Childhood-onset movement disorders

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Chapter 2

Modelling in miniature:
Using *Drosophila melanogaster*
to study human neurodegeneration

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ABSTRACT

Despite great advances in clinical diagnostics, genetics and molecular biology, neurodegenerative diseases like Parkinson's disease (PD), Alzheimer's disease (AD) and Huntington's disease (HD) still pose great challenges, both in terms of understanding their pathophysiology as well as their treatment. Organisms able to adequately model the intricacies of the disease mechanism and response to potential treatment, whilst not compromising on ease of handling, studying and manipulating in order to study them, represent the holy grail of translational biology and medicine. Here, we review the suitability of the fruit fly, *Drosophila melanogaster*, as a model organism in the field of neurodegeneration. We briefly summarize the history of scientific research concerning this organism, review the molecular, genetic and pharmacological toolbox available and we discuss the ways this toolbox has been applied to research in neurodegeneration. Finally, by reviewing some findings in the fruit fly which were subsequently translated to and validated in other organisms on their way to the clinic, the power and robustness of *Drosophila melanogaster* is highlighted.

Keywords: *Drosophila melanogaster*, fruit fly, neurodegenerative diseases, model organism, neurodegeneration

INTRODUCTION

Human neurodegenerative diseases are an increasing burden to the current aging society. Scientific developments, necessary in order to start treating these disorders, include the identification of suitable drugs and drug targets, and lead to increased insights into the pathophysiology underlying these diseases. For this purpose, relevant aspects of disease need to be modelled in a system that allows the appropriate degree of simplification, comes with reasonable costs and convenience, without compromising on the findings' value. Different models provide different advantages and disadvantages, commonly associated with the organism's evolutionary proximity to humans on one hand versus the efficacy with which they can be handled, manipulated and investigated on the other. In the scientific field of neurodegeneration in particular, the complexity of the organism's central nervous system (CNS) is another important aspect to consider. In this review, we will discuss the contribution of *Drosophila melanogaster*, one specific species of the *Drosophila* or fruit fly family, to the field of research in neurodegeneration; demonstrating how its extensively developed toolbox, complex nervous system and facile handling have led and will lead to increasing knowledge regarding human neurodegenerative diseases. In this review, *Drosophila* refers specifically to *Drosophila melanogaster* unless stated otherwise .

Drosophila as a versatile model to study the brain

Over a century ago the first paper using the fruit fly *Drosophila ampelophila Loew* for biological research was published[1], which was the beginning of extensive research with the fruit fly as a model for developmental biology, behavior and disease. Their relatively short life span, easy and cheap culturing conditions, a quick reproduction time and the ability to produce a large number of offspring that is genetically identical make *Drosophila* an attractive model to use. In addition, the great majority of human disease associated or causative genes is conserved in the *Drosophila* genome further affirming the power of the fruit fly as a model for human disease[2,3]. Finally, the *Drosophila* brain is well organized and described with about 200.00 neurons employing various neurotransmitters comparable to human neurons.

The first steps on the path leading to *Drosophila* neuroscience were made when Seymour Benzer identified fruit fly strains with aberrant behaviour [4]. Rather than solely describing the behaviour, which included "staggering" and early lethality, he attempted to make correlations between the observed behaviour and neuropathological findings, discovering that a mutant named *drop dead* displays locomotor abnormalities at the time the fly brain starts degenerating, leading to a vacuolized brain, "shot full of holes"[4].

From the notion of Benzer that the neurodegeneration he observed resembled human neurological disease, *Drosophila* neuroscience remained a subject of interest. *Drosophila* research at this time depended heavily on mutant screens, which introduced mutations using chemical mutagens or radiation in a variably random manner along the genome, leading to the resulting mutants being "found" rather than specifically generated[5,6]. Using this strategy, two other neurodegenerative fly mutants were found and named *eggroll* and *spongecake*[7].

The modelling of particular human genetic defects in *Drosophila* stimulated the development of other sophisticated genetic techniques, such as expression of specific transgenes: this led to the first dedicated models of spinocerebellar ataxia type (SCA3, or Machado-Joseph disease) and Huntington's disease (HD) in 1998[8,9]. An example of the powerful genetics available for *Drosophila* research is the binary UAS-GAL4 system (Figure 1) which offers the possibility of spatiotemporally controlled expression of a construct of choice (Figure 3a), enabling overexpression of disease-related genes. When combined with RNA interference (RNAi, Figure 2), the system can be used to induce tissue specific gene knockdown (Figure 3b). Both techniques are often utilized to study human (neurodegenerative) diseases. Over time different techniques were developed and applied to specifically target and mutagenize a gene of interest, including homologous recombination, ZFN and TALEN and most recently the very popular gene editing technique CRISPR/Cas9[10–12]. In addition to mimicking the pathophysiology of human disease, *Drosophila* has an elaborate range of complex behaviour, like learning and memory, aggression and behaviour influenced by olfactory stimuli. This complex behaviour can contribute to the study of a disease of interest since the underlying molecular pathways are highly conserved[13,14].
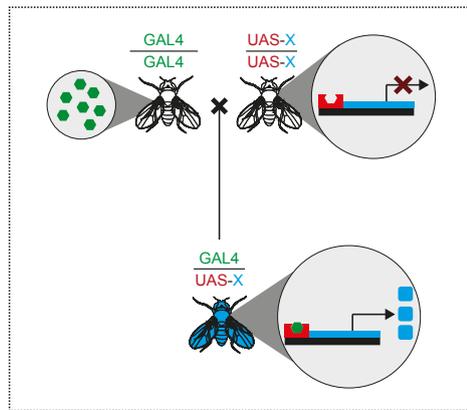

Figure 1. **The UAS/GAL4 system for targeted gene expression**

The UAS/GAL4 system allows the targeted expression of a genetic construct of choice. The system contains two constructs: a driver, which is a genetic construct that leads to expression of the transcription factor GAL4, and a responder element (UAS) to which GAL4 can bind. Binding leads to the expression of the genetic construct (X) coupled to the UAS. By crossing a driver fly line to a responder line the subsequently expressed GAL4 binds to the UAS and results in expression of gene X.

Drosophila models of neurodegenerative diseases

A wide range of neurodegenerative disorders has been studied with the use of *Drosophila* models so far, among which polyglutamine (polyQ) disorders, Alzheimer's (AD) and Parkinson's disease (PD) and various rare neurodegenerative diseases like Chorea-Acanthocytosis (ChAc) and Panthothenate Kinase Associated Neurodegeneration (PKAN). Figure 4 shows a number of assays that are often used to assess neuronal dysfunction and neurodegeneration in *Drosophila*. Dominantly inherited polyQ diseases, including the aforementioned Huntington's disease (HD) and SCA3, are caused by the expansion of CAG
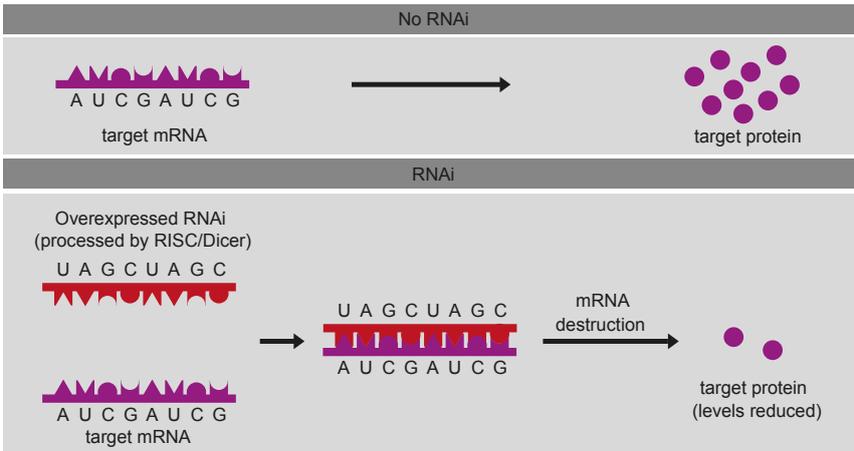
Figure 2. **RNA interference**

Schematic representation of RNA interference (RNAi), which is a biological process where protein translation is inhibited by RNA molecules leading to knockdown of a target protein. Single-stranded mRNA, generated by RISC-mediated processing of short hairpin RNA, hybridizes with a complementary mRNA of the target protein to form double-stranded RNA (dsRNA). This dsRNA is subsequently degraded. Due to the destruction of this mRNA, translation of the target protein is reduced and protein levels are decreased.
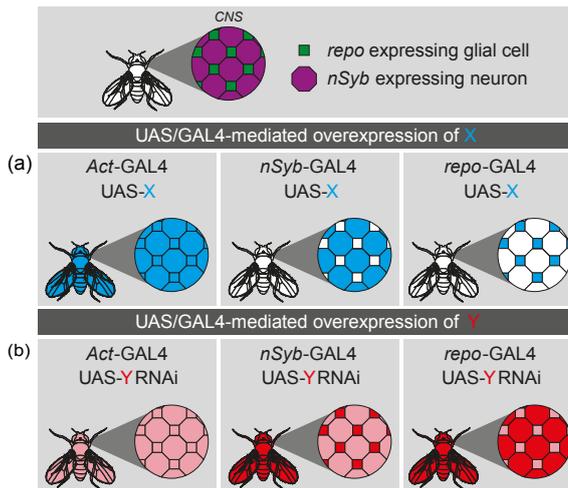
Figure 3**. Applications of the UAS/GAL4 system**

In *Drosophila*, the GAL4 is placed under the control of a native gene promotor and therefore GAL4 is only expressed in cells where the gene is naturally active.

(**a**) The use of the driver element *Actin-GAL4* leads to production of GAL4 wherever and whenever actin is expressed. In practice, this means that *Actin*-GAL4 is a ubiquitous driver. However, the driver element *neuronal Synaptobrevin-GAL4* (*nSyb*-GAL4) leads to a production of GAL4 that is restricted to neurons since neuronal Synaptobrevin is only expressed in neurons. Similarly, *reversed polarity*-GAL4 (*repo*-GAL4) drives expression only in glial cells since Repo is a glial protein.

(**b**) By coupling the RNAi technique to the UAS/GAL4 system, the knockdown can be targeted to specific cell types and/or developmental stages. The degree of knockdown can be regulated with the temperature, where increased temperature leads to increased expression of the GAL4 driver and therefore increased expression of the RNAi construct and a stronger knockdown.

Figure 4. **Frequently used assays to study neuronal dysfunction and degeneration in *Drosophila***

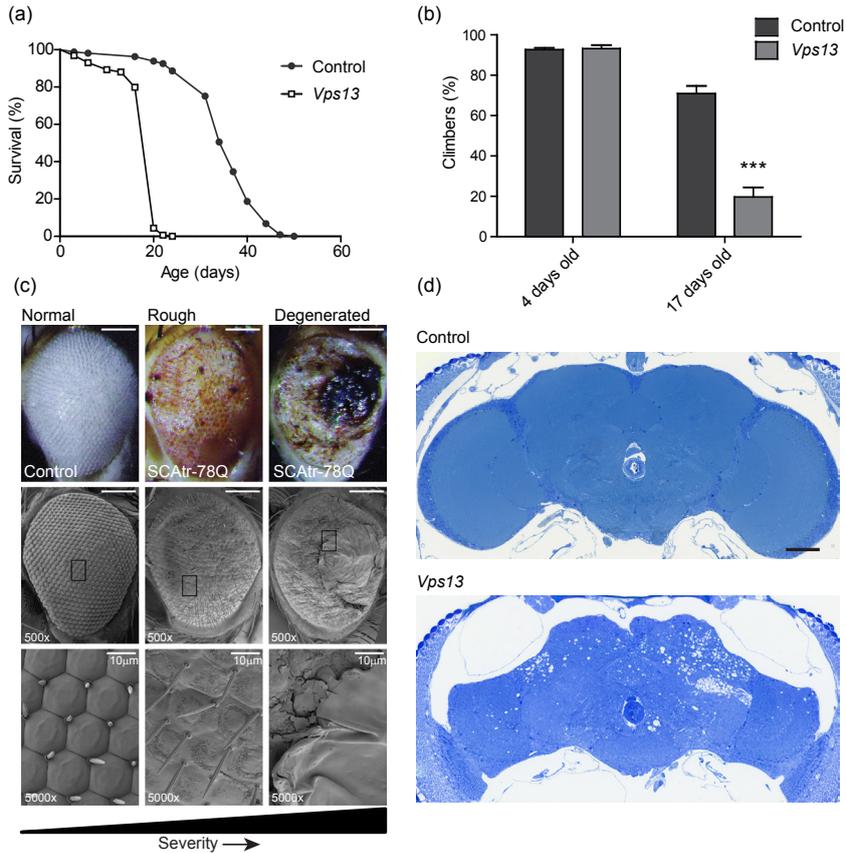(**a**) Lifespan analysis of a *Drosophila* neurodegenerative disease model for Chorea-Acanthocytosis (ChAc) compared to controls, where neurodegenerative mutants typically have a shortened lifespan.

(**b**) A climbing assay, also known as negative geotaxis, is often used to examine age- and disease-related motor deficits. Flies are tapped to the bottom of the vial after which healthy flies will immediately start climbing up the walls. The number of flies that climb above a certain height within a certain time period is recorded. For this assay a *Drosophila* neurodegenerative disease model for Chorea-Acanthocytosis was used.

(**c**) Disease-related genes can be expressed in the eye using the UAS/GAL4 system (Figure 2) after which the eyes are scored for retinal degeneration. Flies can develop a 'rough' eye phenotype and sometimes rough-eyed flies also have black patches with increased degeneration, here due to expression of SCAtr-78Q. Eyes can be analyzed using light microscopy (top images). Scanning electron microscopy shows loss of tissue integrity.

(**d**) Neurodegeneration in *Drosophila* is often accompanied by the presence of vacuoles in the brain and can be visualized in sections of the brain. Fly heads or brains are embedded in plastic or paraffin and the morphology can be examined. For this assay a *Drosophila* neurodegenerative disease model for Chorea-Acanthocytosis was used.

A,B & D are reprinted from[33]; C is reprinted from[34].

repeats in the gene-coding DNA which is translated into abnormal proteins[15–17]. Those aberrant proteins form insoluble aggregates that are associated with neuronal dysfunction and degeneration[18,19]. An *in vivo* model of SCA3 in *Drosophila* was established by the overexpression of a truncated C-terminal domain of the gene affected in SCA3: *MJD1*[8]. Both a pathogenic protein (SCA3tr-78Q) with an extended polyQ repeat length and a control protein (SCA3tr-27Q) were expressed in different tissues using the UAS-GAL4 system. Expression of the SCA3tr-78Q protein in the developing eye disrupts eye morphology and targeted expression of the pathogenic protein to the peripheral and central nervous system is lethal, while SCA3tr-27Q expression does not have any effects, indicating severe consequences of SCA3tr-78Q expression. Moreover, the expanded ataxin-3 protein forms nuclear inclusions in a time- and concentration-dependent manner, which recapitulates the pathological aspects of SCA3[8]. After that, subsequent studies used the *Drosophila* eye to further investigate the pathogenesis underlying SCA3, *e.g.*[20–22]. As for HD, the *Drosophila* eye also proved to be a valuable model, where it was shown that expression of the first exon (exon1) of the HD gene with disease-associated polyQ expansions (Q75 and Q120) causes late-onset progressive degeneration. Severity of the degeneration is dependent on the polyQ repeat length[9], thereby resembling the human disease.

AD is the most prevalent neurodegenerative disease. Clinically, it is associated with progressive memory loss and pathologically by the presence of extracellular amyloid beta (Aβ42) plaques as well as intracellular tangles containing hyperphosphorylated tau in brains of AD patients[23,24]. The majority of AD cases is of sporadic aetiology; mutations in the genes encoding *Amyloid precursor protein* (*APP*), *Presenilin 1* and *2* (*PS1* and *PS2*) cause familial AD, making up only 1% of all AD patients[25]. Mutations in one of those genes cause production of large amounts of the aggregate-prone Aβ42 that accumulates into extracellular plaques in the brain[26,27]. Many different *Drosophila* models for AD have been established over the last two decades[13]. Flies deficient for the *APP* ortholog, *Appl*, present with behavioural deficits that can be rescued by expression of the human *APP* in the *Appl* mutant background, indicative of functional conservation[28]. Since overexpression of AD related Aβ42 and tau protein in flies was shown to cause memory deficits and neuronal loss, resulting in a shortened life span and reduced locomotion, *Drosophila* also demonstrated to be a feasible model organism to study AD pathology[29–32], although currently the causative role for Aβ42 in AD is under debate.

The rare neurodegenerative disease Chorea-Acanthocytosis (ChAc), caused by mutations in the *Vacuolar protein sorting 13 homolog A* (*VPS13A*), has recently been modelled and described in *Drosophila*[33]. The pathophysiology underlying this disease is largely unknown and models for this disease are limited due to the large size of the gene and protein, which make it hard to study. The *Drosophila Vps13* mutant presents with multiple neurodegenerative characteristics including a shortened lifespan, decreased climbing ability and vacuoles in the brain. In addition, a defect in protein homeostasis was found. Rescue of some of these phenotypes by overexpression of human VPS13A in the *Drosophila* mutant background supports functional conservation of both genes and emphasizes the relevance of a fly model for ChAc[33].

Drosophila has been of major importance in the study of PD, a movement disorder featuring bradykinesia, resting tremor and rigidity amongst other symptoms. Pathological findings include neuronal loss of dopaminergic nigrostriatal neurons and presence of intraneuronal aggregations called Lewy bodies,

that contain α-synuclein[35]. As with AD, most PD cases are sporadic and only a small fraction, less than 10%, is caused by genetic mutations in *PINK1*, *parkin* and others[36]. However, these genetic cases provide understanding about the underlying disease mechanisms[37]. *Drosophila parkin* mutants exhibit muscle degeneration, locomotor defects and structural alterations of mitochondria[38]. Flies mutant for the *Drosophila PINK1* ortholog share phenotypic similarities with degeneration of flight muscles accompanied by mitochondrial defects[39,40]. Subsequent overexpression of *parkin* compensates for the absence of PINK1, suggesting both to function in one single pathway, where Parkin functions downstream from PINK1 since PINK1 overexpression could not compensate for loss of Parkin[39,40]. Furthermore, Lewy bodies are present in flies overexpressing mutant α-synuclein, which also cause age-related degeneration of dopaminergic neurons and climbing defects, thereby recapitulating the most important features of human PD[41]. PD patients are often treated with dopamine agonists, which also have a beneficial effect in different *Drosophila* PD models reinforcing the power of *Drosophila* models for PD[42–44].

Genetic and pharmacological screening possibilities

Due to its short life cycle and straightforward handling, *Drosophila* enables high-throughput analysis of complex neurophysiological traits such as locomotor function. This facilitates its use as a platform to find genetic and chemical modifiers of neurodegeneration on a large scale, and when required in a fully unbiased manner. A recent screen of antioxidants in a *Drosophila* model of Parkinson's disease (PD) identified compounds that ameliorated the locomotor phenotype of mutant flies, which were subsequently validated in neuronal cell culture studies[45]. More recently, a genome-wide association study was performed to investigate the influence of genetic background variation in a fly model of PD, which identified new genes that influence loss of dopaminergic neurons and locomotor dysfunction[46]. In this latter study, the Drosophila Genetic Reference Panel (DGRP, http://dgrp2.gnets.ncsu.edu/) was used as a resource. The DGRP is a collection of 148 lines with a genetically diverse background which have all been sequenced. This collection, available to the community, is especially powerful to identify genetic modifiers of phenotypes of interest. In another study, using a library of deletion constructs, an unbiased genetic screen was carried out to find modifiers of Aβ42-neurotoxicity[47]; a similar approach had been taken to find modifiers of tau using a library of transposable elements[48], both with the aim of elucidating pathophysiological mechanisms behind Alzheimer's disease. Exploiting the ease with which the SCA3 eye phenotype (as mentioned before) can be screened, transposable elements were used to find modifiers for the disease process in an unbiased way[49]. A similar eye phenotype, induced by expression of TDP-43 as a model for amyotrophic lateral sclerosis (ALS), was used to selectively screen regulators of chromatin remodelling for effects on the degenerative phenotype; the resulting insights regarding the role of chromatin regulation in the neurodegenerative process active in ALS were supported by findings in patient tissue[50]. These strategies illustrate the potential of *Drosophila*, unmatched by other more classical model systems such as the mouse, to identify novel pathophysiological and therapeutic aspects of neurodegeneration.

Application of *Drosophila* findings to human disease

The value of *Drosophila* in the study of neurodegenerative diseases is reflected by the insights provided over recent years, which have been translated to more complex model organisms and in some cases approach clinical application. The recognition of histone deacetylase (HDAC) inhibition as a beneficial intervention in Huntington's disease[51] exemplifies the robustness of *Drosophila* in the identification of therapeutic strategies; once this principle was well-established in *Drosophila*, it was transferred to mouse models where it exerted the same effects in models of Huntington's disease[52–54] and other polyglutamine diseases[55,56]. Preliminary studies have shown that this efficacy may extend to patients as well[57]. Similarly, strategies for neurodegeneration secondary to metabolic disease have been devised in *Drosophila*. The neurodegenerative disease pantothenate kinase-associated neurodegeneration (PKAN) is caused by mutations in pantothenate kinase 2, the first enzyme in the pathway that transforms vitamin B5 into the ubiquitous cofactor coenzyme A. Flies that lack *fumble*, the sole *Drosophila* orthologue of pantothenate kinase, feature neurodegeneration, motor defects and a shortened lifespan. In this model, pantethine was identified as a rescue compound capable of reversing not only the reduced coenzyme A levels, but also ameliorating the phenotypes reminiscent of the human disease[58,59]. This was subsequently validated in a mouse model of PKAN[60]. Continuing the development of therapeutics that supply a source for coenzyme A synthesis in the absence of pantothenate kinase, studies in *Drosophila* brought forth 4'-phopshopantetheine as a potential therapeutic[61]. This lead compound was further derivatised to acetyl-4'-phosphopantetheine, which showed a favourable pharmacokinetics as well as therapeutic efficacy in both *Drosophila* and mice models of the disease[62]. Acetyl-4'-phosphopantetheine currently has the orphan drug status and is further developed for clinical use.

Conclusion and future perspectives

Major advances in the field of genetics have revolutionized the study of neurodegenerative diseases, enabling the identification of causative mutations in patients and as a consequence, the creation of model organisms by means of genetic rather than phenotypical similarity. The powerful new possibility to precisely edit the genome by CRISPR/Cas9 enables the generation and study of patient-specific mutations in model systems. In addition, findings from unbiased studies such as GWAS can be verified with the modelling power of *Drosophila*. Although the first descriptions of neurodegenerative phenotypes in the fruit fly already approach their fiftieth anniversary, the disease-directed modelling of neurodegenerative pathology in *Drosophila* is still a flourishingly developing field, where novel insights are readily translated to more classical systems and in some cases, patient care. The ease to study, manipulate and screen an organism of such biological and neurological complexity places *Drosophila* at the centre of novel discoveries regarding pathophysiology and therapy of neurodegenerative disorders in years to come.

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