A fruitfly's guide to keeping the brain wired

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The behaviour of all animals is governed by the connectivity of neural circuits in the brain. Neurodevelopmental and neurodegenerative diseases, as well as traumatic injuries to the nervous system, can alter or disrupt the normal connectivity of the brain and result in disability. In this review, we highlight the contributions of the genetic model organism *Drosophila melanogaster* to our understanding of neural connectivity in health and disease. In this context we also discuss the research areas in which we believe the fruitfly is likely to be a useful model system in the future.

Keywords: axon injury; *Drosophila melanogaster*; neural circuit; neurodegeneration; neurodevelopment

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Introduction

The central nervous system (CNS) of higher animals comprises between approximately 200,000 (in the fruitfly *Drosophila melanogaster*) and approximately 100 billion (in humans) neuronal cells (Zars *et al*, 2000). Rather than being an amorphous collection of individual cells, neurons are functionally interconnected by their neurites—that is, their axons and dendrites. These neuronal networks form the basis of the correct functioning of the brain. Their sensory modalities receive inputs from the environment, central components process this information and a behavioural response is generated by output motor neurons (Fig 1A). Producing an appropriate response therefore depends crucially on the correct formation and maintenance of interneuronal connections.

During brain development, the correct formation of connections is assured by the tight regulation of neurite outgrowth. Axons are induced from neuronal cell bodies, grow along specified paths and form synapses when they reach their correct target neurons (da Silva & Dotti, 2002; Dickson, 2002). Once connectivity is established, it needs to be maintained throughout life to ensure the correct function of the organism. Death of neurons in a circuit or disruption of their connections leads to functional impairment, and can result in various

neurological disorders. In this review, we briefly summarize why and how the genetic model organism *D. melanogaster* has contributed to unprecedented insights into the formation and function of normal neuronal circuits in the brain (Fig 1). We then highlight three types of neurological disorder in which defects in neural circuit connectivity or circuit malfunction seem to contribute to the neuropathology; for each type, we give one example to illustrate how *D. melanogaster* has been used to study the pathophysiology. Finally, we identify opportunities for further study.

D. melanogaster: a genetic model organism...

There are several reasons why *D. melanogaster* is widely used in biological studies (Bier, 2005). First, *D. melanogaster* has the practical advantages of being small, highly fertile and having a short generation time. These characteristics allow large-scale experiments, such as genetic screens, to be performed in a relatively short period of time and at low cost. Second, sophisticated genetic-transformation techniques provide a flexible tool for genetic studies of the molecular pathways underlying a biological process of interest (Venken & Bellen, 2005). Third, the *D. melanogaster* genome has been fully sequenced and annotated, and is publicly available (www.flybase.org). From these data, remarkable genetic conservation has emerged with species as distant as humans. This is of particular interest for the study of human disease-related genes.

...with a brain

Apart from the genetic similarities between the fruitfly and humans, fundamental similarities in their brains are also apparent. Although morphologically distinct and of lower complexity, the *D. melanogaster* CNS comprises the same basic building blocks as the mammalian CNS. Neurons and glial cells form the main constituents, and many of their characteristics, such as the neurotransmitter systems, are also conserved. These properties make the fruitfly a powerful tool in neurobiological research, and *D. melanogaster* has made a significant contribution to the understanding of the genetic and molecular mechanisms of neural development (Thor, 1995). Particularly important examples include the discovery of neural precursor determination and specification (Hassan & Bellen, 2000), as well as the identification of genes that regulate axon guidance (Dickson, 2002). Apart from providing fundamental insights into the morphological development of nervous systems, the fruitfly is also increasingly being used to study the neural basis of behaviour. Taking advantage of the powerful genetic tools offered by the fruitfly, the organization of the neural circuitry

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that underlies olfactory perception (Jefferis & Hummel, 2006), sexual behaviour (Manoli *et al*, 2006) and circadian rhythmicity (Hardin, 2005) are also being unravelled. These and other neural circuits can now act as a basis for the future study of the molecular pathology of neurodevelopmental and neurodegenerative diseases.

Malformed connections: neurodevelopmental diseases

In addition to providing fundamental insights into how neural circuits form and function, *D. melanogaster* can be used to model genetic neurological disorders in which neural circuits are malformed (Bier, 2005). In view of the high level of similarity between the human and *D. melanogaster* genomes, fruitflies that are mutant for the homologue of a disease-related protein can provide insights into the function and mode of action of this particular protein in brain connectivity.

One relatively recent and well-studied example is the *D. melanogaster* homologue of the fragile X mental retardation 1 gene, *dfmr1*. Fragile X syndrome is the most common cause of inherited mental retardation in humans and results from the lack of expression of the *Fmr1* gene (Turner *et al*, 1996). The most typical anatomical defect found in patients and mouse models of the disease so far are dendritic spine defects (Nimchinsky *et al*, 2001). Analysis of fruitflies that are mutant for the *dfmr1* gene has shown it to be a negative regulator of axon extension and synapse differentiation. Behavioural defects in the circadian rhythm and learning ability of the fruitfly are associated with morphological malformations of the underlying CNS neurons (Zarnescu *et al*, 2005). As well as helping to explain the causes of behavioural phenotypes, studies of specific neural circuits allow the underlying molecular mechanisms to be unravelled. Using genetic-interaction studies in combination with biochemical analysis, it has become clear that *dfmr1* is a translational regulator of several proteins involved in the organization of the cytoskeleton, which probably explains the neuromorphological defects observed in the mutant fruitflies (Schenck *et al*, 2003; Reeve *et al*, 2005). Finally, therapeutic approaches have also been tested in the fruitfly model of fragile X disease: when *dfmr1* mutant fruitflies are treated with metabotropic glutamate receptor antagonists or lithium, some of the morphological and behavioural phenotypes revert to those of wild-type fruitflies (McBride *et al*, 2005).

Whether these findings can be extended to the human disease remains to be seen. The fruitfly neuromorphological phenotypes seem to be more severe than those detected in mice and humans. This might be due to the presence of two other FMR-related genes in mammals or to the fact that the higher level of resolution in the fruitfly CNS allows the detection of subtle phenotypes. In any case, it is clear that fruitfly models provide an excellent platform on which to form and test novel hypotheses about the molecular pathogenesis of neurodevelopmental diseases.

Degenerating connections: neurodegenerative diseases

In addition to neurodevelopmental disorders, neurodegenerative disease models have been developed in *D. melanogaster* (Bilen & Bonini, 2005). Most of these studies use the ectopic expression of disease-related mutant human proteins in the fruitfly CNS. Another approach to understanding the pathogenesis of the disease is to study the normal physiological function of the fruitfly homologues of the proteins that are involved.

One example of a neurodegenerative disease that has been investigated using both of these approaches is Alzheimer's disease

Fig 1 | Three classes of connectivity problems in a neural circuit. (A) Simple neural circuit: the input (sensory) neuron receives a signal and activates the output (motor) neuron through a chain of activating interneurons (green and blue). (B) Malformed circuit: the first neuron connects to an inhibitory neuron (red), which reduces the output of the circuit. (C) Degenerated circuit: the axons of the first neuron have degenerated and fail to activate interneurons and the output neuron. (D) Severed circuit: the direct axonal connection between the first neuron and the output neuron is disrupted.

(AD). Clinically, AD is characterized by a progressive decline of higher cognitive function that correlates with a reduction in synaptic communication in neuronal circuits (Selkoe, 2002). In addition, extracellular amyloid plaques that consist mainly of the amyloid β peptide (A β)—a cleavage fragment of the large amyloid precursor protein (APP)—are a typical anatomopathological feature of the disease. Rare inherited forms of AD are linked to aberrant cleavage or increased expression of APP (Selkoe, 2002; Rovelet-Lecrux *et al*, 2006). The overproduction of A β is believed to initiate an amyloid cascade that leads to the degeneration of axonal tracts and neuronal death. Finally, neurofibrillary tangles consisting of intracellular accumulations of hyperphosphorylated tau protein are found in affected neurons. Which of the pathological lesions in the brains of patients are the cause of the clinical symptoms of the disease remains a matter of intense debate and controversy (Blennow *et al*, 2006).

To model the extracellular anatomopathological features of AD in the fruitfly, aggregation-prone forms of the human A β peptide, or its precursor APP in combination with the cleaving enzymes, have been ectopically expressed. These genetic manipulations cause progressive loss of learning ability and apoptotic cell death (Greeve *et al*, 2004; Iijima *et al*, 2004). In addition, typical amyloid-like accumulations were found in the brains of these fruitflies.

Intracellularly, tau-associated neurotoxicity has also been mimicked in the fruitfly. The expression of human tau protein in the fruitfly brain caused neurodegeneration, but no neurofibrillary tangles were found (Wittman *et al*, 2001). This prompted the idea that the cause of death of the degenerated neurons was probably not the

aggregated form of the protein (tangles), as was previously believed, but rather the pre-aggregate oligomeric species of tau protein are the cause of cellular toxicity. Further insights into the molecular mechanisms of toxicity also came from directed genetic-interaction studies (Jackson *et al*, 2002), as well as from unbiased interaction screens—mainly kinases and phosphatases—identified in these studies—should prove useful for manipulating the pathogenic cascades.

In addition to modelling the late pathological features of AD, research in fruitflies has led to the discovery of a new physiological function of APP that might have an important role early in the disease. Continuing from biochemical studies that showed binding between APP and axonal motor proteins (Kamal *et al*, 2000), the fruitfly APP homologue, APP-like (APPL), was identified as an important factor in anterograde axonal transport, which is essential for the health of axonal connections (Gunawardena & Goldstein, 2001). These findings were extended to mammals and mouse models of AD, as well as human AD brains, were found to exhibit defects in axonal transport (Stokin *et al*, 2005). Defects in axonal transport have therefore emerged as a putative early event in AD, which lead to better known pathological features, such as plaques and tangles, at later stages. However, it is important to state that there is still controversy about the putative causative roles of the different pathological features, such as amyloid plaques, hyperphosphorylated tau and axonal transport blockage, in the pathogenesis of the disease.

The case of APP exemplifies how fundamental insights into protein function from the fruitfly can lead to novel hypotheses about the pathophysiology of human diseases. As is clear from the example of AD, as well as many other neurodegenerative diseases (Bilen & Bonini, 2005), the fruitfly CNS can act as a model for human brain diseases that are caused by aberrant proteins. In addition, genetic-interaction studies are a powerful tool for identifying underlying molecular components.

It is surprising that the level of resolution routinely used in the analysis of neurodevelopmental diseases is, with a few exceptions, not exploited in the study of neurodegeneration. Most of the focus has been on the recreation of the overt pathological features of the human disease, as well as cell death. It is also clear that early events, such as electrophysiological dysfunction, subtle disruptions in axonal connectivity or the blockade of axonal transport, are of fundamental importance in the correct functioning of neuronal circuits. These early dysfunctions might be more informative about the principles of disease pathogenesis than a common outcome of all degeneration, such as cell death. The tools are now available to express disease-causing peptides and proteins in well-characterized neural circuits of the fruitfly brain, and to perform detailed behavioural, morphological and physiological analysis of these animals. It will therefore be of interest to perform this type of study on existing disease models, to reveal new insights into the early mechanisms of human brain pathogenesis.

Severed connections: traumatic injury

Neural connectivity can be disrupted not only by molecular injuries but also by acute mechanical forces. This type of traumatic injury to the brain and spinal cord often leads to disabilities, such as paralysis and sensory deficits in humans. The failure of CNS axonal tracts to regenerate prevents the efficient repair of broken neural circuits that govern behaviour, which leads to the long-term functional defects associated with these conditions.

The morphological responses of neurons after injury to the spinal cord were first described by Ramon y Cajal in 1890. During the past 20 years, almost all insight into the molecular mechanisms of CNS axonal injury has come from vertebrate models. On the distal side of the injury, the axonal fragments lose connection to the cell body and are removed in a process called Wallerian degeneration, which is characterized by the cleavage of the axon into small vesicles that are cleared by glial cells (Luo & O'Leary, 2005). On the proximal side, severed axons are maintained and form a dystrophic end bulb that, although alive and dynamic, fails to grow beyond the lesion site (Silver & Miller, 2004); a large body of research has focused on unravelling the molecular mechanisms behind this failed regrowth (Schwab *et al*, 2006). The most well-known inhibiting factors in the CNS environment are the myelin-associated proteins Nogo, myelin-associated glycoprotein (MAG) and oligodendrocyte-myelin glycoprotein (OMgp). Other inhibitory extracellular factors are the chondroitin sulphate proteoglycans and several axon-guidance molecules. Insights into the intracellular mechanisms that prevent injured axons from growing have also been obtained. Two important factors in this process are RhoA and protein kinase A. The challenge for the future is to expand this list and, importantly, to integrate these factors into a functional framework. As for the case of neurodegenerative disease pathophysiology, this process could be facilitated by the use of simple genetic models of CNS axonal injury.

Whether the success of *D. melanogaster* in solving questions related to neural development and degeneration will apply to the field of acute CNS injury depends on how accurately the fruitfly can model this condition. In this respect, it is important to be aware of some of the obvious differences between a fruitfly and a human. For the large vertebrates, extensive myelination of axon sheaths is required for efficient saltatory transmission of action potentials over long distances. The short *D. melanogaster* axons are tightly enveloped by glial cells (Bhat, 2003), but the fruitfly does not synthesize myelin in its CNS and lacks the myelin-associated inhibitors of axonal outgrowth. However, this does not mean that new insights into axonal regeneration cannot be gained from fruitflies. Several recent studies have focused on the molecular mechanisms that underlie the degeneration of severed axons and the pruning of unnecessary axons in the fruitfly brain (Luo & O'Leary, 2005). It is now clear that severed invertebrate axonal fragments respond morphologically in a similar manner to their mammalian counterparts, and that, perhaps more importantly, some of the molecular mechanisms that govern this process are conserved. In one spontaneous mouse mutant—Wallerian degeneration slow (WldS)—the process of Wallerian degeneration of severed axons is significantly delayed. The WldS protein is a fusion of nicotinamide mononucleotide adenylyltransferase-1 (Nmnat1) and the 70 amino acids at the amino terminus of the ubiquitylation assembly factor Ube4b. Ectopic expression of this artificial fusion protein in the fruitfly significantly delays axon degeneration after severing (MacDonald *et al*, 2006; Hoopfer *et al*, 2006). It seems that WldS in *D. melanogaster* suppresses a signal on the severed axon that induces glial cells to clear them. An important factor in this process is the glial cell-surface receptor Draper. Whether, in addition to WldS, Draper also has a role in mammals awaits confirmation. Further work on this model will allow a more complete understanding of the underlying mechanisms and might lead to new approaches to slow down or even stop this type of degeneration.

What is happening on the side of the cell body? So far, no high-resolution model for axonal injury and regeneration in the fruitfly CNS has been described. An initial attempt to address the genetics of axonal regeneration in *D. melanogaster* relied on the induction of relatively large traumatic injuries in the fruitfly brain (Leyssen *et al.*, 2005). In this study, increased APPL expression in response to brain injury was important for their survival, although it is not yet clear why injured neurons need to upregulate APPL. Remarkably, however, when APPL or human APP is expressed specifically in postmitotic adult brain neurons, it can induce *de novo* axonal outgrowth. This indicates that APPL might be required for some level of axonal regeneration of the injured neuron or for the reorganization of surrounding axons after injury. Interestingly, the upregulation of APPL/APP in response to traumatic injury is not restricted to the fruitfly; APP expression also increases after brain trauma in mammals (Van den Heuvel *et al.*, 1999). Furthermore, epidemiological and anatomopathological studies suggest a link between brain injury and AD (Borenstein *et al.*, 2006). As brain lesions also affect axonal transport, this might be an additional factor in the pathophysiology of AD. Another study showed that after ablation of olfactory receptor neurons on one side of the brain, the terminal density of the contralateral counterparts increased, possibly in an attempt to regenerate functional performance (Berdnik *et al.*, 2006).

Although these initial studies on the response of *D. melanogaster* neurons to injury indicate that many molecular mechanisms are conserved, they also highlight the difficulties in inducing consistent axon-tract injury in the fruitfly CNS. The current models rely on either the removal of peripheral cell bodies of neurons with axonal projection into the brain (Berdnik *et al.*, 2006; MacDonald *et al.*, 2006; Hoopfer *et al.*, 2006) or the induction of large brain lesions (Leyssen *et al.*, 2005). The main obstacle to developing a more precise axon-severing model is the combination of the small size of the *D. melanogaster* brain and the cuticle that encapsulates it, which makes the brain inaccessible. In addition, the fact that the injury cannot be examined until the brain is dissected means that it is impossible to ascertain the reproducibility of the injury, let alone to follow the evolution of regenerating axons over time.

Similar problems in mammalian models for spinal cord injury have recently been overcome by the expression of the green fluorescent protein (GFP) in a small number of axons in the spinal cord of mice. The morphological consequences of injury can be directly observed and the axonal response can be repetitively imaged in a living animal (Kerschensteiner *et al.*, 2005). The challenge for the future is to combine the resolution and levels of insight obtained from this type of injury model with the fast and flexible genetics of the fruitfly. Many axonal subpopulations in the fruitfly CNS have been studied to single-axon resolution, and could be marked with GFP and genetically manipulated. In addition, reliable access to the living brain of fruitflies can be gained, for example, through studies in which the activity of neuronal circuits is directly observed (Wang *et al.*, 2003). It is therefore only a matter of imagination, time and effort before specific lesions can be made in the fruitfly brain, and their response to this manipulation can be visualized over time. Once this crucial barrier has been overcome, the fruitfly brain might prove to be as powerful for investigating the molecular mechanisms of axonal regeneration as it has been in unravelling neurodegenerative and neurodevelopmental diseases.

Concluding remarks

Ever since Thomas Hunt Morgan isolated the *white* mutation in *D. melanogaster* in 1910, the tiny fruitfly has made large contributions to the understanding of the genetic and molecular mechanisms of heredity and development. More recently, the remarkable power of fruitfly genetics has been applied to study the basic mechanisms of human diseases, including those debilitating pathologies that affect the human brain. Interestingly, two parallel developments in neurobiology are occurring. First, there is an increased appreciation of the concept of the neuronal circuit as a basic unit of behavioural control; the discovery of neuronal circuits regulating specific behaviours is a rapidly expanding field in fruitfly research. Second, the fruitfly is becoming an established model system for the study of the neuropathology of human disease. These two exciting fields will probably overlap in the near future, and the sophisticated and high-resolution techniques used in neural circuit research might be applied to the problem of nervous system disease. Many of these disorders might result from disruptions in the anatomical and/or functional connectivity of neuronal circuits. It will be interesting to see whether molecular similarities between causative and/or protective factors will be found in these different diseases. Modelling axonal regeneration after acute injury is likely to be the next, but by no means final, frontier. The fruitfly is therefore emerging as a powerful model for how the brain makes, unmakes and possibly even remakes neuronal circuits.

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