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Expression in *Drosophila* of Tandem Amyloid β Peptides Provides Insights into Links between Aggregation and Neurotoxicity*[S]

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Background: Investigating the kinetics of Aβ 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 Aβ increases amyloid deposition but not necessarily toxicity.
Significance: The toxicity of Aβ depends on the mechanism and not just the rate of amyloid formation.

The generation and subsequent aggregation of amyloid β (Aβ) 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 Aβ peptides to probe the manner in which changes in the aggregation kinetics of Aβ 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 β (Aβ)[7] 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 Aβ peptides in the brain, is the major species found in plaques (7–9). However, the aggregation reactions for the two Aβ 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

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[7] The abbreviations used are: Aβ, amyloid β; 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 Aβ peptide *in vivo* and its links to neurotoxicity. We demonstrate that expression of these tandem Aβ peptides in the brains of *Drosophila melanogaster* provides insights into the mechanisms of Aβ 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 Aβ transgenes were synthesized (Eurofins MWG Operon, Munich, Germany) using insect optimized codons. Aβ peptides within tandem constructs are linked either directly or by a 12- (GGGGSGGGGSGG) or a 22- (GGGGSGGGGSGGGGSGGGGSGG) 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 φC31 system (15). The transgenic lines were backcrossed for six generations into w_{1118} flies to obtain isogenic lines. To analyze the effect of Aβ expression, transgenic UAS-Aβ 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 × *g* for 20 min at 4 °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 μl 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 μl 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 Aβ using a mouse monoclonal anti-Aβ antibody (6E10; Covance) as described previously (17).

Filter Retardation Assay—Protein aggregates in brain extracts from flies reared at 25 °C and collected 24 h after eclosion were detected using a filter retardation assay. Fifteen fly heads were homogenized in 60 μl 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 × *g* for 20 min at 4 °C. The supernatant was removed and loaded on a SDS-polyacrylamide gel, transferred onto a membrane, and probed with a β-actin antibody. The pellet was washed with PBS and resuspended in 20 mM Tris-HCl, pH 8.0, 15 mM $MgCl_2$, 0.5 mg/ml DNase I and incubated for 1 h at 37 °C. The samples were then loaded onto a nitrocellulose membrane (0.11-μm 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 Aβ 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 β-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 Aβ variants were crossed with the $elav_{c155}$-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 Aβ peptides under the control of *GMR-GAL4* 24 h after eclosion and immunostained with the mouse anti-Aβ antibody 6E10 as described previously (17). Confocal scanning images were collected using identical acquisition parameters at intervals of 5 μm using a Nikon Eclipse C1si on Nikon E90i upright stand (Nikon Corporation) with a 20× objective. Images were projected and processed using ImageJ software.

RESULTS

We designed tandem Aβ constructs in which two copies of the Aβ monomer were linked together by a 12-amino acid linker ($T_{12}A\beta$; Fig. 1*A*, *ii* and *iv*). The length and glycine-rich composition of this linker peptide were designed to provide sufficient flexibility to allow the individual Aβ 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 Aβ sequences within the tandem construct compared with two freely diffusing monomeric Aβ molecules. Identical sets of tandem constructs were made for $A\beta_{40}$ and $A\beta_{42}$ peptides (Fig. 1*A*, $T_{12}A\beta_{40}$ and $T_{12}A\beta_{42}$).

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

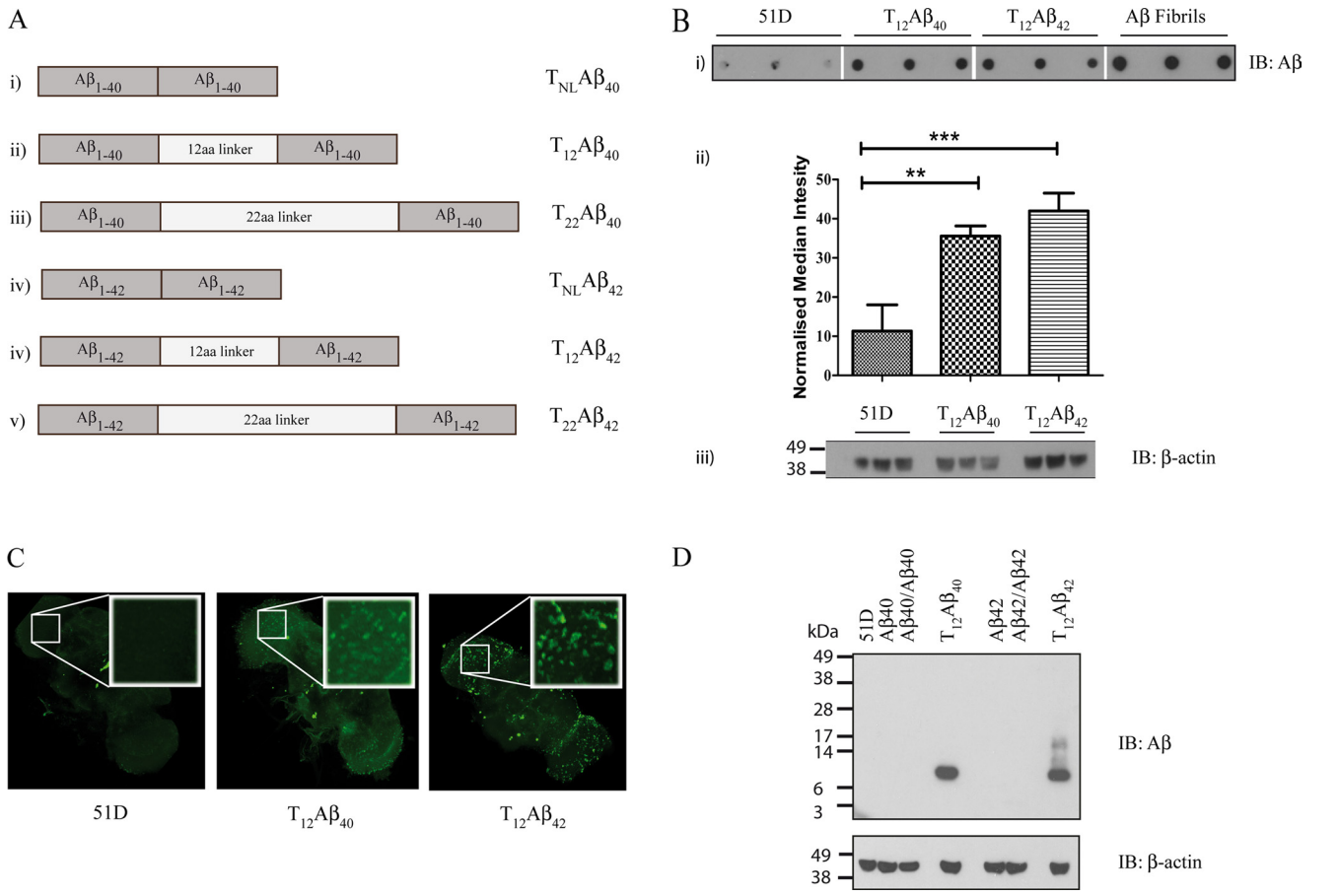

FIGURE 1. **Tandem Aβ peptides deposit as insoluble aggregates in neuronal tissue.** *A*, series of tandem $A\beta_{40}$ or $A\beta_{42}$ peptides were designed and introduced transgenically into *D. melanogaster*. *B*, *i*, filter trap assays demonstrate that expression of both $T_{12}A\beta_{40}$ and $T_{12}A\beta_{42}$ at 25 °C results in the accumulation of large (>0.11-μm diameter) SDS-insoluble aggregates. Fibrillar aggregates of synthetic Aβ 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 β-actin staining. *C*, anti-Aβ 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 Aβ 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 Aβ 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 β-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 Aβ 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 Aβ aggregation in each case. Aβ 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. 1*B*) as measured by this assay. By contrast, nontransgenic flies (Fig. 1*B*, 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. 1*C*).

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. 1*D*, $T_{12}A\beta_{40}$ and $T_{12}A\beta_{42}$), whereas the negative control (*i.e.* Fig. 1*D*, 51D) along with the corresponding monomeric Aβ peptides, whether expressed from one (Fig. 1*D*, $A\beta_{40}$ and $A\beta_{42}$) or two (Fig. 1*D*, $A\beta_{40}/A\beta_{40}$ 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-

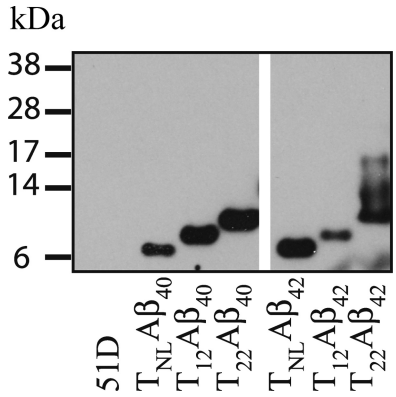

FIGURE 2. **Western blotting reveals presence of SDS-insoluble Aβ_{42} and Aβ_{40} aggregates for flies expressing all the tandem constructs regardless of linker length.** Nontransgenic control flies (51D) show no insoluble Aβ.

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

Whereas joining two copies of the Aβ peptide by a linker sequence increases the effective concentration of the Aβ 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 Aβ peptides in which the linker between the two Aβ sequences was either removed ($T_{NL}A\beta$) or extended to 22 amino acids ($T_{22}A\beta$). 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 Aβ peptides increases the rate of formation of insoluble aggregates. Material from flies expressing $T_{22}A\beta_{42}$ 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 Aβ 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 Aβ peptides with the results from the corresponding monomeric Aβ 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. 3*A*, *vii–ix*). However, $T_{12}A\beta_{42}$ expression in the retina causes severe eye malformation with loss of the ommatidial arrays and the appearance of melanized patches of necrotic tissue (Fig. 3*A*, *v*). This severe rough eye phenotype is also observed upon expression of $T_{22}A\beta_{42}$ (Fig. 3*A*, *vi*) but is notably less severe upon expression of $T_{NL}A\beta_{42}$ (Fig. 3*A*, *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. 3*A*, *i–iii*) and despite the accumulation of insoluble material (Fig. 2).

A quantitative measure of the toxic effects of tandem Aβ 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 $T_{12}A\beta_{42}$ construct (Fig. 4*A*, *red line*) have a significantly shorter lifespan compared with controls expressing two transgenes (Fig. 4*A*, *blue line*) of the unlinked $A\beta_{42}$ (median survival 6 days *versus* 19 days, $n = 100$ for both genotypes, $p < 0.0001$). Likewise, $T_{22}A\beta_{42}$ expression in the brain causes a further reduction in longevity (Fig. 4*A*, *green line*). Moreover, the longevity assay revealed significant neurotoxicity for the $T_{NL}A\beta_{42}$ (Fig. 4*A*, *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. 4*B*). 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 Aβ 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 Aβ 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 Aβ 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 $T_{NL}A\beta$ peptides. Quantification of the SDS-soluble Aβ 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 Aβ 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 Aβ 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

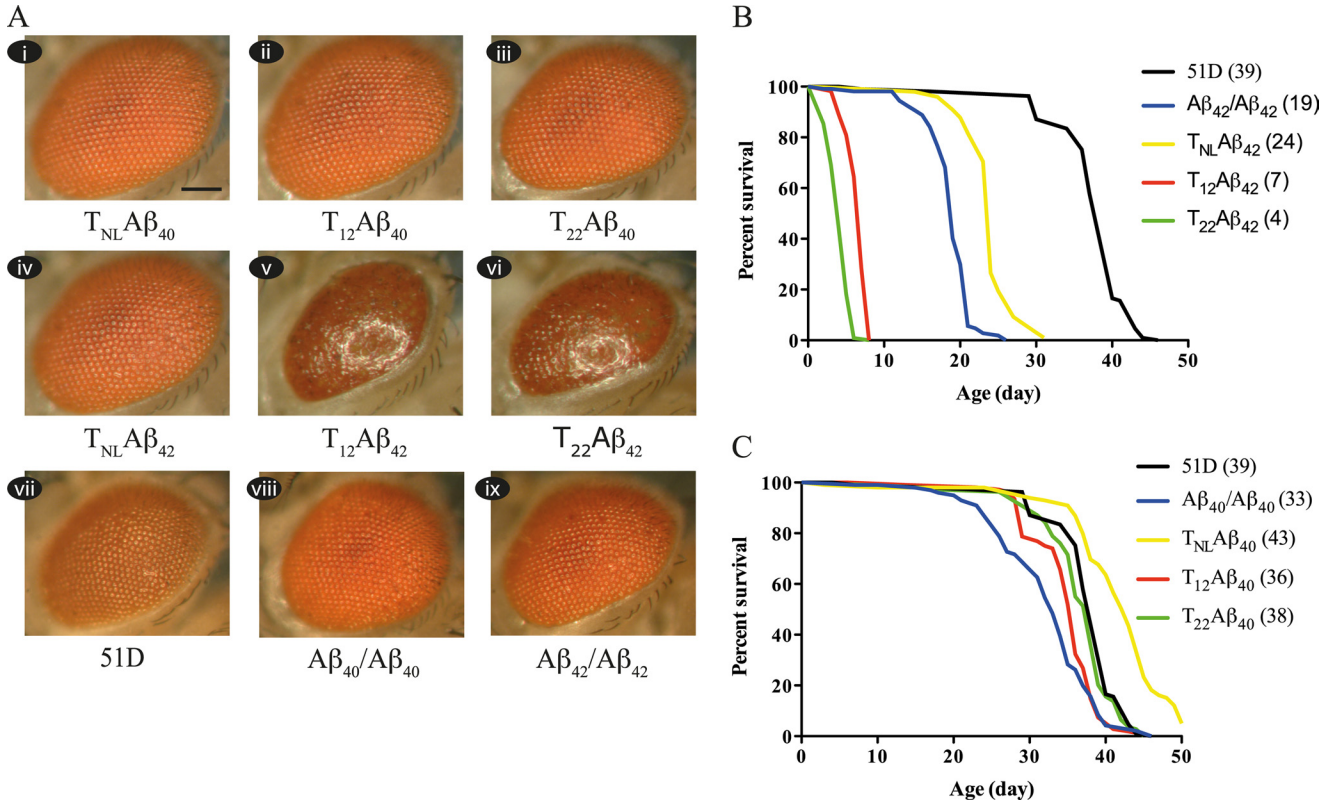

FIGURE 3. **Differential toxic effects are observed for tandem constructs of $Aβ_{40}$ and $Aβ_{42}$ peptides.** *A*, tandem constructs of $Aβ_{40}$ do not cause developmental deficits when expressed in the retina using GMR-GAL4 (*i–iii*). In contrast, all of the tandem constructs of $Aβ_{42}$ (*iv–vi*)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

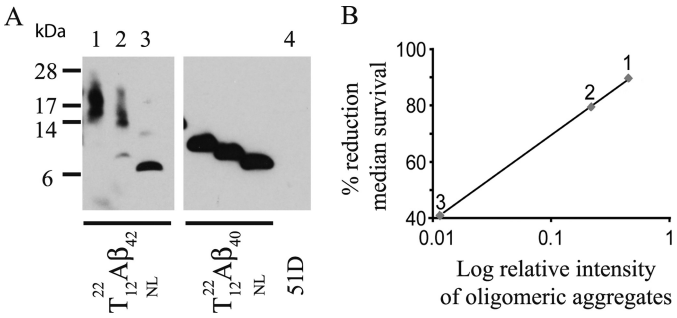

FIGURE 4. **Neurotoxicity correlates with the levels of soluble oligomeric aggregates of Aβ.** *A*, retinal expression of Aβ constructs, followed by Western blotting using the 6E10 monoclonal antibody, reveals SDS-soluble Aβ aggregates in flies expressing $T_{22}Aβ_{42}$ and $T_{12}Aβ_{42}$. In contrast, $T_{NL}Aβ_{42}$ generates very few soluble aggregates whereas all tandem constructs based on $Aβ_{40}$ generate no detectable soluble oligomers. *B*, there is a correlation (R_2 = 0.98) between the logarithm of the levels of SDS-soluble Aβ aggregates resulting from expression in the eye and the reduction in the median survival in flies expressing the corresponding Aβ constructs in their brain (see *A*). The degree of increase in aggregate levels was determined for $T_{22}Aβ_{42}$ (*1*), $T_{12}Aβ_{42}$ (*2*), and $T_{NL}Aβ_{42}$ (*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 $Aβ_{42}$ dimers to populate soluble oligomeric species is significantly greater than that of $Aβ_{40}$ 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 $Aβ_{42}$ but not dimeric $Aβ_{40}$ is consistent with recent observations from another *in vitro* study in which these two peptides, when constrained by an intramolecular disulfide bond (Aβcc peptides), were found to aggregate via different pathways, resulting in very different propensities to populate stable oligomers and protofibrils (23). In this case, $Aβ_{42}$cc was observed to be more prone to form oligomers and larger protofibrils rich in β-sheet content than $Aβ_{40}$cc and to be more neurotoxic than its more disordered counterparts. Two other recent studies that have linked $Aβ_{40}$ 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 Aβ 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 Aβ deposition *in vivo* and toxicity is consistent with the observation that many elderly humans are able to accumulate large numbers of β-amyloid plaques in their brains without suffering significant clinical consequences (26). The comparable flies are those expressing tandem $A\beta_{40}$, where abundant Aβ 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.

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