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Research Article

A Fruitful fly forward: The role of the fly in drug discovery for neurodegeneration

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Abstract. Neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease (PD) and Huntington's disease (HD) are increasing in prevalence and the need for novel disease-modifying therapies is critical. Identifying compounds that modify disease progression has been a struggle - mainly due to the insufficient knowledge regarding the underlying pathophysiological mechanisms of these diseases. Traditional high-throughput screening *in vitro* have previously identified positive hits. However, subsequent validation experiments *in vivo*, rendered them ineffective and/or toxic. *Drosophila* models of neurodegenerative disease can be effectively exploited in drug screens for the identification of compounds and target disease mechanisms. This review sheds light on how *Drosophila* models of neurodegeneration can aid the therapeutic discovery process through the use of chemical and genetic suppressor/enhancer screens and other existing techniques. Integrating *Drosophila* models of neurodegeneration to the drug discovery process holds great promise for the enhanced rate of therapeutic-modifying compound discovery.

Keywords Drosophila – fruit fly – drug discovery – neurodegeneration – Alzheimer's disease – Parkinson's disease – Huntington's disease.

1 Introduction

Drug discovery is the process by which new candidate compounds and molecules that have a therapeutic effect on disease symptoms are discovered. Traditionally, the process of drug discovery begins with identifying a disease-causing protein, followed by a screening of a large library of known chemical compounds in order to identify drugs that ameliorate or alter the function of the disease-causing proteins. Subsequently, compounds are optimized and then testedin animal models (Pandey and Nichols, 2011). The traditional high-throughput screening (HTS) approach has been the source of many documented successes, ranging from identifying therapeutic compounds to cell functions and pathological pathways (Giacomotto and Ségalat, 2010). However, despite the numerous triumphs brought about by HTS, the past couple of year shave seen a slow-down in the development of new therapeutic drugs, due to a variety of reasons, mainly the positive hits generated by initial screening are being rendered invalid when tested in animal models and therapeutically ineffective when tested on humans; furthermore, the method depends on predetermined targets and therefore, does not leave space for new target discovery (Giacomotto and Ségalat, 2010; Lindsay, 2003).

Lately, there has been an increased interest in target discovery. The identification of novel targets and early validation techniques will help reduce subsequent failure rates of positive hits,and address the problems being faced by the pharmaceutical companies (Lindsay, 2003). The limited predictive value of HTS for clinical outcome can be overcame with the use of animal models for a primary drug screening platform (Pandey and Nichols, 2011).

Drosophila melanogaster is one such organism that is gaining momentum as a valid screening tool for the drug discovery process (Lieschke and Currie, 2007). *Drosophila* has been used since the 20th century and provides the advantage of combing genetic acquiescence and a rapid lifecycle. In addition, culture conditions are compatible with large-scale screening and their use permits high-throughput screening in a whole animal context which is not dependent on a predetermined tar-

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get (Giacomotto and Ségalat, 2010). Another advantageous aspect to keep in mind is that key biological features are conserved from the flies to humans, including a highly structured brain, elaborate neuromuscular junctions, and underlying mechanisms of synaptic transmission. This context provides a good foundation for developing an appropriate model for human disease characterising the pathological phenotype (Lenz et al., 2013). Screening for novel drugs in *Drosophila* enables the selection of high-quality hits that display keys features such as transdermal availability, metabolic stability and low toxicity (Pandey and Nichols, 2011). This in turn reduces the expenses spent on primary screening and filters the quality of positive hits for secondary screening.

2 Why The Fly?

2.1 Brief history of the fly

Drosophila is an old player in the biomedical field with a rich history spanning over 100 years. Fruit flies have been indirectly associated with medical progress and drug discovery for many years, mainly through genetic, biochemical and disease pathology-related discoveries (Ségalat, 2007). Indeed, Nobel prizes have been awarded to people for their pioneering research in flies, starting with Thomas Hunt Morgan for the role of chromosomes in heredity (1933), Hermann Muller for the production of mutations by x-rays (1946), Edward B. Lewis, Christiane Nusslein-Volhard and Eric F. Wieschaus for genetic control of early structural development (1995) and finally Jules Hoffman for the discoveries around innate and adaptive immunity (2011).

In the modern era, *Drosophila* was amongst first complex organisms to have its entire genome sequenced (Adams et al., 2000). A couple of years later when the human genome was sequenced, the observed homologies between the two genomes was recognised and this strengthened its role as a model to understand disease processes (Pandey and Nichols, 2011). Recently, their use as direct HTS tools gained momentum mainly due to their unique life cycle, form, function and experimental manipulation (Rand, 2010).

2.2 Basic biology of the fly

Flies are reared in small vials on a simple solid food medium of cornmeal, yeast and agar. At the optimum temperature of 25 °C their generation time is 10-11 days from egg to adult (Rand, 2010). The fly has a very rapid lifecycle which is comprised of four distinct stages: embryo, larva, pupa and adult. Each stage presents a unique opportunity to assess the susceptibility to the nervous system to xenobiotics (Rand, 2010). The embryo stage lasts around 24 hours at 25 °C and during this time neurogenesis and differentiation give rise to a fully functioning nervous system, capable of sensory and motor behaviours characteristic to the larva (Rand, 2010). In fact, the embryo is typically used in developmental studies examining pattern formation, cell fate determination, organ formation, neuronal development and axon guidance (Pandey and Nichols, 2011). The larval stage lasts over 4 days and is characterised by a period of growth that results in a 10-fold increase in body size (Truman and Bate, 1988). The larva is used to study physiological processes and simple behaviours like foraging (Pandey and Nichols, 2011). The following 5-6 days of pupal metamorphosis is characterised by tissue reorganisation and the fusing together of the adult structures from the precursor imaginal disc tissues. At the same time, the central and periphery nervous system neurons undergo pruning and regrowth while newly born adult neurons migrate to their final position and extend their synapses to their targets (Williams and Truman, 2004). The emerged adult fly is a complex organism capable of showing behaviours like flight, chemo-, photo- and geo-taxis, foraging and mating (Rand, 2010). The brain of the adult fly has more than 100,000 neurons that form discreet circuits and mediate complex behaviours including circadian rhythms, sleep, learning and memory, courtship, feeding, aggression, grooming, and flight navigation (Pandey and Nichols, 2011) (Figure 1).

2.3 Genetic Workhorse

Drosophila has been primary regarded as a model for the study of genetics. The first genetic principles of chromosomal heredity and mutagenesis, were primary discovered using the fly (Rubin and Lewis, 2000). The large polytene chromosomes served as a template for gene mapping and the mutagenesis screens rapidly advanced the knowledge of gene function. The fly has a relatively simple genetic make-up of four chromosomes encoding roughly 13,600 genes (half the number found in humans) (Adams et al., 2000). More than 95 % of its genetic content is on three of its four chromosomes, with the first being the sex chromosome and the other three being the autosomes (Rand, 2010). The sequence identification of genes brought about by the emergence of molecular cloning and recombinant DNA technology, opened doors to functional analysis of transgenes *in vivo* (Rand, 2010). The method of creating transgenic flies through transposable element transformation, opened the door to manipulating the expression of endogenous or exogenous genes over the course of development and provided a means of integrating foreign DNA into the chromosome (Duffy, 2002). Variations in the method has led to the creation of the "workhorse" of fly transgenic models, the Gal4-UAS gene expression

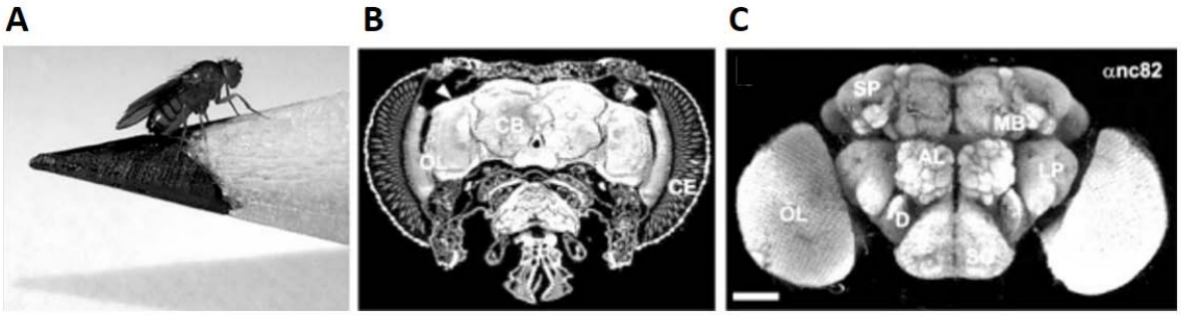

Figure 1: Drosophila as a model organism.(a) The arthropod Drosophila fits on a pencil tip and can be easily kept in the laboratory. Their anatomy displays characteristic features that can be used as phenotypes to study neurodegeneration and as endpoints in high-throughput screening. (b) Confocal image of a cross-section through the adult Drosophila head; auto-immunofluorescence visualises the ommatidia of the compound eye (CE), the optic lobe (OL) and the central brain (CB). The cell bodies (arrowheads) are topologically separated from axonal extensions which make up the neuropil. (c) Confocal image of a whole mount adult brain immunolabelled with anti-nc82 which recognises the Bruchpilot protein that is specifically enriched in active zones of synaptic terminals. This allows the visualisation of cortical areas in the fly brain, including optic lobes (OL), antennal lobes (AL), superior protocerebrum (SP), lateral protocerebrum (LP), mushroom bodies (MB), deuterocerebrum (D), and subesophageal ganglion (SG) (adapted from Hirth (2010)).

system (Duffy, 2002). This bipartite approach keeps the yeast transcription factor Gal4 and the Upstream Activating Sequence (UAS) promoter (which holds the transgene) to which Gal4 binds, on different parental strains. When the two strains are mated, the progeny expresses the transgene in the specific tissues defined by the Gal4 driver (Pandey and Nichols, 2011). Other modifications and enhancers further refined tissue specificity as well as temporal expression specificity (McGuire et al., 2004). This methodology has been crucial in creating "humanized" versions of the flies that express disease genes that invoke neuropathology mimicking human disease in the fly (Bilen and Bonini, 2005; Cauchi and van den Heuvel, 2006).

3 Modelling Neurodegeneration in the Fly

The genetic advantages and tools that come with the use of *Drosophila* make it very easy to rapidly generate models of human disease, which can be used for therapeutic target discovery. The generation of *Drosophila* models that express human genes is a popular approach for studying neurodegenerative diseases. Fly models for neurodegenerative diseases have been generated, studied and used for many years. Neurodegenerative diseases are considered to be predominately diseases that occur at an older age, implying that processes that are altered due to aging may contribute to their pathogenesis (Newman et al., 2011). They are characterised by the misfolding, aggregation and deposition of normal or abnormal proteins that become aberrant (Newman et al., 2011). Many late-onset neurodegenerative diseases are generally associated with the formation of intracellular aggregates of toxic proteins (Taylor et al., 2002). The common hypothesis is that neurodegenerative diseases come about when the production of neurotoxic proteins exceeds the cell's capacity for clearing them or when they evade autophagic clearance altogether (Pandey and Nichols, 2011).

3.1 Parkinson's disease

PD is a progressive, disabling, heterogeneous neurodegenerative disorder that clinically presents with motor and non-motor features (Henchcliffe et al., 2011). PD is characterized by loss of dopaminergic neurons in the substantia nigra and formation of filamentous intraneuronal inclusions (Lewy bodies [LBs]) (Feany and Bender, 2000). The major components of LBs are amyloid fibrils of the protein α-synuclein (Spillantini et al., 1998). Mutations in the α-synuclein gene (SNCA) are associated with familial PD and have an increased aggregation propensity *in vitro* (Conway et al., 2000; Greenbaum et al., 2005). Overexpression of human α-synuclein in different model systems, have provided a pathologically true model of PD. In several of these models, the rate of fibril and inclusion body formation does not correlate with neurotoxicity (Chen and Feany, 2005; Volles and Lansbury, 2007). This lack of correlation formed the basis for the hypothesis that small oligomers, but not fibrils, are the most toxic species of α-synuclein (Lashuel and Lansbury, 2006). One great advantage of using *Drosophila* to model PD is their ability to reprise some of the key neuropathological characteristics of PD. Transgenic flies show age-dependent, progressive degeneration of dopamine neurons, inclusion-like formation, progressive locomotor deficits and a decrease in lifespan (Muqit and Feany, 2002).

3.2 Alzheimer's disease

Alzheimer's disease is the most prevalent form of senile dementia in humans, with its manifestation being age-dependent and its incidence in the general population expected to rise from 6 % to 30 % over the next 65 years (Puglielli et al., 2003). AD is diagnosed by the presence of neuritic plaques, composed mainly of Aβ peptides and neurofibrillary tangles composed of tau protein (Finelli et al., 2004). There is evidence that the β-amyloid peptides are central to the pathogenesis of AD (Crowther et al., 2006). These peptides are generated by β- and ɣ-secretase cleavage of amyloid precursor protein (APP) to yield peptides of either 40 or 42 amino acids ($A\beta_{1-40}$ or $A\beta_{1-42}$) (Crowther et al., 2006). Recently, studies have shown that monomeric peptides are not toxic but that oligomeric peptides gain toxicity that is subsequently lost when mature fibres are formed (Lashuel and Lansbury, 2006). In order to understand the pathway of neurodegeneration in AD, a faithful animal model is required. Current mouse models have successfully recapitulated AD-like phenotypes (Crowther et al., 2006; Hsiao et al., 1996). Despite this, mouse models are laborious to characterize and develop. *Drosophila* models of AD drive the expression of the Aβ peptides in temporal and tissue specific manner. Several fly models of AD have been made, including ones expressing Arctic Aβ peptides (E693G; an aggressive mutation for the AD model) and show progressive neurodegeneration, amyloid deposits, reduction in lifespan and locomotor dysfunction (Crowther et al., 2006; Sofola et al., 2010; Iijima et al., 2004; Finelli et al., 2004).

3.3 Huntington's Disease

A large and diverse group of neurodegenerative diseases are characterised by the abnormal function of long tracks on tri-nucleotide repeats. The repeats can be of two kinds; part of the protein-coding sequences which result in the production of long stretch of polyglutamine-containing (polyQ) peptides or in non-coding sequences (Konsolaki, 2013). Huntington's disease (HD) is associated with expanded polyQ repeats in the gene Huntington within exon 1. Translated stretches of polyglutamines disrupt cellular processes including nucleolar stress, the functions of miR34 (its disruption is linked to the occurrences of some types of cancer), autophagic processes involved in the immune system and the Akt/GSK3b pathway (involved in cellular growth) (Konsolaki, 2013). In addition, a form of mutant Huntington may interfere with specific components of transcriptional machinery in early stages of HD (Cui et al., 2006).

Mutant Huntington is expressed ubiquitously but selective cell loss is observed in the brain (Vonsattel and DiFiglia, 1998). In clinical settings, HD is characterised by involuntary movements and psychiatric disturbances (Vonsattel and DiFiglia, 1998). Due to the fact, that polyglutamine diseases are brought about by single-gene defects (e.g. Huntington gene in HD), *Drosophila* models are commonly used to study this neurodegenerative disease. Pathological hallmarks of HD, such as intracellular and cytoplasmic aggregates of expanded Huntington are present in Drosophila models of HD (Pandey and Nichols, 2011). *Drosophila* models can be generated through the expression of truncated wild-type and mutant forms of Huntington, however, it has been noted that increased polyQ expression leads to increased severity of degeneration, age-dependent degeneration and repeat length-dependent protein aggregation in *Drosophila* models (Spada and Taylor, 2010). These models have provided a platform to demonstrate that human disease genes can yield parallel neurodegenerative effects in *Drosophila* models (Pandey and Nichols, 2011).

4 Drug Discovery Takes Flight

4.1 Drug delivery

The mode of drug or compound delivery is an important aspect to take into consideration when performing drug discovery screens (Figure 2). Exposure of the compound to the cells or organs of interest in flies can take a variety of forms depending on the developmental stage of the fly. For embryos, drugs can be administered via maternal feeding, injection or *in vitro* incubation (Rand, 2010). Due to the unknown metabolic and delivery characteristics of the embryo, maternal feeding requires the determination of the experimental dosage (Rand, 2010). Embryo injection methods have recently been optimized for use in high-throughput screens using an automated system based on microelectromechanical systems injectors, which allows successful mass-injections of *Drosophila* embryos (Zappe et al., 2006). Chemical exposure through *in vitro* incubation of fly embryos has been effective, however, one limitation of this system is the permeabilization of the vitelline membrane of the *Drosophila* egg chamber (Rand, 2010). For larva and adult flies, drugs can be supplied through dosages in food media (Peng et al., 2009). For longer exposures, the drug can be mixed in the solid media and the larva can be reared on it; when shorter exposure to the drug is required, the compound can be diluted and mixed in a yeast paste (Pandey and Nichols, 2011). Drugs (e.g. cocaine) have also been successfully delivered to both larva and adult flies through injection methods (Dimitrijevic et al., 2004). The routes for drug administration in adult flies are numerous. Drugs can be presented as vapour or aerosols in a controlled environment (Moore et al., 1998; Parr et al., 2001), in the solid media (Peng

et al., 2009), from a drug-saturated filter paper (Nichols et al., 2002), dropped directly onto the exposed nerve cord of decapitated flies (Torres and Horowitz, 1998), or injected directly into the fly abdomen (Dzitoyeva et al., 2003). The route of administration is also dependent on the taste of the drug being administered: if the drug has a poignant smell the flies might not eat it. If ingesting the drug is the route of administration chosen, then it might be necessary to introduce the drug to the fly through a rewarding substrate (e.g. yeast, sucrose or banana) (Pandey and Nichols, 2011).

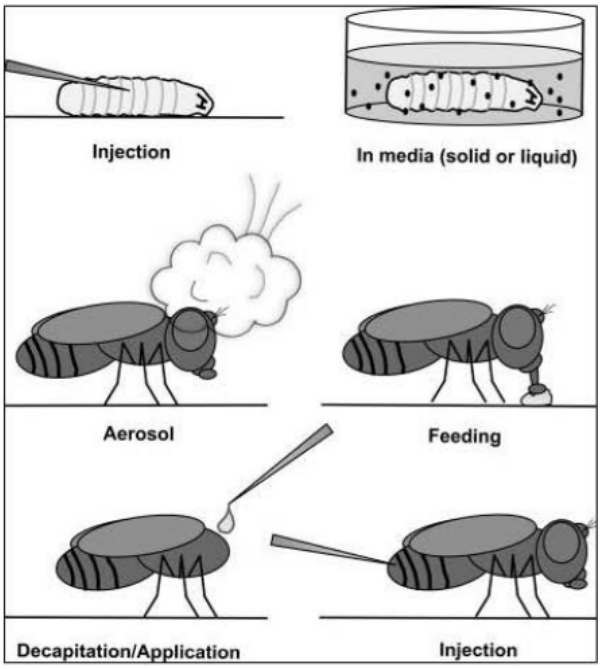

Figure 2: Routes for drug administration for drug delivery in *Drosophila*. For larva (top), drug can be directly injected or drug can be mixed with media. Media can be either solid or liquid with 2 % yeast paste to encourage feeding behaviour. Adults can have drug delivered as an aerosol or gas, as a mixture with food substrate, as a direct application to exposed nerve cord, or as an injection. Drug administration through feeding generally has the highest throughput (Pandey and Nichols, 2011).

Feeding assays can also be performed in order to determine whether the presence of a drug is influencing food intake. One such assay is based on measuring the feeding frequency by observing the proboscis-extension. This method can then be validated by short-term measurements of food dye intake (Wong et al., 2009). When administering drugs, it is important to keep in mind that female flies feed more frequently than males and therefore, their food intake is higher, a consideration that is especially important for drug delivery. Flies feed more often when housed in larger groups, and their feeding times vary on the time of the day (Wong et al., 2009). There are also different strategies to be considered when feeding drugs to adult flies. Firstly, the flies will generally consume a large amount of food if previously starved for up to 18 hours. This strategy allows for the observation of the acute effects of the drugs, however, there is a significant amount of dosage variability of the ingested drug and is relatively low-throughput. Secondly, maintaining flies on drug/food for longer than 24 hours allows for steady-state levels to be achieved before testing. This method allows high-throughput administration of the drug across large populations, however, the possibility of adaptive mechanisms (such as down-regulation of target genes) to prolonged exposure may occur. When interpreting the data, it is important to keep in mind that if the flies are removed from the drug-supplemented food before being subjected to tests, the rate of drug elimination may need to be accounted for (Pandey and Nichols, 2011).

Once the method of administration has been chosen, the next issue to tackle is the compound concentration that will be delivered to the flies. A key consideration to take into account are the potential differences in the pharmacokinetics and pharmacodynamics of compounds and small molecules. These molecules can produce significant discrepancies in drug levels, tissue distribution profiles and toxicity, between mammals and flies (Pandey and Nichols, 2011). Since the target is not always known and the treatment is usually delivered through food media, it is difficult to predict the range of doses that need to administered in order to avoid a low ineffective concentration, or a high toxic one (Giacomotto and Ségalat, 2010). A conceivable approach would be to test compounds at several concentrations and log dilutions, established by existing data and the chemical properties of the compound. Physiologically effective concentrations can vary from 0.01 to 100 mM in the food media, although the commonly used concentrations are within the range of 1 to 10 mM (Pandey and Nichols, 2011; Giacomotto and Ségalat, 2010). The best approach is to start with high concentrations of the compounds in the medium, since this will ensure that a lot of the compounds will display a toxic effect on the flies and they could be further re-tested at a lower concentration (Giacomotto and Ségalat, 2010). Despite this, pilot studies have also been done using three different log dilutions of 1 mM, 10 mM and 100 mM in order to ensure the efficacy of a given assay and that drugs are being ingested.

4.2 The screening process

Once the drug has been delivered to the fly, the next step is to screen the flies in an attempt to find hits showing an activity or an affinity on a selected target or in a disease model (Spring, 2005). This usually involves an alternation of the pathogenic phenotype that could

be directly attributed to the administered drug. Traditional HTS involves massive parallel analysis of the effects of molecules from a large library of compounds based on *in vitro* cell culture, biochemical assays or receptor binding assays (Pandey and Nichols, 2011) (Figure 3). This approach has contributed to the discovery of therapeutically effective compounds, identification of cellular pathways and pathophysiological mechanisms. Its ultimate goal is to discover and explain relationships between chemical structures and biological activities (Giacomotto and Ségalat, 2010). Despite the varied documented successes, this approach has three major limitations. Firstly, it provides a biased approach dependent on the existence of detectable targets (Lindsay, 2003). Secondly, most disease mechanisms cannot be reproduced *in vitro* due to a difference in the complexity between cells and multicellular organisms (Giacomotto and Ségalat, 2010). Thirdly, most of the positive hits generated by the initial screening, fail to reproduce the results once tested in organisms such as rodents. The latter is due to a number of difficulties resulting from poor absorption, solubility, distribution, metabolism, excretion and toxicity characteristics, which result in a waste of funds and efforts and a dead-end for most hits (Bleicher et al., 2003). One such example is that of a recent screen of 184,880 novel compounds on Huntington's disease (HD) aggregates which led to the identification of positive hits, including a number of benzothiazoles that inhibited polyglutamine-mediated aggregation of toxic and misfolded proteins (Heiser et al., 2002). In a cell culture model of aggregation, all the primary hits were found to be toxic to cells, and in an animal model of HD, none of the compounds were therapeutically effective (Hockly et al., 2006). The advantages of integrating the fly in the primary screening process include; cost-effectiveness and high-quality positive hits to streamline the pool of candidates, before moving on to secondary screening on expensive mammalian-based models (Pandey and Nichols, 2011).

Drug discovery studies from models of disease can be done in two approaches: the disease model can be used in the chemical enhancer/suppressor drug screens or the model can be used to further understand the pathological processes of the disease and in turn, highlight potential therapeutic targets. Chemical enhancer/suppressor (or candidate drug delivery) can be unbiased and they consist in testing chemical libraries for their ability to reverse the disease phenotype (Newman et al., 2011). For this purpose, mutant strains of *Drosophila* which mimic human disease and have clear and easy readouts of phenotypic modulation, are an attractive package for medium to high-throughput drug screening (Giacomotto and Ségalat, 2010) (Figure 3). An example of such a screen is that reported by Zhang et al. (2005), in which primary screening of a 16,000 compound library that was done in a yeast model of HD, identified 4 compounds capable of preventing polyQ aggregation and toxicity. A cell-based assay and a *Drosophila* rough eye phenotype HD model, were then used to test the anti-aggregation properties of these 4 compounds. One of them, C2-8 was found to suppress the rough eye phenotype and polyQ inclusion formation, atrophy and motor impairment in a rodent model (Chopra et al., 2007). Several biased candidate drug studies have been undertaken in *Drosophila* models of neurodegeneration in which, compounds were used to interfere with protein misfolding (Fujikake et al., 2008), aggregation (Apostol et al., 2003), clearance (Sarkar et al., 2008) and neuronal dysfunction (Steffan and Thompson, 2003) (Table 1).

Drosophila models of neurodegeneration can be used in two ways to study the underlying pathological characteristics of a disease: unbiased genetic screens can be used to identify novel players, or genetic/pharmacological manipulation of proteins or pathways implicated in the disease can shed light on the downstream consequences (Newman et al., 2011). In traditional forward genetic screens, randomly mutagenized flies are screened for disturbances of a pre-defined phenotype or process. The mutagenized flies can be produced by a chemical mutagen (e.g. ethylnitrosourea) or mediated mutagenesis through transposon elements (e.g. P-element and piggyBac) (Lindsay, 2003; Lenz et al., 2013). Models of disease can be used in this genetic enhancer/suppressor screen by expressing the disease gene with a random mutant gene, which may or may not interact and modify the phenotype (Newman et al., 2011). One such screen involved using transgenic flies with the Aβ pathology expressed in the eye, crossed against 1,963 mutant fly stocks. This screen identified 23 modifiers of the rough eye phenotype including proteins in the secretory pathway, cholesterol homeostasis pathway, and proteins involved in chromatin structure and function (Cao et al., 2008). This unbiased screen identified some pathways and proteins previously implicated in the disease pathology, but more importantly, it helped shed light on some new participants of the disease process which can now be targeted for therapeutic interventions. Strategic expression or pharmacological manipulation of implicated proteins or pathways, can help with validating hypotheses about pathogenic mechanisms (Newman et al., 2011). The 'Tau microtubule' hypothesis is one of the proposed theories to explain the pathological Tau processes underlying AD. *In vitro* cell-based screens produced convincing evidence to support this theory, however, there was no such evidence *in vivo* (Cowan et al., 2010). All aspects of this hypothesis were tested in one *in vivo* experimental paradigm

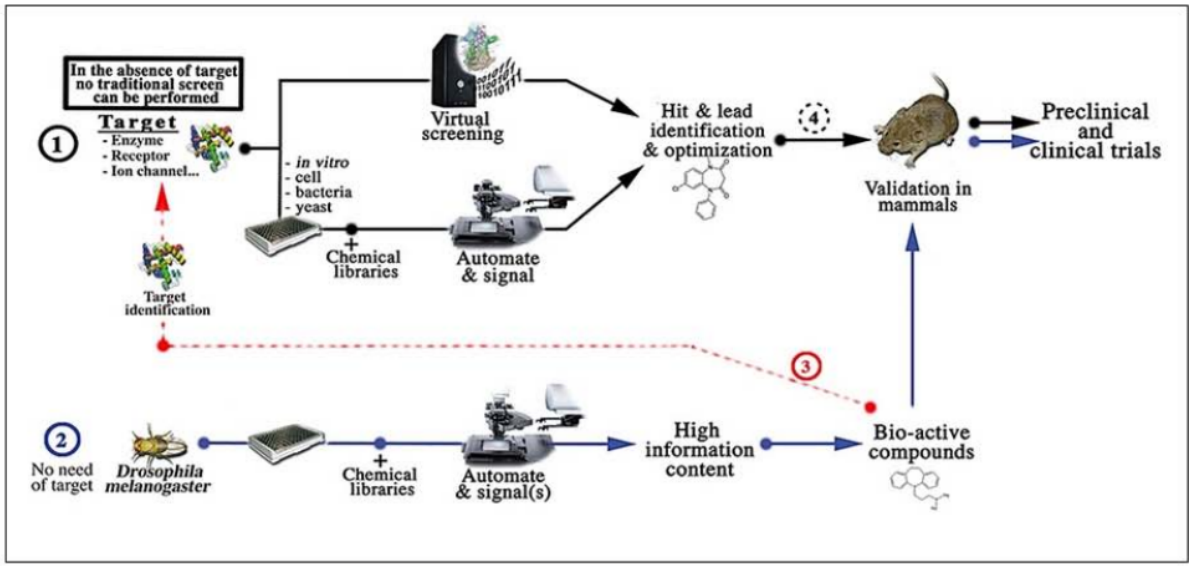

Figure 3: High-throughput screening using Animal Models for Drug Discovery.(1, black lines) A schematic view of the different stages which came upon a drug discovery process based on traditional HTS. In the absence of target or in complex mechanism the HTS can hardly be set up. (2, blue line) An alternative approach is utilising phenotypic chemical screens with small animal models like Drosophila. (3, red line) Identification of hits in these models may reveals new molecular mechanisms and targets. The target could be further used in traditional HTS. (4), Drosophila may also bridge the gap between traditional high-throughput screening and validation in mammalian models (adapted from Giacomotto and Ségalat (2010)).

Table 1: Overview of *Drosophila* Models of Neurodegeneration and their successes in Drug Discovery. **β-Amyloid**, Beta Amyloid; **HD**, Huntington's Disease; **HSP**, Heat shock protein; **REP**, Rough eye phenotype.

Disease	Genes Involved	Phenotypes	Drug Discovery/Target Discovery	Positive Hits/Targets involved	References
Alzheimer's Disease (AD)	B-Amyloid Protein	Eye degeneration, accumulation of amyloid plaques, vacuolation of brain, reduced lifespan and locomotor defects.	1963 mutant fly stock tested in Aβ42 *Drosophila* model with REP modifications. 23 modifiers were identified.	Proteins involved in: • Secretory pathway • Cholesterol homeostasis • Regulators of chromatin	(Cao et al. 2008)
	Tau	Eye degeneration, disruption of the microtublular network at presynaptic never terminals, axonal degeneration, neuromuscular junctions morphological defects	Mutant tau flies with a library of 1250 mutants and identified 30 lines involved in REP modification.	Proteins included: • 4 cytoskeletal • 3 molecular chaperones • Cysteine string protein • 2 members of the HSP40 co-chaperone family	(Blard et al. 2007)
Parkinson's Disease (PD)	α- Synculein	Age-dependent loss of dopaminergic neurons ad progressive climbing defects.	Mutant flies overexpressing α- Synculein were fed with: • Geldanamycin (enhancer of Hsp70 expression and modulator of stress response) • Ubiquitin • Cathepsin D • Nicotinamide • Polyphenols	• Geldanamycin, ubiquitin and cathepsins D suppresses α-Synculein toxicity. • Nicotinamide suppresses α-Syn toxicity through improvement of oxidative mitochondrial dysfunction.	(Auluck and Bonini 2002, Lee et al. 2009) (Cullen et al. 2009) (Jia, et al., 2008) (Long et al. 2009)
	Parkin and Pink	Dopaminergic neuron loss, age-dependent motor deficits, reduced lifespan, locomotor defects, male sterility and mitochondrial pathology	Parkin and Pink1-deficient flies were treated with: • Vitamin E • Rapamycin	• Vitamin E offered neuroprotection against degeneration. • Rapamycin suppressed parkin and pink1 related phenotypes.	(Wang, et al., 2006) (Tain, et al., 2009)
Huntington's Disease (HD)		Axonal transport defect, lethality, neurodegeneration, behaviour and electrophysiological defects	Library of 16,000 compounds (Chembridge, San Diego, CA) in primary screens using yeast, PC12 cells and mouse hippocampal slice. Secondary screen was performed on fly models of HD.	C2-8	(Meriin et al. 2002) (Apostol et al. 2003, Zhang et al. 2005)

using a *Drosophila* model of tauopathies (Mudher et al., 2004). *Drosophila* models of neurodegeneration play an important role in drug discovery design as they provide a vital step for the primary screening of drugs in whole organisms (for examples refer to Table 1).

4.3 Measurable endpoints

Another crucial step which has a profound impact upon the quality of the information produced from the screening process, is the output measurements or endpoints (Giacomotto and Ségalat, 2010) (Table 2). The requirement of HTS makes choosing endpoints a bal-

ance between the complexity of the measurable outcome (e.g. behavioural phenotype, changes in morphology or molecular composition), and the simplicity in detection and quantification (Rand, 2010). As automated animal screening is not always possible, identifying and quantifying endpoints usually depends on laborious observations and manual scoring (Evanko, 2006). *Drosophila* screens vary depending on the assay and the degree to which it can be mechanised (Pandey and Nichols, 2011). The higher throughput assay depends on the scoring of visible phenotypes, dead/alive, or a visible marker. One of the highest throughput quantitative strategies involves measuring the fluorescent markers in embryos by flow cytometry (Pulak, 2006). Viability or lethality in both larval and pupal stages has proven to be a very effective high-throughput endpoint in toxicology screening in *Drosophila* (Christie et al., 1985), together with measurement of fluorescent markers (e.g. green fluorescent protein [GFP]-tagged genes). Medium throughput endpoints which involve manual scoring techniques include observation of overt morphological development (Tögel et al., 2013), or indication of a rough eye phenotype (Pandey and Nichols, 2011). The latter techniques, despite being medium throughput endpoints are still able to screen thousands of drugs per week. Neurodegenerative disorders often result in locomotion or behavioural defects such as circadian activity, which produce quantifiable measures of neurodegeneration. Lower throughput behavioural assays, such as learning, memory and social interaction assays (including measuring aggression and courtship), require time to train the flies and produce a throughput of 25 to 50 drugs per week (Scott et al., 2002). Lower throughput assays are mostly used for validation of leads and target determination and include assays which require a more detailed analysis of the fly using biochemical techniques such as affinity chromatography coupled to mass spectrometry, microarray technologies, and genome-wide RNA interference (RNAi) screening or metaboprofiling studies (Sleno and Emili, 2008; Lindsay, 2003). The challenge that lies ahead with improving the screening process involves optimising the sensitivity of quantifiable endpoints for pathway-specific screening.

5 Considerations and Limitations

Whole-animal screening is a new and useful tool in the drug discovery processes. Despite this, animal models do come with their limitations. The profitability of an animal model of disease lies in its ability to manifest the relevant disease phenotype, together with the similarity of the underlying pathological changes, to human disease. Recent analyses point to the conservation of approximately 77 % of human disease genes in *Drosophila* (Reiter et al., 2001). Despite this, nearly all diseases are multifactorial and involve a variety of interactions from different genes and complexes, hence, the consequential ramifications is the failure of animal models to replicate the human disease gene network (Venter et al., 2001).

An additional limitation to keep in mind is the fact that *Drosophila* are surrounded by a thick cuticle that serves as a physical barrier to the penetration of molecules (Ségalat, 2007). Compounds penetrate through the animal's epidermis by both ingestion and diffusion (Kaletta and Hengartner, 2006). As a result, the concentration of a given compound within the animal is never accurately known and it varies, depending on the chemical properties of the compounds. Consequently, it is close to impossible to determine if a negative result is due to poor penetration, docking problems or a true absence of biological activity in the model; on the other hand, positive results can only be qualitatively interpreted (Ségalat, 2007). In an unbiased screen, the target is not known; since the treatment is delivered through the media, it is difficult to predict the range of doses which have to be tested. To avoid missing hits, a feasible approach may be to test compounds at several concentrations, starting with a high concentration and then retesting with lower ones (Giacomotto and Ségalat, 2010; Pandey and Nichols, 2011).

The anatomical and molecular differences between small model organisms and humans with respect to the metabolism of compounds, may result in the exclusion of a significant fraction of positive hits (Giacomotto and Ségalat, 2010). One such example is the anatomical differences between insects and vertebrates in relation to the blood brain barrier (BBB). The *Drosophila* humoral/central nervous system (CNS) is protected from the open circulatory system through a thin layer of glial-deprived epithelial cells (Stork et al., 2008). This makes the BBB in *Drosophila* much simpler than that of vertebrates, hence resulting in differences between humans and flies with regards to drug delivery to the brain tissues. The question, 'how well is the translation from fly to human?' still remains due to, pharmacokinetics and pharmacodynamics of small molecules as well as species-specific metabolic issues. Some drugs may be toxic in flies but not in human and vice versa. This can be due to a difference in metabolism of the drugs across species. In addition, the biochemical and physiological differences may lead to significant discrepancies in drug levels and tissue distribution profiles between mammal and fly (Pandey and Nichols, 2011). Despite this, the animal models are likely to reflect a comparative toxicity to mammals, which would substantiate future parallel studies in other invertebrate and vertebrate models (Rand, 2010).

Table 2: Throughput endpoints in *Drosophila melanogaster* models. (adapted from Pandey and Nichols (2011)).

High Throughput	Medium Throughput	Low Throughput	References
Lethality	Olfactory	Locomotor defect	(Pandey and Nichols, 2011)
Body Size	Overt morphological development	Body wall contraction	(Rand, 2010)
Necrotic Patches	Rough Eye Phenotype (Retinal degeneration)	Body wall muscle	(Pulak, 2006)
Viability	Negative Geotaxis Assay	Response to pain	(Christie et al., 1985)
Lethality	Circadian activity – sleep, arousal & rest behaviour	Phototaxis	(Tögel et al., 2013)
Visible & Fluorescent Markers	Body weight	Rotorod Test (Sleno and Emili, 2008)	
Flow cytometry	Fecundity	Electrophysiology	(Scott et al., 2002)
Visible phenotypes	Aggression	Prepulse inhibition	(Lindsay, 2003)
Lifespan Assay (dead/alive)	Wing expansion behaviour	Courtship behaviour	
Flight ability		Feeding behaviour	
Stress test		Learning and memory behaviour	
Anaesthesia Response		Seizure behaviour	
		Visual discrimination	
		Social interaction assay	
		Aggressive behaviour	
		Biochemical techniques (i.e. affinity chromatography, mass spectrometry, micro-arrays genome wide RNAi screening & metaboprofiling)	

When using *Drosophila* to model neurodegenerative diseases that are common in old age, several challenges can be encountered. Despite the fact that *Drosophila* is readily used to address questions about tissue-specific functional decline and genetic perturbations on ageing, the techniques used including lifespan and locomotor analysis can also be fraught with pitfalls if not carefully applied (He and Jasper, 2014). Some of the suggestions to keep in mind when modelling ageing in *Drosophila* include: careful control of genetic backgrounds due to the heterosis effect, which generally results in a longer lifespan in outcrossed animals; proper maintenance of source cultures with defined larval densities to avoid infection; synchronised populations with controlled mating status in order to have same-aged flies; gender differences which account for asymmetric inheritance of mitochondrial genomes, hormonal and metabolic differences, and maternal effects (Tower and Arbeitman, 2009); diet consisting of a standardized amount of yeast and hydration in food (Ja et al., 2009); control of temperature, humidity and circadian light exposure, which can all have an effect on the ageing process in the flies (He and Jasper, 2014).

6 Conclusion

Animal models are increasingly being incorporated into drug discovery screens. Due to their small size, they provide a more efficient alternative to *in vitro* screens as they fulfil the requirements of large-scale screens, whilst providing a system in which the physiological context is preserved. Despite this, *Drosophila* models have some limitations when it comes to HTS and therefore, they are only fully exploited when they are integrated in a mixed approach for drug discovery. For example, a combination of unbiased enhancer/suppressor screens (used to identify the novel targets), together with subsequent validation using candidate genetic or pharmacological tools, has been previously used in AD *Drosophila* models (Cao et al., 2008). Another example is the use of pharmacological agents in order to identify suspected cellular processes affected by the administered compounds, followed by genetic validation of the candidate players (Mudher et al., 2004; Pallos et al., 2008). In conclusion, *Drosophila* models of neurodegeneration hold a lot of promise as they provide not only a way of modelling the disease mechanism at a subcellular level, but they also allow for the study of pathological mechanisms through behavioural consequences. This information can be easily used for the design of disease-modifying therapeutic drug discovery that can be potentially applied to treat human conditions. Furthermore, the integration of *Drosophila* models in the drug discovery process allows for the selection of potential therapeutic models with an improved safety profile earlier in the drug discovery process, resulting in less funds and time being wasted on false positive hits. All in all, the future should see the incorporation of *Drosophila* in drug discovery strategies increasing drastically, especially within the areas of neurodegenerative diseases.

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