Phenotypic differences between *Drosophila* Alzheimer’s disease models expressing human Aβ42 in the developing eye and brain

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**ABSTRACT**

*Drosophila melanogaster* expressing amyloid-β42 (Aβ42) transgenes have been used as models to study Alzheimer’s disease. Various Aβ42 transgenes with different structures induce different phenotypes, which make it difficult to compare data among studies which use different transgenic lines. In this study, we compared the phenotypes of four frequently used Aβ42 transgenic lines, UAS-AB42^2X, UAS-AB42^BL33770, UAS-AB42^11C39, and UAS-AB42^29.3. Among the four transgenic lines, only UAS-AB42^2X has two copies of the upstream activation sequence-amyloid-β42 (UAS-Aβ42) transgene, while remaining three have one copy. UAS-Aβ42^BL3370 has the 3′ untranslated region of *Drosophila* α-tubulin, while the others have that of SV40. UAS-Aβ42^11C39 and UAS-Aβ42^29.3 have the rat pre-proenkephalin signal peptide, while UAS-Aβ42^2X and UAS-AB42^BL3370 have that of the fly argos protein. When the transgenes were expressed ectopically in the developing eyes of the flies, UAS-AB42^2X transgene resulted in a strongly reduced and rough eye phenotype, while UAS-Aβ42^BL3370 only showed a strong rough eye phenotype; UAS-AB42^11C39 and UAS-Aβ42^29.3 had mild rough eyes. The levels of cell death and reactive oxygen species (ROS) in the eye imaginal discs were consistently the highest in UAS-Aβ42^2X, followed by UAS-AB42^BL3370, UAS-AB42^11C39, and UAS-Aβ42^29.3. Surprisingly, the reduction in survival during the development of these lines did not correlate with cell death or ROS levels. The flies which expressed UAS-AB42^11C39 or UAS-Aβ42^29.3 experienced greatly reduced survival rates, although low levels of ROS or cell death were detected. Collectively, our results demonstrated that different *Drosophila* AD models show different phenotypic severity, and suggested that different transgenes may have different modes of cytotoxicity.

**Abbreviations:** Aβ42: amyloid-β42; AD: Alzheimer’s disease; UAS: upstream activation sequence

**Introduction**

Alzheimer’s disease (AD) is the most common neurodegenerative disorder and is characterized by amyloid plaques, neurofibrillary tangles, and loss of neurons (Mattson 2004). There are several hypotheses to explain the cause of AD (Hardy & Higgins 1992; Markesbery 1997; Francis et al. 1999; Hardy & Selkoe 2002; Berridge 2010; Maccioni et al. 2010). Among them, the amyloid hypothesis states that most of AD pathologies are caused by deposition of amyloid-β42 (Aβ42) peptide, which is generated by proteolytic processing of the amyloid precursor protein (Hardy & Higgins 1992).

Based on well-developed genetic tools, such as the upstream activation sequence (UAS)-GAL4 system, by which the expression of desired genes can be regulated, *Drosophila* has been used as an animal model to study AD (Lee et al. 2014, 2016; Bang et al. 2016). To date, different groups have generated several different transgenic lines that can be used for the ectopic expression of human Aβ42. In the present study, we selected four lines to investigate the relationship between transgene structure and their functions (Table 1 and Figure 1). UAS-Aβ42^2X (Casas-Tinto et al. 2011) contains two serially concatenated copies of the transgene with an argos signal peptide and SV40 poly A tail (Figure 1). UAS-Aβ42^BL3370 (Singh & Mahoney 2011) contains one copy of the transgene with the argos signal peptide and the *Drosophila* α-tubulin 3′ untranslated region (UTR) (Figure 1). The α-tubulin 3′ UTR is thought to provide stability to transgenes linked with it (Ollmann et al. 2000; Liu et al. 2015); it is supposed to increase Aβ42 protein levels by prolonging the RNA half-life. UAS-Aβ42^11C39 (Iijima et al. 2008) and
UAS-β\textsubscript{42}\textsuperscript{2X} (Casas-Tinto et al. 2011. Hum Mol Genet 20(11):2144–2160) both contain a copy of same transgene with a pre-proenkephalin signal peptide and an SV40 poly A tail (Figure 1).

Although several Drosophila Aβ\textsubscript{42} transgenic lines were developed and used in a variety of studies, their phenotypic differences have not been studied in detail. Therefore, we compared the phenotypes of the four representative UAS-Aβ\textsubscript{42} lines under the same experimental conditions. They showed different Aβ\textsubscript{42} expression levels and phenotypic severity in eyes and neurons. Interestingly, the level of reactive oxygen species (ROS) generation did not correlate with survival rate in this comparative study.

### Materials and methods

#### Drosophila strains

Glass multimer reporter (GMR)-GAL4 (BL9146), embryonic lethal abnormal vision (elav)-GAL4 (BL458), and UAS-Aβ\textsubscript{42}BL33770 (BL33770) were acquired from the Bloomington Drosophila Stock Center. UAS-Aβ\textsubscript{42}\textsuperscript{2X}, UAS-Aβ\textsubscript{42}H\textsuperscript{29.3}, and UAS-Aβ\textsubscript{42}11C\textsuperscript{39} were provided by Dr Pedro Fernandez-Funez (University of Florida, USA), Dr Mary Konsolaki (University of Rutgers, USA), and Dr Koichi M. Iijima (University of Thomas Jefferson, USA), respectively.

### Table 1. The list of studies in that used the UAS-Aβ\textsubscript{42} transgenic lines.

| Line   | Publication                                      |
|--------|-------------------------------------------------|
| 2X     | Casas-Tinto et al. 2011. Hum Mol Genet 20(11):2144–2160 |
| BL33770| Liu et al. 2015. Biol Pharm Bull 38(12):1891–1901 |
| N1C39  | Iijima et al. 2008. PLoS One 3(2):e1703          |
| H\textsuperscript{29.3} | Finelli et al. 2004. Mol Cell Neurosci 26(3):365–375 |

#### Figure 1. Constructs in four different UAS-Aβ\textsubscript{42} lines. The schematic figures show the constructs in the four UAS-Aβ\textsubscript{42} lines, UAS-Aβ\textsubscript{42}\textsuperscript{2X}, UAS-Aβ\textsubscript{42}BL33770, UAS-Aβ\textsubscript{42}11C\textsuperscript{39}, and UAS-Aβ\textsubscript{42}H\textsuperscript{29.3}, which have differences in the number of copies, signal peptides, and poly A tails. UAS-Aβ\textsubscript{42}\textsuperscript{2X} has two copies of the UAS-Aβ\textsubscript{42} sequence, while the others have one copy. UAS-Aβ\textsubscript{42}\textsuperscript{2X} and UAS-Aβ\textsubscript{42}BL33770 have the signal peptide-encoding region of the fly argos gene, whereas UAS-Aβ\textsubscript{42}\textsuperscript{11C\textsuperscript{39}} and UAS-Aβ\textsubscript{42}H\textsuperscript{29.3} have that of the rat pre-proenkephalin gene. UAS-Aβ\textsubscript{42}BL33770 carries the poly A tail of α-tubulin, and the others contain that of SV40.
**Thioflavin S staining**

Thioflavin S staining was performed as described previously by Iijima et al. (2004). Whole brains were dissected, permeabilized, and incubated overnight at 4°C in 50% ethanol containing 0.125% thioflavin S (Sigma-Aldrich). The samples were rinsed with 50% ethanol and phosphate buffered saline (PBS) containing 0.5% Triton X-100, and examined using confocal microscopy.

**Immunohistochemistry**

Immunohistochemistry was performed as described previously by Jeong et al. (2015). Whole brains were dissected and blocked with 5% normal goat serum and 2% bovine serum albumin in PBS containing 0.5% Triton X-100. They were incubated for 48 h with anti-Aβ42 antibodies (1:200; Santa Cruz Biotechnology) at 4°C and washed four times with PBS containing 0.5% Triton X-100. Samples were then incubated overnight with Alexa-Fluor-488-labeled anti-mouse antibody (1:200; Invitrogen) at 4°C and washed four times with PBS containing 0.5% Triton X-100.

**Acridine Orange staining**

Acridine orange (AO) staining was performed as described previously by Hong et al. (2012) and Park et al. (2013). The eye discs of stage L3 larvae were dissected rapidly in PBS and incubated for 5 min with $1.6 \times 10^{-6}$ M AO (Sigma-Aldrich). After rinsing twice for 5 min in PBS, the samples were analyzed using a fluorescence microscope (Carl Zeiss).

**Dihydroethidium staining**

For dihydroethidium (DHE) staining, the eye discs of stage L3 larvae were dissected in Schneider’s medium at room temperature, and incubated with Schneider’s medium containing the $3.0 \times 10^{-6}$ M DHE dye (Invitrogen Molecular Probes) for 5 min in the dark. They were then washed with Schneider’s medium, and observed under a fluorescence microscope (Carl Zeiss).

**Analysis of Drosophila development**

Fifty embryos of each genotype were collected in vials that contained standard cornmeal media and incubated at 25°C. Survival scores (the ratio of the number of adult male flies raised from collected embryos against half the total number of collected embryos) were obtained for each group. The experiment was repeated six times.

**Climbing assay**

The climbing assay was performed as previously reported by Hwang et al. (2013) with some modifications. The experiment was conducted with 80 male flies. Ten male flies were collected in each climbing assay vial, and the flies were tapped down to the bottom of the vial. Then, the number of flies that climbed to the top of the vial within 15 s was counted. Ten trials were performed for each group. Climbing scores (the ratio of the number of flies that reached the top against the total number of flies) were obtained for each group, and the mean climbing scores for the 10 repeated tests were compared.

**Statistics**

In all experiments, data were analyzed using one-way ANOVA followed by a Tukey–Kramer multiple comparison test. Statistical results were exhibited as means ± SEM. Decisive values were expressed by asterisks (*p < .05, **p < .01, and ***p < .001). Eye size was gauged using ImageJ software (National Institutes of Health).

**Results**

**The levels of Aβ42 protein and its aggregates in the developing eyes and brains of Drosophila AD models**

To characterize the four different Aβ42 transgenic lines, we measured the levels of Aβ42 aggregates and its protein abundance. As expected, the Aβ42 protein and its aggregation level in both the developing eyes and brain were the highest in the UAS-Aβ422X line (Figure 2), which contains two copies of the Aβ42 transgene (Figure 1). The second highest was the UAS-Aβ42BL33770 line (Figure 2), which has an argos signal peptide and poly A tail of fly α-tubulin (Figure 1). The expression level of Aβ42 protein by the UAS-Aβ4211C39 line was higher than that of UAS-Aβ42H29.3 (Figure 2), despite having Aβ42 transgenes with the same structure (Figure 1), which suggested that their difference might be caused by a position effect.

We also measured Aβ42 aggregate levels by thioflavin S staining, which is a commonly used method to detect amyloid fibrils, but not monomers (Yamamoto & Hirano 1986). The levels of Aβ42 aggregates were proportional to the protein levels (Figure 2), which indicated that the aggregation properties of the protein produced by the transgenes were similar.
The levels of cell death induced by the four different \( \text{A}\beta 42 \) transgenes

Next, we examined the cell death induced by the transgenes in developing eyes, which have been used frequently to measure cell death (Lee et al. 2014). Ectopic \( \text{A}\beta 42 \) expression resulted in severely reduced and rough eyes in the UAS-\( \text{A}\beta 42^{2X} \) lines when reared at both 25°C and 29°C (Figure 3(a)–(d)). However, the eye phenotype of flies expressing the UAS-\( \text{A}\beta 42^{BL33770} \) transgene depended on the rearing temperature. The reduced and rough eye phenotype appeared only at 29°C, while the rough eye phenotype without size reduction appeared at 25°C (Figure 3(a)–(d)). The flies expressing UAS-\( \text{A}\beta 42^{11C39} \) and UAS-\( \text{A}\beta 42^{H29.3} \) showed very mild rough eye phenotype at 29°C (Figure 3(b)).

The number of dead cells in the developing eyes was consistently the highest in UAS-\( \text{A}\beta 42^{2X} \), followed by UAS-\( \text{A}\beta 42^{BL33770} \), UAS-\( \text{A}\beta 42^{11C39} \), and UAS-\( \text{A}\beta 42^{H29.3} \) (Figure 3(e) and 3(f)).

The levels of ROS in the flies expressing the four different \( \text{A}\beta 42 \) transgenes

ROS generation is an important pathological characteristic of AD, and ROS is closely associated with neuronal cell death (Markesbery 1997); therefore, we also measured the ROS levels using DHE staining in the eye imaginal discs expressing the \( \text{A}\beta 42 \) transgenes. A prominent amount of ROS was detected in the eye imaginal discs expressing the \( \text{A}\beta 42 \) transgenes. A prominent amount of ROS was detected in the eye imaginal discs expressing UAS-\( \text{A}\beta 42^{2X} \) and UAS-\( \text{A}\beta 42^{BL33770} \), while
little was observed in the discs expressing UAS-\(\beta_42^{11C39}\) and UAS-\(\beta_42^{H29.3}\) (Figure 4).

The phenotypes of the flies expressing the \(\beta_42\) transgenes in neurons

We also examined the effects of transgene expression in neurons during development by calculating the survival rates, which were the ratio of emerged adults from eggs. Interestingly, the trend of decreased survival in each \(\beta_42\)-expressing line was different from the levels of \(\beta_42\) expression or the eye phenotype (Figure 5(a)). The survival rate of the UAS-\(\beta_42^{BL33770}\) flies was the lowest, while the UAS-\(\beta_42^{11C39}\) and UAS-\(\beta_42^{H29.3}\) flies also showed significantly reduced survival (Figure 5(a)).

To compare the effects of \(\beta_42\) expression on adult neurological function, the locomotor activities of the flies expressing the transgenes were measured. Surprisingly, the trend in the locomotor dysfunction levels in the UAS-\(\beta_42^{X}\) and UAS-\(\beta_42^{BL33770}\) lines was quite different from that of their survival rates (Figure 5(b)). Although the survival rate of UAS-\(\beta_42^{BL33770}\) flies was extremely low (12%), upon emerging, they only showed a moderate locomotor defect (Figure 5(b)), which suggested that the surviving flies may be relatively healthy.

Discussion

In this study, we compared the expression levels of \(\beta_42\) and the phenotypes of flies expressing four frequently used UAS-\(\beta_42\) transgenes. The relative expression levels of \(\beta_42\) in the transgenic lines are similar in both the developing eyes and brain. Both the \(\beta_42\) proteins and its aggregation levels were consistently the highest in the developing eyes and brain of UAS-\(\beta_42^{X}\) line, followed by UAS-\(\beta_42^{BL33770}\), UAS-\(\beta_42^{11C39}\), and UAS-\(\beta_42^{H29.3}\). However, the effects of \(\beta_42\) expression on the phenotypes in these lines were different in these tissues. The eyes of flies expressing UAS-\(\beta_42^{2X}\) or UAS-\(\beta_42^{BL33770}\) showed severe defects, while UAS-\(\beta_42^{11C39}\) or UAS-\(\beta_42^{H29.3}\) flies had very mild rough eye phenotypes, which correlated with \(\beta_42\) protein levels. In contrast, the severity of neuronal phenotypes in each transgenic line did not correlate with \(\beta_42\) protein levels. When the transgenes were expressed pan-neuronally using the elav-GAL4 driver, the survival rate was reduced significantly in both UAS-\(\beta_42^{11C39}\) and
UAS-\(A\beta42^{29,3}\), unlike their eye phenotypes. This discrepancy in the effects of \(A\beta42\) in the different tissues might be caused by the difference in susceptibility between neurons and non-neuronal cells. In support of this notion, a previous study showed that A\(\beta\) oligomer administration induced cell death in primary cultures of

**Figure 4.** The ROS levels in the flies expressing four different \(A\beta42\) transgenes. (a) Fluorescent microscopic images of DHE-stained eye imaginal discs expressing human \(A\beta42\) using four different transgenic lines at 29°C. Magnification of the pictures, ×200. (b) A graph showing ROS levels, which were detected by DHE staining (Tukey–Kramer test, \(n \geq 18\), **\(p < .01\), ***\(p < .001\); NS, not significant).

**Figure 5.** Survival rates and climbing ability of neuronal \(A\beta42\)-expressing flies with four different \(A\beta42\) transgenes. (a) A graph showing the survival rates of *Drosophila* expressing human \(A\beta42\) in their brains using four different transgenic lines at 25°C (Tukey–Kramer test, \(n \geq 180\), ***\(p < .001\)). (b) A graph showing the climbing ability of \(A\beta42\)-expressing flies at 25°C (Tukey–Kramer test, \(n \geq 80\), *\(p < .05\), ***\(p < .001\)).
rat cortical neurons, but not in astrocytes (Ebenezer et al. 2010). The hypersensitivity of neuronal cells to Aβ oligomers might reflect the high level of Aβ oligomer receptors, such as the receptor for advanced glycation end products (Du Yan et al. 1996) and prions (Laurén et al. 2009), or erroneous cell cycle activation by the Aβ protein in neurons (Caricasole et al. 2003). Although the detailed mechanism is not clear, our data suggest that the Aβ hypersensitivity of neuronal cells is conserved in Drosophila.

We also found that the survival rate of the flies expressing UAS-Aβ42 BL33770 in neurons was the lowest, while Aβ42 expression levels of these flies are much lower than that of flies expressing UAS-Aβ42 x transgene. This result suggests that the neurotoxicity of Aβ42 is not simply determined by Aβ42 levels. This phenomenon is also well known in human brain. That is, the degree of cognitive impairment in AD patients does not correlate well with the brain Aβ deposits number (Hardy & Selkoe 2002). However, the soluble Aβ concentrations were inversely correlated with synapse loss in AD patients and distinguished AD patients from high pathology control patients (Lue et al. 1999), which suggests that soluble Aβ42 oligomers, but not insoluble Aβ42 deposits, are responsible for AD pathology such as synapse loss. Therefore, the unexpected highly decreased survival rate of Aβ42 BL33770-expressing flies would be the result from the high level of soluble Aβ42 oligomer generation in this line.

The difference between the constructs of the transgenes in different UAS-Aβ42 lines might also be associated with their phenotypic variation. The different secretory abilities of the Aβ42 peptide expressed from each transgenic line might explain the unexpected strong reduction of survival during the development of flies expressing UAS-Aβ42 11C39 or UAS-Aβ42 29.3 in neurons. As these lines contain a mammalian signal peptide, Aβ42 proteins might be secreted less efficiently in these lines compared to UAS-Aβ42 x and UAS-Aβ42 BL33770 lines, which contain a Drosophila signal peptide. In that case, flies with UAS-Aβ42 11C39 and UAS-Aβ42 29.3 might secrete little Aβ42 out of the cells, resulting in intracellular Aβ42 accumulation that would damage mitochondria. In contrast, the UAS-Aβ42 x and UAS-Aβ42 BL33770 lines secreted most of the Aβ42 proteins outside the cells, while relatively little accumulates in the cytoplasm. Further studies on the Aβ42 secretion for each transgenic line are needed to reveal the detailed mechanism of Aβ42 cytotoxicity.

In addition, the effect of different genetic backgrounds should be considered. Although we used the same GAL4 lines to express the four different Aβ42 transgenes ectopically, the transgenic lines have different genetic backgrounds, which could affect the phenotypes produced by the transgenes. Therefore, to exclude this possibility completely, further studies should be conducted with the new transgenes with clear genetic backgrounds, which can be achieved by backcrossing to the same control line, such as w1118.

In conclusion, our data demonstrate that different Drosophila AD models show different phenotypic severity in different tissues, and suggest that different Aβ42 transgenes might have different modes of cytotoxicity. Therefore, AD models should be designed for the specific aims of each study.

Disclosure statement
No potential conflict of interest was reported by the authors.

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