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Review Article

α-Synuclein Transgenic *Drosophila* As a Model of Parkinson's Disease and Related Synucleinopathies

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α-Synuclein (α-Syn) is a major component of protein inclusions known as Lewy bodies, which are hallmarks of synucleinopathies such as Parkinson's disease (PD). The α-Syn gene is one of the familial PD-causing genes and is also associated with an increased risk of sporadic PD. Numerous studies using α-Syn expressing transgenic animals have indicated that α-Syn plays a critical role in the common pathogenesis of synucleinopathies. *Drosophila melanogaster* has several advantages for modeling human neurodegenerative diseases and is widely used for studying their pathomechanisms and therapies. In fact, *Drosophila* models expressing α-Syn have already been established and proven to replicate several features of human PD. In this paper, we review the current research on synucleinopathies using α-Syn *Drosophila* models and, moreover, explore the possibilities of these models for comprehensive genetic analyses and large-scale drug screening towards elucidating the molecular pathogenesis and developing therapies for synucleinopathies.

1. Introduction

Protein inclusions known as Lewy Bodies (LBs) are one of the hallmarks of Parkinson's disease (PD), in which the major component is now known to be α-synuclein (α-Syn) [1, 2]. LBs are found in the substantia nigra in PD and also more extensively in other brain regions in other synucleinopathies including multiple system atrophy and dementia with Lewy bodies (DLB) [3, 4]. The α-Syn encoding gene, SNCA, is the first gene in which missense mutations such as A30P and A53T were found to cause familial PD [5, 6]. Furthermore, the multiplication mutations of α-Syn gene were also found to cause familial PD [7]. Most importantly, single nucleotide polymorphisms (SNPs) of α-Syn have been reported to associate with an increased risk of sporadic PD, which comprises the majority of PD patients [8–11]. α-Syn expression has been experimentally shown to mimic several aspects of PD in transgenic animals, such as motor dysfunction, α-Syn aggregation/accumulation, and neurodegeneration [12–14]. These phenotypes are manifested not only by mutations in the α-Syn gene but also by overexpression of wild-type α-Syn [15], indicating that α-Syn plays a critical role in the common pathogenesis of synucleinopathies.

Drosophila melanogaster, commonly known as the fruit fly, has been recognized as a powerful organism for modeling human neurodegenerative diseases [16]. At least ~75% of human disease genes have *Drosophila* homologues [17]. Using *Drosophila* for modeling human neurodegenerative diseases has various advantages as follows: (1) analysis of gene functions *in vivo*, (2) rapid generation cycle (10–14 days) with a short life span (50–60 days), (3) suitability for genetic analysis, (4) abundant genetic information, and (5) little labor and cost-effective to maintain fly stocks (Table 1). In fact, *Drosophila* models of several neurodegenerative diseases including PD, Alzheimer's disease, and the polyglutamine diseases have already been established and have successfully provided valuable insights into the elucidation of pathomechanisms and development of therapies for these diseases.

Feany and Bender first developed transgenic *Drosophila* models expressing either wild-type or familial PD-linked mutants (A53T and A30P) of human α-Syn [12]. These

Table 1: Advantages of using *Drosophila* for modeling human neurodegenerative diseases.

(1) Analysis of gene functions in vivo
At least ~75% of human disease genes have *Drosophila* homologues.
(2) Rapid generation cycle with a short life span
10–14 days from embryo to adults.
Average life span is ~50–60 days.
(3) Suitable for genetic analyses
Stock centers maintain a variety of mutant fly libraries as public resources.
Various genetic screening methods have been established.
(4) Abundant genetic information
Whole genome sequence is available.
(5) Little labor and cost-effective to maintain fly stocks
Transgenic flies can be established at low cost.
Mutant flies are available from public stock centers at low cost.
Only small space is required for their maintenance.

α-Syn expressing flies replicate several features of human PD, including (1) locomotor dysfunction, (2) LB-like inclusion body formation, and (3) age-dependent loss of dopaminergic neurons and are therefore widely used for studying the molecular pathogenesis of α-Syn-induced neurodegeneration in not only PD but also synucleinopathies. In this paper, we will discuss what has been revealed in the pathogenesis of synucleinopathies using α-Syn *Drosophila* models, focusing on "misfolding and aggregation of α-Syn", "posttranslational modifications of α-Syn", and "oxidative stress" (Table 2).

2. Misfolding and Aggregation of α-Synuclein

Recent accumulating evidence has implicated that misfolding and subsequent aggregation of α-Syn play a central role in the pathogenesis of synucleinopathies [37]. Indeed, α-Syn has been demonstrated to be aggregated and deposited as inclusion bodies in flies expressing either wild-type or mutant α-Syn (A53T and A30P), the latter of which has accelerated aggregation propensity. Recently, Karpinar et al. showed that structurally-engineered α-Syn mutants with an increased propensity to form soluble oligomers exhibit enhanced neurotoxicity in *Drosophila* [18]. Moreover, a recent study demonstrated that histone deacetylase 6 (HDAC6) suppresses α-Syn-induced dopaminergic neuron loss and locomotor dysfunction by reducing α-Syn oligomers and instead promoting inclusion formation in α-Syn flies, further supporting a critical role of toxic oligomers in α-Syn-induced neurodegeneration in the pathogenesis of synucleinopathies [19].

Protein quality control systems function as a defense mechanism against protein misfolding and aggregation, which consist of molecular chaperones and protein degradation systems [38]. Molecular chaperones assist proper protein folding and hence are considered as essential proteins for protecting cells against the detrimental effects of the misfolding and aggregation of proteins such as α-Syn. Most molecular chaperones are induced upon heat stress to promote the refolding of misfolded proteins, and hence they are called heat shock proteins (HSPs) [39]. On the other hand, once proper protein folding has been altered, the resulting misfolded and aggregated proteins must be eliminated by their degradation. Two major protein degradation systems are the ubiquitin-proteasome system (UPS) and the autophagy-lysosome system [40]. UPS degrades short-lived and misfolded proteins through selective deubiquitination of substrate proteins and their targeting to the proteasome, whereas the autophagy-lysosome system is a nonselective bulk degradation system for long-lived and misfolded proteins, which involves engulfment of substrate proteins into the autophagosome and their delivery to the lysosome. The role of molecular chaperones and protein degradation systems in protecting against α-Syn misfolding in the pathogenesis of synucleinopathies has been investigated using α-Syn *Drosophila* models.

2.1. Molecular Chaperones. As molecular chaperones are expected to protect against protein misfolding and aggregation, their roles in the pathogenesis of PD have been investigated so far [41]. Extensive colocalization with LBs has been demonstrated for several HSPs [42], and expression levels of HSPs have been reported to be elevated in synucleinopathy brains [43]. HSP70 has been shown to inhibit α-Syn aggregation *in vitro* [44], and HSPs, such as HSP27 or HSP70, have been reported to protect against α-Syn-induced neurotoxicity in cultured cells and transgenic mice [45, 46], suggesting an important role of HSPs in PD pathology.

Indeed, Auluck et al. demonstrated that coexpression of HSP70 ameliorated the toxicity of α-Syn to dopaminergic neurons without changing the number of inclusions [20]. They also confirmed that coexpression of Hsc4.K71S, a dominant negative form of *Drosophila* HSP70, accelerated dopaminergic neuron loss in α-Syn expressing flies. Furthermore, they subsequently showed that geldamamycin, an Hsp90 inhibitor and heat shock transcription factor 1-activator compound, protects against neurotoxicity through induction of Hsp70 in α-Syn flies [21]. Taken together, these results confirmed that the molecular chaperone HSP70 suppresses α-Syn toxicity *in vivo* by using the *Drosophila* system.

2.2. Protein Degradation. The UPS and the autophagy-lysosome system can degrade misfolded proteins, and impairment of these systems has been reported to cause neurodegeneration [40, 47]. Furthermore, the UPS has been suggested to coordinate with the autophagy system to eliminate misfolded proteins. Lee et al. have shown protective effects of the UPS on α-Syn-induced toxicity using cell culture and *Drosophila* models [22]. A cell culture-based study indicated that K48-linked polyubiquitination is protective against α-Syn-induced toxicity in a UPS-dependent manner. In α-Syn flies, coexpression of ubiquitin has been shown to suppress loss of dopaminergic neurons and locomotor dysfunction and to extend life-span. These

Table 2: Summary of studies on α-Syn-induced neurodegeneration using *Drosophila* models.

Mechanisms/modifiers of α-Syn toxicity	Effect	Findings	References
α-Syn expression		α-Syn expression causes dopaminergic neuron loss, LB-like inclusion body formation and locomotor dysfunction in *Drosophila* (wild-type < familial PD-linked mutants).	[12]
Misfolding and aggregation			
α-Syn oligomer formation	Enhance	α-Syn mutants which tend to form oligomers enhance α-Syn toxicity.	[18]
HDAC6	Suppress	Expression of HDAC6 reduces α-Syn oligomers and suppresses α-Syn toxicity.	[19]
HSP70	Suppress	Expression of HSP70 reduces α-Syn toxicity, and a dominate negative form of HSP70 enhances toxicity.	[20]
Geldanamycin	Suppress	Geldanamycin induces HSP70 expression and suppresses α-Syn toxicity.	[21]
Ubiquitin	Suppress	Expression of ubiquitin reduces α-Syn toxicity.	[22]
Cathepsin D	Suppress	Deficiency of cathepsin D enhances α-Syn-induced neurodegeneration.	[23]
Posttranslational modifications			
α-Syn phosphorylation at Ser129	Enhance	A phosphomimic S129D α-Syn mutant enhances α-Syn toxicity and a phospho-resistant S129A α-Syn mutant reduces toxicity.	[24]
α-Syn phosphorylation at Tyr125	Suppress	Expression of shark increases α-Syn Y125 phosphorylation and reduces α-Syn toxicity. Blocking of Y125 phosphorylation enhances toxicity.	[25]
α-Syn C-terminal truncation	Enhance	Expression of C-terminal truncated α-Syn (1–120) enhances α-Syn aggregation and toxicity.	[26]
α-Syn cleavage by Calpain I	Enhance	Calpain I-cleaved α-Syn fragments were identified in the brains of α-Syn flies as well as PD/DLB patients.	[27]
Oxidative stress			
Reactive oxygen species	Enhance	Hypoxia-induced oxidative stress enhances α-Syn toxicity, and expression of superoxide dismutase suppresses toxicity.	[28]
Dopamine	Enhance	Decreased dopamine levels by tyrosine hydroxylase RNAi reduces α-Syn toxicity.	[29]
Glutathione metabolism	Suppress	Defect of glutathione metabolism genes enhances α-Syn toxicity and expression of glutathione metabolism genes suppresses toxicity.	[30]
Nicotinamide	Suppress	Nicotinamide suppresses α-Syn toxicity through improvement of oxidative mitochondrial dysfunction.	[31]
Polyphenols	Suppress	Grape extracts containing various polyphenols suppress α-Syn toxicity.	[32]
Other PD-causing genes			
Parkin	Suppress	Expression of Parkin suppresses α-Syn toxicity.	[33–35]
PINK1	Suppress	Expression of PINK1 suppresses α-Syn toxicity.	[36]

results suggest that UPS-mediated degradation of α-Syn is a potential therapeutic approach for synucleinopathies including PD.

Cathepsin D (CathD) is a major lysosomal aspartyl protease and its defect results in fatal neurodegenerative diseases [48]. CathD has been shown to efficiently degrade recombinant α-Syn in *in vitro* experiments, and knockdown of CathD in cultured cells increased α-Syn levels, indicating a role of CathD in α-Syn degradation [49]. Using α-Syn expressing flies, Cullen et al. demonstrated that a CathD defect enhanced α-Syn-induced neurodegeneration *in vivo* [23]. CathD knock-out mice have also been shown to facilitate insoluble α-Syn accumulation and α-Syn-induced neurotoxicity, confirming that CathD may protect neurons against α-Syn-induced toxicity through degradation.

3. Posttranslational Modifications of α-Synuclein

Posttranslational modifications including phosphorylation, ubiquitination, or C-terminal truncation of α-Syn have been observed in LBs in the postmortem brain of synucleinopathy patients [37]. *In vitro* studies suggest that these modifications can accelerate oligomerization or aggregation of α-Syn.

Accordingly, the role of posttranslational modifications of α-Syn on toxicity has been studied using α-Syn expressing flies.

3.1. α-Synuclein Phosphorylation. Phosphorylation at Ser129 has been identified in α-Syn deposited as LBs in synucleinopathy brains [50]. To explore the pathological role of this phosphorylation *in vivo*, accumulation and phosphorylation of α-Syn was studied in flies expressing wild-type or mutant α-Syn. Indeed, α-Syn accumulated in these flies was phosphorylated at Ser129 as reported in human patients, and the order of the degree of phosphorylation was A53T > A30P > wild-type [51]. Mutagenesis studies demonstrated that the phosphomimic S129D mutant increases α-Syn-induced toxicity, whereas the phospho-resistant S129A mutant reduces the toxicity accompanied with an increased number of inclusion bodies [24]. Furthermore, GPRK2 has been shown to be responsible for the α-Syn phosphorylation in *Drosophila*. These studies revealed that Ser129 phosphorylation plays an important role for α-Syn-induced neurotoxicity and inclusion body formation.

Chen et al. recently reported that Tyr125 of α-Syn is also phosphorylated in α-Syn expressing flies [25]. This phosphorylation occurs at a young age but diminishes during the aging process in both humans and flies. They showed that soluble oligomers of α-Syn were increased by phosphorylation at Ser129 and decreased by phosphorylation at Tyr125. In addition, blocking Tyr125 phosphorylation increased α-Syn toxicity. Taken together, these studies suggest that α-Syn toxicity in synucleinopathies results from an imbalance between the detrimental action of Ser129 phosphorylation by accelerating toxic oligomer formation and a neuroprotective action of Tyr125 phosphorylation by suppressing oligomer formation.

3.2. α-Synuclein Truncation. Truncated small species of α-Syn have been detected in purified LBs and insoluble fractions from synucleinopathy brains [52, 53], suggesting that truncation of α-Syn contributes to aggregation and LB formation. Several studies have implicated that C-terminal truncation of α-Syn accelerates its aggregation [54, 55], and the NAC domain (residues 61–95) of α-Syn has been demonstrated to be essential for α-Syn aggregation *in vitro* [56, 57]. Indeed, flies expressing α-Syn with an NAC domain deletion (α-Syn Δ71–82) did not show any loss of dopaminergic neurons with no evidence of α-Syn aggregation, confirming an essential role of the NAC domain in α-Syn aggregation and toxicity *in vivo* [26]. On the other hand, expression of C-terminal truncated α-Syn (α-Syn 1–120) resulted in increased α-Syn aggregation and significantly greater loss of dopaminergic neurons than wild-type in *Drosophila*, suggesting a potential role of the C-terminal region of α-Syn in suppressing aggregation.

α-Syn has been shown to be a substrate for proteolytic cleavage by calpain *in vitro*, which is one of a family of intracellular calcium-dependent proteases [58, 59]. The calpain-cleaved α-Syn species exhibit a similar molecular size to truncated α-Syn fragments that have been shown to promote aggregation and to enhance toxicity [54, 55, 60]. Dufty et al. have identified calpain I-cleaved α-Syn fragments in the brains of human PD/DLB patients as well as α-Syn expressing flies using a specific antibody [27]. These results suggest that calpain I-mediated cleavage of α-Syn may be involved in the disease-linked aggregation of α-Syn in synucleinopathies.

4. Oxidative Stress and Antioxidants

Oxidative stress has been believed to play a central role in the progression of neurodegenerative diseases although its relationship with α-Syn toxicity has not been well elucidated. Dopaminergic neurons of α-Syn expressing flies have been shown to be sensitive to hyperoxia-induced oxidative stress [28]. Importantly, overexpression of Cu/Zn superoxide dismutase rescued both the dopaminergic neuron loss and locomotor dysfunction in mutant α-Syn flies. The same group also demonstrated that reduction of dopamine levels by RNAi silencing of the tyrosine hydroxylase gene decreases the neurotoxicity in α-Syn expressing flies, implying that dopamine which produces reactive oxygen species might be involved in the α-Syn-induced neurotoxicity through oxidative stress [29]. These results suggest that oxidative stress plays a significant role in the pathogenesis of PD *in vivo*.

Trinh et al. examined the involvement of the phase II detoxification pathway, specifically glutathione metabolism, in α-Syn-induced neurotoxicity in *Drosophila* models [30]. They found that the loss-of-function gene mutations affecting glutathione metabolism pathways enhance dopaminergic neuron loss in α-Syn expressing flies. Moreover, the dopaminergic neuron loss can be rescued by genetic or pharmacological interventions that increase glutathione biosynthesis or glutathione conjugation activity, suggesting that oxidative stress is involved in α-Syn-induced neurotoxicity and that induction of the phase II detoxification pathway may be a potential therapy for synucleinopathies.

In addition, feeding Nicotinamide, the principal form of niacin (vitamin B3), has been shown to improve the motor dysfunction in α-Syn expressing flies through improvement of oxidative mitochondrial dysfunction [31]. Grape extracts, which contain various polyphenols and exhibit scavenging effects on reactive oxygen species, also showed a significant improvement in locomotor function and average lifespan in α-Syn flies [32].

5. Association with Other PD-Causing Genes

Loss of function gene mutations of Parkin, an E3 ubiquitin ligase, is responsible for a rare familial form of PD, autosomal recessive juvenile Parkinsonism, which develops typical Parkinsonian symptoms as a result of midbrain dopaminergic neuron loss, but usually lacks LBs [61]. Although a direct molecular interaction between Parkin and α-Syn remains controversial, several studies have shown that coexpression of Parkin rescues α-Syn-induced dopaminergic neurodegeneration and motor dysfunction in α-Syn flies.

These studies suggest that up-regulation of Parkin expression may provide a novel therapy for PD [33–35].

Mutations in the PTEN-induced putative kinase 1 (PINK1) gene cause another form of autosomal recessive PD [62]. PINK1 has been shown to be located in mitochondria and is thought to be involved in cellular protection. Overexpression of PINK1 has been shown to rescue loss of climbing ability and neurodegeneration induced by α-Syn expression in *Drosophila* [36]. Furthermore, it has been suggested that Parkin and PINK1 function in a common pathway in maintaining mitochondrial integrity and morphology, as demonstrated using *Drosophila* models [63, 64].

6. Genomics and Proteomics Studies

One of the advantages of using *Drosophila* models in studying human diseases is the easiness to handle numerous samples at one time, which can provide us with reliable amounts of data for unbiased statistical analyses. In addition, shortness of their life span makes it convenient to perform time course analyses in relatively short time periods.

Scherzer et al. performed expression profiling analysis of α-Syn A30P flies at different disease stages using microarray and found that expression of genes involved in lipid processing, energy production, and membrane transport is significantly altered by α-Syn expression [65]. Xun et al. performed proteomic analysis of α-Syn flies at different disease stages using liquid chromatography coupled with mass spectrometry [66, 67]. They found cytoskeletal and mitochondrial protein changes in the presymptomatic and early disease stages in the α-Syn A30P expressing flies [66]. They further reported dysregulated expression of proteins associated with membrane, endoplasmic reticulum, actin cytoskeleton, mitochondria, and ribosome in the presymptomatic α-Syn A53T flies, consistent with the α-Syn A30P flies [67]. These unbiased genomics and proteomics studies especially in the presymptomatic α-Syn flies will provide us with further insight into pathomechanisms and potential therapeutic targets of synucleinopathies.

7. Concluding Remarks

As described above, α-Syn *Drosophila* models have been widely employed to uncover the molecular pathogenesis of synucleinopathies (Table 2). Most of the results reviewed here have indeed been confirmed in transgenic mouse models expressing α-Syn. As we described in the introduction, *Drosophila* is a powerful *in vivo* model to study human neurodegenerative diseases with various advantages (Table 1), especially its short life span since human neurodegenerative diseases gradually appear and progress in middle-late ages.

Genetic analyses using α-Syn expressing flies have revealed pathological associations between α-Syn and various synucleinopathy-related genes and have provided novel insights into the molecular pathogenesis of synucleinopathies. *Drosophila* models of other neurodegenerative diseases such as the polyglutamine diseases have also been established, and numerous comprehensive genetic screenings have been conducted and have elucidated previously unknown pathomechanisms, taking advantage of the characteristics of *Drosophila* [68]. Similarly, comprehensive genetic screenings using *Drosophila* models will further lead to the elucidation of the pathomechanisms of synucleinopathies including PD in the future.

On the other hand, *Drosophila* models are also suited for drug screening. Indeed, L-DOPA and dopamine agonists have been shown to exert therapeutic effects against α-Syn-induced neurotoxicity using α-Syn flies [69]. In addition, HDAC inhibitors such as sodium butyrate or SAHA, and SIRT2 inhibitors have been identified as novel therapeutic agents that protect against α-Syn-induced neurotoxicity using *Drosophila* [70, 71]. In the future, novel therapeutic candidates for synucleinopathies are expected to be developed by extensive large-scale drug screening using *Drosophila* models.

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