AD. In a study carried out by Wang and collaborators, 2,000 synthetic compounds presumed to be kinase modulators were tested for their ability to rescue memory loss in Aβ42-expressing flies, which was dependent on EGFR activation (Wang et al. 2012). In this screen, 45 compounds were effective in reducing memory loss in the *Drosophila* AD model and four of them had the same effect in a mouse AD model. These results support the conservation of the molecular mechanism mediating Aβ-induced memory loss

Table 2 Compounds identified using *Drosophila* models of Alzheimer's disease

Alzheimer's disease			
Drosophila model	Compound	Phenotype	Reference
$A\beta_{42}$[a]	*Coriandrum sativum* (coriander) extract	Eye size	Liu et al. (2015)
	Nardostachys jatamansi (nard) extract		
	Polygonum multiflorum (Fo-ti) extract		
	Rehmannia glutinosa (gān dì huáng) extract		
	Sorbus commixta (rowan) extract		
	Opuntia ficus-indica (prickly pear) extract	Life span	Briffa et al. (2017)
	Padina pavonica (peacock's tail) extract		
	Gardenia jasminoides (gardenia) extract	Cognition deficits	Ma et al. (2017)
	Gefitinib	Memory loss	Wang et al. (2012)
	Memantine		
	Erlotinib		
	D737	Locomotor defects	McKoy et al. (2012)
	Doxycycline	Locomotor defects and eye degeneration	Costa et al. (2011)
	Nordihydroguaiaretic acid	Life span, locomotor defects, and GSH levels	Siddique and Ali (2017)
$A\beta_{40}$, $A\beta_{42}$, and *tau*	Curcumin	Locomotor defects, survival, and amyloid deposits	Caesar et al. (2012)
APP/BACE-1	Acacetin	Eye phenotype, locomotor defects, and Aβ levels	Wang et al. (2015)
$A\beta_{42}$ and *APP/BACE-1*	Salidroside	Life span, locomotor defects, and Aβ aggregation	Yao et al. (2015)

[a]There are two isoforms of the Aβ peptide, $A\beta_{40}$ and $A\beta_{42}$. $A\beta_{42}$ is the larger and more fibrillogenic form of Aβ and is associated with Alzheimer's disease states (Yin et al. 2007).
Aβ amyloid beta, *APP* amyloid beta peptide, *BACE* beta secretase, *GSH* glutathione-S-transferase

in both *Drosophila* and mice. (Wang et al. 2012). *Drosophila* AD models can be also employed to validate compounds identified in HTS performed in other organisms or cell lines. Since Aβ42 aggregation is thought to lead to cellular dysfunction and neuronal death in AD, (McKoy et al. 2012) a collection of 65,000 small molecules were tested in *Escherichia coli* cells expressing an Aβ42-GFP fusion protein for identifying inhibitors of Aβ42 aggregation (McKoy et al. 2012). The most effective compound identified in the *E.coli* was subsequently tested in transgenic flies

expressing Aβ42 and was able to improve their life span and locomotor abilities (McKoy et al. 2012).

As mentioned above, the most common strategy in AD drug discovery is to find compounds able to inhibit β- and γ-secretases, which could lead to a reduction in Aβ aggregation. However, several studies have used different approaches in order to identify new therapeutic compounds for this disease. One example is doxycycline, an antibiotic that has been found to be neuroprotective in several models of NDs. This compound was able to improve locomotor ability and to reduce the eye degeneration in flies overexpressing Aβ42. In this work, doxycycline was found to exert its function by clearance of aggregated forms of Aβ and not by interfering with their production (Costa et al. 2011).

Huntington's Disease

Huntington's disease (HD) is an autosomal dominant ND that produces motor dysfunctions, cognitive deficits, and psychiatric disturbances, such as depression, dementia, and personality changes, and leads to death approximately 15–20 years after disease onset (Bates et al. 2002). Its symptom is mainly due to the loss of neurons in the striatum and cortex. HD is caused by the expansion of a trinucleotide repeat (CAG)n in the *HTT* gene. It encodes an endogenous polyglutamine (polyQ) tract in the N-terminus of the Htt (huntingtin) protein. In unaffected individuals, the number of CAG repeats varies from 6 to 35, while those with 36 repeats or more develop HD (Rosas-Arellano et al. 2018). The Htt protein participates in human development and normal brain function. Some post-translational modifications, such as phosphorylation, can play a significant role in regulating toxicity of the Htt protein. HD pathogenesis involves cleavage of the protein and is associated with neuronal accumulation of aggregated forms due to the presence of the polyQ tracts in HD alleles (Bates et al. 2002). These aggregations preferentially occur in the CNS and cause the preferential death of medium spiny neurons within the striatum (Clinical and Genetics 1997) leading to the symptoms of the disease. Although Htt function is not well known, it is thought to play a role in transcriptional regulation and transport of vesicles within the cell and in the endosome–lysosome pathway, and also has pro-survival properties (Landles and Bates 2004). As a result of this, it has been hypothesized that the mechanism underlying HD is a noxious gain-of-function mutation, which leads to transcriptional deregulation, protein misfolding, and degradation as well as impairment of mitochondrial function (Landles and Bates 2004; Sugars and Rubinsztein 2003; Cha 2000).

The *Drosophila* Htt protein (DmHtt) is similar in size to the human one and contains five different regions also very similar in sequence (Li et al. 1999). There are several *Drosophila* models of HD expressing different numbers of polyQ repeats in exon 1 of human *HTT* (Doumanis et al. 2009; Kaltenbach et al. 2007; Lee et al. 2004; Steffan et al. 2001; Jackson et al. 1998). In addition, there is a model overexpressing full-length human HTT with a 128Q repeat expansion. All these models mimic HD in terms of decreased life span, impaired locomotion, and progressive

degeneration of photoreceptor neurons, when repeats are expressed in the fly eye. Therefore, they are being effectively used in the identification of compounds with therapeutic potential for this disease (see Table 3).

To date, two drugs (tetrabenazine and deutetrabenazine) have been approved by the FDA; however, they only provide symptomatic treatment (Rodrigues et al. 2017). In the last years, different *Drosophila* models of HD have been used in an attempt to discover new therapeutic targets and treatments for this disease. Like other ND, HD is characterized by altered Ca^{2+} homeostasis. A *Drosophila* model of HD expressing the full-length human Htt protein with 128Q showed that store-operated Ca^{2+} entry pathway is up-regulated in neurons (Romero et al. 2008). This

Table 3 Compounds identified using *Drosophila* models of Huntington's disease

Huntington's disease			
Drosophila model	Compound	Phenotype	Reference
Transgenic flies that express human huntingtin with a long polyQ (glutamine) repeat of 128 amino acids	Meldonium	Life span, survival rate, locomotor defects, and ROS levels	Di Cristo et al. (2018)
Transgenic flies that express exon 1 of the Huntington gene with 93 polyQ repeats	Congo red	Integrity of photoreceptor neurons and locomotor defects	Apostol et al. (2003)
	Cystamine bitartrate		
	Rhodiola rosea (roseroot) extract	Life span, locomotor defects, rhabdomere number, percent of eclosion, eclosion rate, and larval crawling distance	Arabit et al. (2018)
	Curcumin	Degeneration of photoreceptor neurons and eye morphology	Chongtham and Agrawal (2016)
	Dexamethasone	Huntingtin aggregates in eye imaginal discs, eye degeneration, locomotor defects, and life span	Maheshwari et al. (2014)
	UPF 648	Retinal degeneration	Campesan et al. (2011)
Transgenic flies that express the first 171 residues of huntingtin with 120 polyQ repeats	Rapamycin	Retinal degeneration	Ravikumar et al. (2004)
Transgenic flies that express human huntingtin with 46, 72, or 102 polyQ repeats	Resveratrol	Cardiac dysfunction	Melkani et al. (2013)
Transgenic flies that express human huntingtin with 103 polyQ repeats	Ebselen	Retinal degeneration	Mason et al. (2013)

PolyQ polyglutamine, *ROS* reactive oxygen species

phenotype can be suppressed by the inhibition of genes encoding voltage-gated Ca^{2+} channels. Accordingly, a series of quinazoline-derived compounds that inhibited Ca^{2+} release were found to be potentially therapeutic for HD using model flies (Wu et al. 2011). Another important pathophysiological mechanism in HD is mitochondrial dysfunction as a consequence of Htt-dependent transcriptional dysregulation (Kumar et al. 2014). Di Cristo et al. (2018) demonstrated that meldonium, a cardioprotective drug that modulates cardiac energy metabolism pathways, can ameliorate some mitochondrial dysfunction in both in vitro and in vivo models of HD (Di Cristo et al. 2018). Indeed, it was found that meldonium was able to restore mitochondrial morphology and decreased intracellular Htt aggregation in a cellular model of HD. Besides, meldonium was also able to suppress locomotor dysfunction as well as lead to increased life span and reduced ROS levels in HD model flies expressing human *HTT* with 128Q repeats in all neurons (Di Cristo et al. 2018).

Although current researches are directed to find therapies to halt or reduce the progression of the disease, symptomatic treatments can ameliorate living conditions of HD patients. In this scenario, *Drosophila* was used to test the potential efficacy of the root extract of *Rhodiola rosea* in HD (Arabit et al. 2018). Its putative active compound, salidroside, has been shown to inhibit the mTOR pathway and induce autophagy in bladder cancer cell lines (Liu et al. 2012). Moreover, salidroside was able to increase life span in several organisms, including *Drosophila*, *Caenorhabditis elegans*, and *Saccharomyces cerevisiae (*Bayliak et al. 2014*;* Wiegant et al. 2009*)*. To test the therapeutic potential of this compound for HD, an age-related disease, Arabit et al. (2018) used flies in which human *HTT* exon 1 with 93Q was expressed pan-neurally *(*Arabit et al. 2018*)*. Their results show that it was able to prevent neurodegeneration, improve locomotion, and increase life span in affected flies when compared to that of controls indicating that it could be a promising preventive treatment for HD.

Friedreich's Ataxia

Friedreich's ataxia (FRDA) is an autosomal recessive ND and the most common hereditary ataxia among populations of European origin (2–4/100,000) (Harding 1993). FRDA usually manifests before age 25, with progressive neurodegeneration of the dorsal root ganglia, sensory peripheral nerves, corticospinal tracts, and dentate nuclei of the cerebellum. Many patients develop hypertrophic cardiomyopathy, diabetes mellitus and impaired glucose tolerance (reviewed in Bidichandani and Delatycki 2018). The mutation responsible for this disease is, in the majority of patients, an expansion of a GAA triplet repeat located in the first intron of the *FXN* gene (Campuzano et al. 1996). This gene codes for the mitochondrial protein frataxin, whose levels are reduced from 5% to 30% of the normal levels in the FRDA patients (Campuzano et al. 1997). Frataxin function is not yet fully characterized, but a crucial role in iron–sulfur cluster biogenesis is the most generally accepted (Maio and Rouault 2015; Martelli and Puccio 2014). The defects associated with Frataxin deficiency include mitochondrial iron accumulation, OS

hypersensitivity, impaired iron–sulfur cluster biogenesis, reduced activity of aconitase as well as respiratory chain dysfunction (reviewed in (González-Cabo and Palau 2013; Bayot and Rustin 2013; Santos et al. 2010)).

Frataxin is an evolutionary conserved protein, which has allowed the development of several models of FRDA in different organisms, including *Drosophila*. The GAL4/UAS transgene-based RNA interference (RNAi) methodology has successfully been used to induce tissue-specific and ubiquitous knockdown of the *Drosophila FXN* ortholog, the *fh* gene (Calap-Quintana et al. 2018). It was able to reduce rather than totally eliminate frataxin levels, a situation resembling the condition of the FRDA patients. Flies with a reduction of ~70% of the normal levels of frataxin recapitulate FRDA phenotypes at biochemical, cellular, and physiological levels (Llorens et al. 2007). Decreased life span, impaired motor performance, and cardiac dysfunction in adulthood were selected for drug testing and analysis in fly models (see Table 4).

Table 4 Compounds identified using *Drosophila* models of Friedreich's ataxia

Friedreich's ataxia			
Drosophila model	Compound	Phenotype	Reference
RNAi *fh*	Idebenone	Life span, locomotor defects, aconitase activity, and heart defects	Soriano et al. (2013) and Tricoire et al. (2014)
	Methylene blue	Heart defects	Tricoire et al. (2014) and Palandri et al. (2018)
	Paclitaxel	Heart defects	Palandri et al. (2018)
	Deferiprone Desferrioxiamine	Life span, locomotor defects, transition from larva to pupa, and iron levels	Soriano et al. (2013) and Seguin et al. (2015)
	Zn and copper chelators: Bathocuproine disulfonate TPEN Tetrathiomolybdate	Locomotor defects	Soriano et al. (2016)
	Rapamycin	Life span, motor defects, ATP levels, aconitase activity, ROS levels, and antioxidant gene expression	Calap-Quintana et al. (2015)
	Tauroursodeoxycholic acid	ER stress, cellular degeneration, and aconitase activity	Edenharter et al. (2018)
fh^{1} [a]	Myriocin	Neurodegeneration	Chen et al. (2016c)

[a] fh^{1} is a point mutation in the *frataxin* (*fh*) gene that results in an amino acid change (S135R)

The first chemical treatments were performed in *Drosophila* to validate the use of this organism as a tool for identifying therapeutic molecules in FRDA (Soriano et al. 2013). Expressing a *fh*-RNAi allele in a ubiquitous pattern or in the peripheral nervous system, the authors tested two drugs that were initially proposed as potential treatments of the disease. One of them was Idebenone (IDE), a synthetic analog of Coenzyme Q10 that is able to enhance mitochondrial respiration by improving the electron flux along the ETC and with free-radical scavenger properties (Hargreaves 2014). IDE was administered in the fly food using two regimens, in an early treatment (from larva to adult stage) and in an adult treatment (in adult phase). This compound improved life span and motor performance in model flies, especially with the early treatment, and also enhanced aconitase activity in flies subjected to an external oxidative damage (Soriano et al. 2013). These results indicate that early treatments with IDE might be more beneficial for FRDA patients. In line with this finding, the patient age at which the treatment is commenced was suggested to be a crucial factor for the efficacy of this therapy (Pineda et al. 2008). However, IDE was not able to ameliorate cardiac defects such as dilatation or defective systolic function in a *Drosophila* heart model of FRDA (Tricoire et al. 2014). Therefore, the efficacy of IDE treatments might be dependent on the type of tissue. In contrast, methylene blue, a compound showing electron carrier properties, was also able to reduce the cardiac phenotypes in a dose-dependent manner in this fly model (Tricoire et al. 2014). Moreover, this compound could also reduce heart dilatation in flies mutant for genes encoding some components of mitochondrial complexes III and I. These findings suggest that drugs with electron transfer qualities might be useful to treat diseases with mitochondrial respiratory chain dysfunction as FRDA.

Reduction of frataxin levels in flies also produces iron accumulation in the mitochondria (Chen et al., 2016c; Soriano et al. 2013) as occurs in the FRDA patients and other model organisms (Calap-Quintana et al. 2017). Therefore, the efficacy of iron chelators to rescue several phenotypes was tested in two *Drosophila* models for the disease. Longevity, motor performance, and transition from larva to pupa were examined under the treatments with deferiprone (Soriano et al. 2013) and deferoxamine B (Seguin et al. 2015). Deferiprone is a small molecule with the capability to relocate and transfer iron to cellular acceptors such as transferrin (Sohn et al. 2008) and deferoxamine B chelates iron by forming a stable complex and preventing the entrance of iron into other chemical reactions (Mobarra et al. 2016). It was found that deferiprone improved survival and climbing abilities of the model flies and these improvements were associated with chelation of mitochondrial iron (Soriano et al. 2013), while deferoxamine B increased the proportion of larva undergoing pupariation as well as the timing of this biological process (Seguin et al. 2015). However, there are several reports of the effect of iron chelators in FRDA patients, where some have shown either improvement of the cardiac and/or neurological conditions (Boddaert et al. 2007; Velasco-Sánchez et al. 2011; Elincx-Benizri et al. 2016), some have reported no significant effect (Arpa et al. 2014), while others have reported the worsening of ataxia symptoms (Pandolfo et al. 2014).

Studies using *Drosophila* as a model organism for FRDA proposed a new pathological mechanism that links this disease with neurodegeneration and iron toxicity. The expression of a mutant allele of *fh* activated sphingolipid synthesis and *3-phosphoinositide dependent protein kinase-1* (*Pdk1*) and *myocyte enhancer factor-2* (*Mef2*) pathway downstream of the iron accumulation (Chen et al. 2016c). Inhibition of the serine palmitoyltransferase activity, an enzyme needed for de novo synthesis of sphingolipids, by feeding flies with the compound myriocin could rescue the degeneration of photoreceptor neurons. These authors found that *Pdk1-Mef2* signaling is also activated in heart tissues of FRDA patients and in a FRDA mouse model (Chen et al. 2016b). These results suggest a conserved pathological mechanism that might be susceptible to pharmacological intervention in FRDA, underlining the utility of *Drosophila* for identifying new therapeutic strategies for human diseases.

Frataxin deficiency could also provoke dysregulation of other metals in addition to iron. In line with this, the yeast frataxin protein has been reported to bind copper and manganese with higher affinities than iron (Han et al. 2017). It was found that copper, manganese, and zinc also accumulate in frataxin-deficient flies (Soriano et al. 2016) and that the copper chelators bathocuproine disulfonate and tetrathiomolybdate as well as the zinc chelator N,N,N′,N′-tetrakis (2-pyridinylmethyl)-1,2-ethanediamine improved the locomotor ability of the FRDA flies (Soriano et al. 2016). These findings suggest that these metals might be involved in the pathophysiology of FRDA and that their chelation might be of potential therapeutic interest in this disease.

A genetic screen performed in *Drosophila* to identify modifiers of FRDA (Calap-Quintana et al. 2015) pointed to the TORC1 pathway as a possible target for FRDA treatment. Chemical inhibition of TORC1 signaling by rapamycin, a macrolide compound, increased motor performance, longevity, and ATP levels in frataxin depleted flies (Calap-Quintana et al. 2015). Rapamycin also increased the nuclear translocation of the transcription factor cap-n-collar, the fly ortholog of the human Nrf2, allowing the expression of different antioxidant genes and consequently enhanced defenses against OS. Moreover, rapamycin also protected the frataxin-depleted flies from external OS by inducing autophagy. Edenharter and collaborators showed that mitochondrial clearance by mitophagy was enhanced in frataxin-deficient glia and muscles in *Drosophila* (Edenharter et al. 2018). Therefore, drugs promoting autophagy might speed up the removal of damaged mitochondria, reducing OS and other deleterious defects in the frataxin-deficient cells. The use of rapamycin and its derivatives (rapalogs) are approved in humans; hence, they may be potentially used in FRDA.

Finally, other kinds of drugs tested in *Drosophila* models of FRDA are tauroursodeoxycholic acid (the more hydrophilic form of ursodeoxycholic acid, a bile acid) and 4-phenyl butyric acid. These compounds reduced ER stress and cellular degeneration and partially restored aconitase activity in frataxin-deficient flies (Edenharter et al. 2018), thus highlighting the role of ER stress in the pathophysiology of FRDA.

Concluding Remarks

As it has been shown in the previous sections, *Drosophila* has become a very powerful screening platform in the initial drug discovery process in which multiple compounds can be assessed in vivo and in a relatively short period of time (Strange 2016; Tickoo and Russell 2002). A schematic of the different steps to be followed in this process is shown in Fig. 3. Due to the potent genetic tools available in *Drosophila*, fly models of NDs can be easily obtained independently of the inheritance pattern of the disease of interest. Once the fly model is generated, multiple assays to study neuronal dysfunction and degeneration can be used in different developmental stages of *Drosophila* life cycle. Moreover, several experimental approaches to perform chemical screens and to test the therapeutic potential of candidate compounds can be designed. Obviously, these experimental approaches are more complex than those used in traditional in vitro screening methods; however, screening in living animals such as *Drosophila* can filter out unwanted leads often identified in cell culture or biochemical assays (Fernández-Hernández et al. 2016). As a consequence, *Drosophila* is currently considered as a useful and economical tool in the drug discovery process to develop new treatments for NDs (Neri 2011). Of course, it should be assumed that despite the similarity in the essential physiological pathways between flies and humans, lead compounds identified in *Drosophila* have to be validated in traditional mammalian models before confirming its therapeutic potential and being used in human patients. Thus, this chapter clearly

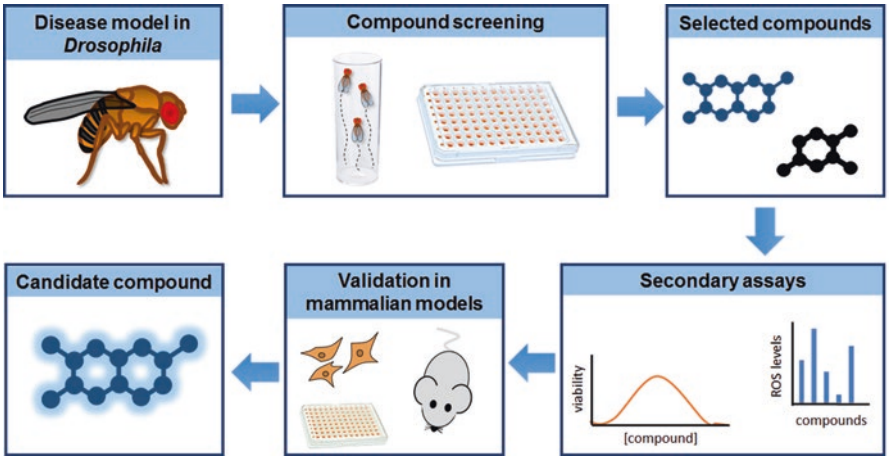

Fig. 3 *General steps followed in the drug discovery process using Drosophila models***.** The first step is to obtain or generate an appropriate *Drosophila* model of the corresponding ND that is able to reproduce disease-like phenotypes. The effectiveness of the compounds is evaluated in the *Drosophila* model using different assays (behavioral, developmental, phenotypic, or biochemical assays). Once a result is obtained, the beneficial compounds can be validated in the *Drosophila* model with support from additional assays and subsequently validated in a mammalian model. After this process, the candidate compound might be further evaluated or used in clinical trials

demonstrates that in the future, further research in *Drosophila* models of NDs will definitely contribute to the development of effective therapeutic strategies for mitigating these devastating disorders.

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