Chapter 13

Drosophila melanogaster as a Model for Studies on the Early Stages of Alzheimer's Disease

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Abstract

Fruit flies (*Drosophila melanogaster*) have been widely used to study the cellular and molecular basis of human neurodegenerative disease. The biological similarities between the human and the fly have been explored successfully to further investigate the pathological basis of Alzheimer's disease (AD). Here, we discuss transgenic *Drosophila* models systems and the methodologies that have been employed in the study of AD.

Key words Alzheimer's disease, *Drosophila melanogaster*, Amyloid β peptide, Protein aggregation, Invertebrate animal model

1 Introduction

Alzheimer disease is one of the most prevalent causes of dementia in the elderly, with an estimated 35.6 million people affected worldwide in 2009. The AD population is expected to rise to 66 million by 2030 [1, 2]. Pathologically, AD is characterized by the accumulation of two very different proteins, each with a distinct distribution. Amyloid β (Aβ) peptide accumulates extracellularly in amyloid plaques while hyperphosphorylated tau, a microtubule binding protein, accumulates intracellularly in neurofibrillary tangles [3–5]. In particular, the Aβ peptide is formed by the sequential cleavage of the amyloid precursor protein (APP) when β-secretase cuts extracellularly followed by intramembraneous cleavage by γ-secretase [6]. The precise location of γ-secretase cutting determines the nature of the Aβ generated; predominant production of the shorter, less aggregation prone isoform, $A\beta_{1\text{-}40}$, has been linked to health, while the generation of the longer peptide, $A\beta_{1\text{-}42}$, is a risk for AD [7, 8]. Before Aβ peptides form plaques they appear as oligomeric aggregates and it is these forms that are thought to be the neurotoxic intermediate products that cause neuronal dysfunction and death. The biological similarities between human and *Drosophila* have been exploited with great success in the modeling of Aβ

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toxicity [9]. The fly has a brain, containing approximately 200,000 neurons, and like the vertebrate central nervous system, it is composed of a series of functionally specialized substructures [10–12]. Efforts at modeling AD in *Drosophila* have been predicated in large part on the amyloid cascade hypothesis which states that Aβ aggregation is the first step in a chain of pathological events [13, 14]. Human Aβ peptides can be expressed in the fly in two main ways. Firstly a partially humanized system has been created by Greeve and colleagues by engineering triple transgenic flies that express human wild type APP, human β-secretase (BACE) and the catalytically active subunit of fly γ-secretase (dPsn) [15]. These lines exhibit modest elevations in Aβ40 and Aβ42 peptides, thioflavin S positive amyloid plaques in the retina and an age-dependent degeneration of the photoreceptors. Retinal pathology is particularly marked in flies expressing the FAD related dPsn variant. A second model consists of flies expressing a truncated APP coupled to a secretion signal peptide but still requiring endogenous fly γ-secretase to generate Aβ [16]. Both these approaches result in the secretion of Aβ peptides from neurons and generate similar phenotypes. In this review we will focus on the simpler approach of expressing only Aβ peptides, coupled to a secretion signal peptide. In these fly models of Aβ toxicity, we have taken advantage of the widely-used gal4-UAS system that allows one line of flies that carries a gal4-responsive transgene, to be crossed to any number of tissue-specific gal4-expressing driver lines (*see* Fig. 1 and Table 1) [17].

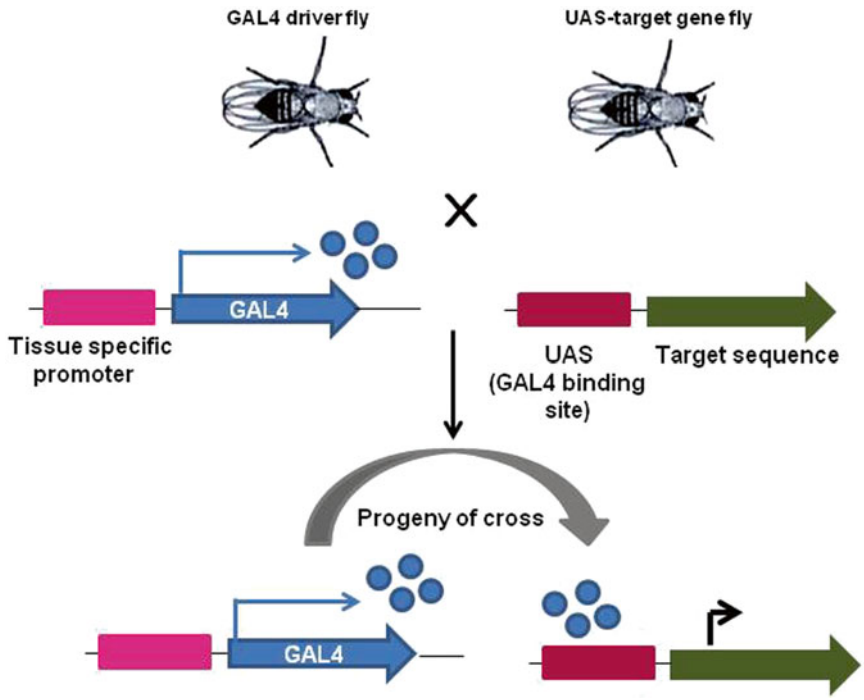

Fig. 1 Schematic representation of the GAL4-based systems for transgene expression

Table 1
Gal4 drivers for tissue specific expression in *Drosophila*

Tissue specificity	Driver line
Brain	elav-gal4
Retina	GMR-gal4
Ubiquitous	act5C-gal4

Once flies expressing Aβ peptides have been generated we then undertake molecular and biochemical analyses, along with phenotype assessment at both the micro- and macroscopic levels.

2 Materials

2.1 *Drosophila* Husbandry

1. Fly stocks: the transgenic lines carrying the Aβ transgene have been generated by a number of groups and are described elsewhere (Crowther et al. ref. 18). Other fly stocks (e.g. driver lines such as *elav^{C155}-GAL4*) are obtained from the Bloomington *Drosophila* Stock Center (Indiana University; http://flystocks.bio.indiana.edu/).
2. *Drosophila* culture medium: a mixture of 1.25 % (w/v) agar, 10.5 % (w/v) dextrose, 10.5 % (w/v) maize, 2.1 % (w/v) yeast to 80 °C is heated and then 5 mL are dispensed into tubes (12 cm × 2 cm diameter) or 30 mL into bottles (10 cm diameter), allowing the mix to cool and solidify.
3. The flies are then incubated at an appropriate temperature (range 18–29 °C) with a 12:12 h light:dark cycle at constant humidity (60 %) (*see* **Note 1**).

2.2 Protein Extraction

1. Control *w^{1118}* flies or flies expressing the Aβ transgene under the control of neuron specific driver *elav-GAL4* (*elav-GAL4*; *w^{1118}* vs *elav-GAL4*; *UAS-Aβ$_{1-42}$*).
2. Disposable pestle.
3. Electric homogenizer.
4. Bath sonicator.
5. Soluble fraction buffer: 2 % (w/v) sodium dodecyl sulfate (SDS) solution in distilled water (dH_2O) with protease inhibitors (Complete, Roche).
6. Insoluble fraction buffer: 80 % (w/v) dimethyl sulfoxide (DMSO), 50 mM Tris–HCl, pH 8.8.
7. Bench top centrifuge.

2.3 Immunoblotting

1. SDS-PAGE gel: Bis-Tris 4–12 % (w/v) gels (Invitrogen).
2. 1 × LDS sample buffer: glycerol, lithium dodecyl sulfate (LDS) sample buffer.
3. Heat block at 70 °C.
4. 2-[morpholino]ethanesulfonic acid (MES) SDS running buffer (Invitrogen).
5. Transfer membrane: Nitrocellulose membrane 0.11 μm pore (Whatman, GE Healthcare).
6. Semi-dry transfer kit (Bio-Rad).
7. Transfer buffer (Tris-glycine buffer): 0.025 M Tris, 0.192 M glycine, 20 % methanol.
8. Washing buffer: 0.05 % (v/v) Triton X-100 solution in phosphate buffer saline (PBS).
9. Blocking buffer: 5 % (w/v) non-fat milk in 0.05 % (v/v) Triton-PBS.
10. Shaker.
11. Primary antibody: anti-$A\beta_{1\text{-}16}$ (6E10) monoclonal antibody (Covance) in blocking buffer.
12. Secondary antibody: HRP-conjugated goat anti-mouse antibody (DAKO) in blocking buffer.
13. Detection: SuperSignal Chemiluminescent Pico and Femto substrates (Thermo Scientific).
14. Kodak X-Omat LS film.
15. Stripping buffer: 10 % (w/v) SDS, 0.5 M Tris–HCl, pH 6.8, 0.8 % ß-mercaptoethanol.
16. Fume hood.

2.4 Brain Dissection

1. Control w^{1118} flies or flies expressing the Aβ transgene under the control of neuron specific driver *elav-GAL4* (*elav-GAL4*; w^{1118} vs *elav-GAL4*; *UAS-*$A\beta_{1\text{-}42}$).
2. Petri dish.
3. 0.5 mL microcentrifuge tubes.
4. Fixation solution: 0.1 % (v/v) Triton X-100 in phosphate buffer (PB), pH 7.4. Prepare 4 % (w/v) paraformaldehyde (PFA) in Triton-PB.
5. Washing buffer: 0.1 % (v/v) Triton-PB.
6. Dissecting microscope.
7. Two pairs of sharp forceps.

2.5 Immunohistochemistry

1. Washing buffer: 0.1 % (v/v) Triton-PB.
2. Blocking buffer: 5 % (w/v) normal goat serum in 0.5 % (v/v) Triton-PB.

3. Primary antibody: anti-$A\beta_{1\text{-}16}$ (6E10) monoclonal antibody (Signet) in blocking buffer.
4. Secondary antibody: Alexa Fluor 488-conjugated goat anti-mouse (Invitrogen) in blocking buffer.
5. TOTO-3 iodide stain (Invitrogen) diluted in 0.5 % (v/v) Triton-PB.
6. Glass slide.
7. Cover slip.
8. Mounting solution Vectashield (Vectors Lab).
9. Nikon Eclipse C1si confocal microscope on Nikon E90i upright stand and imaging software.

2.6 Longevity Assay

1. Control w^{1118} flies or flies expressing the Aβ transgene under the control of neuron specific driver *elav-GAL4* (*elav-GAL4*; w^{1118} vs *elav-GAL4*; *UAS-*$A\beta_{1\text{-}42}$) (*see* **Notes 2** and **3**).
2. 12 cm × 2 cm diameter glass vials containing standard fly food [18].
3. Barcode that can be transferred from tube to tube (*see* **Note 4**).
4. Barcode.
5. Scanner.
6. Database software such as Flytracker2 software designed and programmed by Damian C. Crowther (www.flytracker.gen.cam.ac.uk) (*see* ref. 18).

2.7 Climbing Assay

1. Control w^{1118} flies or flies expressing the Aβ transgene under the control of neuron specific driver *elav-GAL4* (*elav-GAL4*; w^{1118} vs *elav-GAL4*; *UAS-*$A\beta_{1\text{-}42}$).
2. 25 cm × 1.5 cm diameter sterile plastic column.
3. Timer (*see* ref. 19).

2.8 Locomotor Assay

1. Control w^{1118} flies or flies expressing the Aβ transgene under the control of neuron specific driver *elav-GAL4* (*elav-GAL4*; w^{1118} vs *elav-GAL4*; *UAS-*$A\beta_{1\text{-}42}$).
2. 10 cm × 2 cm diameter glass tube.
3. iFly apparatus (iFly chamber, camera, mirror) (*see* ref. 20).

3 Methods

3.1 *Drosophila* Crossing

Figure 2 shows the crossings set up to obtain flies expressing an Aβ transgene under the control of the neuron specific driver, *elav-Gal4*, in the X chromosome. In this instance male flies, homozygous for UAS-Aβ transgenes, are crossed with *elav-Gal4* virgin females (*see* **Note 5**). Flies are then reared at an appropriate temperature (range 18–29 °C) on 12:12 h light:dark cycle at constant humidity (60 %) until they eclose (Fig. 2).

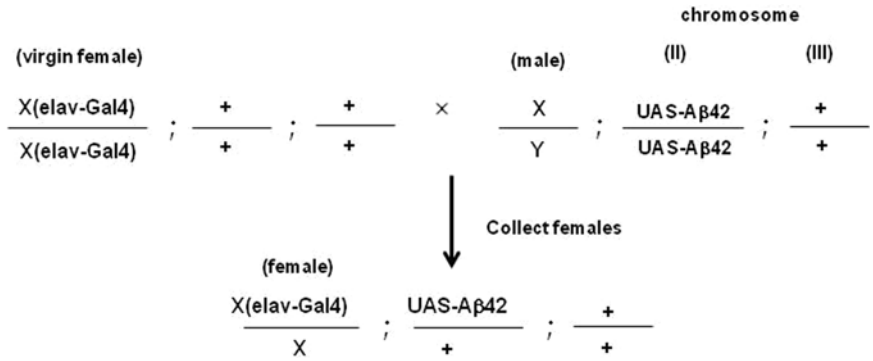

Fig. 2 Crossing scheme for generation of transgene flies. Crossing for generating flies expressing Aβ protein under the control of neuron specific driver *elav-GAL4* (*see* **Note 6**)

3.2 Soluble and Insoluble Fraction of Total Aβ Peptide in the Fly Brain

1. Male flies carrying *UAS-Aβ* transgenes are crossed with *elav-Gal4* virgin females. Developing flies are then reared at 18–29 °C. Progeny are collected 24 h after eclosion and aged for up to 30 days before being collected for the assay of soluble vs insoluble Aβ.
2. At least 30–50 flies are decapitated and the heads homogenised in 50 μL 2 % (w/v) SDS/water solution supplemented with protease inhibitors.
3. The samples are then sonicated in an ice water bath for 8 min, followed by centrifugation at 4 °C for 20 min, at 18,000×*g*.
4. After the centrifugation step, the supernatant is collected and labelled as the 'soluble fraction'. The remaining pellet is washed in PBS which is then removed after a further round of centrifugation for 20 min at 18,000×*g*. The pellet is then resuspended in 5 μL of a solution containing 80 % (w/v) DMSO and left at 55 °C in a sealed tube for 1 h.
5. 15 μL of 50 mM Tris–HCl are added, and a brief centrifugation step (18,000×*g*) is performed to eliminate debris. The supernatant is retained and labelled as the 'insoluble fraction'.
6. Soluble and insoluble samples are denatured in lithium dodecyl sulfate (LDS) sample buffer for 10 min at 70 °C. Proteins are then separated by polyacrylamide gel electrophoresis on 4–12 % (w/v) Bis-Tris gels and transferred onto a 0.1 μm pore nitrocellulose membrane using semi-dry transfer, at 15 V for 35 min.

3.3 Immunoblotting

1. The membranes are boiled for 5 min in PBS and then blocked for 1 h in 5 % (w/v) dried milk in PBS (*see* **Note 7**). The primary antibody, 6E10 is diluted 1:2,500 in 5 % (w/v) dried milk in 0.05 % (v/v) Triton-PBS and incubated with the blot overnight at 4 °C, followed by washing five times with PBS, 5 min each time.

2. The secondary antibody, HRP-labelled anti-mouse IgG, diluted 1:2,500 in 5 % (w/v) dried milk in 0.05 % (v/v) Triton-PBS is incubated with the blot for 2 h at room temperature (RT).
3. The membrane is then washed five times with PBS, 5 min each time, before developing the blot using Super Signal West Pico (for insoluble fraction) or Femto (for soluble fraction).
4. After developing, the membrane is washed with PBS and then incubated with the stripping solution at 50 °C for up to 45 min with some agitation (*see* **Note 8**).
5. Dispose of the solution and the membrane is washed five times with PBS, 5 min each time, and then blocked for 1 h in 5 % (w/v) dried milk in 0.05 % (v/v) Triton-PBS.
6. Repeat **steps 2** and **3** with new antibody. This step often includes anti-β-actin to provide a loading control for the western blot (Fig. 3).

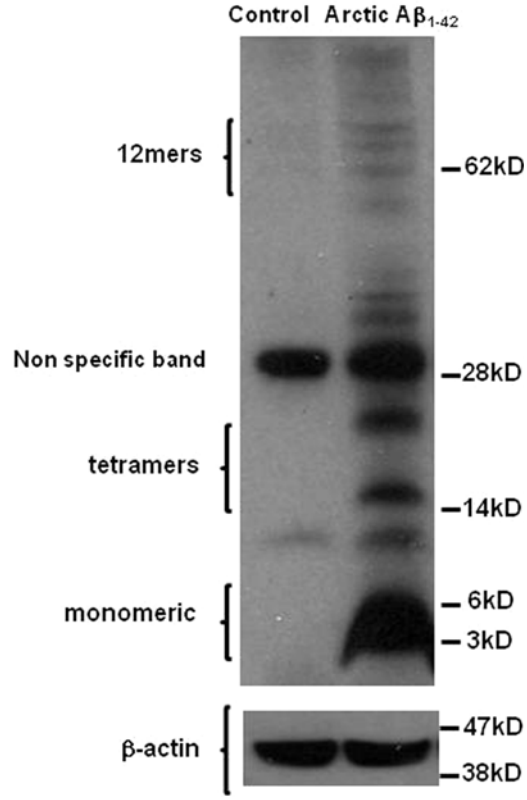

Fig. 3 Western blotting analysis of flies expressing Aβ transgenes. The western blot is performed with head extracts of *Drosophila*. The SDS soluble fraction is probed for Aβ using the monoclonal 6E10 antibody. The E22G variant of $A\beta_{1\text{-}42}$ (*Arctic* $A\beta_{1\text{-}42}$) exhibits a variety of higher molecular weight (25–65 kDa) immunoreactive species that are not present in the negative control (*Drosophila* brain extracts without Aβ expression). Loading of equal amounts total protein are confirmed using anti-β-actin antibody

3.4 Brain Dissection

1. Anaesthetize flies with ice and place them in a petri dish.
2. Prepare a 500 µL tube containing 500 µL of fresh 4 % (w/v) paraformaldehyde and place the flies into the tube and fix them for 2.5 h at room temperature (RT).
3. The samples are washed three times (10 min) with 0.1 % (v/v) Triton-PB at RT.
4. Place the samples into the petri dish filled with PB buffer and put it under a dissecting microscope.
5. The brain is removed from the head cuticle using gentle manipulations with dissection forceps. Firstly the fly should be held with forceps in the petri dish filled with PB buffer, belly up. With the second pair of forceps, gently insert one side into the cavity just below the eye to obtain a grip on the eye. Be careful to avoid internal head structures such as the brain. Gently pull the head off of the fly and discard the body. With the free forceps, obtain a grip on the other eye from the underside. Gently pull the two pairs of forceps away from each other to open the head cuticle.
6. The tissue is now ready for immunohistochemistry.

3.5 Immunohistochemistry

1. Brains are washed in 0.1 % (v/v) Triton-PB and blocked (to prevent non-specific staining), in 5 % (w/v) normal goat serum in 0.5 % (v/v) Triton-PB for 2 h at room temperature (RT). Brains are stained for Aβ using a dilution 1:1,000 of the anti Aβ monoclonal antibody 6E10 in blocking buffer for 48 h at 4 °C.
2. Brains are then washed three times for 10 min with 0.1 % (v/v) Triton-PB and left in a solution containing the secondary antibody, goat anti-mouse Alexa Fluor 488 diluted 1:1,000 in blocking buffer at 4 °C overnight.
3. After removal of the secondary antibody, brains are washed in 0.1 % (v/v) Triton-PB and stained for DNA using TOTO-3 iodide diluted in 0.1 % (v/v) Triton-PB 1:5,000 for 10 min.
4. After an additional wash in 0.1 % (v/v) Triton-PB, brains are orientated on a glass slide under the light microscope and covered with a drop of the Vectashield mounting solution.
5. Confocal laser scanning images are collected at intervals of 5 µm using a Nikon Eclipse C1si on Nikon E90i upright stand, 20× objective. Laser intensities are set at the beginning of each image acquisition session and kept constant to allow comparison of the fluorescence intensity between different samples. Images are processed using ImageJ software (Fig. 4).

3.6 Longevity Assays

1. Male flies carrying *UAS-Aβ* transgene are crossed with *elav-Gal4* virgin females. Flies are then reared at an appropriate temperature (range 18–29 °C) for survival analysis.

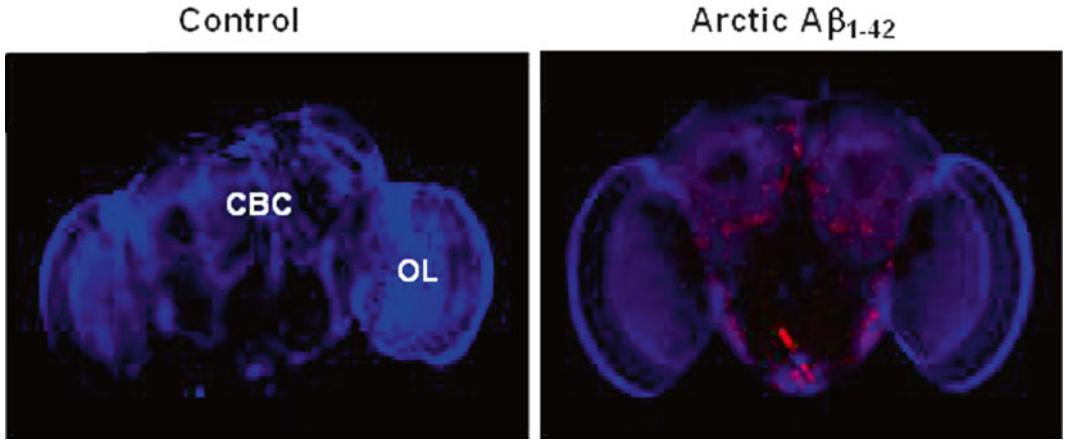

Fig. 4 Confocal micrographs of control or flies expressing Aβ transgenes under the control of the *elav-Gal4* driver. Fly brains are counter-stained with TOTO-3 (DNA staining, *blue*) and probed for amyloid with the 6E10 antibody (*red*). The *left panel* illustrates a typical *Drosophila* brain without beta-amyloid expression. Roughly, the brain is divided into the central brain complex (CBC) and the optic lobes (OL) area to each side. Flies that overexpress the E22G variant of $A\beta_{1\text{-}42}$ (*Arctic* $A\beta_{1\text{-}42}$) display more brain plaques (*right panel*). High resolution color images appear in the online version of this work

2. From the progeny, mated females flies are collected 24 h post-eclosion and divided between ten tubes, each containing ten flies. The flies are then incubated on standard fly food and yeast at an appropriate temperature [18].
3. The number of flies surviving is documented with a frequency appropriate to the lifespan of the particular flies: For short-lived flies, daily observations are made; however, for most strains, the flies are counted on days 1, 3, and 5 of a 7 days cycle. At each time point, the number of flies that are observed to die and the number of flies that are lost to follow up (for example, flies that escape or are accidentally killed) are noted. A computer database may be required to maintain large amounts of data.
4. When all the test and control flies are either dead or lost, the data can be visualized using Kaplan–Meier survival plots and statistical comparisons are performed using the log rank test (GraphPad Prism), assuming that the total population of 100 flies is homogenous. A more conservative approach is to calculate the median survival for each of the ten tubes and use a non-parametric test to assess differences in these survival estimates as described by Crowther et al. [18]. This analysis provides median and mean survival times for a population and determines the significance of any difference in survival times (Fig. 5). Typically, reliable data are derived from the assessment of at least 50 flies from three or more independent crosses.

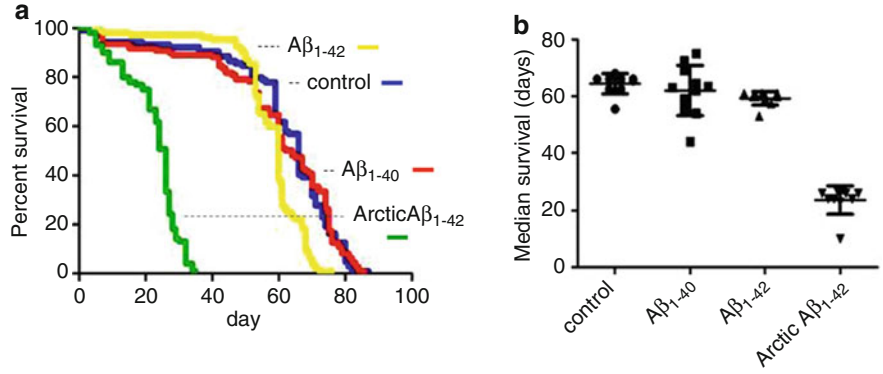

Fig. 5 Longevity assay of flies expressing Aβ transgenes. The longevity of control w^{1118} flies is compared with flies expressing *Arctic* $A\beta_{1\text{-}42}$, $A\beta_{1\text{-}42}$ or $A\beta_{1\text{-}40}$ peptide under the control of *elav-GAL4* driver. The survival curves are drawn using GraphPad Prism (**a**). Expression of the $A\beta_{1\text{-}42}$ peptide in the fly's nervous tissue reduces the longevity of the flies and this is further accelerated by expression of the transgene containing the E22G variant of $A\beta_{1\text{-}42}$ (*Arctic* $A\beta_{1\text{-}42}$). The analysis of the survival data shows that expression of the $A\beta_{1\text{-}42}$ peptide in the fly's nervous tissue results in reduction in the median survival (**b**). In this example there are no remarkable differences in the survival profiles for flies expressing $A\beta_{1\text{-}40}$ compared to the control flies indicating no toxic effect on adult neurons; however $A\beta_{1\text{-}42}$, and more potently *Arctic* $A\beta_{1\text{-}42}$, reduce fly longevity. Color figure appears in the online version of this work

3.7 Climbing Assays

1. Male flies carrying *UAS-Aβ* transgenes are crossed with *elav-Gal4* virgin females. Flies are then reared at an appropriate temperature (range 18–29 °C).
2. From the progeny, mated females flies are collected 24 h post-eclosion, and sets of 15 flies are placed at the bottom of a clean plastic column of 1.5 cm diameter and 25 cm height.
3. The flies are bought down to the bottom of the column by firmly tapping the tube on the bench and start the timer.
4. After 45 s the flies at the top of the column (N_{top}) and the flies remaining at the bottom (N_{bot}) are counted.
5. The flies are bought down again by firmly tapping the tube on the bench and repeat this for a total of three climbing opportunities at 1 min intervals.
6. Another test run is performed with the same parameters.
7. A performance index is calculated for each group of flies and repeated tests are performed to allow statistical comparison of different fly populations. The performance index (PI) is calculated as $PI = (15 + N_{top} - N_{bot})/30$. Statistical analysis are performed using the two-tailed Student's *t*-test (*see* ref. 19).

3.8 Locomotor Assay

1. Male flies carrying *UAS-Aβ* transgenes are crossed with *elav-Gal4* virgin females. Flies are then reared at an appropriate temperature (range 18–29 °C).
2. From the progeny, mated females flies are collected 24 h post-eclosion and, for each condition, five flies are placed in each of four different tubes of 2 cm diameter and 10 cm height. Videos are taken on days 1, 3, 5, 8, 10, 12, 15 and 18.
3. Test tubes are tapped down into the iFly apparatus at time zero and videos recorded for 90 s, with the tube tapped down again into the apparatus after 30 and 60 s to give a total of three climbing opportunities. All videos are processed with the iFly software (*see* ref. 20). Tapping of the test tube results in flies dropping to the bottom of the tube on impact and immediate initiation of upwards movement is triggered by innate negative geotaxis reflexes in the flies. The trajectories of all flies are recorded by the computer for each of the three 30 s movie chips, each in form of time-stamped Cartesian coordinates of the derived fly positions in 3D space.
4. The Cartesian coordinates for the locomotion behavior are analysed to extract statistical descriptors of the fly populations. The statistical properties of these parameters are useful to discriminate flies at various stages of their lives with a high level of confidence (Fig. 6).

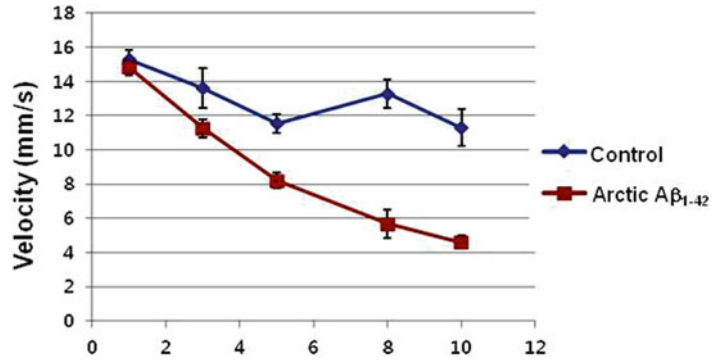

Fig. 6 Locomotor assay of flies expressing Aβ transgenes. The locomotor result for flies of control w^{1118} is compared with flies expressing *Arctic* $A\beta_{1\text{-}42}$, peptide under the control of the *elav-Gal4*. Velocities are assigned to bins of width 3 mm per second, with the first bin starting at zero millimetres per second. Error bars indicate the bin-wise standard deviation over three replicates of 20 flies each. The analysis of the locomotor data shows that expression of the $A\beta_{1\text{-}42}$ peptide in the fly's nervous tissue resulted in reduction in the locomotor activity

4 Notes

1. Higher temperature induces stronger Aβ expression, which reduces the life span of flies expressing Aβ transgenes under the control of neuron-specific driver *elav-GAL4*.
2. Ensure that flies are entered into longevity assays within a few days to avoid differences in food and environmental conditions affecting the outcome.
3. Once virgin females have been collected, add the males. Females will then begin laying fertile eggs soon after. Check the flies in 2–3 days to see if larvae are present and then remove the parent flies.
4. Anonymize tubes by using barcode labels and also randomize tubes within trays so that the operator does not know the identity of the flies being followed.
5. Virgin females are required for each crossing. In a mixed culture females remain virgin for only 8 h at 25 °C after eclosion and must be collected within this time frame. To confirm that females are virgin they can be cultured on standard food for 2–3 days at 25 °C. The presence of larvae on the food indicates that at least one female is non-virgin. In this case all the females in the particular tube will be discarded. If only eggs are present, the females are all likely to be virgin.
6. There is a choice of collecting females or virgin females. We choose to allow 24 h mating so they are all females.
7. Boiling of the nitrocellulose is required for western blot detection of Aβ from fly brains. Bring the blot to boil in the microwave and allow it to stand for 5 min before placing it in the blocking buffer.
8. Put the membrane with the buffer into a small plastic box with a tight lid. Use a volume of buffer that covers the whole membrane.

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