Review article

Understanding and treating neurodegeneration: insights from the flies

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Abstract

Drosophila has recently emerged as a model system for studying mechanisms of neurodegeneration. Genetic models for most of the major neurodegenerative diseases, including Alzheimer's disease (AD), Parkinson's disease (PD), polyglutamine diseases, and tauopathies, have been successfully established. Pharmacological models of some of these diseases have also been created. Genetic modifier screens using these models have uncovered previously implicated mechanisms and molecules as well as novel ones. Fly models have turned out to be excellent system for the *in vivo* testing of therapeutic potentials of candidate compounds. It is anticipated that further exploration of the fly models will not only provide novel insights into mechanisms of neurodegeneration but also lead to the development of rational treatment of those debilitating degenerative diseases.

Introduction

Of all the diseases affecting human health, neurodegenerative diseases were once considered the most obscure and intractable ones. This situation is about to change, thanks in a large part to breakthroughs in human genetics and genomics that help pinpoint genes associated with familial forms of these diseases such as AD and PD. The identification of these disease-associated genes has provided the much-needed entry points for characterizing mechanisms of neurodegeneration at the molecular level. One of the powerful approaches for studying disease mechanism has been the establishment of animal models. Invertebrate animals, especially *Drosophila*, have proven to be excellent models for human neurodegenerative diseases and have attracted particular attention in recent years. A number of excellent reviews have been written on this topic recently (Bonini and Fortini 2003; Driscoll and Gerstbrein 2003; Muqit and Feany

2002). The aim of this review is to summarize recent new developments in the building of neurodegenerative disease models in *Drosophila*, initial insights on disease mechanisms we have gained from these models, and their relevance to mammalian systems. In the end, I will present some perspective on how further studies of these models can help us understand and ultimately treat those debilitating neurodegenerative disorders.

Building of new disease models

Four approaches have been applied successfully in *Drosophila* to study neurodegenerative diseases. First, forward genetic screens have been carried out to identify mutations that cause brain degeneration. Some pioneering studies in the Benzer lab led to the identification of a number of interesting mutants such as *bubblegum*, *swisscheese*, *drop-dead* (Kretzschmar et al. 1997; Rogina et al. 1997). The mammalian

homologues of some of these genes also cause neurodegeneration phenotypes when mutated, indicating that this approach can identify conserved mechanisms that maintain nervous system integrity (Akassoglou et al. 2004). The advantage of this approach is that it allows unbiased searching of the entire genome for genes that are essential for the maintenance of the integrity of the nervous system. The flip side of this approach is that the neurodegeneration phenotypes in these mutants tend to be quite widespread and the relevance of these mutants to specific neurodegenerative disease is not that obvious at the onset, since most of these diseases show cell type selectivity of neuronal death.

In the second approach, disease-associated human genes are ectopically expressed in fly neurons as transgenes. Generally these disease genes act in a dominant manner in humans and are assumed to have gained toxic functions. This gain of function disease mechanism appears to be very prevalent in most of the neurodegenerative diseases studied. Consequently, models of this dominant type of diseases have been successfully established in *Drosophila* by transgenic expression of the toxic proteins (Feany and Bender 2000; Fernandez-Funez et al. 2000; Jackson et al. 1998, 2002; Kazemi-Esfarjani and Benzer 2000; Warrick et al. 1998; Wittmann et al. 2001). In many cases, transgenic animals overexpressing wild type version of the diseases-associated genes also developed neurodegeneration phenotypes. This is frequently associated with protein misfolding/aggregation, supporting the notion that defects in the disposal of abnormal proteins maybe the underlying cause of many of these neurodegenerative diseases.

The third approach to model neurodegenerative diseases is to disrupt the function of endogenous fly gene homologous to human disease genes. This approach is used to model those familial diseases that transmit in a recessive fashion, which are likely caused by a loss of function mechanism. The wide array of techniques for inhibiting gene function in *Drosophila* makes this approach a particularly attract one for modeling those recessive familial diseases. Transposon-mediated mutagenesis, transgenic RNA interference (RNAi), and homologous recombination-based gene knockout can be used for this purpose (Greene et al. 2003; Pesah et al. 2004; Yang et al. 2003).

Finally, a pharmacological approach can be used to model neurodegenerative diseases and to test potential therapeutics in flies. The pharmacological approach has been used widely in mammalian system

to model Parkinson's disease and some of the drugs have been successfully used to treat flies (Betarbet et al. 2002; Coulom and Birman 2004). Drugs can be mixed with the fly food and easily taken by the flies. One advantage of the fly system is the lack of a stringent blood brain barrier. This allows compounds to gain access to the nervous system easily. Together with the small size, low cost, and short life cycle, this makes *Drosophila* an attractive model for drug studies and screenings.

AD models

AD is the most common neurodegenerative diseases and the leading cause of dementia worldwide. Clinically, AD is manifested as a gradual decline of cognitive functions such as learning and memory. Pathologically, the disease is characterized by two brain lesions-the amyloid plaques and neurofibrillary tangles (NFT). The mechanistic relationship between NFT and amyloid plaque in disease pathogenesis has remained a source of considerable debate (Davies 2000; Hardy and Selkoe 2002). Studies in transgenic mice have shown that expression of mutant human tau (h-tau) alone could result in tangle pathology and neuronal loss without accompanying plaque pathology (Ishihara et al. 1999; Lewis et al. 2000). Induction of amyloid deposits in these animals could further promote NFT formation, suggesting that the two lesions might be causally linked (Gotz et al. 2001; Lewis et al. 2001; Oddo et al. 2003). The notion that amyloid plaque and tau NFT pathologies may be causally associated was further supported by recent studies which demonstrated that blocking A β peptide production in transgenic mouse models could remove amyloid pathology as well as early stage tau lesions (Oddo et al. 2004). Together, these studies seem to support the amyloid cascade hypothesis, which postulates that the initiating event in AD pathogenesis is the accumulation of A β peptide, and that through a mechanistically still undefined process, excess A β peptide promote NFT pathology, which plays a major role in inducing neuronal dysfunction, cell death, and dementia (Hardy and Selkoe 2002). Since NFT pathology is also evident in non-AD dementias which do not show clear amyloid plaque pathology, there must be initiating events other than A β peptide accumulation in the pathogenesis of those diseases (Hou et al. 2004).

A β is generated by endoproteolysis of the amyloid precursor protein (APP) via the sequential action of β - and γ -secretase activities. β -secretase activity is provided by the β -site APP-cleaving enzyme (BACE) whereas γ -secretase activity depends on a protein complex consists of presenilin (Psn), nicastrin, aph-1, and pen-2 (De Strooper 2003). Autosomal dominant mutations in APP, Psn-1, and Psn-2 can accelerate the onset of neurodegeneration and progression of the disease in familial AD. At the molecular level, these mutations promote the generation of amyloidogenic A β peptides (Haass and De Strooper 1999). This has provided one of the strongest genetic evidences for the amyloid cascade hypothesis. The γ -secretase complex components are conserved in *Drosophila*, and are required for the processing of Notch (Struhl and Greenwald 1999; Ye et al. 1999). Fly is lacking a clear homologue of mammalian BACE. There is a fly homologue of APP (APPL) but it is lacking the amyloidogenic A β peptide sequence at the C-terminus and there is no evidence that it is processed *in vivo* as human APP.

Several labs have recently generated fly models of A β -induced amyloid formation and neurodegeneration. In the first report, a human BACE transgene was coexpressed with a human APP transgene and a fly Psn transgene, both containing FAD-linked mutations. Transgenic flies expressing all three transgenes developed β -amyloid plaques and age-dependent neurodegeneration. Genetic manipulation and pharmacological treatment with γ -secretase inhibitors can modulate the severity of the disease phenotypes (Greeve et al. 2004). In another approach, A β -40 and A β -42 peptides were directed fused to signal peptides and expressed as transgenes. Transgenic flies expressing A β -42 developed diffuse amyloid deposits, age-dependent learning defects and neurodegeneration, whereas A β -40 expressing flies showed only age-dependent learning defects but no amyloid formation or neurodegeneration. These studies confirmed the importance of A β -42 toxicity in the pathogenesis of AD (Finelli et al. 2004; Iijima et al. 2004). The development of these A β toxicity models should allow future studies aimed at understanding the molecular mechanisms in the pathogenic cascade of AD and the screening for drug targets and potential therapeutics.

The major component of NFT is the tau protein. Tau is normally a highly soluble microtubule-binding protein enriched in axons (Cleveland et al. 1977), but

it becomes abnormally phosphorylated and insoluble in NFT (Greenberg and Davies 1990; Grundke-Iqbal et al. 1986; Lee et al. 1991). While these observations provide circumstantial evidence that tau abnormalities may lead to neuronal dysfunction and degeneration, direct evidence was not available until the discovery that mutations in tau are associated with frontotemporal dementia with Parkinsonism linked to chromosome 17 (FTDP-17) (Foster et al. 1997; Hutton et al. 1998; Poorkaj et al. 1998; Spillantini et al. 1998). NFT is also a pathological hallmark of AD, where NFTs often surround amyloid plaques composed of A β -42 peptides (Braak and Braak 1991; Mandelkow and Mandelkow 1998).

Drosophila has been successfully used for studying tau-associated neurodegeneration. The first evidence of tau neurotoxicity in flies was provided by transgenic studies of a bovine tau-GFP reporter protein which caused axonal degeneration in sensory neurons (Williams et al. 2000). Subsequently, fly models of tauopathy were created by expressing wild type or FTDP-linked mutant forms of human tau (Jackson et al. 2002; Wittmann et al. 2001). As discussed later, these tauopathy models have been used in genetic studies to test the role of candidate kinases in the regulation of tau phosphorylation and toxicity and in genetic screens for modifiers of tau toxicity.

PD models

PD is the most common movement disorder and the second most common neurodegenerative disease. The movement abnormality in PD is largely attributed to the deficiency of brain dopamine contents caused by the degeneration of dopaminergic neurons in the substantia nigra. The most common forms of PD are sporadic with no known cause. Nevertheless, postmortem studies have identified defects in mitochondrial complex I function and oxidative damages of nigralstriatal dopaminergic neurons (Dexter et al. 1994; Dunnett and Bjorklund 1999). The oxidative damages were proposed to reflect both increased burden of free radical production due to dopamine metabolism and mitochondrial dysfunction, and the limited scavenging ability of these cells (Jenner 2003; Zhang et al. 2000). Oxidative stress was also considered a major factor in the aging process (Sohal 2002), and in the pathogenesis of other age-depen-

dent diseases (Albers and Beal 2000). The identification of environmental toxins that cause PD-like symptoms by inhibiting complex I function and promoting oxidative stress has further supported the oxidative stress model of PD pathogenesis and provided pharmacological tools for disease mechanism investigation (Beal 2001; Betarbet et al. 2002).

The contribution of genetic factors in the pathogenesis of PD, though initially controversial, has been firmly established by better-controlled human genetic studies. At least 10 distinct loci (PARK1-11) have been linked to rare familial forms of PD (FPD) (Bertoli-Avella et al. 2004; Dawson and Dawson 2003). It is anticipated that understanding the molecular lesions associated with these familial PD genes will shed lights on the pathogenesis of the more common forms of the disease. To date, six FPD genes have been molecularly cloned. These include α -Synuclein (α -Syn), Parkin, Ubiquitin C-terminal hydrolase-1 (UCHL-1), DJ-1, PTEN induced kinase-1 (PINK-1), and Park 8 (LRRK2) (Bonifati et al. 2003; Kitada et al. 1998; Leroy et al. 1998; Paisan-Ruiz et al. 2004; Polymeropoulos et al. 1997; Valente et al. 2004; Zimprich et al. 2004). Of these six genes, α -Syn and Parkin have been most intensively studied. Intriguingly, although biochemical and biophysical studies have primarily linked dysfunction of both genes to aberrant protein handling, *in vivo* genetic and *in vitro* cell culture studies have revealed their connection to mitochondrial impairment and oxidative stress, thus reinforcing the involvement of these processes in PD pathogenesis (Greene et al. 2003; Hsu et al. 2000; Palacino et al. 2004; Shen and Cookson 2004; Sherer et al. 2002).

DJ-1, PINK-1, and Park 8 are three recently cloned FPD genes whose molecular functions relevant to PD pathogenesis are still ill defined. PINK-1 is a putative Ser/Thr kinase that is localized to the mitochondria (Canet-Aviles et al. 2004; Valente et al. 2004). Although PINK-1 was initially isolated as a gene induced by overexpression of the tumor suppressor PTEN (Unoki and Nakamura 2001), its functional relationship with PTEN and whether it acts in PI3K/PTEN signaling have not been determined. Nevertheless, the subcellular localization of PINK-1 emphasizes mitochondrion as potential sites of PINK-1 action. DJ-1 encodes a highly conserved protein belonging to the ThiJ/PfpI superfamily. Human DJ-1 was initially discovered as a candidate oncoprotein that can transform cells in cooperation

with activated *ras* (Nagakubo et al. 1997). It was later found as a component of an RNA-binding protein complex, and was associated with male infertility (Hod et al. 1999; Nagakubo et al. 1997; Takahashi et al. 2001). Under oxidative stress conditions, DJ-1 was found to be modified by oxidation and the modified form associate with mitochondria in cell culture (Canet-Aviles et al. 2004; Kinumi et al. 2004; Mitsumoto and Nakagawa 2001; Mitsumoto et al. 2001). Further, knocking down DJ-1 expression with siRNA enhanced cell death induced by oxidative stress, ER stress, and proteasome inhibition (Taira et al. 2004; Yokota et al. 2003); and pathogenic DJ-1 mutations possessed reduced antioxidative stress activities (Takahashi-Niki et al. 2004). However, these studies are largely based on transfection and cell culture assays; the *in vivo* physiological function of DJ-1 and the cellular signaling pathway(s) mediated by DJ-1 that is directly related to PD pathogenesis remain to be defined. Park 8 encodes a 2482 amino acid protein that contains a leucine rich repeat, a kinase domain, a RAS domain, and a WD 40 domain. The subcellular localization of the protein and the loss of function and overexpression phenotypes of Park 8 have not been described.

In the sequenced *Drosophila* genome, there are clear homologues of *Parkin*, *DJ-1*, *Pink-1*, and *Park8*. Given the recessive nature of Parkin, DJ-1, and Pink-1 associated familial Parkinson's diseases (FPD) in humans, a loss of function approach can be applied to model these disease, whereas a transgenic overexpression approach can be used to model Park8-associated FPD, which transmits in a dominant fashion. Although *Drosophila* does not have a clear α -Syn homologue, overexpression of wild type and FPD-associated mutant α -Syn in *Drosophila* has produced one of the best animal models of PD. These models are reported to reproduce several cardinal features of the human disorder, including Lewy body-like aggregate formation, selective degeneration of dopaminergic neurons, and locomotor behavior abnormality (Auluck et al. 2002; Feany and Bender 2000). The fact that both mutant and wild type α -Syn form aggregates in fly neurons and cause disease phenotypes supports the notion that improper disposal of aggregation-prone abnormal proteins can result in neurodegeneration. Two labs independently generated *parkin* null mutant flies (Greene et al. 2003; Pesah et al. 2004). A prominent feature of *parkin* mutant flies is mitochondrial pathology and

apoptotic muscle degeneration. In addition, these mutant flies exhibit sterility, reduced lifespan, reduced cell number and size, and hypersensitivity to oxidative stress. However, *parkin* mutant flies do not show degeneration of brain dopaminergic neurons. Using a different approach, *parkin* loss of function phenotype was created by transgenic RNAi, which allows tissue and cell type-specific gene knockdown (Yang et al. 2003). Targeted expression of *parkin* dsRNA in *Drosophila* dopaminergic neurons did not result in neuronal loss, suggesting that fly Parkin is not absolutely essential for the maintenance of dopaminergic neurons under normal physiological conditions. Targeted overexpression of human Pael-R protein, a Parkin substrate protein (Imai et al. 2001), did result in a reduction of TH-positive dopaminergic neurons in certain regions of fly brain, and this phenotype was exacerbated by the co-expression of *parkin* dsRNA (Yang et al. 2003). Conversely, overexpression of human Parkin can suppress Pael-R-induced toxicity. Together, these *in vivo* genetic interaction studies confirmed the biochemical relationship between Parkin and Pael-R and indicated that accumulation of Pael-R protein in Parkin deficiency dopaminergic neurons may be one of the causes of neuronal death.

Pharmacological PD model in mammals have been instrumental in recapitulating oxidative stress-induced dopaminergic neurodegeneration and are good models for sporadic forms of the disease (Betarbet et al. 2002). A recent study indicated that pharmacological treatment could be used in *Drosophila* to model sporadic PD (Coulom and Birman 2004). Sublethal chronic exposure to rotenone in *Drosophila* resulted in PD-related neurodegenerative and behavioral effects. Rotenone-treated flies presented characteristic locomotor impairments that increased with the dose of rotenone. At the cellular level, a selective loss of dopaminergic neurons was detected in all of the brain clusters. Feeding the flies with L-dopa (3,4-dihydroxy-L-phenylalanine), the precursor to dopamine in the biosynthetic pathway, rescued the behavioral deficits but not neuronal death. In contrast, the antioxidant melatonin (N-acetyl-5-methoxytryptamine) alleviated both symptomatic impairment and neuronal loss, suggesting that antioxidant agents such as melatonin may be beneficial in the treatment of PD. Thus, pharmacological treatment in *Drosophila* provides a new avenue for studying the mechanisms of dopaminergic degeneration.

Polyglutamine disease models

The molecular cloning of the gene responsible for Huntington's disease more than a decade ago revealed that the disease causing mutation is CAG trinucleotide repeats that encodes polyglutamines (polyQs) in the Huntingtin (Htt) protein. Additional studies showed that a diverse group of dominantly inherited neurodegenerative diseases are caused by CAG repeat expansion within disease-specific proteins. These polyQs confer toxic properties to the resident proteins. There is a good correlation between repeat length and disease severity and the number of polyQ repeats needs to be expanded above a threshold level in order for disease to occur (Gusella and MacDonald 2000). These polyQ repeats form intranuclear inclusions in neurons and cultured cells and are intrinsically toxic. However, when placed in different protein-context, the toxicity of polyQ repeats could be altered (Marsh et al. 2000). This has led to the suggestion that this protein context-dependent toxicity of polyQ may explain the neuronal subtype specificity of the different diseases. As will be discussed later, protein co-factors binding to the non-polyQ region of the disease gene product can have profound effects on the toxicity of polyQs.

A number of polyQ disease models have been established in *Drosophila*. These include models for Spinocerebellar Ataxia-3 (SCA-3), SCA-1, Huntington's diseases, and spinal and bulbar muscular atrophy (SBMA) (Chan et al. 2002; Kazemi-Esfarjani and Benzer 2000; Fernandez-Funez et al. 2000; Jackson et al. 1998; Takeyama et al. 2002; Warrick et al. 1998). In all four cases, expression of truncated, expanded polyQ-containing protein fragments leads to retinal degeneration. In the case of SCA-1, overexpression of the non-expanded wild type ataxin-1 protein also resulted in retinal degeneration, indicating that ataxin-1 protein is intrinsically toxic (Fernandez-Funez et al. 2000). Interestingly, in SBMA case, ingestion of androgen promoted neurodegeneration in flies expressing full-length mutant human androgen receptor (hAR), which is otherwise non-toxic (Takeyama et al. 2002). This suggested that hormone binding, subsequent structural alterations, and nuclear localization of mutant hAR protein are critical steps in the pathogenesis of SBMA. The retinal degeneration phenotypes in these polyQ disease models has allowed large-scale genetic screen to identify second-site genetic modifiers.

These models have also been widely used in testing the therapeutic effects of candidate small molecule compounds.

Other disease models

In addition to the polyQ diseases where trinucleotide repeat expansion leads to toxic protein production, there are other diseases caused by trinucleotide repeat expansion within non-coding regions of gene transcripts. Examples include SCA-8, -10, -12, Myotonic Dystrophy type 1 and 2, and Fragile X permutation. Unlike the polyQ diseases, the mechanism by which the non-coding region trinucleotide repeats cause pathology is not clear and there are no good mammalian models for studying these diseases.

To understand how rCGG repeats in the 5'-UTR of FMR1 gene cause neurodegeneration in premutation carriers, 90 rCGG repeats, which is intermediate between the number of repeats found in patients (>200) and normal individuals (<60 repeats), was expressed in the fly eye. This 90 non-coding rCGG repeats was sufficient to cause degeneration of photoreceptors (Jin et al. 2003). Although no mutant protein was produced, the degenerating neurons form neuronal inclusion bodies, which are HSP70 and ubiquitin positive. Further, overexpression of HSP70 was able to suppress rCGG repeats-induced neuronal death. This demonstrates that abnormal RNAs can also cause neurodegeneration. In another fly model of RNA-mediated neurodegeneration, human SCA8 gene, which encodes a non-coding RNA, was expressed in fly retina (Mutsuddi et al. 2004). It induced a late-onset, progressive neurodegeneration phenotype. Interestingly, both wild type and trinucleotide expanded SCA8 induced neurodegeneration phenotypes. Using these SCA8 models, a genetic screen was carried out, which resulted in the identification of four neuronally expressed RNA binding proteins as modifiers of SCA8-induced neurodegeneration. Further characterization of the biochemical interaction between these RNA binding proteins and the wild type and expanded SCA8 RNAs and the physiological significance of these interactions will help understand the mechanism of non-coding region trinucleotide repeats induced-neurodegeneration. In a recent effort to model the pathogenesis of myotonic dystrophy using *Drosophila*, transgenic flies incorporating CTG repeats in the 3'-UTR of a reporter gene were generated. Expanded CUG repeats form

discrete ribonuclear foci in muscle cells. However, flies expressing expanded CUG repeats had no detectable pathological phenotype, suggesting that in contrast to expanded polyglutamine-containing proteins, neither the expanded CUG repeat RNA nor the ribonuclear foci are directly toxic (Houseley et al. 2005).

Molecular insights gained from these disease models

Tauopathy models

Role of phosphorylation in regulating tau toxicity

The presence of abnormally phosphorylated tau in NFT suggests that an imbalance in tau phosphorylation/dephosphorylation may be pathogenic. A number of protein kinases and phosphatases have been shown to regulate tau phosphorylation *in vitro*. These include GSK-3 β MAP kinase, Cdk2/5, PKA, PKB, CaMKII, microtubule-affinity regulating kinase (MARK), and protein phosphatase 1, 2A, 2B, and 2C (Baumann et al. 1993; Billingsley and Kincaid 1997; Drewes et al. 1997; Goedert et al. 1992; Jicha et al. 1999; Mandelkow et al. 1992; Singh et al. 1996). Despite a large body of biochemical studies, surprisingly little is known about the *in vivo* roles of these enzymes in determining tau toxicity. The fact that many of these genes play essential roles during development and that for others there is considerable genetic redundancy may have impeded *in vivo* genetic analysis of these genes in regulating tau phosphorylation and toxicity in mammalian systems.

Identifying the physiological tau kinases and *in vivo* loss of function genetic analysis of these kinases are required in order to evaluate the importance of phosphorylation in tau-mediated neuropathology. Through loss-of-function and overexpression genetic studies and biochemical analysis, PAR-1 was found to be a physiological tau kinase that plays a central role in regulating tau phosphorylation and toxicity in *Drosophila* (Nishimura et al. 2004). PAR-1 is a Ser/Thr kinase originally identified in *C. elegans* for its role in regulating cell polarity and asymmetric cell division (Guo and Kemphues 1995). PAR-1 homologues have been found in eukaryotes ranging from yeast to mammals and they exert essential cellular and developmental functions. MARK kinases, the mammalian homologue of PAR-1, regulates MT dynamics, epithelial cell polarity, and neuronal differentiation un-

der normal conditions and were found to be associated with NFT in AD brain (Biernat et al. 2002; Bohm et al. 1997; Drewes et al. 1997). *Drosophila* PAR-1 plays important roles in MT organization, oocyte differentiation, anterior-posterior axis formation, and Wingless signaling (Cox et al. 2001; Shulman et al. 2000; Sun et al. 2001; Tomancak et al. 2000). Overexpression of PAR-1 leads to elevated tau phosphorylation at S262 and S356 sites and enhanced toxicity, whereas removing PAR-1 function or mutating PAR-1 phosphorylation sites in tau abolishes tau toxicity (Nishimura et al. 2004). Importantly, an initiator role for PAR-1 in a multisite phosphorylation process that generates pathogenic tau was discovered by genetic and mutagenesis studies. In this process, phosphorylation by PAR-1 at the S262/S356 sites precedes and is obligatory for downstream phosphorylation events, including the T208 site that is phosphorylated by GSK-3 β and Cdk5, in the generation of neurotoxic tau (Nishimura et al. 2004). These results, together with the result of a large-scale genetic screen that uncovers protein kinases and phosphatases as main modifiers of tau toxicity (Shulman and Feany, 2003), clearly established the importance of phosphorylation in determining tau toxicity *in vivo*.

In some disease models, there is a correlation between the extent of macroscopically visible protein aggregate formation and disease severity, whereas in other models disease symptoms can occur without the large aggregate formation. In some cases, genetic manipulations that interfere with protein aggregate formation actually exacerbate disease symptoms (Cummings et al. 1999). The role of protein aggregate formation in disease pathogenesis thus remains uncertain. In fly tauopathy models, neurodegeneration can occur in the absence of NFT formation, suggesting that some soluble, non-tangle form of phosphorylated tau is intrinsically toxic (Wittmann et al. 2001). Interestingly, when fly GSK-3 β was co-expressed with tau, large NFT-like aggregates formed in the photoreceptor neurons and neurodegeneration was enhanced (Jackson et al. 2002). While this observed suggested that NFT-like aggregates may contribute to tau toxicity, it was also consistent with the notion that soluble, non-tangle form of phosphorylated tau is the neurotoxic species and that in the process of NFT formation by GSK-3 β overexpression, these toxic forms of tau also accumulated. Further studies are needed to resolve this important question regarding NFT formation and tau toxicity.

The importance of individual sites in determining tau toxicity *in vivo* has not been clearly defined. Post-mortem analysis indicated that in AD patient brain tau is hyperphosphorylated in more than 20 Ser/Thr sites (Hanger et al. 1998). In theory, these phosphorylation sites could act in concert to confer tau toxicity or that a small subset of sites play a dominant role in determining tau toxicity. The analysis of the PAR-1-triggered sequential tau phosphorylation process has implicated the importance of a small subset of phosphorylation events in determining tau toxicity (Nishimura et al. 2004). One of these sites, the AT8 site, has been regarded as a disease-diagnostic marker because it recognizes late-stage NFT pathology (Augustinack et al. 2002). However, mutating the T208 residue at the AT8 recognition site into non-phosphorylatable Ala residue had little influence on tau toxicity in the fly retina. This suggests that other sites may play more significant roles in determining tau toxicity (Nishimura et al. 2004). Further studies are required to pinpoint the exact phosphorylation events that play critical roles in conferring tau toxicity. This information will help identify which species of phospho-tau corresponds to the neurotoxic ones and thus will have important implications for revealing critical steps in the pathogenic cascade, which would be rational targets for therapeutic intervention.

Role of apoptosis in tau toxicity

In the large-scale genetic screen for modifiers of tau toxicity, two enhancers with known function in the apoptosis pathway were identified (Shulman and Feany 2003). Expression of *thread* (*th*), a *Drosophila* homologue of inhibitor of apoptosis (IAP), was recovered as a suppressor of tau toxicity, whereas a *Drosophila* homologue of *C. elegans* Fem-1, a CED-3 substrate protein, was found as an enhancer. These data suggest that pro-apoptotic caspases play important roles in tau-induced neuronal death. This is also consistent with earlier studies that demonstrated partial suppression of tau-induced eye degeneration by the expression of p35, a baculovirus-derived IAP (Jackson et al. 2002). Pro-apoptotic caspases could act in the final execution step of the cell death reaction, or at an earlier step in the pathogenic cascade of tau-induced cell death. There is evidence that tau can be cleaved by caspases and that the processed tau may be more toxic (Rissman et al. 2004). It

would be interesting to test whether the apoptotic pathway genes affect the processing of tau in the fly system.

PolyQ models

Forward genetic screen reveal novel mechanisms and molecules in SCA-1 pathogenesis

Significant insights into the pathogenic pathways of polyglutamine diseases were obtained from the SCA-1 model. In loss-of-function and overexpression forward genetic screens for genes that can modify expanded ataxin-1-induced neurodegeneration phenotypes, genes involved in a number of biological processes were identified (Fernandez-Funez et al. 2000). Not surprisingly, molecular chaperones, which have previously been implicated by candidate gene testing and forward genetic modifier screens for modifiers of polyglutamine toxicity, were recovered in the SCA-1 screen. This highlights the importance of protein folding and clearance of abnormal proteins in the development of polyQ pathology. This screen also identified modifier genes involved in RNA processing, nuclear transport, transcriptional regulation (histone deacetylation), and cellular detoxification. The identification of these new genes and mechanisms demonstrated the power of fly genetics in dissecting diseases mechanism. Further analysis of the roles of these proteins in mammalian disease models will help validate the relevance of fly models to human diseases and may provide new therapeutic strategies.

Phosphorylation of ataxin-1 and 14-3-3 binding

In another study using the SCA-1 model, it was found that 14-3-3 protein, an abundant and multifunctional protein, could bind to ataxin-1 (Chen et al. 2003). This leads to the stabilization and accumulation of the toxic protein. 14-3-3 proteins are known to bind other proteins in a phosphorylation-dependent manner and indeed the interaction between ataxin-1 and 14-3-3 is also phosphorylation-dependent. The PI3K/AKT pathway was shown to regulate the association between ataxin-1 and 14-3-3. AKT phosphorylates ataxin-1 at a conserved Ser residue and this phosphorylation event provides a binding site for 14-3-3. In support of the role of AKT and 14-3-3 in SCA-1 pathogenesis, *in vivo* genetic manipulation of AKT and 14-3-3 activities modulates ataxin-1-induced neurodegeneration. These results

suggest that manipulating PI3K/AKT pathway activity or interfering with 14-3-3/ataxin-1 interaction may provide new strategies for therapeutic intervention. Significantly, phosphorylation of ataxin-1 was also found to play an important role in regulating disease pathogenesis in a mouse SCA-1 model (Emamian et al. 2003).

Modulation by sumoylation and ubiquitination

Studies of a fly model of SBMA have emphasized the roles of the ubiquitin-proteasome pathway and sumoylation pathway in polyglutamine pathogenesis. Expression of a pathogenic AR protein with an expanded polyQ repeat in *Drosophila* results in nuclear and cytoplasmic inclusion formation, and cellular degeneration (Chan et al. 2002). The influence of ubiquitin-dependent modification and the proteasome pathway on neural degeneration was studied in this model. Compromising the ubiquitin/proteasome pathway enhances degeneration and decreases polyQ protein solubility. Further, overexpression of a mutant of the SUMO-1 activating enzyme Uba2, showed that polyQ-induced degeneration is intensified when the cellular SUMO-1 protein conjugation pathway is altered, although the exact molecular nature of the effects of mutant Uba2 overexpression on polyQ modification was not addressed in this study. This study implicated the ubiquitin/proteasome and the SUMO pathways in modulating polyQ pathogenesis. A recent study on expanded Huntingtin (Htt) protein provided more conclusive evidence for the involvement of sumoylation in regulating polyQ protein toxicity (Steffan et al. 2004). It was found that sumoylation and ubiquitination compete for the same Lys residue in Htt. Sumoylation stabilizes the protein, prevents aggregate formation, and enhanced the transcriptional repressor activity of Htt. In a fly HD model, sumoylation enhanced neurodegeneration, whereas ubiquitination reduced neurodegeneration. Mutating the Lys residue prevented both sumoylation and ubiquitination and abrogated neurodegeneration. Sumoylation thus appears to be an essential step in polyQ pathogenesis and may present a unique opportunity for therapeutic intervention. It should be mentioned that a pathogenic fragment of Htt (Httex1p) was used in this study. It would be interesting and important to see whether the same conclusion can be drawn when the full-length or longer protein is used.

Role of specific cell death regulators in polyQ disease pathogenesis

The role of programmed cell death in polyQ disease pathogenesis is a matter of debate. In fly models, expression of the anti-apoptotic protein p35 has been shown to have little effect on polyQ toxicity (Jackson et al. 1998). Further, apoptosis-related genes have not been reported as modifiers from various genetic screens. However, testing on candidate cell death genes have revealed a common role for Dark in polyQ pathogenesis. Dark is the fly homolog of human Apaf-1, a key regulator of apoptosis in mammals. PolyQ-induced cell death was dramatically suppressed in flies lacking Dark. This was accompanied by a suppression of caspase activation and aggregate formation. Dark colocalized with ubiquitinated protein aggregates. Further, mammalian Apaf-1 also was found to colocalize with Huntingtin-containing aggregates in a murine model and Huntington disease brain, suggesting a common role for Dark/Apaf-1 in polyglutamine pathogenesis (Sang et al. 2005). The molecular pathway mediating Dark/Apaf-1 function in modulating aggregates formation and cell death remains to be defined.

PD models

Role of molecular chaperones

Compared to the other diseases, familial genes that are associated with PD were discovered relatively recent and most of the research is still in the model building stage. Available models do not exhibit obvious neurodegeneration phenotypes such as eye degeneration that will allow large-scale genetic modifier screening. Using a candidate gene approach, it was shown that directed expression of the molecular chaperone HSP70, which has been shown to be effective in ameliorating neurodegeneration in polyQ models, prevented dopaminergic neuronal loss associated with α -Syn expression in *Drosophila* (Auluck et al. 2002). Further, interference with endogenous chaperone activity accelerated α -Syn toxicity. To demonstrate the relevance of these findings to human diseases, the authors found that Lewy bodies in human postmortem tissue immunostained for molecular chaperones, suggesting that chaperones may play a role in Parkinson's disease progression. These observations suggest that abnormal protein folding and aggregation may be a common

mechanism in the pathogenesis of polyQ and α -Syn-associated neurodegenerative diseases. A recent human genetics study indicated that polymorphisms in the human *Hsp70* genes may affect susceptibility to PD, thus corroborating the previous study (Wu et al. 2004).

Recently, the potential of Hsp70 for gene therapy in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model of idiopathic PD was tested. Hsp70 gene transfer to dopamine neurons by a recombinant adeno-associated virus significantly protects the mouse dopaminergic system against MPTP-induced dopamine neuron loss and the associated decline in striatal dopamine levels and degeneration of TH-positive dopaminergic fibers. At the cellular level, Hsp70 expression reduced MPTP-induced apoptosis in the substantia nigra; at the behavioral level, unilateral protection of the dopaminergic system by Hsp70 was associated with increased amphetamine-induced turning toward the uninjected side (Dong et al. 2005). The effectiveness of HSP70 in preventing dopaminergic degeneration in an acute pharmacological model and a chronic genetic model suggests that increasing chaperone activity may be beneficial for the treatment of PD.

Neuroprotective function of Parkin

The candidate gene approach was also used to study the relationship of Parkin and α -Syn in dopaminergic neuron degeneration. There are indications that these two genes may functionally interact in the disease process. Parkin co-localizes with α -Syn in Lewy bodies and an O-glycosylated form of α -Syn was reported to be a substrate protein for Parkin (Shimura et al. 2001). Overexpression of Parkin was found to be effective in suppressing α -Syn-induced degeneration of dopaminergic neurons (Yang et al. 2003). This suppression was not associated with significant changes in total cellular α -Syn protein levels, suggesting that either the toxic species of α -Syn, which may be eliminated by Parkin overexpression, may represent only a small portion of total α -Syn protein. Alternatively, Parkin suppression of α -Syn toxicity may act through a mechanism unrelated to α -Syn degradation by the ubiquitin-proteasome pathway. Using two other assays of α -Syn-induced toxicity, premature decline of climbing ability and degeneration of ommatidial arrays, overexpression of Parkin was also found to be effective in suppressing α -Syn toxicity (Haywood and Staveley 2004).

The neuroprotective effect of Parkin was also demonstrated in mammalian systems. In cell culture systems, overexpression of mutant α -Syn increases sensitivity to proteasome inhibitors by decreasing proteasome function, which can be counteracted by overexpression of Parkin. Mutant α -Syn or application of proteasome inhibitors caused selective toxicity to catecholaminergic neurons in primary midbrain cultures, an effect that can also be rescued by Parkin overexpression. Therefore, Parkin and α -Syn are linked by common effects on a pathway associated with selective cell death in catecholaminergic neurons (Petrucelli et al. 2002). In an *in vivo* study carried out in a rat lentiviral model of PD, overexpression of wild-type rat Parkin could protect against the toxicity of mutated human A30P α -Syn. Animals overexpressing Parkin showed significant reductions in α -Syn -induced neuropathology, including preservation of TH-positive cell bodies in the substantia nigra and sparing of TH-positive nerve terminals in the striatum. Interestingly, Parkin-mediated neuroprotection was associated with an increase in hyperphosphorylated α -Syn inclusions, supporting a role for Parkin in the genesis of Lewy bodies (Lo Bianco et al. 2004). These studies suggest that upregulation of Parkin activity may represent a promising treatment for PD.

Gene expression changes in the course of disease pathogenesis

In the absence of an easy assay system for a forward genetic screen for genes involved in PD, whole genome analysis of gene expression changes is an alternative approach for defining disease mechanism and identifying new players. To define the transcriptional program encoding molecular machinery involved in α -Syn-induced neuropathology, expression of the entire *Drosophila* genome at pre-symptomatic, early and advanced disease stages was characterized by DNA microarray analysis (Scherzer et al. 2003). Fifty-one signature transcripts, including lipid, energy and membrane transport mRNAs, were found tightly linked with disease pathogenesis. The advantage of the DNA microarray approach is that it allows detection of gene expression changes at the pre-symptomatic stage. Indeed, expression changes for genes involved in catecholamine biosynthesis, mitochondrial function, and lipid-binding were observed in histologically and behaviorally normal 1-

day-old α -Syn transgenic flies. Interestingly, in age-matched tau transgenic *Drosophila*, the transcription of α -Syn-associated genes was normal. This analysis, together with other studies comparing the effects of the genetic modifiers identified from the SCA-1 screen to the tauopathy model (Ghosh and Feany 2004; Shulman and Feany 2003), suggests that the pathways of neurodegeneration are highly specific for different diseases. If this observation is confirmed in other disease models, it will have important implications for the designing of therapeutic approaches targeting different diseases.

Using fly models to test therapeutic compounds

Drosophila models of neurodegenerative diseases have turned out to be excellent systems for *in vivo* testing of therapeutic effects of various compounds. In a successful application of the chemical approach to neurodegeneration in *Drosophila*, feeding the fly neurodegeneration mutant *bubblegum* with one of the components of 'Lorenzo's oil', glyceryl trioleate oil, prevented neurodegeneration (Min and Benzer 1999). The *bubblegum* mutant exhibits adult neurodegeneration, shows elevated levels of very long chain fatty acids, as seen in the human disease adrenoleukodystrophy (ALD). In ALD, the excess of very long chain fatty acids can be lowered by dietary treatment with 'Lorenzo's oil', a mixture of unsaturated fatty acids. This study demonstrates the relevance of fly models for studying mechanisms of neurodegenerative disease and screening drugs for therapeutic intervention.

Subsequent studies using other disease models further proved the power of the chemical approach in flies for understanding disease mechanism and for testing therapeutic strategies. In Huntington's disease, biochemical studies in mammalian cell culture showed that the polyglutamine-containing domain of Htt directly binds the acetyltransferase domains of certain transcriptional factors, including CREB-binding protein (CBP) and p300/CBP-associated factor (P/CAF), resulting in inhibition of the acetyltransferase activity of these enzymes. Expression of Htt in cultured cells reduces the level of the acetylated histones H3 and H4, an effect that can be reversed by administering inhibitors of histone deacetylase (HDAC). Significantly, in two *Drosophila* models of polyQ disease, HDAC inhibitors could reduce

cellular toxicity associated with polyglutamine repeat expansion and arrest neuronal degeneration (Steffan et al. 2001). Supporting a role for histone acetylation in polyQ disease pathogenesis, application of vitamin B3, a vitamin with histone deacetylase inhibiting activity, potentially suppressed polyQ-mediated toxicity in the adult brain (Ghosh and Feany 2004). Several other compounds with anti-polyQ aggregation properties were identified in cell-based screens and subsequently shown to be effective in preventing neurodegeneration in fly polyQ models (Nagai et al. 2003; Pollitt et al. 2003; Zhang et al. 2005). These compounds and the HDAC inhibitors represent strong lead compounds for the development of therapeutics for human polyQ diseases.

Compound testing in fly PD and AD models have also begun. Based on the evidence that Hsp70 overexpression can prevent α -Syn-induced toxicity in fly dopaminergic neurons, the same authors tested the effect of feeding flies with geldanamycin, a naturally occurring benzoquinone ansamycin that specifically binds and inhibits HSP90, a negative regulator of heat shock factor, and can induce stress-response in cell culture. Geldanamycin treatment was shown to induce HSP70 expression and prevented α -Syn-induced dopaminergic neuronal loss (Auluck and Bonini 2002). As mentioned before, melatonin has been shown to be effective in preventing dopaminergic degeneration in a fly rotenone model of sporadic PD (Coulom and Birman 2004). If geldanamycin and melatonin's neuroprotective effect can be confirmed in mammalian PD models, these compounds warrant further exploration as novel therapeutics for PD. Given the importance of protein phosphorylation in regulating tau toxicity, protein kinase inhibitors are excellent candidates for therapeutics of tauopathies. Overexpression of tau disrupts axonal transport, causing vesicle aggregation and loss of locomotor function. Co-expression of constitutively active GSK-3 β enhances these toxic effects of tau. Two GSK-3 β inhibitors, lithium and AR-A014418, reverse both the axon transport and locomotor phenotypes, supporting that the pathological effects of tau are phosphorylation dependent and that the inhibition of GSK-3 β might have therapeutic benefits in tauopathies (Mudher et al. 2004). Lithium has been used for sometime to treat depression. If the effects of lithium on tauopathies can be further validated in mammalian systems, it can be quickly moved to clinical trials to treat diseases with as-

sociated tau pathology, such as AD, progressive supranuclear palsy, and frontotemporal dementia.

Future directions

The development of highly relevant fly models of human diseases have ushered in a new era in neurodegenerative diseases research. The power of the fly models lie in the ability to perform large-scale forward genetic screens to dissect the cellular signaling pathways involved in the disease process, without making assumptions about what kind of molecules or mechanisms are involved. Once novel mechanisms or molecules are identified in fly models, their relevance to mammalian system will be tested. It is anticipated that ongoing and future genetic modifier screens will generate more mechanistic insights into the disease process and help understand some of the unresolved questions in the field, such as the identity of the neurotoxic species for each disease; the nature of their effect on neuronal function; and what determines the cell type-specific vulnerability underlying each disease. The genetic modifiers identified in flies may also help reveal disease susceptibility genes in humans.

It is expected that the candidate gene approach will continue to be fruitful in elucidating disease mechanisms. For example, aging is a major risk factor for many of the neurodegenerative diseases. Oxidative stress may be the functional link between aging and neurodegeneration (Sohal 2002). The role of oxidative stress in neurodegeneration is beginning to be unraveled in the fly models, especially PD models (Pesah et al. 2004), and will be a focus in future studies. Candidate genes identified in DNA microarray studies of oxidative stress response and aging can be tested in neurodegenerative disease models (Landis et al. 2004; Zou et al. 2000). In mammalian systems, inflammation and immune response have been recognized as contributing factors in the pathogenesis of neurodegenerative diseases such as PD and AD (Hirsch et al. 2003; Wyss-Coray and Mucke 2002). It will be interesting to see whether similar physiological processes play a role in neurodegeneration in flies.

Further application of the chemical approach will continue to provide insights of disease mechanism and for identifying potential drug candidates. Some

candidate compounds that could be tested include: heavy metals, which are implicated in the pathogenesis of AD, PD, and ALS; dopamine and its metabolites, which are involved in PD; and oxidative stressors and antioxidants. The small size, high fecundity, and low cost make *Drosophila* an excellent system to perform high throughput screens from random chemical libraries for compounds with therapeutic potentials. One added advantage of the fly system is target validation. Once a drug that shows effect is found, one can use genetics to validate the pathway by showing that the drug effect is dependent upon the function of a presumed target. In the end, if a drug originally isolated using fly models can be used to cure a disease in human, it will be the best validation of the medical relevance of the *Drosophila* system to human neurodegenerative diseases.

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