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Invited Review

Drosophila models for studying iron-related neurodegenerative diseases

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Abstract: In recent years, iron has been regarded as a common pathological feature of many neurodegenerative diseases, including Alzheimer's disease (AD), Parkinson's disease (PD) and Friedreich's ataxia (FRDA). A number of genes involved in iron transport, storage and regulation have been found associated with initiation and progression of neurodegeneration. However, whether iron abnormalities represent a primary or secondary event still remains unknown. Due to the limitation in transgenic rodent model construction and transfection systems, the progress in unraveling the pathogenic role of different iron-related proteins in neurodegenerative diseases has been slow. *Drosophila melanogaster*, a simple organism which has a shorter lifespan and smaller genome with many conserved genes, and captures many features of human nervous system and neurodegeneration, may help speed up the progress. The characteristics that spatial- and temporal-specific transgenic *Drosophila* can be easily constructed and raised in large quantity with phenotype easily determined turn *Drosophila* into an excellent *in vivo* genetic system for screening iron-related modifiers in different neurodegenerative conditions and hence provide a better picture about the pathogenic contribution of different iron-related protein abnormalities. It is believed that identification of important iron-related genes that can largely stop or even reverse degenerative process in *Drosophila* models may lead to development of novel therapeutic strategies against neurodegenerative diseases.

Key words: *Drosophila*; iron; neurodegenerative diseases

果蝇模型在研究铁代谢相关神经退行性疾病发病机制中的应用

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摘 要：铁代谢紊乱一直被视为是许多神经退行性疾病共同的病理特征，如阿尔茨海默氏症(Alzheimer's disease, AD)、帕金森氏病(Parkinson's disease, PD)以及弗里德赖希共济失调(Friedreich's ataxia, FRDA)等均与脑铁代谢紊乱密切相关。随着分子生物学的进展，迄今为止也已经发现许多参与铁运输、储存和调控的基因与神经退行性病变的发生和发展有关，然而铁代谢紊乱在疾病发病过程中的致病机制仍不十分清楚。近年来许多研究者利用各种转基因动物模型来研究铁代谢相关神经退行性疾病的发病机制，但是啮齿类动物模型由于模型构建系统周期较长且比较复杂，从而限制了铁相关蛋白在神经退行性疾病中作用机制的研究进展。果蝇具有生活周期短暂、染色体数目少以及表型易于观察等优点，同时果蝇与人在很多基因和通路上都高度保守，且神经系统也可表现出与人相似的复杂的功能，因此被广泛地应用在铁代谢相关神经退行性疾病发病机制的研究中。果蝇还以其独特的分子遗传学优势，更容易构建缺失、插入、敲除或转基因模型，可在不同神经退行性病理情况下进行遗传学筛选铁相关的调控基因，从而为解决铁代谢紊乱在疾病发病过程中的致病机制提供更多的线索。因此在果蝇模型中发现可以中止甚至是逆转神经元退化进程的铁相关基因，以期为神经退行性疾病的研究和治疗提供策略。

关键词：果蝇；铁；神经退行性疾病
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1 Introduction

Neurodegenerative diseases are a subgroup of human diseases with clinical conditions involving the progressive loss of certain neuronal populations, eventually leading to cognitive and behavioral defects of the patients [1]. Iron is the cofactor of many proteins and its homeostasis is essential for maintaining the normal function of neurons. Recently, there is increasing evidence showing that a number of neurodegenerative diseases are linked to high level of iron in the brain, and progressively increased iron level in the brain contributes to neurodegenerative processes, possibly through generation of free radicals via Fenton reaction [2,3]. Iron-related neurodegenerative disorders can be due to abnormal iron transport, storage and regulation in specific brain regions [4]. Two prominent examples of iron-related neurodegenerative disorders are Friedreich's ataxia (FRDA) and neurodegeneration with brain iron accumulation (NBIA).

The studies contributing to the identification of the pathophysiology of iron-related neurodegenerative diseases used samples taken from patients. However, human brain samples are limited in amount and availability for experimental manipulation, such as those for elucidating signaling pathways and cellular processes that underlie the neurodegenerative process [5]. Animal models are powerful tools to elucidate neurodegeneration at genetic and molecular level and hence disease mechanisms. Despite the advantages of using transgenic mouse models, genetic manipulation in mice is costly and time-consuming. Alternative animal models that allow easy genetic manipulation are needed [6]. Nowadays, simple systems such as yeast, *Caenorhabditis elegans* and *Drosophila melanogaster*, which possess many genes that are highly conserved with humans, have been established to study the molecular mechanisms of human neurodegenerative diseases [7]. Among these models, *Drosophila* is most well-known for its complex nervous system and behaviors that are generated by conserved mechanisms [8]. *Drosophila* genome contains many conserved genes, including those that participate in iron metabolism [9]. Previous studies also demonstrated that flies can mimic iron accumulation during aging, similar to human [10]. All these prove that *Drosophila* is an excellent model for studying iron-related neurodegenerative diseases.

The aim of this review is to introduce the feasibility of and summarize the advances in using *Drosophila* models for investigating iron-related neurodegenerative disorders. Understanding the early molecular pathophysiology of these diseases should help unravel the role of iron and shed light on the design of new therapeutic approaches.

2 Advantages of using *Drosophila* in neurodegenerative disease studies

A fundamental aim in the study of neurodegenerative diseases is to elucidate the underlying pathogenic pathways, and in turn to develop treatment strategies to stop or at least slow down the neurodegeneration process [11]. *Drosophila* serves as a powerful model system to study neurodegeneration due to the fact that many genes and core pathways are conserved in *Drosophila*, such as iron metabolism [12]. Comparative analysis of whole genome sequencing also revealed significant similarities in the structural composition between individual genes of human and *Drosophila* [7]. Same types of neurotransmitter system like GABA, glutamate, dopamine, serotonin and acetylcholine are found in *Drosophila*. Studies have also shown that *Drosophila* is able to accomplish complex behaviors, making it a good model for studying basic neuronal functions, such as memory formation [12].

Besides the aforementioned features of *Drosophila* that allow human disease modeling, there are many advantages of using *Drosophila* to model neurodegenerative diseases. First, manipulation of gene expression in *Drosophila*, usually achieved by the yeast transcription Activator Protein-Upstream Activating Sequences (Gal4-UAS) system, is easy and extremely flexible [13]. Gal4 can bind onto UAS and activate expression of gene linked to UAS. Gal4, cloned after different specific promoter or enhancer, allows promoter- or enhancer-specific expression of Gal4 and hence time- and tissue-specific manipulation of any gene of interest in *Drosophila* [13]. Thousands of publicly available fly lines make it possible to modulate the expression level of any protein, in specific tissues or cell types, and even in specific neurons. It is noteworthy that the compound eye-specific Gal4 driver is predominantly used because it allows the generation of neurodegenerative rough eye phenotype that can be easily scored without affecting the survival of the fly [14]. Transgenic expression of disease-causing genes and silencing or mutation of *Drosophila* endogenous genes homologous to human disease-causing genes are very useful in studying pathogenic mechanisms and interaction between genes

implicated in the disease. In addition to the genetic approaches, *Drosophila* disease models can also be constructed via pharmacological approaches and be used to test the potential therapeutic effect of candidate compounds. Fly food, supplemented with defined concentration of drugs, can be easily prepared in the laboratory. However, the greatest advantage of using *Drosophila* to model neurodegenerative diseases is the ease of growing flies in large number and their relatively short lifespan. All these facilitate genome-wide and unbiased genetic screenings for identifying enhancers and suppressors that are able to modify a phenotype caused by and to discover new disease-related genetic factors, with the factor of aging taken into account, which might be difficult to be achieved in rodent models (Table 1).

A shortcoming of using *Drosophila* is the risk that a critical factor in the disease pathology is only specific to mammals, which therefore cannot be studied in the *Drosophila* model. Despite this limitation, many human disease-causing proteins that are elusively found in human are found to be successfully expressed in *Drosophila* and exhibit their toxicity similar to what is found in human. These include proteins with polyglutamine expansions and mutant proteins found in Parkinson's disease (PD) and Alzheimer's disease (AD) patients [8]. Hence, *Drosophila* has remained one of the most commonly used neurodegenerative disorder model systems.

3 Iron metabolism in *Drosophila*

Iron is an indispensable micronutrient for the development of *Drosophila* [15, 16]. Enzymes that bind iron, heme or iron-sulfur clusters are important in numerous physiological functions, including respiration and the synthesis of DNA and neurotransmitters such as dopamine [17–20]. Early studies have identified many conserved genes that are involved in iron uptake, transport, storage and regulation in *Drosophila*. The first iron-related genes identified in *Drosophila* include those encoding the subunits of ferritin for iron storage, transferrin (Tsf1) for iron transport and two iron regulatory protein-1 (IRP1) homologs [21–24]. Studies on the phenotypic alteration caused by the genetic mutation of iron uptake protein divalent metal transporter-1 (DMT1) had led to the characterization of the DMT1 homolog Malvolio (Mvl) and melanotransferrin homolog Tsf2 which are involved in iron transport in flies [25, 26]. *Drosophila* genome sequencing has also led to discovery of a mitochondrial form of ferritin known as mitoferritin and multi-copper oxidases (MCOs) [27–29].

DMT1 is currently the only known transporter for cellular uptake of non-heme iron [30]. Mvl is the *Drosophila* DMT1 homolog and Mvl mutant flies have iron depletion and loss of wild-type sugar-preference trait while these phenotypes can be rescued by exposure to excess dietary iron [25, 31, 32]. Mvl mutation has also be found to reverse iron accumulation in the fly intestine caused by silencing of ferritin [33]. Hence, there is sufficient evidence supporting that Mvl is an iron uptake protein in *Drosophila*.

Ferritin is present in four intracellular compartments: cytosol, nucleus, mitochondria and vacuoles. In vertebrates, cytosolic ferritin is the principal site of iron storage while ferritin in *Drosophila* is largely secretory and responsible for not just iron storage but also iron absorption [16, 33–35]. There are two subunits of ferritin identified in *Drosophila*, ferritin-1 heavy chain homolog (Fer1HCH) and ferritin-2 light chain homolog (Fer2LCH). Fer1HCH possesses ferroxidase activity required for iron loading and Fer2LCH provides iron nucleation sites required for the mineralization of the ferrihydrite iron core and iron storage [16, 22, 23, 26]. Radioactive tracing showed that most ingested iron is stored in *Drosophila* ferritin [27]. Using single insertion mutants to disrupt either Fer1HCH or Fer2LCH gene product can reduce total ferritin level in whole flies and lead to embryonic or early larval death [16]. Mid-gut-specific silencing of ferritin resulted in iron accumulation in the intestine and systemic iron deficiency [33]. These studies showed that ferritin is an important iron storage and mobilization protein in *Drosophila*.

Despite the fact that many key iron-related genes are

Table 1. Advantages of using *Drosophila* in neurodegenerative disease studies

Relevance	Genetic manipulation	Favorable experimental characteristics
Conserved genes and core pathways	Time- and tissue-specific manipulation of any gene of interest	Short lifespan
Complex nervous system and behaviors	Thousands of publicly available fly lines	Easy to expand into a large quantity
	Easy to construct transgenic flies	Easy for screening by phenotypes

conserved in *Drosophila*, significant difference remains. For example, the different functions of iron-related protein homologs and the absence of master iron regulator hepcidin homolog in *Drosophila*.

4 Progress made by *Drosophila* models of iron-related neurodegenerative diseases

4.1 FRDA *Drosophila* models

FRDA is the most common autosomal recessive ataxia in the Caucasian population that occurs in 1/50 000 and typically begins before 25 years of age [36]. Symptoms range from gait disturbance and other locomotion disabilities to speech problems, heart disease and diabetes [37]. The neurological symptoms are mainly caused by the degeneration of sensory neurons in the dorsal root ganglia (DRG) and spino-cerebral tracts, and lesions in the dentate nucleus of the cerebellum [38]. Iron misdistribution and accumulation have been identified in FRDA patients. To be specific, FRDA results in significantly higher level of mitochondrial iron but much lower level of cytosolic iron [39]. Such iron misdistribution is believed to be caused by the deficiency of frataxin in the mitochondria and cells, which may be due to the presence of homozygous guanine-adenine-adenine (GAA) tri-nucleotide repeat expansion within the first intron of the frataxin gene. At present, the function of frataxin remains unclear but implicated in iron-sulfur cluster assembly, iron chaperoning and storage [40].

The *Drosophila* frataxin homolog (dfh) was discovered in 2000 and found to have similar functions as in humans [41]. This led to the development of first *Drosophila* model of FRDA by using RNA interference (RNAi) gene silencing technology to knock down dfh expression. It is found that suppression of the dfh expression in *Drosophila* recapitulates several features of the disease, including higher susceptibility to iron-induced toxicity, lower activity of mitochondrial [4Fe-4S]-containing aconitase and respiratory complexes. Only a small percentage of larvae with dfh-silencing are able to form viable adults at a more permissive temperature (18 °C), which limits the utilization of this model [42]. In this regard, the researchers tried to specifically silence dfh in the peripheral nervous system in *Drosophila* by using PNS-Gal4 line. This method significantly improved the availability of adult flies for experiments while relevant FRDA-like phenotypes are still constantly observed. Overexpression of H_2O_2 scavenging enzymes has been found to restore the lifespan and aconitase enzymatic activity in dfh-silenced flies, suggesting a role of oxidative stress in pathology of FRDA [43]. Meanwhile, another laboratory also generated a ubiquitous moderate dfh reduction *Drosophila* line, which had a weaker silencing of dfh (30%–40%) and was compatible with normal embryonic development but resulted in shortened lifespan and motor defects. These dfh-deficient flies were found to have a hypersensitive response to hyperoxia, which further supports a causative role of oxidative stress in FRDA [44].

In short, the above FRDA *Drosophila* models supported the hypothesis that frataxin plays a physiological role in protection against oxidative stress. However, further studies are needed, possibly by screening for genetic modifiers that might reverse GAA repeat-induced frataxin transcriptional reduction, iron misdistribution and elevated oxidative stress, to unravel the unknown in frataxin functioning and the pathogenic mechanisms of FRDA upstream and downstream of frataxin deficiency.

4.2 Pantothenate kinase-associated neurodegeneration (PKAN) *Drosophila* models

PKAN previously known as Hallervorden–Spatz syndrome accounts for approximately half of all cases of NBIA, which is a disease manifested as dystonia, seizures and dementia, and characterized by excessive iron deposition in the brain (mainly globus pallidus), progressive degeneration of the nervous system [45]. It is the best-known example of neuronal brain iron accumulation associated with neurological impairments [46]. The typical feature of PKAN is the specific MRI pattern known as the “eye of the tiger” - a region of hyper-intensity surrounded by an area of hypo-intensity, which is a characteristic of iron accumulation [47]. It is believed that PKAN is caused by the mutations in the gene encoding pantothenate kinase II (PANK2), which is a key enzyme involved in the biosynthesis of coenzyme A from pantothenate [48]. However, the etiological link between loss of PANK2 and the neurodegenerative phenotype is still not well understood.

PANK2 only has a single homolog in *Drosophila*, which is named fumble (fbl) [49]. Recent work on *Drosophila* models has provided important insight into the cellular lesions that play important roles in PKAN pathology. An early work demonstrated that hypomorphic

mutation in fbl resulted in cytogenetic, eclosion and metamorphosis defects in *Drosophila* [49, 50]. Later studies have made progress in modeling PKAN in *Drosophila* and demonstrated that fbl-deficient flies have brain lesion that can be largely rescued by the expression of human PANK2 [51]. Specific reduction of the metabolite, cofactor A (CoA), increased protein oxidation, as well as mitochondrial dysfunction, were subsequently identified in the fbl mutant flies. When the fbl mutant flies were fed with the compound pantethine that can allow an alternative (PANK2-independent) CoA biosynthetic pathway, it was found that the health of mutant flies could be largely improved, including reduced defects in brain morphology, as well as ameliorated climbing and mitochondrial defects [52]. Recently, some researchers exclusively overexpressed endogenous fbl in circadian tissues by using tim-Gal4 driver. These flies displayed aberrant circadian rhythms, increased sensitivity to oxidative stress and a unique transcriptional profile characterized by significant reduction in the expression of key components in the photoreceptor recycling pathways, which could lead to retinal degeneration, a hallmark of PKAN. These results suggest that retinal lesions may not be solely due to oxidative stress and highlight the role of the transcriptional response to CoA deficiency in the defects observed in fbl-deficient flies [53]. However, the relationship between iron abnormalities and the pathological features of PKAN still remains unclear and represents an important future research direction, which can be facilitated by genetic screening in *Drosophila* models.

5 Future perspectives

Nowadays, elevated brain iron content has been regarded as a common feature of many neurodegenerative disorders, including AD and PD [47, 54]. However, it remains a hot topic whether iron accumulation is a primary or secondary event. Identification of iron-related genes that are associated with neurodegeneration suggests a pathogenic role of iron accumulation [55, 56]. For example, increased non-transferrin-bound iron (NTBI) uptake via DMT1 has been found to possibly contribute to iron accumulation and initiate neurodegenerative process in PD [57]. There are many other iron-related genes, such as transferrin receptor 2 (TfR2), that are found disrupted in the process of neurodegeneration [58]. However, the progress in unravelling the mystery of iron as a primary event in neurodegeneration and the pathological contribution of iron-related protein abnormalities has been slow. According to the advantageous features mentioned previously, *Drosophila* may represent an excellent *in vivo* screening platform that can solve the questions in not-too-distant future and at a low cost. It is believed that the results obtained may uncover the pathogenic role of certain iron-related proteins in neurodegeneration and shed light on the discovery of novel therapeutic strategies against neurodegenerative diseases.

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