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

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Background: Investigating the kinetics of Aβ peptide aggregation in vivo is vital to understanding Alzheimer disease.

Results: Linking two Aβ40 or Aβ42 peptides together increases their aggregation rates in Drosophila, but only increases the neurotoxicity of Aβ42.

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 (Aβ40) or 42 residues (Aβ42), 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 Aβ40 and Aβ42 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 Aβ42 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 Aβ42 peptide in vivo can therefore be attributed to the higher kinetic stability of the oligomeric intermediate states that it populates relative to those of Aβ40 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) 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, Aβ40 and Aβ42. The longer isoform, Aβ42, 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 pre fibrillar 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...
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 Aβ40 and Aβ42 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- (GGGGSGGGGSGG-GGGGGSGG) 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 Q31 system (15). The transgenic lines were backcrossed for six generations into w1118 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) allowing a tissue-specific expression. Double transgenic lines were subsequently crossed to GAL4 driver line. From the progeny, 100 mated female flies 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.

**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 MgCl2, 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<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 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<sub>12</sub>Aβ; 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 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 Aβ<sub>40</sub> and Aβ<sub>42</sub> peptides (Fig. 1A, T<sub>12</sub>Aβ<sub>40</sub> and T<sub>12</sub>Aβ<sub>42</sub>).

To determine the effects on aggregation in vivo of linking pairs of Aβ peptides together, we generated transgenic Dro-
sophila in which either the tandem Aβ₄₀ or the tandem Aβ₄₂ construct was inserted into an identical genomic locus; controls were also generated in which single copies of the monomeric Aβ₄₀ or Aβ₄₂ 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 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 Aβ₄₀ or Aβ₄₂ 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 T₁₂Aβ₄₀ and T₁₂Aβ₄₂ 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 T₁₂Aβ₄₂ compared with the corresponding monomeric Aβ peptide. T₁₂Aβ₄₀ and T₁₂Aβ₄₂ generated abundant SDS-insoluble aggregates (Fig. 1D, T₁₂Aβ₄₀ and T₁₂Aβ₄₂), whereas the negative control (i.e. Fig. 1D, 51D) along with the corresponding monomeric Aβ peptides, whether expressed from one (Fig. 1D, Aβ₄₀ and Aβ₄₂) or two (Fig. 1D, Aβ₄₀/Aβ₄₀ and Aβ₄₀/Aβ₄₂) 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-
Aggregation and Neurotoxicity of Tandem Aβ in Drosophila

In this study, we have created tandem dimers of the most common forms of the AD-related Aβ peptide, Aβ40 and Aβ42, in which two copies of the peptide are conjoined head-to-tail by a flexible linker of varying length, and we have expressed them in Drosophila melanogaster. Concordant with the eye phenotype described above, flies expressing a single copy of the T12Aβ42 construct (Fig. 4A, red line) have a significantly shorter lifespan compared with controls expressing two transgenes (Fig. 4A, blue line) of the unlinked Aβ42 (median survival 6 days versus 19 days, n = 100 for both genotypes, p < 0.0001). Likewise, T22Aβ42 expression in the brain causes a further reduction in longevity (Fig. 4A, green line). Moreover, the longevity assay revealed significant neurotoxicity for the T34Aβ42 (Fig. 4A, yellow line), median survival 23 days versus 35 days for the unlinked Aβ42/Aβ42 control, n = 100 for both genotypes, p < 0.0001). Despite the deposition of insoluble aggregates, however, the expression of all tandem Aβ40 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 Aβ42 and Aβ40, high levels of neurotoxicity are evident only for the Aβ42 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 Aβ42 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 Aβ40 constructs and were likewise absent for both T34Aβ peptides. Quantification of the SDS-soluble Aβ aggregates demonstrated a remarkably close logarithmic correlation (R² = 0.98) between the abundance of these oligomeric Aβ42 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, Aβ40 and Aβ42, in which two copies of the peptide are conjoined head-to-tail by a flexible linker of varying length, and we have expressed them.
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 Aβ42 dimers to populate soluble oligomeric species is significantly greater than that of Aβ40 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 Aβ42 but not dimeric Aβ40 is consistent with recent observations from another *in vitro* study in which these two peptides, when constrained by an intramolecular disulfide bond (Aβ42cc peptides), were found to aggregate via different pathways, resulting in very different propensities to populate stable oligomers and protofibrils (23). In this case, Aβ42cc was observed to be more prone to form oligomers and larger protofibrils rich in β-sheet content than Aβ40cc and to be more neurotoxic than its more disordered counterparts. Two other recent studies that have linked Aβ40 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 Aβ_{40} and Aβ_{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 Aβ_{40} constructs failed to generate any detectable developmental eye abnormalities despite their significantly accelerated aggregation, compared with monomeric Aβ_{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 Aβ_{40} constructs were found to have negligible effects on longevity whereas all of the tandem Aβ_{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 Aβ_{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 Aβ_{42} constructs. Whereas the most neurotoxic conformations are denied to tandem repeats of Aβ_{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 Aβ_{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 Aβ_{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 Aβ_{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 Aβ_{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 Aβ_{40} oligomers relative to those of Aβ_{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 Aβ_{42} oligomers toward the more inert fibrillar state.

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