

Phenotypic differences between *Drosophila* Alzheimer's disease models expressing human A β 42 in the developing eye and brain

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ABSTRACT

Drosophila melanogaster expressing amyloid- β 42 (A β 42) transgenes have been used as models to study Alzheimer's disease. Various A β 42 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 A β 42 transgenic lines, UAS-A β 42^{2X}, UAS-A β 42^{BL33770}, UAS-A β 42^{11C39}, and UAS-A β 42^{H29.3}. Among the four transgenic lines, only UAS-A β 42^{2X} has two copies of the upstream activation sequence-amyloid- β 42 (UAS-A β 42) transgene, while remaining three have one copy. UAS-A β 42^{BL33770} has the 3' untranslated region of *Drosophila a-tubulin*, while the others have that of SV40. UAS-A β 42^{11C39} and UAS-A β 42^{H29.3} have the rat pre-proenkephalin signal peptide, while UAS-A β 42^{2X} and UAS-A β 42^{BL33770} have that of the fly argos protein. When the transgenes were expressed ectopically in the developing eyes of the flies, UAS-A β 42^{2X} transgene resulted in a strongly reduced and rough eye phenotype, while UAS-A β 42^{BL33770} only showed a strong rough eye phenotype; UAS-A β 42^{H29.3} and UAS-A β 42^{11C39} 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-A β 42^{2X}, followed by UAS-A β 42^{BL33770}, UAS-A β 42^{11C39}, and UAS-A β 42^{H29.3}. 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-A β 42^{11C39} or UAS-A β 42^{H29.3} 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: A β 42: amyloid- β 42; AD: Alzheimer's disease; UAS: upstream activation sequence

ARTICLE HISTORY

Received 25 December 2016
Revised 6 March 2017
Accepted 17 March 2017

KEYWORDS



Alzheimer's disease; amyloid- β 42; *Drosophila*; UAS-A β 42

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; Bertridg 2010; Maccioni et al. 2010). Among them, the amyloid hypothesis states that most of AD pathologies are caused by deposition of amyloid- β 42 (A β 42) 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 A β 42. In the present study, we selected four lines to investigate the relationship between transgene structure and their functions (Table 1 and Figure 1). UAS-A β 42^{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-A β 42^{BL33770} (Singh & Mahoney 2011) contains one copy of the transgene with the argos signal peptide and the *Drosophila a-tubulin* 3' untranslated region (UTR) (Figure 1). The *a-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 A β 42 protein levels by prolonging the RNA half-life. UAS-A β 42^{11C39} (Iijima et al. 2008) and

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Table 1. The list of studies in that used the *UAS-Aβ42* transgenic lines.

Line	Publication
2X	Casas-Tinto et al. 2011. <i>Hum Mol Genet</i> 20(11):2144–2160 Ambegaokar & Jackson 2011. <i>Hum Mol Genet</i> 20(24):4947–4977 Fernandez-Funez et al. 2015. <i>Hum Mol Genet</i> 24(21):6093–6105
BL33770	Liu et al. 2015. <i>Biol Pharm Bull</i> 38(12):1891–1901 Lee et al. 2016. <i>Dis Model Mech</i> 9(3):295–306 Chouhan et al. 2016. <i>Acta Neuropathol Commun</i> 4(1):62 Liu et al. 2016. <i>Am J Chin Med</i> 44(7):1325–1347
11C39	Iijima et al. 2008. <i>PLoS One</i> 3(2):e1703 Iijima-Ando et al. 2008. <i>J Biol Chem</i> 283(27):19066–19076 Chiang et al. 2009. <i>FASEB J</i> 23(6):1969–1977 Chiang et al. 2010. <i>Proc Natl Acad Sci USA</i> 107(15):7060–7065 Iijima et al. 2010. <i>Hum Mol Genet</i> 19(15):2947–2957 Lee et al. 2012. <i>Nat Commun</i> 3:1312 Wang et al. 2012. <i>Proc Natl Acad Sci USA</i> 109(41):16743–16748 Lang et al. 2012. <i>PLoS Genet</i> 8(4):e1002683 Lang et al. 2013. <i>Neurobiol Aging</i> 34(11):2604–2612 Lin et al. 2014. <i>Aging Cell</i> 13(3):507–518 Ando et al. 2016. <i>PLoS Genet</i> 12(3):e1005917
H29.3	Finelli et al. 2004. <i>Mol Cell Neurosci</i> 26(3):365–375 Cao et al. 2008. <i>Genetics</i> 178(3):1457–1471 Ling et al. 2009. <i>PLoS One</i> 4(1):e4201 Sanokawa-Akakura et al. 2010. <i>PLoS One</i> 5(1):e8626 Ling & Salvaterra 2011. <i>Acta Neuropathol</i> 121(2):183–191 Lee et al. 2011. <i>Mol Cells</i> 31(4):337–342 Hong et al. 2012. <i>Biochem Biophys Res Commun</i> 419(1):49–53 Lüchtenborg & Katanaev 2014. <i>Mol Brain</i> 7:81 Liu et al. 2015. <i>Biol Pharm Bull</i> 38(12):1891–1901 Lee et al. 2016. <i>Dis Model Mech</i> 9(3):295–306 Gerstner et al. 2016. <i>J Neurosci Res</i> DOI:10.1002/jnr.23778 Liu et al. 2016. <i>Am J Chin Med</i> 44(7):1325–1347

UAS-Aβ42^{H29.3} (Finelli et al. 2004) both contain a copy of same transgene with a pre-proenkephalin signal peptide and an SV40 poly A tail (Figure 1).

Although several *Drosophila* *Aβ42* 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-Aβ42* lines under the same experimental conditions. They showed different *Aβ42* 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-Aβ42^{BL33770}* (BL33770) were acquired from the Bloomington *Drosophila* Stock Center. *UAS-Aβ42^{2X}*, *UAS-Aβ42^{H29.3}*, and *UAS-Aβ42^{11C39}* 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.

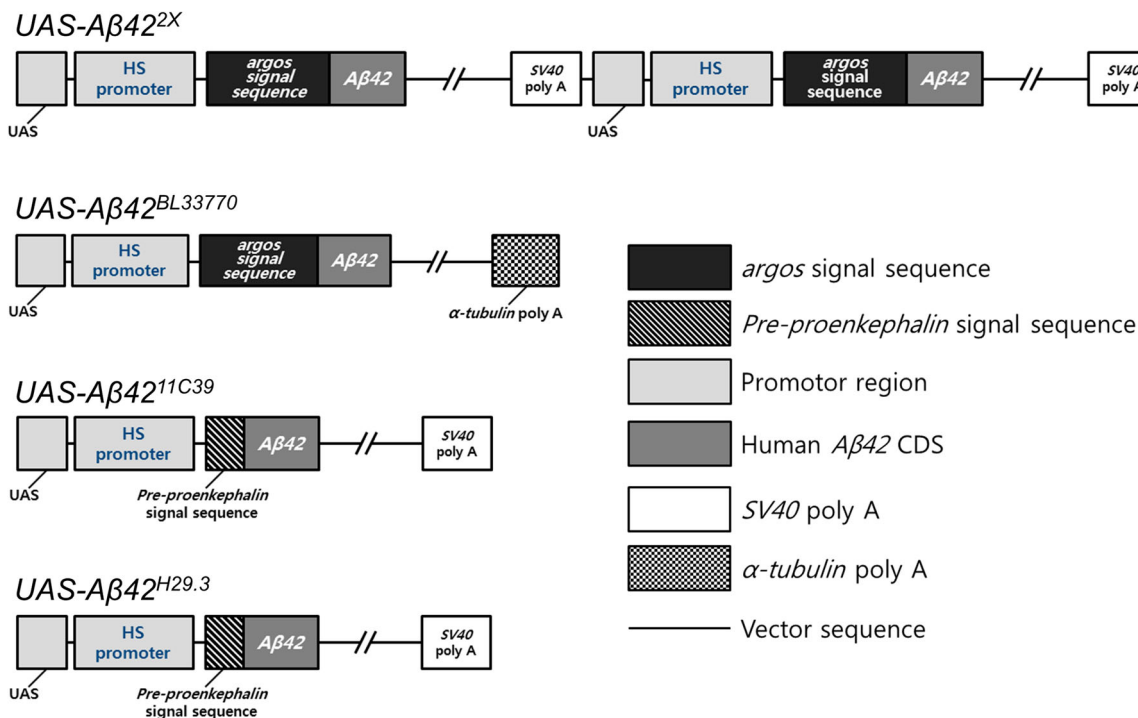


Figure 1. Constructs in four different *UAS-Aβ42* lines. The schematic figures show the constructs in the four *UAS-Aβ42* lines, *UAS-Aβ42^{2X}*, *UAS-Aβ42^{BL33770}*, *UAS-Aβ42^{11C39}*, and *UAS-Aβ42^{H29.3}*, which have differences in the number of copies, signal peptides, and poly A tails. *UAS-Aβ42^{2X}* has two copies of the *UAS-Aβ42* sequence, while the others have one copy. *UAS-Aβ42^{2X}* and *UAS-Aβ42^{BL33770}* have the signal peptide-encoding region of the fly *argos* gene, whereas *UAS-Aβ42^{11C39}* and *UAS-Aβ42^{H29.3}* have that of the rat *pre-proenkephalin* gene. *UAS-Aβ42^{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-A β 42 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 1.6×10^{-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 3.0×10^{-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 \pm 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 A β 42 protein and its aggregates in the developing eyes and brains of Drosophila AD models

To characterize the four different A β 42 transgenic lines, we measured the levels of A β 42 aggregates and its protein abundance. As expected, the A β 42 protein and its aggregation level in both the developing eyes and brain were the highest in the UAS-A β 42^{2X} line (Figure 2), which contains two copies of the A β 42 transgene (Figure 1). The second highest was the UAS-A β 42^{BL33770} line (Figure 2), which has an argos signal peptide and poly A tail of fly α -tubulin (Figure 1). The expression level of A β 42 protein by the UAS-A β 42^{11C39} line was higher than that of UAS-A β 42^{H29.3} (Figure 2), despite having A β 42 transgenes with the same structure (Figure 1), which suggested that their difference might be caused by a position effect.

We also measured A β 42 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 A β 42 aggregates were proportional to the protein levels (Figure 2), which indicated that the aggregation properties of the protein produced by the transgenes were similar.

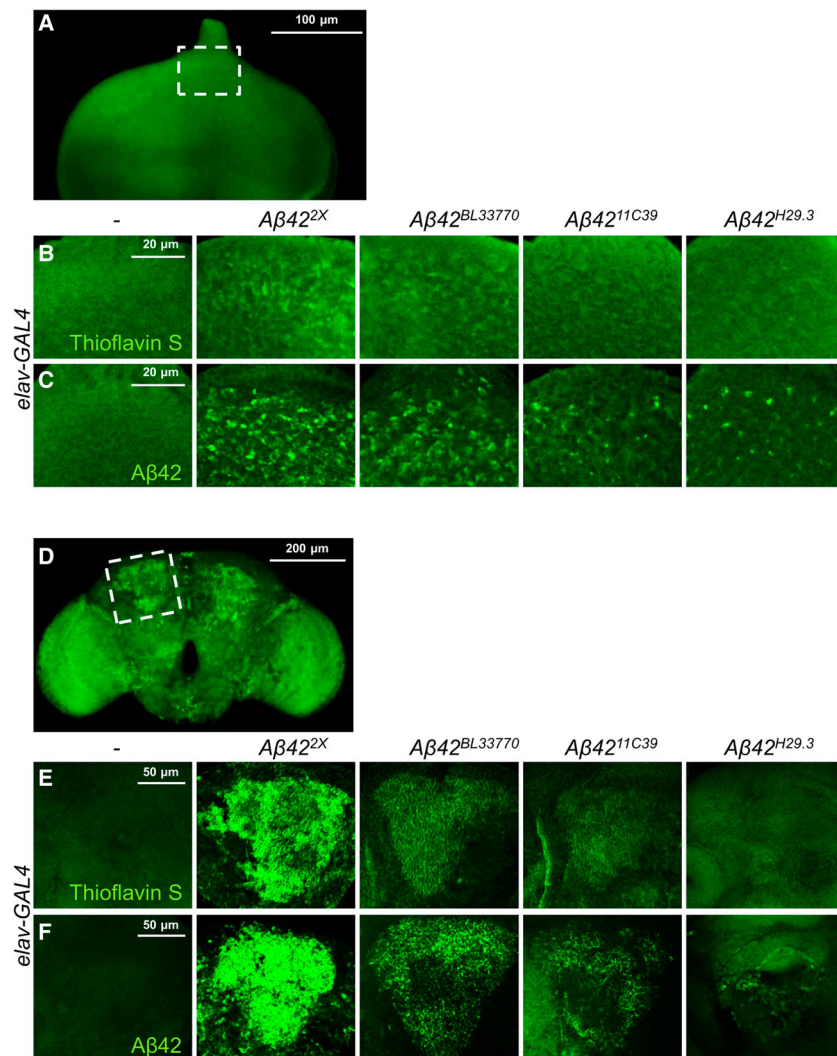


Figure 2. The A β 42 levels and extent of aggregation in the eye imaginal discs (a–c) and the adult brains (d–f) of the flies expressing different A β 42 transgenes. Representative images of thioflavin S staining (a, b, d, e) and A β 42-immunostaining (c, f) in eye imaginal discs and adult brains. (b) and (c) correspond to the dotted area in (a), while (e) and (f) correspond to the dotted area in (d). The human A β 42 transgenes were expressed in *Drosophila* eye imaginal discs at 29°C and neurons at 25°C, respectively. Magnification of the pictures: (a) $\times 200$, (b, c, e, f) $\times 400$, and (d) $\times 100$.

The levels of cell death induced by the four different A β 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 A β 42 expression resulted in severely reduced and rough eyes in the *UAS-A β 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-A β 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-A β 42^{11C39}* and *UAS-A β 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-A β 42^{2X}*, followed by *UAS-A β 42^{BL33770}*, *UAS-A β 42^{11C39}*, and *UAS-A β 42^{H29.3}* (Figure 3(e) and 3(f)).

The levels of ROS in the flies expressing the four different A β 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 A β 42 transgenes. A prominent amount of ROS was detected in the eye imaginal discs expressing *UAS-A β 42^{2X}* and *UAS-A β 42^{BL33770}*, while

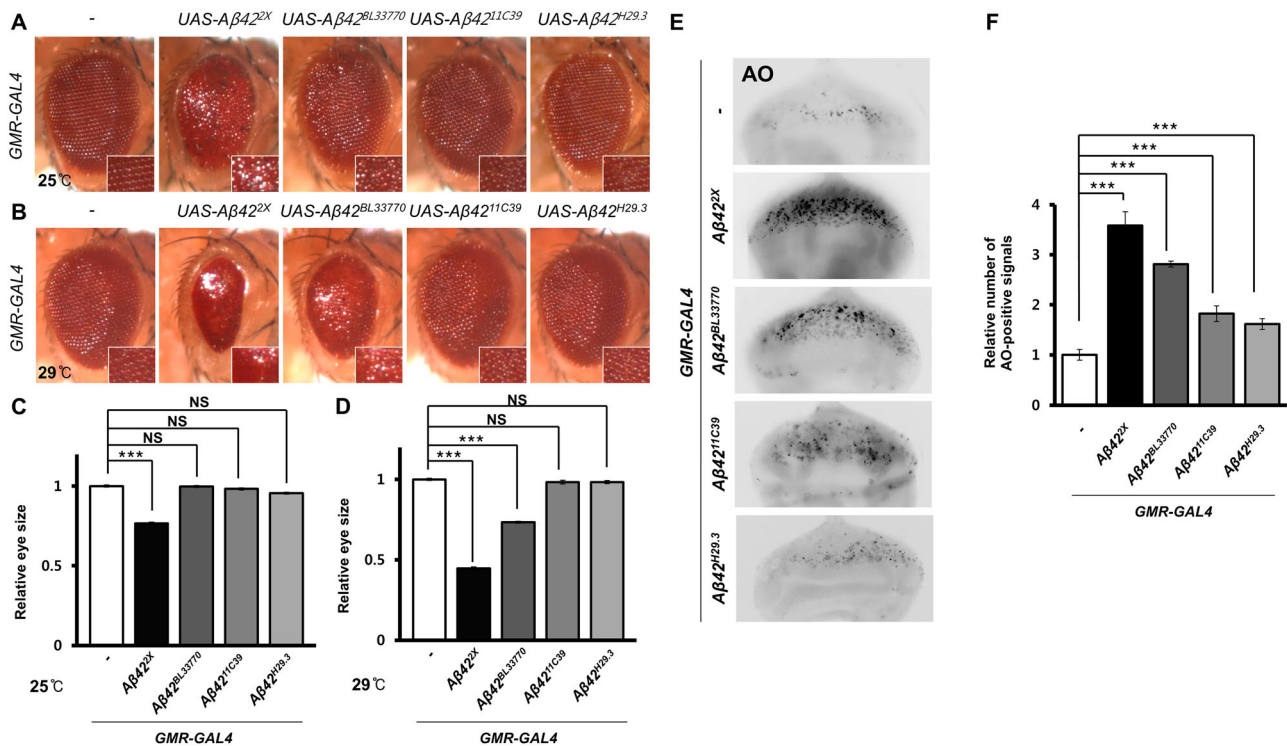


Figure 3. The morphology and cell death of *Drosophila* eyes expressing four *Aβ42* transgenes. (a, b) Pictures showing the eyes of flies expressing different *Aβ42* transgenes at different temperatures. Magnification of the pictures, $\times 50$. (c, d) Graphs showing the relative sizes of the eyes of each experimental group (Tukey–Kramer test, $n \geq 19$, $***p < .001$, NS, not significant). (e) Fluorescent microscopic images of AO-stained eye imaginal discs expressing human *Aβ42* using four different transgenic lines at 29°C. Magnification of the pictures, $\times 200$. (f) A graph showing the relative number of AO-positive signals in the eye imaginal disc of each experimental group (Tukey–Kramer test, $n \geq 17$, $***p < .001$).

little was observed in the discs expressing *UAS-Aβ42^{11C39}* and *UAS-Aβ42^{H29.3}* (Figure 4).

The phenotypes of the flies expressing the *Aβ42* 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 *Aβ42*-expressing line was different from the levels of *Aβ42* expression or the eye phenotype (Figure 5(a)). The survival rate of the *UAS-Aβ42^{BL33770}* flies was the lowest, while the *UAS-Aβ42^{11C39}* and *UAS-Aβ42^{H29.3}* flies also showed significantly reduced survival (Figure 5(a)).

To compare the effects of *Aβ42* 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-Aβ42^{2X}* and *UAS-Aβ42^{BL33770}* lines was quite different from that of their survival rates (Figure 5(b)). Although the survival rate of *UAS-Aβ42^{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 *Aβ42* and the phenotypes of flies expressing four frequently used *UAS-Aβ42* transgenes. The relative expression levels of *Aβ42* in the transgenic lines are similar in both the developing eyes and brain. Both the *Aβ42* proteins and its aggregation levels were consistently the highest in the developing eyes and brain of *UAS-Aβ42^{2X}* line, followed by *UAS-Aβ42^{BL33770}*, *UAS-Aβ42^{11C39}*, and *UAS-Aβ42^{H29.3}*. However, the effects of *Aβ42* expression on the phenotypes in these lines were different in these tissues. The eyes of flies expressing *UAS-Aβ42^{2X}* or *UAS-Aβ42^{BL33770}* showed severe defects, while *UAS-Aβ42^{11C39}* or *UAS-Aβ42^{H29.3}* flies had very mild rough eye phenotypes, which correlated with *Aβ42* protein levels. In contrast, the severity of neuronal phenotypes in each transgenic line did not correlate with *Aβ42* protein levels. When the transgenes were expressed pan-neuronally using the *elav-GAL4* driver, the survival rate was reduced significantly in both *UAS-Aβ42^{11C39}* and

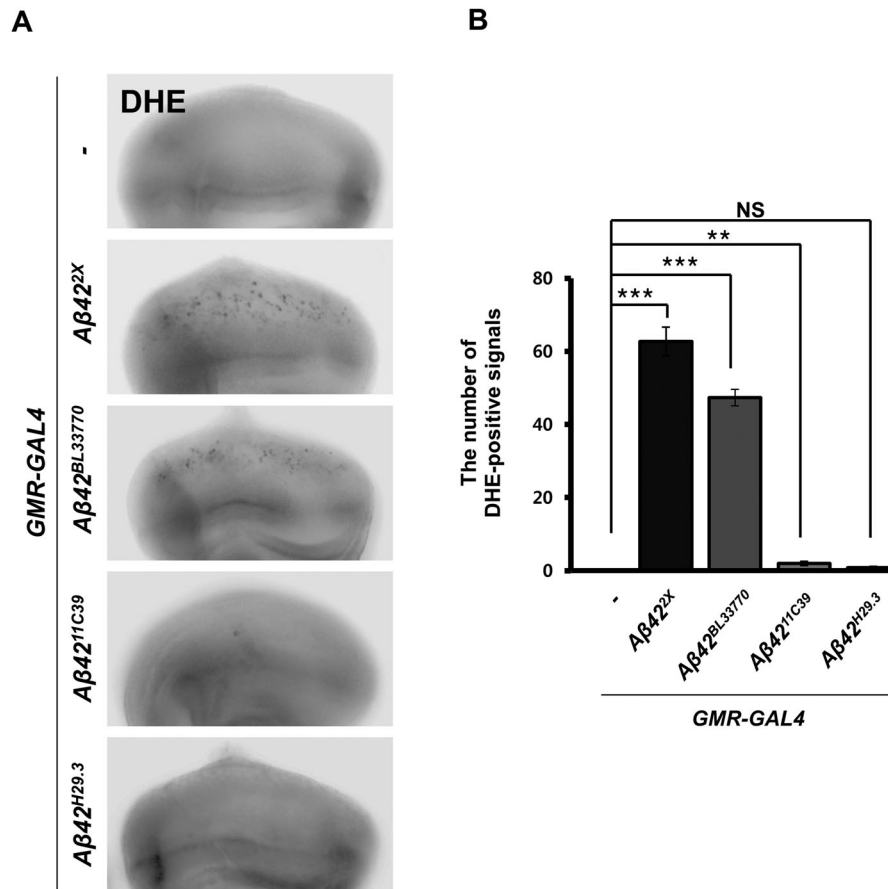


Figure 4. The ROS levels in the flies expressing four different $A\beta 42$ transgenes. (a) Fluorescent microscopic images of DHE-stained eye imaginal discs expressing human $A\beta 42$ using four different transgenic lines at 29°C. Magnification of the pictures, $\times 200$. (b) A graph showing ROS levels, which were detected by DHE staining (Tukey–Kramer test, $n \geq 18$, ** $p < .01$, *** $p < .001$; NS, not significant).

$UAS-A\beta 42^{H29.3}$, unlike their eye phenotypes. This discrepancy in the effects of $A\beta 42$ 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

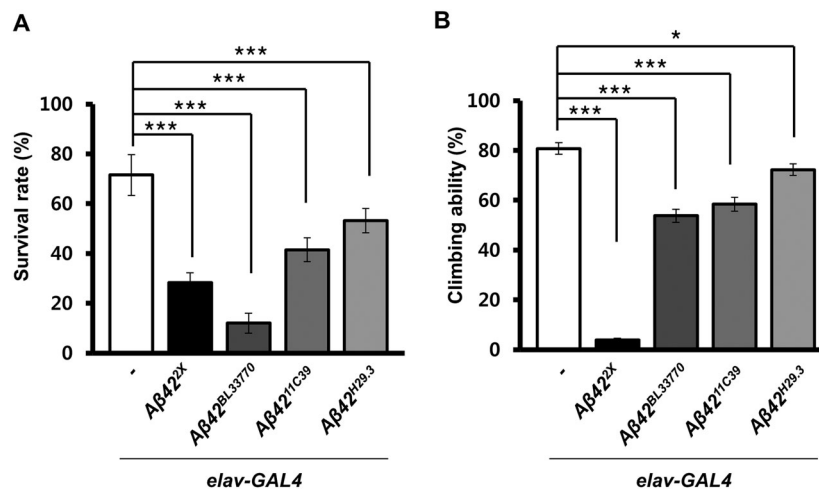


Figure 5. Survival rates and climbing ability of neuronal $A\beta 42$ -expressing flies with four different $A\beta 42$ transgenes. (a) A graph showing the survival rates of *Drosophila* expressing human $A\beta 42$ in their brains using four different transgenic lines at 25°C (Tukey–Kramer test, $n \geq 180$, *** $p < .001$). (b) A graph showing the climbing ability of $A\beta 42$ -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 A β oligomers might reflect the high level of A β 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 A β protein in neurons (Caricasole et al. 2003). Although the detailed mechanism is not clear, our data suggest that the A β hypersensitivity of neuronal cells is conserved in *Drosophila*.

We also found that the survival rate of the flies expressing *UAS-A β 42^{BL33770}* in neurons was the lowest, while A β 42 expression levels of these flies are much lower than that of flies expressing *UAS-A β 42^{2X}* transgene. This result suggests that the neurotoxicity of A β 42 is not simply determined by A β 42 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 A β deposits number (Hardy & Selkoe 2002). However, the soluble A β 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 A β 42 oligomers, but not insoluble A β 42 deposits, are responsible for AD pathology such as synapse loss. Therefore, the unexpected highly decreased survival rate of *A β 42^{BL33770}*-expressing flies would be the result from the high level of soluble A β 42 oligomer generation in this line.

The difference between the constructs of the transgenes in different *UAS-A β 42* lines might also be associated with their phenotypic variation. The different secretory abilities of the A β 42 peptide expressed from each transgenic line might explain the unexpected strong reduction of survival during the development of flies expressing *UAS-A β 42^{11C39}* or *UAS-A β 42^{H29.3}* in neurons. As these lines contain a mammalian signal peptide, A β 42 proteins might be secreted less efficiently in these lines compared to *UAS-A β 42^{2X}* and *UAS-A β 42^{BL33770}* lines, which contain a *Drosophila* signal peptide. In that case, flies with *UAS-A β 42^{11C39}* and *UAS-A β 42^{H29.3}* might secrete little A β 42 out of the cells, resulting in intracellular A β 42 accumulation that would damage mitochondria. In contrast, the *UAS-A β 42^{2X}* and *UAS-A β 42^{BL33770}* lines secreted most of the A β 42 proteins outside the cells, while relatively little accumulates in the cytoplasm. Further studies on the A β 42 secretion for each transgenic line are needed to reveal the detailed mechanism of A β 42 cytotoxicity.

In addition, the effect of different genetic backgrounds should be considered. Although we used the same *GAL4* lines to express the four different A β 42 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 A β 42 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.

Funding

This paper was supported by Konkuk University in 2014.

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