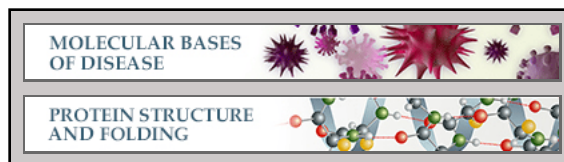


**Molecular Bases of Disease:**  
**Expression in *Drosophila* of Tandem  
Amyloid  $\beta$  Peptides Provides Insights into  
Links between Aggregation and  
Neurotoxicity**



Elena Speretta, Thomas R. Jahn, Gian  
Gaetano Tartaglia, Giorgio Favrin, Teresa P.  
Barros, Sara Imarisio, David A. Lomas, Leila  
M. Luheshi, Damian C. Crowther and  
Christopher M. Dobson

*J. Biol. Chem.* 2012, 287:20748-20754.

doi: 10.1074/jbc.M112.350124 originally published online March 29, 2012

---

Access the most updated version of this article at doi: [10.1074/jbc.M112.350124](https://doi.org/10.1074/jbc.M112.350124)

Find articles, minireviews, Reflections and Classics on similar topics on the [JBC Affinity Sites](#).

Alerts:

- [When this article is cited](#)
- [When a correction for this article is posted](#)

[Click here](#) to choose from all of JBC's e-mail alerts

Supplemental material:

<http://www.jbc.org/content/suppl/2012/03/29/M112.350124.DC1.html>

This article cites 29 references, 9 of which can be accessed free at  
<http://www.jbc.org/content/287/24/20748.full.html#ref-list-1>

# Expression in *Drosophila* of Tandem Amyloid $\beta$ Peptides Provides Insights into Links between Aggregation and Neurotoxicity<sup>\*[5]</sup>

Received for publication, February 13, 2012, and in revised form, March 26, 2012. Published, JBC Papers in Press, March 29, 2012, DOI 10.1074/jbc.M112.350124

Elena Speretta<sup>‡§1</sup>, Thomas R. Jahn<sup>‡§1,2</sup>, Gian Gaetano Tartaglia<sup>§3</sup>, Giorgio Favrin<sup>¶1</sup>, Teresa P. Barros<sup>‡§5</sup>, Sara Imarisio<sup>‡</sup>, David A. Lomas<sup>||</sup>, Leila M. Luheshi<sup>§4</sup>, Damian C. Crowther<sup>||5</sup>, and Christopher M. Dobson<sup>§6</sup>

From the <sup>‡</sup>Department of Genetics, University of Cambridge, Downing Street, Cambridge CB2 3EH, United Kingdom, the <sup>§</sup>Department of Chemistry, University of Cambridge, Lensfield Road, Cambridge CB2 1EW, United Kingdom, the <sup>¶</sup>Department of Biochemistry, University of Cambridge, Sanger Building, 80 Tennis Court Road, Cambridge CB2 1GA, United Kingdom, and the <sup>||</sup>Department of Medicine, University of Cambridge, Cambridge Institute for Medical Research, Hills Road, Cambridge CB2 0XY, United Kingdom

**Background:** Investigating the kinetics of A $\beta$  peptide aggregation *in vivo* is vital to understanding Alzheimer disease.

**Results:** Linking two A $\beta_{40}$  or A $\beta_{42}$  peptides together increases their aggregation rates in *Drosophila*, but only increases the neurotoxicity of A $\beta_{42}$ .

**Conclusion:** Increasing the rate of aggregation of A $\beta$  increases amyloid deposition but not necessarily toxicity.

**Significance:** The toxicity of A $\beta$  depends on the mechanism and not just the rate of amyloid formation.

The generation and subsequent aggregation of amyloid  $\beta$  (A $\beta$ ) peptides play a crucial initiating role in the pathogenesis of Alzheimer disease (AD). The two main isoforms of these peptides have 40 (A $\beta_{40}$ ) or 42 residues (A $\beta_{42}$ ), the latter having a higher propensity to aggregate *in vitro* and being the main component of the plaques observed *in vivo* in AD patients. We have designed a series of tandem dimeric constructs of these A $\beta$  peptides to probe the manner in which changes in the aggregation kinetics of A $\beta$  affect its deposition and toxicity in a *Drosophila melanogaster* model system. The levels of insoluble aggregates were found to be substantially elevated in flies expressing the tandem constructs of both A $\beta_{40}$  and A $\beta_{42}$  compared with the equivalent monomeric peptides, consistent with the higher effective concentration, and hence increased aggregation rate, of the peptides in the tandem repeat. A unique feature of the A $\beta_{42}$  constructs, however, is the appearance of high levels of soluble oligomeric aggregates and a corresponding dramatic increase in their *in vivo* toxicity. The toxic nature of the A $\beta_{42}$  peptide *in vivo* can therefore be attributed to the higher kinetic stability of the oligomeric intermediate states that it populates relative to those of A $\beta_{40}$  rather than simply to its higher rate of aggregation.

The misfolding and subsequent aberrant aggregation of proteins into a range of potentially toxic conformers underlie many

age-related neurodegenerative diseases (1). Although a relatively small fraction of proteins is found to be associated with such diseases, the intrinsic ability to self-assemble into stable and organized amyloid aggregates is a generic feature of all such molecules (2). Whereas organisms ranging from *Escherichia coli* to humans can derive functional advantage from the amyloidogenic propensity of some proteins (3), amyloid formation is associated predominantly with cytotoxicity and disease (1). The kinetics and thermodynamics of protein aggregation and amyloid formation have been studied extensively *in vitro* (4, 5), but less is understood about the critical steps, particularly those relating to the formation of toxic species, that govern the analogous processes *in vivo*. A detailed molecular description of these processes is essential if we are to intervene in a rational manner to prevent or treat the many diseases that are linked to protein misfolding and aggregation.

In this paper we describe an *in vivo* approach for investigating the aggregation behavior of amyloid  $\beta$  (A $\beta$ )<sup>7</sup> peptides, which in their fibrillar amyloid forms are the primary constituent of the senile plaques in patients with Alzheimer disease (AD). Such amyloid deposits are present in the brains of many elderly people, whether or not they are suffering from dementia (6), and are composed of two predominant isoforms, A $\beta_{40}$  and A $\beta_{42}$ . The longer isoform, A $\beta_{42}$ , has been found to aggregate more rapidly into fibrils and, despite constituting only a small fraction of the soluble forms of A $\beta$  peptides in the brain, is the major species found in plaques (7–9). However, the aggregation reactions for the two A $\beta$  isoforms *in vitro* have been shown to differ not only in their overall rates but also in the nature of the prefibrillar intermediates they generate (10–12).

We have shown previously that it is possible to investigate the significance of kinetic factors in fibril formation *in vitro* by conjugating multiple copies of a protein to one another in a

\* This work was supported by a combined grant from the Medical Research Council and Engineering and Physical Sciences Research Council G0700990.

⌘ Author's Choice—Final version full access.

[5] This article contains supplemental Fig. 1 and Materials and Methods.

<sup>1</sup> Both authors are joint first authors.

<sup>2</sup> Supported by the Wellcome Trust.

<sup>3</sup> Present address: Centre for Genomic Regulation, Universitat Pompeu Fabra, Barcelona, Spain.

<sup>4</sup> To whom correspondence may be addressed. E-mail: lml25@cam.ac.uk.

<sup>5</sup> Alzheimer's Research UK Senior Research Fellow. To whom correspondence may be addressed. E-mail: dcc26@cam.ac.uk.

<sup>6</sup> To whom correspondence may be addressed. E-mail: cmd44@cam.ac.uk.

<sup>7</sup> The abbreviations used are: A $\beta$ , amyloid  $\beta$ ; AD, Alzheimer disease; GMR, glass multimer reporter.

head-to-tail fashion using flexible linkers that allow the individual peptides of the tandem repeats to interact in an optimal manner, while substantially increasing their effective local concentration (13, 14). Here, we use this strategy to design tandem repeats to probe the aggregation behavior of the A $\beta$  peptide *in vivo* and its links to neurotoxicity. We demonstrate that expression of these tandem A $\beta$  peptides in the brains of *Drosophila melanogaster* provides insights into the mechanisms of A $\beta$  aggregation *in vivo*, the extent to which such mechanisms differ for the A $\beta_{40}$  and A $\beta_{42}$  peptides, and how these differences relate to their relative neurotoxicities.

## EXPERIMENTAL PROCEDURES

### Generation of Transgenic *Drosophila*

Tandem A $\beta$  transgenes were synthesized (Eurofins MWG Operon, Munich, Germany) using insect optimized codons. A $\beta$  peptides within tandem constructs are linked either directly or by a 12- (GGGGSGGGSGG) or a 22- (GGGGSGGGSGGGSGGGSGGG) amino acid linker, as indicated in the text.

These linker regions were chosen on the basis of previous work (13) and of Monte Carlo simulations designed to estimate the degree of conformational flexibility associated with the various linkers. Each construct was subcloned into the pUASTattB (GenBank Accession number EF362409) plasmid downstream of a secretory signal peptide derived from the *Drosophila* necrotic gene (28). Site-specific transgenesis to the 51D locus (yM{int.Dm}ZH2Aw\*;M{3xP3RFP.attP}ZH-51D) was achieved using the  $\phi$ C31 system (15). The transgenic lines were backcrossed for six generations into w<sup>1118</sup> flies to obtain isogenic lines. To analyze the effect of A $\beta$  expression, transgenic UAS-A $\beta$  lines were subsequently crossed to GAL4 driver lines, allowing a tissue-specific expression. Double transgenic flies were generated to express two identical transgenes (51D/51D) using the same driver. Importantly, a 51D line without insert but crossed to the GAL4 driver line was used as a negative control for all crosses to account for genetic effects and the possible consequences of GAL4 protein expression in specific tissues.

### Biochemical Analysis

**SDS-PAGE**—Twenty fly heads were homogenized in 2% (w/v) SDS in water, sonicated for 480 s, and centrifuged at 18,000  $\times$  g for 20 min at 4  $^{\circ}$ C. The supernatant was collected as the “SDS-soluble fraction.” The remaining pellet (the “SDS-insoluble fraction”) was washed in PBS before being resuspended in 5  $\mu$ l of a 80% (v/v) dimethyl sulfoxide, 20% (v/v) water solution and incubated for 1 h at room temperature before the addition of 15  $\mu$ l of 50 mM Tris-HCl, pH 8.8. The samples were again sonicated for 480 s in a water bath, and a brief centrifugation was performed to eliminate any visible debris. The supernatant was collected as the SDS-insoluble fraction. These fractions were then separated by SDS-PAGE and probed for A $\beta$  using a mouse monoclonal anti-A $\beta$  antibody (6E10; Covance) as described previously (17).

**Filter Retardation Assay**—Protein aggregates in brain extracts from flies reared at 25  $^{\circ}$ C and collected 24 h after eclosion were detected using a filter retardation assay. Fifteen fly heads were homogenized in 60  $\mu$ l of a 2% (w/v) SDS solution and

sonicated for 8 min on ice. Samples were briefly centrifuged to pull down debris, and total protein concentration was measured by BCA assay (Thermo Scientific Pierce). Equal amounts of protein for each sample were then centrifuged at 18,000  $\times$  g for 20 min at 4  $^{\circ}$ C. The supernatant was removed and loaded on a SDS-polyacrylamide gel, transferred onto a membrane, and probed with a  $\beta$ -actin antibody. The pellet was washed with PBS and resuspended in 20 mM Tris-HCl, pH 8.0, 15 mM MgCl<sub>2</sub>, 0.5 mg/ml DNase I and incubated for 1 h at 37  $^{\circ}$ C. The samples were then loaded onto a nitrocellulose membrane (0.11- $\mu$ m pore size) using a 96-well dot blotting apparatus (Bio-Rad). Once the samples had passed through the membrane under vacuum, the membranes were removed from the apparatus and boiled for 5 min in PBS and incubated in blocking buffer containing 5% (w/v) milk in PBS with 0.05% (w/v) Triton X-100. The immunodetection of A $\beta$  peptides was then performed as described previously (17). The signal intensity of each spot was quantified by densitometry using ImageJ software (distributed by National Institutes of Health) and normalized to the intensity of the corresponding  $\beta$ -actin band from the immunoblotted SDS-polyacrylamide gel. Statistical comparisons between groups were made using ANOVA followed by Dunnett's Multiple Comparison Test. All statistical analysis was performed using GraphPad Prism (GraphPad Software).

### Longevity Assays

Flies expressing A $\beta$  variants were crossed with the elav<sub>c155</sub>-GAL4 driver line. From the progeny, 100 mated female flies were collected on the day of eclosion, and their longevity was analyzed as described previously (29). Differences in survival were analyzed using the Kaplan-Meier survival plots and log-rank analysis (GraphPad Prism). Statistical significance was set at  $p < 0.05$ .

### Immunohistochemistry

Fly brains were dissected from adult flies expressing tandem A $\beta$  peptides under the control of *GMR-GAL4* 24 h after eclosion and immunostained with the mouse anti-A $\beta$  antibody 6E10 as described previously (17). Confocal scanning images were collected using identical acquisition parameters at intervals of 5  $\mu$ m using a Nikon Eclipse C1si on Nikon E90i upright stand (Nikon Corporation) with a 20 $\times$  objective. Images were projected and processed using ImageJ software.

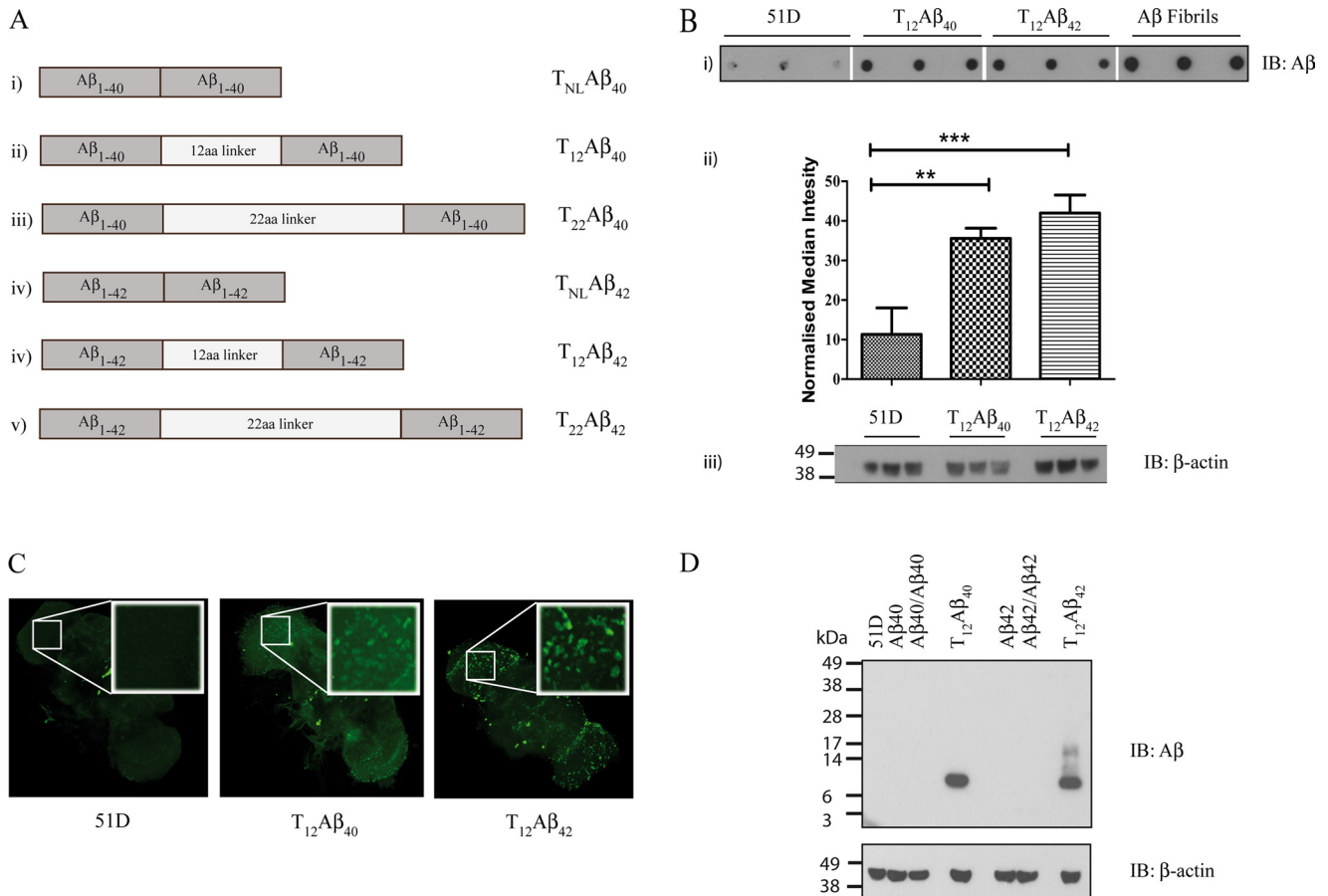
## RESULTS

We designed tandem A $\beta$  constructs in which two copies of the A $\beta$  monomer were linked together by a 12-amino acid linker (T<sub>12</sub>A $\beta$ ; Fig. 1A, *ii* and *iv*). The length and glycine-rich composition of this linker peptide were designed to provide sufficient flexibility to allow the individual A $\beta$  peptides to adopt a wide range of conformations. This objective is achieved while still significantly reducing the entropic barrier associated with aggregate formation for the two A $\beta$  sequences within the tandem construct compared with two freely diffusing monomeric A $\beta$  molecules. Identical sets of tandem constructs were made for A $\beta_{40}$  and A $\beta_{42}$  peptides (Fig. 1A, T<sub>12</sub>A $\beta_{40}$  and T<sub>12</sub>A $\beta_{42}$ ).

To determine the effects on aggregation *in vivo* of linking pairs of A $\beta$  peptides together, we generated transgenic *Dro-*



## Aggregation and Neurotoxicity of Tandem A $\beta$ in *Drosophila*



**FIGURE 1. Tandem A $\beta$  peptides deposit as insoluble aggregates in neuronal tissue.** *A*, series of tandem A $\beta_{40}$  or A $\beta_{42}$  peptides were designed and introduced transgenically into *D. melanogaster*. *B*, *i*, filter trap assays demonstrate that expression of both  $T_{12}A\beta_{40}$  and  $T_{12}A\beta_{42}$  at 25 °C results in the accumulation of large (>0.11- $\mu$ m diameter) SDS-insoluble aggregates. Fibrillar aggregates of synthetic A $\beta$  were used as positive controls. In contrast, extracts from nontransgenic flies (51D) contain no aggregates. *ii*, densitometric analysis of the filter retardation assay shows significant differences among groups:  $T_{12}A\beta_{40}$  versus 51D, \*\*,  $p \leq 0.01$ ;  $T_{12}A\beta_{42}$  versus 51D, \*\*\*,  $p \leq 0.001$ . *iii*, these values were compared after normalization of total protein loaded using the  $\beta$ -actin staining. *C*, anti-A $\beta$  immunofluorescence detects large deposits in  $T_{12}A\beta_{40}$ - and  $T_{12}A\beta_{42}$ -expressing fly brains but not in control (51D) tissue from nontransgenic flies. *IB*, immunoblotting. *D*, flies expressing one or two A $\beta$  transgenes ( $A\beta_{40}$ ,  $A\beta_{40}/A\beta_{40}$ ,  $A\beta_{42}$ ,  $A\beta_{42}/A\beta_{42}$ ) do not accumulate significant amounts of SDS-insoluble A $\beta$  peptide compared with nontransgenic flies (51D). In contrast, the  $T_{12}A\beta_{40}$  peptide and the  $T_{12}A\beta_{42}$  peptides form abundant insoluble aggregates. Equal protein loading was confirmed by  $\beta$ -actin staining.

*sophila* in which either the tandem A $\beta_{40}$  or the tandem A $\beta_{42}$  construct was inserted into an identical genomic locus; controls were also generated in which single copies of the monomeric A $\beta_{40}$  or A $\beta_{42}$  peptides were inserted into the same genomic locus. The use of a single defined genomic locus for insertion of all transgenes ensures that levels of transcription of each transgene are comparable by minimizing variability in expression arising from the different genomic context of transgenes inserted in different locations. We tested the efficacy of this approach by comparing the transcript levels, measured by quantitative RT-PCR, for flies expressing either A $\beta_{40}$  or A $\beta_{42}$  inserted in the same genomic locus. There was no significant difference in their levels of expression. (supplemental Fig. 1 and Materials and Methods) (15, 16).

Retinal expression of a single tandem A $\beta$  peptide transgene or of two transgenes of the monomeric counterparts was achieved using the GMR-GAL4 driver, and total head homogenates were prepared to analyze the extent of A $\beta$  aggregation in each case. A $\beta$  aggregates were quantified by a filter retardation assay. Both  $T_{12}A\beta_{40}$  and  $T_{12}A\beta_{42}$  formed significant quantities of large, insoluble deposits in the fly head (Fig. 1B) as measured

by this assay. By contrast, nontransgenic flies (Fig. 1B, 51D) did not contain detectable levels of such mature SDS-insoluble aggregates, although a very low level of nonspecific binding of the antibody to the insoluble material from these flies was observed. Immunohistochemical staining of the fly brains confirmed the presence of discrete aggregates for both tandem peptides, although their appearances differed somewhat, being more clearly punctate in flies expressing  $T_{12}A\beta_{42}$  compared with  $T_{12}A\beta_{40}$  (Fig. 1C).

Dissolution of SDS-insoluble material in 80% (v/v) dimethyl sulfoxide followed by SDS-PAGE and Western blotting confirmed that aggregation of the tandem peptides is increased very significantly compared with the corresponding monomeric peptides. Both  $T_{12}A\beta_{40}$  and  $T_{12}A\beta_{42}$  generated abundant SDS-insoluble aggregates (Fig. 1D,  $T_{12}A\beta_{40}$  and  $T_{12}A\beta_{42}$ ), whereas the negative control (*i.e.* Fig. 1D, 51D) along with the corresponding monomeric A $\beta$  peptides, whether expressed from one (Fig. 1D, A $\beta_{40}$  and A $\beta_{42}$ ) or two (Fig. 1D, A $\beta_{40}/A\beta_{40}$  and A $\beta_{42}/A\beta_{42}$ ) copies of the respective transgenes failed to generate a signal on the Western blot. Whereas the single dominant band on the Western blot for both tandem species indi-

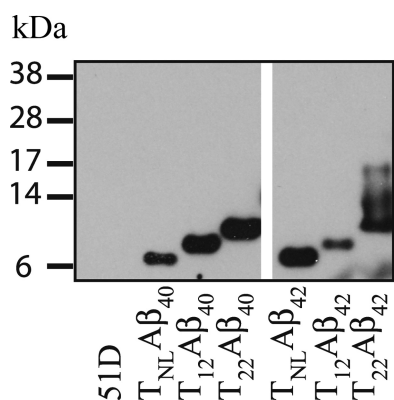


FIGURE 2. Western blotting reveals presence of SDS-insoluble A $\beta_{42}$  and A $\beta_{40}$  aggregates for flies expressing all the tandem constructs regardless of linker length. Nontransgenic control flies (51D) show no insoluble A $\beta$ .

icates that the SDS-insoluble aggregates were largely dissociated from monomeric peptides by treatment with 80% (v/v) dimethyl sulfoxide, interestingly T<sub>12</sub>A $\beta_{42}$  was found to have retained some residual supramolecular structure.

Whereas joining two copies of the A $\beta$  peptide by a linker sequence increases the effective concentration of the A $\beta$  peptides, too short a linker may create steric barriers to the formation of optimally stable aggregates, and, conversely, too long a linker will reduce the favorable entropic factors promoting faster aggregation (14). To investigate further the interplay between these different factors, we proceeded to generate tandem dimeric A $\beta$  peptides in which the linker between the two A $\beta$  sequences was either removed (T<sub>NL</sub>A $\beta$ ) or extended to 22 amino acids (T<sub>22</sub>A $\beta$ ). When analyzed by SDS-PAGE and Western blotting we found that all of the dimer constructs, when expressed using the *GMR-GAL4* driver, generated abundant SDS-insoluble aggregates (Fig. 2). This finding indicates that in all cases the tethering of two copies of A $\beta$  peptides increases the rate of formation of insoluble aggregates. Material from flies expressing T<sub>22</sub>A $\beta_{42}$  was not completely disaggregated to monomeric peptides but generated a ladder of bands on the Western blot; similar bands were not detectable for any tandem A $\beta_{40}$  constructs.

Aggregation-prone A $\beta$  peptides may cause developmental neurotoxicity in the *Drosophila* eye when their expression is driven by *GMR-GAL4*. We therefore compared the severity of the rough eye phenotype elicited by expression of the tandem A $\beta$  peptides with the results from the corresponding monomeric A $\beta$  peptides. The retinal expression of two copies of the monomeric A $\beta_{40}$  or A $\beta_{42}$  transgenes yields flies with eyes that are normal and comparable with nontransgenic controls (Fig. 3A, vii–ix). However, T<sub>12</sub>A $\beta_{42}$  expression in the retina causes severe eye malformation with loss of the ommatidial arrays and the appearance of melanized patches of necrotic tissue (Fig. 3A, v). This severe rough eye phenotype is also observed upon expression of T<sub>22</sub>A $\beta_{42}$  (Fig. 3A, vi) but is notably less severe upon expression of T<sub>NL</sub>A $\beta_{42}$  (Fig. 3A, iv). By contrast, expression of tandem A $\beta_{40}$  in the retina does not result in a detectable rough eye phenotype regardless of linker length (Fig. 3A, i–iii) and despite the accumulation of insoluble material (Fig. 2).

A quantitative measure of the toxic effects of tandem A $\beta$  peptides in the adult CNS was provided by determining the

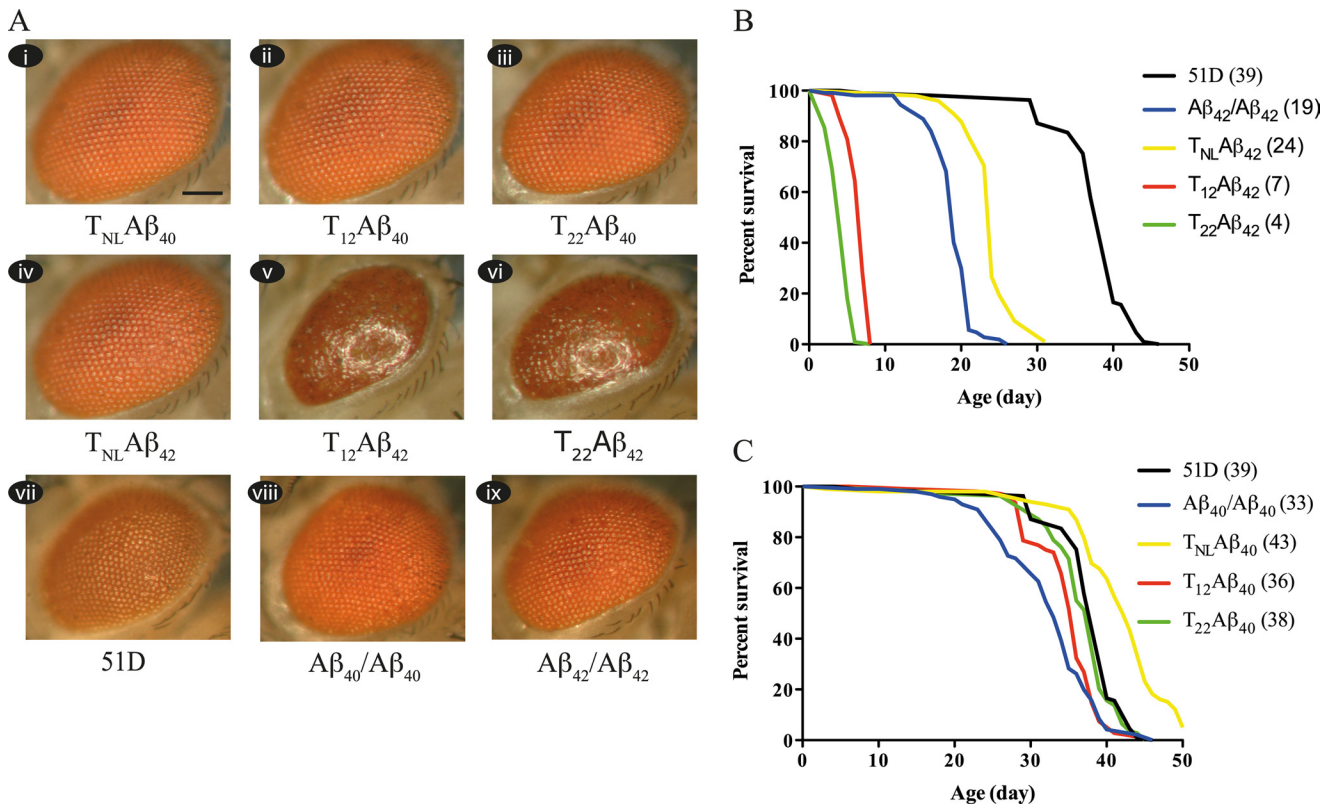
longevity of flies expressing transgenes driven by the pan-neuronal *elav-GAL4* construct. Concordant with the eye phenotypes described above, flies expressing a single copy of the T<sub>12</sub>A $\beta_{42}$  construct (Fig. 4A, red line) have a significantly shorter lifespan compared with controls expressing two transgenes (Fig. 4A, blue line) of the unlinked A $\beta_{42}$  (median survival 6 days versus 19 days,  $n = 100$  for both genotypes,  $p < 0.0001$ ). Likewise, T<sub>22</sub>A $\beta_{42}$  expression in the brain causes a further reduction in longevity (Fig. 4A, green line). Moreover, the longevity assay revealed significant neurotoxicity for the T<sub>NL</sub>A $\beta_{42}$  (Fig. 4A, yellow line, median survival 23 days versus 35 days for the unlinked A $\beta_{42}$ /A $\beta_{42}$  control,  $n = 100$  for both genotypes,  $p < 0.0001$ ). Despite the deposition of insoluble aggregates, however, the expression of all the tandem A $\beta_{40}$  constructs was compatible with a normal lifespan, and equivalent, within experimental error, to that of control flies (Fig. 4B). Thus, longevity assays show that whereas the existence of both 12- and 22-residue linker peptides accelerates the formation of insoluble aggregates for both A $\beta_{42}$  and A $\beta_{40}$ , high levels of neurotoxicity are evident only for the A $\beta_{42}$  peptides.

Although we have shown previously that the systematic introduction of single amino acid substitutions reveals correlations between the intrinsic aggregation rate and toxicity of the A $\beta$  sequence, an increase in the overall aggregation propensity does not always result in a corresponding increase in *in vivo* neurotoxicity (17). Rather, such toxicity has been found to correlate more strongly with the formation of prefibrillar oligomeric aggregates leading to the conclusion, in accord with other studies, that it is these species that are likely to be particularly damaging to neuronal cells (18–22). To investigate the levels of soluble, oligomeric aggregates of A $\beta$  we prepared extracts of SDS-soluble proteins from fly heads and visualized the species present using SDS-PAGE and Western blotting. When we expressed peptides in the neuronal tissue of the retina we observed abundant SDS-soluble oligomeric A $\beta$  aggregates for tandem A $\beta_{42}$  constructs containing linker peptides. Importantly, we observed a correlation between the abundance of such SDS-soluble aggregates and the severity of the rough eye phenotypes when the constructs were expressed in the retina. Likewise, quantification of these retinal SDS-soluble aggregates correlated with the reduction in median survival when the same constructs were expressed throughout the brain. No such SDS-soluble aggregates were detected in flies expressing any of the A $\beta_{40}$  constructs and were likewise absent for both T<sub>NL</sub>A $\beta$  peptides. Quantification of the SDS-soluble A $\beta$  aggregates demonstrated a remarkably close logarithmic correlation ( $R_2 = 0.98$ ) between the abundance of these oligomeric A $\beta_{42}$  aggregates in the retinal tissue and the relative decrease in median survival of flies with these constructs expressed in the brain. These data strongly suggest that the observed neurotoxic effect of A $\beta$  in *Drosophila* is caused by soluble oligomeric species rather than by insoluble aggregates.

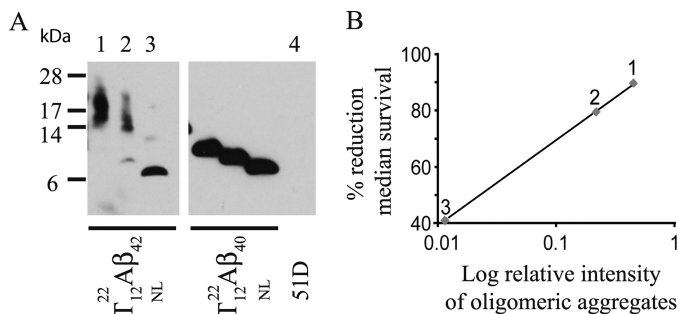
## DISCUSSION

In this study we have created tandem dimers of the most common forms of the AD-related A $\beta$  peptide, A $\beta_{40}$  and A $\beta_{42}$ , in which two copies of the peptide are conjoined head-to-tail by a flexible linker of varying length, and we have expressed them

## Aggregation and Neurotoxicity of Tandem A $\beta$ in *Drosophila*



**FIGURE 3. Differential toxic effects are observed for tandem constructs of A $\beta_{40}$  and A $\beta_{42}$  peptides.** *A*, tandem constructs of A $\beta_{40}$  do not cause developmental deficits when expressed in the retina using GMR-GAL4 (*i–iii*). In contrast, all of the tandem constructs of A $\beta_{42}$  (*iv–vi*) except T<sub>NL</sub>A $\beta_{42}$  (*iv*) cause a severe rough eye phenotype at 25 °C. For comparison, when the untethered A $\beta$  peptides were driven from two transgenes, both A $\beta_{40}$  (*viii*) and A $\beta_{42}$  (*ix*) appeared identical to controls (*vii*). *B* and *C*, survival analyses of dimeric A $\beta$  constructs are shown. Longevity assays confirm that longer linker peptides increased the toxicity for A $\beta_{42}$  (*B*) but not A $\beta_{40}$  (*C*). Flies were reared at 18 °C and transferred to 29 °C after eclosion. The median survival time is given in parentheses.



**FIGURE 4. Neurotoxicity correlates with the levels of soluble oligomeric aggregates of A $\beta$ .** *A*, retinal expression of A $\beta$  constructs, followed by Western blotting using the 6E10 monoclonal antibody, reveals SDS-soluble A $\beta$  aggregates in flies expressing T<sub>22</sub>A $\beta_{42}$  and T<sub>12</sub>A $\beta_{42}$ . In contrast, T<sub>NL</sub>A $\beta_{42}$  generates very few soluble aggregates whereas all tandem constructs based on A $\beta_{40}$  generate no detectable soluble oligomers. *B*, there is a correlation ( $R_2 = 0.98$ ) between the logarithm of the levels of SDS-soluble A $\beta$  aggregates resulting from expression in the eye and the reduction in the median survival in flies expressing the corresponding A $\beta$  constructs in their brain (see *A*). The degree of increase in aggregate levels was determined for T<sub>22</sub>A $\beta_{42}$  (1), T<sub>12</sub>A $\beta_{42}$  (2), and T<sub>NL</sub>A $\beta_{42}$  (3) by densitometry taking control flies (4) as the base line.

in neuronal tissues of *D. melanogaster*. We found that the deposition of insoluble aggregates of these dimeric peptides in neuronal tissue is substantially increased compared with equivalent concentrations of their monomeric counterparts. This finding is consistent with *in vitro* studies undertaken previously on two model protein aggregation systems, the SH3 domain of PI3 kinase and an immunoglobulin domain of cardiac titin (13, 14)

in which such repeat sequences were found to aggregate much more rapidly than the monomeric species; such a result can be attributed to an increase of approximately 1 order of magnitude in the effective concentration that is engendered by the covalent linkage of two peptides (13). Strikingly, the propensity of A $\beta_{42}$  dimers to populate soluble oligomeric species is significantly greater than that of A $\beta_{40}$  dimers, indicating that their mechanisms of aggregation may differ significantly *in vivo*, or alternatively that the peptides have different intrinsic stabilities and thus are present at differing concentrations in the cell. Furthermore, it is the appearance of these soluble oligomeric aggregates, rather than the level of insoluble aggregates, that is associated with high levels of neurotoxicity in our *Drosophila* model.

The detection of stable soluble oligomers of dimeric A $\beta_{42}$  but not dimeric A $\beta_{40}$  is consistent with recent observations from another *in vitro* study in which these two peptides, when constrained by an intramolecular disulfide bond (A $\beta$ cc peptides), were found to aggregate via different pathways, resulting in very different propensities to populate stable oligomers and protofibrils (23). In this case, A $\beta_{42}$ cc was observed to be more prone to form oligomers and larger protofibrils rich in  $\beta$ -sheet content than A $\beta_{40}$ cc and to be more neurotoxic than its more disordered counterparts. Two other recent studies that have linked A $\beta_{40}$  peptides either by cysteine substitution at position Ser-26 or at the N or C terminus have also reached similar conclusions (24, 25). Thus, we conclude that the differential



ability of A $\beta_{40}$  and A $\beta_{42}$  dimers to populate stable oligomeric states observed *in vivo* in the present study is likely to reflect underlying differences in the kinetics of specific steps in their aggregation mechanisms.

A particularly clear finding of the present study is that although A $\beta$  aggregation is required for neurotoxicity, it is clearly not sufficient. This conclusion is evident from the finding that the retinal expression of all the dimeric A $\beta_{40}$  constructs failed to generate any detectable developmental eye abnormalities despite their significantly accelerated aggregation, compared with monomeric A $\beta_{40}$ , in the eye. To confirm these findings in the eye we then used the more quantitative approach of longevity analysis to measure the consequences of ubiquitous expression of the peptide constructs in the nervous system. All A $\beta_{40}$  constructs were found to have negligible effects on longevity whereas all of the tandem A $\beta_{42}$  peptides significantly reduced median survival. Moreover, as determined by the rough eye and longevity phenotypes, the degree of neurotoxicity observed is dependent on the length of the linker peptides; the tandem A $\beta_{42}$  constructs containing linker peptides are clearly more toxic than those without a linker sequence, and longer linkers were found to result in more severe phenotypes than shorter linkers. Moreover, the fractional reduction in fly longevity was observed to be proportional to the abundance of oligomeric aggregates for each of the tandem A $\beta_{42}$  constructs. Whereas the most neurotoxic conformations are denied to tandem repeats of A $\beta_{42}$  in the absence of a linker peptide, presumably because of steric factors, our data indicate that longer linker peptides allow the greater flexibility required to generate stable, neurotoxic, oligomeric aggregates.

In the present study the distinction between A $\beta$  deposition *in vivo* and toxicity is consistent with the observation that many elderly humans are able to accumulate large numbers of  $\beta$ -amyloid plaques in their brains without suffering significant clinical consequences (26). The comparable flies are those expressing tandem A $\beta_{40}$ , where abundant A $\beta$  deposition may occur in the brain without neurotoxic consequences. Our data are consistent with the view that genetic or environmental factors that destabilize oligomeric prefibrillar aggregates, relative to either the monomeric or fibrillar forms of the peptide, could protect the brain in elderly individuals despite ongoing plaque formation. The detailed characterization of the variety and proportions of aggregates formed *in vivo* will, therefore, be essential to understand their specific proteotoxicity.

Taken together, our results demonstrate that the aggregation process for A $\beta_{42}$ , as the peptide undergoes transitions *in vivo* that convert it from a soluble monomeric state to insoluble amyloid deposits, involves the relatively high population of a variety of misfolded oligomeric species that are particularly toxic. This accumulation of prefibrillar intermediates may be attributable to the magnitude of the energy barriers that trap such species in local energy minima on the energy landscape that describes the aggregation process, or to the other differences in the relative importance of the various microscopic processes that contribute to the overall aggregation reaction (4). Our results suggest that the aggregation process for A $\beta_{40}$ , however, appears to progress more rapidly to mature forms of aggregate without populating such intermediate states to an

extent detectable *in vivo*. This observation is consistent with recent studies of A $\beta_{40}$  *in vitro* that show clearly that oligomeric species formed during aggregation are able to be rapidly sequestered by molecular chaperones (27).

It is likely, therefore, that the toxic effects of the lower levels A $\beta_{40}$  oligomers relative to those of A $\beta_{42}$  can be more effectively suppressed by the inherent protective mechanisms present *in vivo*. In any case, the results of this paper suggest that therapeutic advantage could be gained not only by increasing the barrier to initial peptide aggregation but also by facilitating the progression of A $\beta_{42}$  oligomers toward the more inert fibrillar state.

*Acknowledgments*—We thank Ann-Christin Moeller and Rita Esperto Costa for help with fly culture and Tuomas Knowles and Michele Vendruscolo, Department of Chemistry, University of Cambridge, for extremely valuable discussions.

## REFERENCES

- Chiti, F., and Dobson, C. M. (2006) Protein misfolding, functional amyloid, and human disease. *Annu. Rev. Biochem.* **75**, 333–366
- Dobson, C. M. (1999) Protein misfolding, evolution, and disease. *Trends Biochem. Sci.* **24**, 329–332
- Fowler, D. M., Koulouf, A. V., Balch, W. E., and Kelly, J. W. (2007) Functional amyloid: from bacteria to humans. *Trends Biochem. Sci.* **32**, 217–224
- Knowles, T. P., Waudby, C. A., Devlin, G. L., Cohen, S. I., Aguzzi, A., Vendruscolo, M., Terentjev, E. M., Welland, M. E., and Dobson, C. M. (2009) An analytical solution to the kinetics of breakable filament assembly. *Science* **326**, 1533–1537
- Jahn, T. R., and Radford, S. E. (2008) Folding *versus* aggregation: polypeptide conformations on competing pathways. *Arch. Biochem. Biophys.* **469**, 100–117
- Glenn, G. G., and Wong, C. W. (1984) Alzheimer's disease: initial report of the purification and characterization of a novel cerebrovascular amyloid protein. *Biochem. Biophys. Res. Commun.* **120**, 885–890
- Jarrett, J. T., Berger, E. P., and Lansbury, P. T., Jr. (1993) The carboxy terminus of the  $\beta$ -amyloid protein is critical for the seeding of amyloid formation: implications for the pathogenesis of Alzheimer's disease. *Biochemistry* **32**, 4693–4697
- Iwatsubo, T., Odaka, A., Suzuki, N., Mizusawa, H., Nukina, N., and Ihara, Y. (1994) Visualization of A $\beta_{42(43)}$  and A $\beta_{40}$  in senile plaques with end-specific A $\beta$  monoclonals: evidence that an initially deposited species is A $\beta_{42(43)}$ . *Neuron* **13**, 45–53
- Gravina, S. A., Ho, L., Eckman, C. B., Long, K. E., Otvos, L., Jr., Younkin, L. H., Suzuki, N., and Younkin, S. G. (1995) Amyloid  $\beta$  protein (A $\beta$ ) in Alzheimer disease brain: biochemical and immunocytochemical analysis with antibodies specific for forms ending at A $\beta_{40}$  or A $\beta_{42(43)}$ . *J. Biol. Chem.* **270**, 7013–7016
- Bitan, G., Kirkitadze, M. D., Lomakin, A., Vollers, S. S., Benedek, G. B., and Teplow, D. B. (2003) Amyloid  $\beta$  protein (A $\beta$ ) assembly: A $\beta_{40}$  and A $\beta_{42}$  oligomerize through distinct pathways. *Proc. Natl. Acad. Sci. U.S.A.* **100**, 330–335
- Bernstein, S. L., Dupuis, N. F., Lazo, N. D., Wytttenbach, T., Condrón, M. M., Bitan, G., Teplow, D. B., Shea, J. E., Ruotolo, B. T., Robinson, C. V., and Bowers, M. T. (2009) Amyloid  $\beta$  protein oligomerization and the importance of tetramers and dodecamers in the aetiology of Alzheimer's disease. *Nat. Chem.* **1**, 326–331
- Chen, Y. R., and Glabe, C. G. (2006) Distinct early folding and aggregation properties of Alzheimer amyloid  $\beta$  peptides A $\beta_{40}$  and A $\beta_{42}$ : stable trimer or tetramer formation by A $\beta_{42}$ . *J. Biol. Chem.* **281**, 24414–24422
- Bader, R., Bamford, R., Zurdo, J., Luisi, B. F., and Dobson, C. M. (2006) Probing the mechanism of amyloidogenesis through a tandem repeat of the PI3-SH3 domain suggests a generic model for protein aggregation and fibril formation. *J. Mol. Biol.* **356**, 189–208

## Aggregation and Neurotoxicity of Tandem A $\beta$ in *Drosophila*

14. Wright, C. F., Teichmann, S. A., Clarke, J., and Dobson, C. M. (2005) The importance of sequence diversity in the aggregation and evolution of proteins. *Nature* **438**, 878–881
15. Bischof, J., Maeda, R. K., Hediger, M., Karch, F., and Basler, K. (2007) An optimized transgenesis system for *Drosophila* using germ-line-specific  $\phi$ C31 integrases. *Proc. Natl. Acad. Sci. U.S.A.* **104**, 3312–3317
16. Jahn, T. R., Kohlhoff, K. J., Scott, M., Tartaglia, G. G., Lomas, D. A., Dobson, C. M., Vendruscolo, M., and Crowther, D. C. (2011) Detection of early locomotor abnormalities in a *Drosophila* model of Alzheimer's disease. *J. Neurosci. Methods* **197**, 186–189
17. Luheshi, L. M., Tartaglia, G. G., Brorsson, A. C., Pawar, A. P., Watson, I. E., Chiti, F., Vendruscolo, M., Lomas, D. A., Dobson, C. M., and Crowther, D. C. (2007) Systematic *in vivo* analysis of the intrinsic determinants of amyloid  $\beta$  pathogenicity. *PLoS Biol.* **5**, e290
18. Lesné, S., Koh, M. T., Kotilinek, L., Kaye, R., Glabe, C. G., Yang, A., Gallagher, M., and Ashe, K. H. (2006) A specific amyloid  $\beta$  protein assembly in the brain impairs memory. *Nature* **440**, 352–357
19. Shankar, G. M., Li, S., Mehta, T. H., Garcia-Munoz, A., Shepardson, N. E., Smith, I., Brett, F. M., Farrell, M. A., Rowan, M. J., Lemere, C. A., Regan, C. M., Walsh, D. M., Sabatini, B. L., and Selkoe, D. J. (2008) Amyloid  $\beta$  protein dimers isolated directly from Alzheimer's brains impair synaptic plasticity and memory. *Nat. Med.* **14**, 837–842
20. Ono, K., Condron, M. M., and Teplow, D. B. (2009) Structure-neurotoxicity relationships of amyloid  $\beta$  protein oligomers. *Proc. Natl. Acad. Sci. U.S.A.* **106**, 14745–14750
21. Brorsson, A. C., Bolognesi, B., Tartaglia, G. G., Shammas, S. L., Favrin, G., Watson, I., Lomas, D. A., Chiti, F., Vendruscolo, M., Dobson, C. M., Crowther, D. C., and Luheshi, L. M. (2010) Intrinsic determinants of neurotoxic aggregate formation by the amyloid  $\beta$  peptide. *Biophys. J.* **98**, 1677–1684
22. Tomic, J. L., Pensalfini, A., Head, E., and Glabe, C. G. (2009) Soluble fibrillar oligomer levels are elevated in Alzheimer's disease brain and correlate with cognitive dysfunction. *Neurobiol. Dis.* **35**, 352–358
23. Sandberg, A., Luheshi, L. M., Sollvander, S., Pereira de Barros, T., Macao, B., Knowles, T. P., Biverstal, H., Lendel, C., Ekholm-Petterson, F., Dubnovitsky, A., Lannfelt, L., Dobson, C. M., and Hard, T. (2010) Stabilization of neurotoxic Alzheimer amyloid  $\beta$  oligomers by protein engineering. *Proc. Natl. Acad. Sci. U.S.A.* **107**, 15595–15600
24. O'Nuallain, B., Freir, D. B., Nicoll, A. J., Risse, E., Ferguson, N., Herron, C. E., Collinge, J., and Walsh, D. M. (2010) Amyloid  $\beta$  protein dimers rapidly form stable synaptotoxic protofibrils. *J. Neurosci.* **30**, 14411–14419
25. Yamaguchi, T., Yagi, H., Goto, Y., Matsuzaki, K., and Hoshino, M. (2010) A disulfide-linked amyloid- $\beta$  peptide dimer forms a protofibril-like oligomer through a distinct pathway from amyloid fibril formation. *Biochemistry* **49**, 7100–7107
26. Hardy, J., and Selkoe, D. J. (2002) The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. *Science* **297**, 353–356
27. Narayan, P., Orte, A., Clarke, R. W., Bolognesi, B., Hook, S., Ganzinger, K. A., Meehan, S., Wilson, M. R., Dobson, C. M., and Klenerman, D. (2012) The extracellular chaperone clusterin sequesters oligomeric forms of the amyloid- $\beta_{1-40}$  peptide. *Nat. Struct. Mol. Biol.* **19**, 79–83
28. Green, C., Levashina, E., McKimmie, C., Dafforn, T., Reichhart, J. M., and Gubb, D. (2000) The necrotic gene in *Drosophila* corresponds to one of a cluster of three serpin transcripts mapping at 43A1.2. *Genetics* **156**, 1117–1127
29. Crowther, D. C., Kinghorn, K. J., Miranda, E., Page, R., Curry, J. A., Duthie, F. A., Gubb, D. C., and Lomas, D. A. (2005) Intraneuronal A $\beta$ , non-amyloid aggregates and neurodegeneration in a *Drosophila* model of Alzheimer's disease. *Neuroscience* **132**, 123–135