Chapter 15

Monitoring $\alpha$-Synuclein Proteotoxicity in Drosophila Models

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Abstract

Parkinson’s disease is the second most common neurodegenerative disease without cure. It is characterized by $\alpha$-synuclein accumulation and aggregation in dopaminergic and other types of neurons. Because $\alpha$-synuclein accumulation leads to a toxic gain of function, its ectopic expression in Drosophila has been a useful in vivo model for testing modifiers of its toxicity. This chapter describes four assays: the rapid iterative negative geotaxis, rough eye phenotype, quantification of dopaminergic neuronal loss, and measurements of circadian effects.

Key words  Drosophila, Behavior, In vivo models, Morphology analysis, Neurotoxicity

1 Introduction

Parkinson’s disease (PD) is the second most common neurodegenerative disorder, affecting 1% of people over the age of 60. Clinically, it is characterized primarily by a movement disorder causing resting tremor, bradykinesia, rigidity, postural instability, and diverse non-motor symptoms including dementia, which in community-based studies was reported in up to 80% of patients with a long disease duration. This latter finding indicates that PD is a diffuse neurodegenerative disorder. Similarly, detailed neuropathological studies have shown that intraneuronal inclusions called Lewy bodies, which are one of the cardinal histological features of the disease, are detected in numerous cortical areas and often correlate with the extent of cognitive decline [1]. Despite this diffuse evolution, the presentation to health services is commonly due to the loss of a critical number of dopaminergic neurons in the substantia nigra, but in patients with diffuse Lewy bodies disease dementia may be the presenting feature. $\alpha$-Synuclein is also the major component of glial cytoplasmic inclusions that define multiple system atrophy (MSA), a movement disorder characterized by cerebellar ataxia, parkinsonism, and autonomic dysfunction. In MSA, unlike Lewy body disease, $\alpha$-synuclein inclusions are found...
in glial cells. The identification of additional causative genes of familial PD that clinically mimic sporadic disease such as LRRK2, VPS35, Pink1, and Parkin has suggested new mechanisms of pathogenicity [2]. A major limitation in translating this basic understanding into therapies is the lack of animal models that faithfully recapitulate the human condition. In this respect, the use of simple model organisms to interrogate in vivo the function of genes against biochemical and cellular readouts has facilitated the mapping of disease-causing genes with a fly ortholog into pathways (e.g., mitophagy and endosomal-lysosomal trafficking) and suggested novel therapeutic strategies in PD.

Drosophila melanogaster is a tractable organism for modeling epistatic interactions in the context of pathological phenotypes. Some of the advantages of utilizing Drosophila for genetic studies include its short life span of 45–60 days, relatively low cost, and evolutionary conservation of most genes. About 75% of human genes have a Drosophila ortholog [3]. The development of the Gal4-UAS system [4] has transformed the tractability of this organism for gene interaction studies. This combines the yeast transcriptional activator Gal4 with UAS (upstream activation sequence), an enhancer to which Gal4 specifically binds to activate gene transcription. By fusing Gal4 to a tissue-specific promoter, it is possible to drive the expression or knockdown of genes of interest in specific cell populations [5].

Drosophila melanogaster has been extensively used to model pathogenic processes, including neurodegenerative diseases. It has been especially informative in the elucidation of the function of the recessive PD genes Pink1 and Parkin in mitochondrial homeostasis. The earliest evidence that Pink1 and Parkin function in the same pathway came from phenotypic observations in Pink1 and Parkin knockout flies which both exhibited swollen mitochondria with broken cristae, muscle dysfunction, dopaminergic neuron degeneration, and reproductive defects [6, 7]. Parkin overexpression could rescue the Pink1 mutant phenotypes whereas Pink1 overexpression did not rescue the Parkin mutant ones [7–9] suggesting a linear cascade with Pink1 upstream of Parkin. This has been delineated mechanistically in mammalian cells where Pink1 activation phosphorylates ubiquitin and Parkin, activating Parkin-mediated ubiquitination and mitochondrial autophagy [10]. Additional insights came from the study of the LRRK2 ortholog in flies, which provided early in vivo evidence for a role of this kinase in endosomal trafficking, lysosomal function [11, 12], and synaptic autophagy [13].

Flies do not have the α-synuclein (SNCA) gene and therefore are not a suitable system to investigate its function. However, in sporadic PD, neurodegeneration is thought to arise from accumulation and misfolding of α-synuclein into oligomeric fibrillar species, which acquire a toxic gain of function, partly by impairing membrane fusion events [14]. In this context, ectopic expression of
α-synuclein in different tissues using cell-type-specific drivers has been shown to recapitulate cardinal features of Parkinson’s pathology: Feany and colleagues first expressed human α-synuclein in the nervous system of flies using the elav/Gal4 system [15]. Transgenic flies showed age-dependent, progressive degeneration of the dorsomedial (PPM1/2) cluster of dopaminergic neurons. The neurodegeneration was associated with an age-dependent decline in the climbing ability of transgenic flies using a negative geostatic assay. In addition, transgenic flies were shown to have neuronal α-synuclein-immunoreactive cytoplasmic aggregates, which exhibited a fibrillar ultrastructure under electron microscopy. The fly eye consists of 760 unit eyes, called ommatidia, which contain photoreceptors and bristles. Transgenic expression of α-synuclein in the fly eye using GMR/Gal4 causes an architectural distortion, known as rough eye phenotype that is associated with retinal degeneration with vacuolization [15]. The rough eye phenotype induced by α-synuclein toxicity is reversed by specific modifiers that were also shown to directly regulate α-synuclein degradation in mammalian cells [16, 17]. The observation that modifiers such as the E3 ligase NEDD4 are protective in fly as well as rat models of α-synuclein proteotoxicity [17] supports the notion that simplified in vivo assays in flies may be especially helpful in the study of proteostasis, where key enzymes are conserved across species.

Sleep timing is partly governed by the circadian clock, which can be readily assayed in Drosophila models [18]. Sleep disturbance is well documented in Parkinson’s patients and there is emerging evidence that circadian defects occur in PD [19]. Recently, circadian defects were observed in fly models expressing α-synuclein [20] suggesting that this may be another simple behavioral readout for in vivo quantification of α-synuclein toxicity.

Here, we describe four assays of α-synuclein proteotoxicity in Drosophila: (1) rapid iterative negative geotaxis (RING) as a readout of motor activity, (2) rough eye phenotype, (3) tyrosine hydroxylase (TH) immunostaining of the adult brain to track the loss of specific dopaminergic neuronal subpopulations, and (4) measurement of locomotor activity in response to circadian rhythms as a potentially non-motor defect.

## 2 Materials

The following Drosophila lines are required: UAS-α-synuclein wild-type (8146) and A53T mutant (8148), available from the Bloomington Drosophila Stock Center. Driver lines such as GMR-Gal4 for eye-specific expression, ddc-Gal4 or TH-Gal4 for expression in the dopaminergic neurons, elav-Gal4 for pan-neuronal expression, and triple mutant TP-α-synuclein flies [21] are available from a number of laboratories. Transgenic flies expressing α-synuclein
and either RNAi or cDNA construct for the modifier of interest can be generated to assay its knockdown or overexpression against α-synuclein toxicity. Flies are typically aged for 30 days post-eclosion.

2.1 Equipment
1. Dissecting stereomicroscope.
2. Sylgard-coated Petri dishes for dissection.
3. Dumont No. 5 forceps.
4. Insect pins.
5. Microscope slides.
6. No. 1 and No. 2 coverslips.
7. Imaging fluorescent microscope.
9. DAM2 Drosophila Activity Monitor (TriKinetics).
10. Glass tubes and tube caps for the monitors (TriKinetics).
11. PSU9 Power Supply Interface Unit (TriKinetics).
12. DC Power Supply (TriKinetics).
14. FaasX software for data analysis.
15. Digital camera and timer.
16. Custom-made RING apparatus (see Fig. 1) and 50 mL Falcon tubes.
17. JEOL JSM 6390 or similar scanning electron microscope.
19. SEM stubs.

2.2 Reagents
1. Fly food—Recipes can be found at the Bloomington Drosophila Stock Center website (https://bdsc.indiana.edu/information/recipes/index.html).
2. Phosphate-buffered saline (PBS).
3. Triton X-100.
4. Schneider’s Drosophila medium.
5. 4% Paraformaldehyde solution (PFA) in PBS.
7. Primary antibody: Monoclonal mouse anti-tyrosine hydroxylase (TH) (Millipore, MAB318, 1:500).
8. Fluorescently labeled secondary antibody (e.g., Alexa 488-coupled goat anti-mouse IgG (1:1000)).
10. 100% ethanol.
11. Milli-Q-grade water.
3 Methods

3.1 Locomotion Assay

Locomotor function can be assayed using the rapid iterative negative geotaxis (RING) protocol, which is an adaptation from Gargano et al. [22].

1. Transfer 10–20 male flies in a clean vial without anesthetizing them with CO₂ as flies can take up to 24 h to fully recover from anesthesia.

2. Use 5 vials per line in each experiment (total 50 flies per line) and mark each vial with a line at the 6 cm height (see Note 1).

3. Allow 15–20 min for the flies to acclimate to the environment.

4. For simultaneous testing of multiple vials, transfer the vials into a custom-made RING apparatus (Fig. 1).

Fig. 1 Schematic illustration of the custom-made equipment for RING. This is comprised of an open-faced box with a removable lid that can be assembled in the laboratory to hold the geotaxis vials of choice in place. A digital camera is placed approximately 30 cm in front of the RING apparatus containing flies. Gentle tapping of the apparatus causes a startle-induced fall of the flies to the bottom of each vial. Images are taken at 4 s after tapping and the percentage of flies above a predefined height (in our case 6 cm) is estimated.
5. Place a digital camera in front of the apparatus (approximately 30 cm) so that the lens is at mid-height of the vials, focus and zoom the camera to capture the image of the whole vial, and set a timer to 4.0 s.

6. Tap the apparatus three times sharply ensuring that the tapping is hard enough for all the flies to startle and drop to the bottom of each vial.

7. With the third tap simultaneously start the timer and take the picture at 4 s. Repeat at least five times.

8. Quantify the percentage of flies above the 6 cm mark. In our experience this is a more accurate measurement of the locomotion defect in the α-synuclein model compared to the average distance traveled.

9. Repeat the experiment from at least two independently derived transgenic lines (see Note 2).

3.2 Rough Eye Phenotype

Although the rough eye phenotype is visible by light microscopy, for more accurate quantification of the effect of modifiers we used scanning electron microscopy (see Notes 3–6).

1. Fix age-matched, male flies in 70% ethanol.

2. Dehydrate the flies in 100% ethanol. The flies are then critically point dried and mounted on SEM stubs.

3. Sputter coat the samples with 15 nm gold nanoparticles using a Denton DV-503 vacuum evaporator or similar equipment and image on a JEOL JSM 6390 or similar scanning electron microscope.

4. Unless a complete rescue is observed, quantitate the eye phenotype, by randomly selecting 20 flies per genotype. Score the images blindly using objective features of severity. Examine the eyes for abnormal bristle orientation, ommatidial fusion or pitting, and disorganization of the ommatidial array.

5. Score the phenotype by giving one point if any of the above features is present, 2 points if it affects more than 5% of the eye, and 4 points if it affects more than 25% of the eye.

3.3 Quantification of the Loss of PPM1/2 Cluster Dopaminergic Neurons in the Brain

1. Obtain adult flies expressing UAS-α-synuclein A53T with ddc-Gal4 or elav-Gal4 and age them together with the single-transgene controls to 25 days post-eclosion.

2. Anesthetize flies under CO2, rinse them in 100% ethanol for 20 s, and place them in ice-cold PBS transferring as little ethanol as possible (see Note 7).

3. Pin one fly submerged in ice-cold Schneider’s Drosophila medium onto a Sylgard-coated dish with two insect pins.
4. Incise between the eyes on the top of the head using the forceps. Extend the opening and gently peel off the eyes one followed by the other.

5. Remove other appendages such as the proboscis and clean the brain from tracheae and floating membranes (see Note 8).

6. Place brains in 1 mL ice-cold PFA in a microcentrifuge tube and incubate rocking for 1 h at 25 °C (see Note 9).

7. Let the brains settle to the bottom of the tube, aspirate PFA, and rinse quickly twice with PBS + 0.05% triton X-100 (PBST).

8. Wash thrice for 20 min nutating in 1 mL PBST at room temperature (RT).

9. Block with 1 mL 5% NGS in PBST at RT.

10. Add anti-TH primary antibody at 1:500 in 5% NGS, and incubate at 4 °C for 48 h.

11. Wash as for point 8.

12. Add secondary antibody in 5% NGS, and incubate at 4 °C for 48 h in the dark.

13. Wash as for point 8 in the dark.

14. Prepare a microscope slide by placing a drop of SlowFade (40 μL) in the center and create a bridge by positioning broken No. 2 coverslips on the sides of the drop.

15. With a pipette and a cutoff P200 tip, transfer the brains from the tube into the drop of SlowFade.

16. Align the brains so that the posterior side faces upwards.

17. Cover them with a No. 1 coverslip and seal with nail polish.

18. Proceed to imaging on an epifluorescent or confocal fluorescent microscope.

19. Identify TH⁺ dopaminergic neurons of the PPM1/2 cluster as shown in Fig. 2 and count them.

3.4 Analysis of Circadian Rhythms in Locomotor Activity

1. Place anesthetized flies individually into glass tubes containing 5% sucrose and 2% Bacto agar designed for DAM2 monitors and close them with a cotton plug (see Note 10).

2. Fill the 32 channels of DAM2 with tubes representing one genotype.

3. Place the DAM2-s into the incubators and connect them to Power Supply Interface Unit through the phone cables.

4. Entrain flies for 3 days to 12-h light (L):12-h dark (D) cycles and then leave them in constant darkness (DD) for 10 days at a constant temperature of 25 °C.

5. Extract the locomotor activity data from the recordings only for the DD period of the experiment using the DAMSystem3.
Data Acquisition Software and DAMFileScan. Use bins of 5 min.

6. Apply the FaasX software (MacIntosh only, http://neuro.psi.cnrs.fr/spip.php?article298&lang=en) to analyze locomotor activity data. Actogram allows the visualization of activity patterns throughout the days. Cycle_p calculates the exact period length in DD. Follow the instructions in the startup kit (see Note 11).

4 Notes

1. We did not observe any desensitization of the flies on the geotaxis assay up to ten consecutive trials spaced 1 min apart.

2. It is critical not to reuse the testing vials because new flies placed into used vials do not climb to the same extent as in fresh vials.

3. We found that the α-synuclein-induced eye damage is observed more consistently when flies were crossed and larvae kept at 29 °C. Because the Gal4/UAS system is heat sensitive we observed that a more consistent phenotype at 29 °C that correlated with a higher level of α-synuclein expression. Under these conditions the rough eye phenotype was detected from day 1.

4. Expression of the A53T mutant α-synuclein consistently caused a more severe phenotype than wild-type α-synuclein, which can be mild depending on the driver. The mutant construct is more suitable for screening assays.

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**Fig. 2** Localization of PPM 1/2 neurons in the adult *Drosophila* brain. Schematic diagram of the adult fly brain (grey) viewed from the posterior side. The neuropil structures of the mushroom body (blue) and the fan-shaped body (yellow) are indicated as an aid for orientation. Cell bodies of the TH+ PPM 1/2 cluster are shown in the dorsomedial region of the brain. Note that their relative position can vary between brains.
5. Homozygous GMR-Gal4 in flies per se can cause a rough eye phenotype and therefore it is important that the driver is heterozygous.

6. We noticed that the level of expression of α-synuclein varies between different heterozygous GMR-Gal4 lines. It is therefore necessary to test and identify the best line that achieves the highest expression of α-synuclein at 29 °C without toxicity when the driver alone is present.

7. The ethanol wash before brain dissection is critical as too little wash will not soften the cuticle while too much will affect the efficiency of dissection by lysing the fat body.

8. Leaving tracheae on the brain will make it float, and thus reduces evenness of staining later.

9. Conditions for fixation with PFA depend on the antibody used for staining—Shorter times or fixation on ice can increase success of staining.

10. Ideally, flies are raised and aged on a light-dark schedule for circadian experiments to facilitate later synchronization to LD.

11. Besides circadian rhythms, sleep can also be analyzed in LD by applying a bin length of 1 min instead of 5 min and using the Matlab script insomniac (Lesley Ashmore, University of Pennsylvania).

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