Neuroscience 132 (2005) 123–135

INTRANEURONAL Aβ, NON-AMYLOID AGGREGATES AND NEURODEGENERATION IN A *DROSOPHILA* MODEL OF ALZHEIMER'S DISEASE

D. C. CROWTHER,[a]* K. J. KINGHORN,[a] E. MIRANDA,[a] R. PAGE,[a] J. A. CURRY,[c] F. A. I. DUTHIE,[b] D. C. GUBB[b] AND D. A. LOMAS[a]

[a]*Department of Medicine, University of Cambridge, Cambridge Institute for Medical Research, Wellcome Trust/MRC Building, Hills Road, Cambridge CB2 2XY, UK*

[b]*Department of Genetics, University of Cambridge, Downing Street, Cambridge CB2 3EH, UK*

[c]*The Neuroscience Research Centre, Merck Sharp and Dohme, Terlings Park, Eastwick Road, Harlow, Essex CM20 2QR, UK*

Abstract—We have developed models of Alzheimer's disease in *Drosophila melanogaster* by expressing the Aβ peptides that accumulate in human disease. Expression of wild-type and Arctic mutant (Glu22Gly) $A\beta_{1-42}$ peptides in *Drosophila* neural tissue results in intracellular Aβ accumulation followed by non-amyloid aggregates that resemble diffuse plaques. These histological changes are associated with progressive locomotor deficits and vacuolation of the brain and premature death of the flies. The severity of the neurodegeneration is proportional to the propensity of the expressed Aβ peptide to form oligomers. The fly phenotype is rescued by treatment with Congo Red that reduces Aβ aggregation *in vitro*. Our model demonstrates that intracellular accumulation and non-amyloid aggregates of Aβ are sufficient to cause the neurodegeneration of Alzheimer's disease. Moreover it provides a platform to dissect the pathways of neurodegeneration in Alzheimer's disease and to develop novel therapeutic interventions.

Key words: dementia, drug screen, Aβ peptide, arctic, conformational.

Alzheimer's disease is the most common dementia, affecting 15 million people worldwide. By 2050 it is estimated that a quarter of Western populations will be older than 65 years and hence particularly at risk (Puglielli et al., 2003). Current therapies palliate the memory and behavioral deficits of the disease but there is an urgent need to develop drugs that have disease-modifying activity. The classical histopathological lesions in the brain of an individual with Alzheimer's disease are extracellular amyloid plaques and intracellular neurofibrillary tangles. The amyloid plaques are found particularly in the hippocampus, the limbic cortex and the neocortex (Dickson, 1997) and are composed of β-amyloid peptides, which are proteolytic fragments of the larger transmembrane amyloid precursor protein (APP; Selkoe, 2001).

There is growing evidence that it is the β-amyloid peptides that are central to the pathogenesis of Alzheimer's disease (Crowther, 2002). These peptides are generated by β- and γ-secretase cleavage of APP to yield peptides of either 40 or 42 amino acids in length ($A\beta_{1-40}$ or $A\beta_{1-42}$). The difference in peptide length is due to variation in the cleavage site of γ-secretase. Three genetic loci have been linked to familial Alzheimer's disease, APP itself and the presenilin-1 and presenilin-2 genes that code for overlapping, but distinct, γ-secretase activities (Lai et al., 2003). In health the shorter, less aggregatory $A\beta_{1-40}$ peptide predominates; however, in sporadic and most cases of familial Alzheimer's disease either the ratio of $A\beta_{1-42}$ to $A\beta_{1-40}$ is increased or the total concentration of $A\beta_{1-42}$ is raised. In the rare cases of familial Alzheimer's disease where the production of $A\beta_{1-42}$ is unchanged, or reduced, then mutations within the β-amyloid peptide, such as the Arctic mutation, greatly accelerate its aggregation (Nilsberth et al., 2001; Dahlgren et al., 2002; Murakami et al., 2002).

Despite these genetic data the pathogenic role of β-amyloid plaques has been questioned because of the presence of plaques in some healthy, elderly individuals and also because of the poor correlation between the number of amyloid plaques and the degree of dementia in individuals with Alzheimer's disease (Wilcock and Esiri, 1982). The debate has centered on whether the amyloid plaque is the neurotoxic species or whether it represents a relatively inert, end-point of β-amyloid peptide aggregation. The observation that total brain β-amyloid peptide concentration correlates better with dementia than plaque count (Naslund et al., 2000) has underpinned the conclusion from *in vitro* and *in vivo* experiments that it is soluble, oligomeric aggregates of Aβ peptides that are the neurotoxic species (Lambert et al., 1998; Walsh et al., 2002). Oligomeric Aβ has characteristic electron microscopic appearances including spheroids, rings and short fibrils (Lashuel et al., 2002, 2003). Fractionation of the aggregating peptides has shown that monomeric peptide is not toxic but that oligomeric peptide gains toxicity that is subsequently lost when mature fibrils are formed (Lashuel et al., 2003). Recently a polyclonal antibody has been raised that has specific affinity for oligomeric Aβ allowing detection of the putative toxic species *in vitro* and *in vivo* (Kayed et al., 2003).

*Corresponding author. Tel: +44-1223-336825; fax: +44-1223-336827.
E-mail address: dcc26@cam.ac.uk (D.C. Crowther).

Abbreviations: Aβ, amyloid beta peptide; *AlzArc1*, *AlzArc2* and *AlzArc3*, three *Drosophila* lines expressing the Arctic mutant (Glu22Gly) of $A\beta_{1-42}$ peptide; *Alz40.1*, *Alz40.2* and *Alz40.3*, three *Drosophila* lines expressing the $A\beta_{1-40}$ peptide; *Alz42.1*, *Alz42.2* and *Alz42.3*, three *Drosophila* lines expressing the $A\beta_{1-42}$ peptide; APP, amyloid precursor protein; DAB, 3,3′-diaminobenzidine tetrahydrochloride; Glu22Gly, Arctic mutant; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; w^{1118}, strain of flies from which the transgenic flies were derived.

0306-4522/05$30.00+0.00
doi:10.1016/j.neuroscience.2004.12.025

Understanding the pathways of neurodegeneration in Alzheimer's disease and the search for disease-modifying drugs requires a faithful animal model. Ideally this should permit the testing of candidate compounds acting at the various points on the pathogenetic cascade from Aβ generation and aggregation through to the pathways of cell death. Current mouse models replicate the abnormal processing of APP in the human disease and develop clear β-amyloid plaques. Although mouse models show mild behavioral deficits these are laborious to characterize and develop after many months (Westerman et al., 2002). Until recently mouse models of Alzheimer's disease did not demonstrate global neuronal loss despite florid amyloid plaque pathology; however, a recent quadruple-mutant mouse has shown neuronal loss in association with intracellular accumulation of Aβ (Casas et al., 2004). In view of the problems with mouse systems (Davis and Laroche, 2003) we and others (Greeve et al., 2004; Iijima et al., 2004) have generated a *Drosophila* model of Alzheimer's disease by driving Aβ production in the CNS and retina of the fly. Our model has enabled us to correlate the rate of Aβ aggregation with the severity of various Alzheimer's phenotypes and has demonstrated that Congo Red significantly rescues the Alzheimer's fly.

EXPERIMENTAL PROCEDURES

Generation of transgenic flies

The $A\beta_{1-42}$ peptide (underlined) was cloned with a secretion signal peptide from the *Drosophila necrotic* gene (Green et al., 2000) (MASKVSILLLLTVHLLAAQTFAQDAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA) into the *Gal4*-responsive *pUAST* expression vector. The QuikChange XL site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) was used to introduce the Arctic $A\beta_{1-42}$ mutation (Glu22Gly) and to introduce a premature stop codon, shortening the coding sequence by two amino acids, to produce the $A\beta_{1-40}$ peptide. Two of the wild-type transgenic *Alz* lines had the $A\beta_{1-42}$ transgene inserted on chromosome 2 (*Alz42.1* and *Alz42.2*) and one line had the transgene on chromosome 3 (*Alz42.3*). The double transgenic (*Alz42.2*+*Alz42.3*) line was generated by crossing the *Alz42.2* and *Alz42.3* lines. The Arctic $A\beta_{1-42}$ lines had the transgene inserted on either chromosome 2 (*AlzArc1* and *AlzArc3*) or 3 (*AlzArc2*). The insert was on chromosomes 2 (*Alz40.3*) or 3 (*Alz40.1* and *Alz40.2*) in lines expressing the $A\beta_{1-40}$ peptide. The presence of the $A\beta_{1-40}$ pUAST construct in transgenic flies was confirmed by PCR from genomic DNA. The Aβ peptides were expressed by crossing transgenic flies with the *Gal4-elav*^{c155} pan-neuronal driver strain. Correct proteolytic processing of the secretion signal peptide was confirmed by expression of identical $A\beta_{1-42}$ constructs in *Drosophila* S2 cells. The constructs were subcloned into the pMT/V5-His vector (Invitrogen) and transfected into S2 cells using FuGENE 6 (Roche, Lewes, UK). Twenty-four hours after induction the cell pellets and conditioned media were examined by immunoprecipitation and Western blotting of SDS-PAGE gels using the 4G8 monoclonal antibody. Expression and correct processing of $A\beta_{1-42}$ were confirmed by immunoprecipitation and Western blotting from 100 fly heads from transgenic flies and controls. Efficient detection of $A\beta_{1-42}$ derived from fly heads required that the nitrocellulose membrane be boiled for 5 min in phosphate-buffered saline (PBS) following electroblotting.

Longevity and climbing assay

Flies expressing one or two copies of $A\beta_{1-42}$, Arctic $A\beta_{1-42}$ or $A\beta_{1-40}$ peptide were incubated at 29 °C in groups of up to 30 in 4-inch glass vials with new food every 2 days. Viable transgenic and control (*Gal4-elav*^{c155}) flies were counted daily. Flies in Fig. 3 were cultured on standard food with dried yeast. Flies in Figs. 4 and 5 were cultured on instant fly food containing the required test compound until eclosion and thereafter on standard fly food with a 500 mg streak of yeast paste containing the test compound. Appropriate untreated flies were cultured in parallel on the instant food to control for small differences in survival between standard and instant fly food. Differences in survival were analyzed using the SPSS 11 Kaplan-Meier software package. To assess climbing behavior up to 20 flies were placed at the bottom of a clean 3-inch glass vial and a second identical vial was placed above. After 20 s the two vials were separated and the number of flies at the top and bottom were counted. The proportion of Alzheimer's flies in the top vial was compared over time with the proportion of control flies (that expressed only the *Gal4-elav*^{c155} driver). The number of observations of fly locomotor function ranged between 319 and 1036, representing more than five cohorts of flies for each transgenic strain.

Immunohistochemistry and microscopy

At time points up to 3 weeks post-eclosion the Alzheimer's flies were decapitated and the proboscis was dissected away to facilitate penetration of the fixative. For the low power images Zamboni's fixative was used (0.2% v/v picric acid, 2% w/v paraformaldehyde, 0.1 M phosphate buffer pH 7.2–7.6) whereas tissue for higher power images was fixed with 2% w/v paraformaldehyde, 0.1 M phosphate buffer pH 7.2–7.6. After fixation the tissue was embedded in paraffin wax and immunostaining was performed on 6 μm sections with the 4G8 monoclonal antibody (20 ng/μl in PBS) with biotinylated anti-mouse antibody (Vector Laboratories Ltd, Peterborough, UK; 1:200 in PBS) as the secondary antibody and subsequently with avidin–HRP, developing with nickel chloride-enhanced 3,3′-diaminobenzidine tetrahydrochloride (DAB). Fly tissue for oligomer staining was treated as described above. Tg2576 mice (Hsiao et al., 1996), expressing the Swedish mutation of human APP (Lys670Asn, Met671Leu), were anesthetized and exsanguinated before the brain was removed and immersion-fixed in 4% w/v paraformaldehyde before embedding in paraffin and sectioning at 6 μm. Postmortem Alzheimer's disease brain was immersion-fixed in 10% w/v formal saline before embedding in paraffin and sectioning at 6 μm. Oligomers were stained with an Aβ oligomer-specific antibody (Kayed et al., 2003; 1:100 dilution of antiserum) with a biotinylated anti-rabbit antibody as the secondary antibody. Congo Red staining with hematoxylin counterstaining of fly sections and control human renal amyloid sections was performed as described previously (Puchtler and Sweat, 1965). The external surfaces of the eyes were visualized by scanning electron microscopy and the architecture of the retina was viewed in 6 μm sections of araldite-embedded tissue stained with Methylene Blue.

All experiments using human and animal tissue complied with local and international ethical guidelines. We minimized the use of human and animal tissue.

RESULTS

Drosophila transgenesis

We have tested the hypothesis that intracellular and non-amyloid aggregates of $A\beta_{1-42}$ are the neurotoxic species in Alzheimer's disease by expressing $A\beta_{1-42}$ and other Aβ peptides (fused to a secretion signal peptide) in the neural tissue of *D. melanogaster*. Independent transgenic lines were

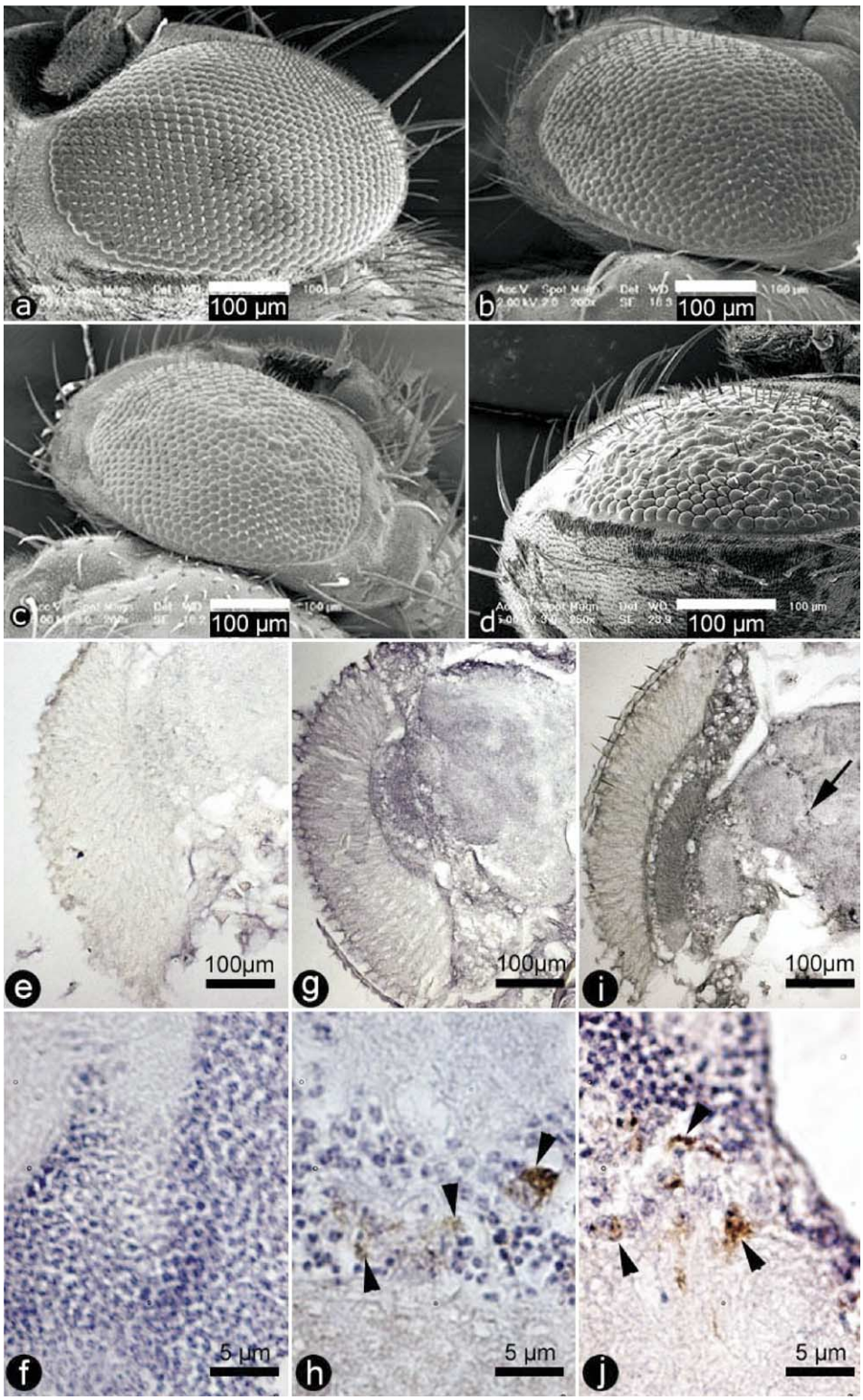

Fig. 1. Plaque formation and neurodegeneration in the brain and retina of Alzheimer's flies. Scanning electron microscopy was used to examine the appearance of the *Drosophila* eyes at eclosion (scale bar=100 µm). Control *Gal4-elav^{c155}* flies (a) and flies expressing one $A\beta_{1-40}$ transgene (data not shown, three independent strains examined) had normal eyes whereas flies expressing one $A\beta_{1-42}$ transgene (b) had a mild rough eye phenotype. Expression of two $A\beta_{1-42}$ transgenes (c) or one Arctic $A\beta_{1-42}$ transgene (d) gave increasingly severe rough eye phenotypes. Immunohistochemistry with the 4G8 monoclonal antibody of the retina and brain of flies expressing a single $A\beta_{1-42}$ transgene (*Alz42.1*) showed undetectable levels of Aβ expression at eclosion that was indistinguishable from control flies (e, f). However by day 10 intracellular accumulation of Aβ could be detected (arrowheads, g, h). By day 15 the intracellular accumulation of Aβ peptide was more marked (arrowheads) and there were large accumulations of peptide (arrow) in the region of the cell bodies bordering the lamina and medulla (i, j).

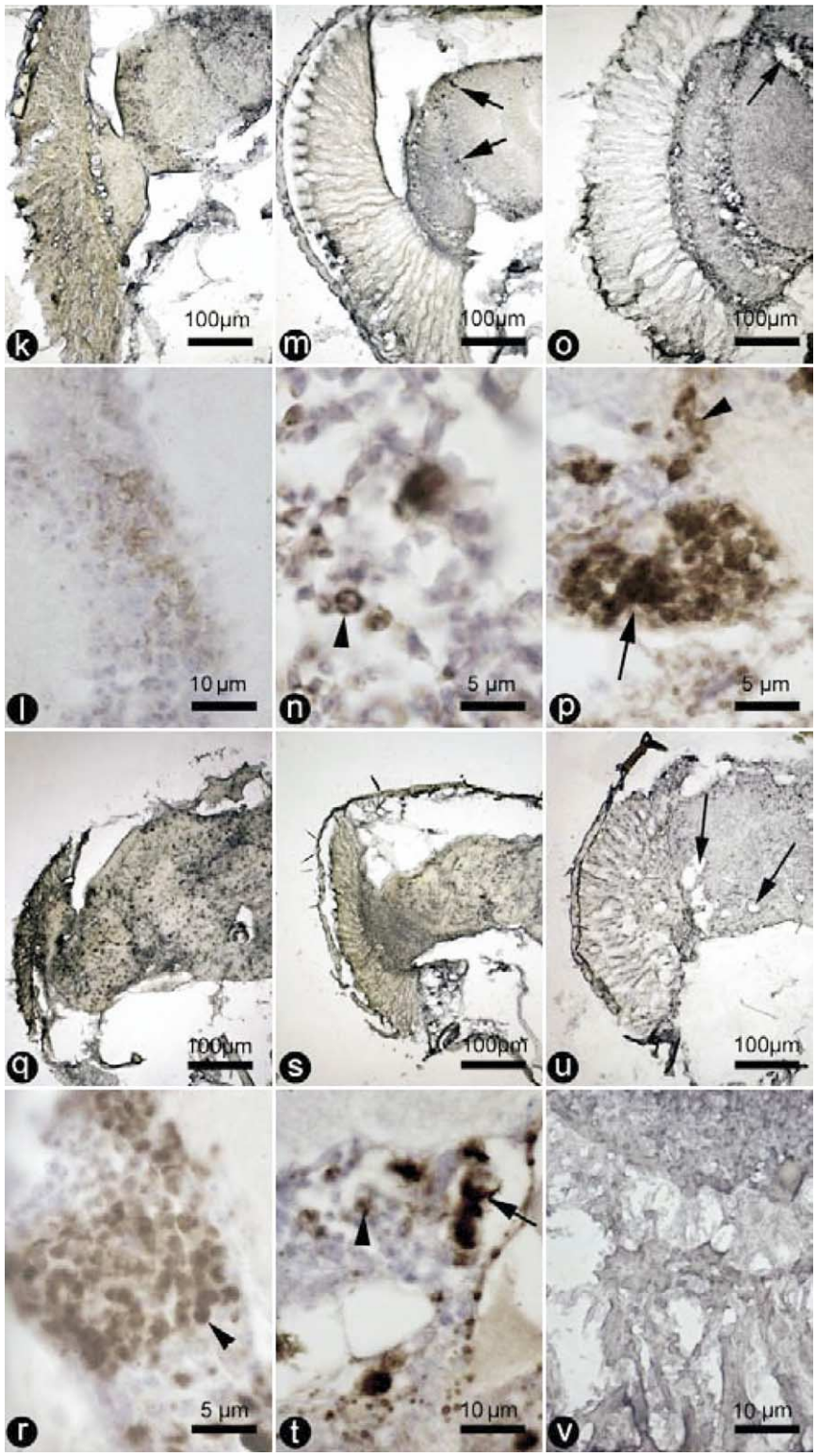

Fig. 1. At day 0 (k, l) there was low-level intracellular accumulation of peptide. By day 5 (m, n) the intracellular staining was more marked (arrowhead) and there were numerous large accumulations (arrows) of peptide. At day 10 (o, p) the intracellular staining remained (arrowheads); however, the larger the foci of Aβ appeared to be extracellular and were accompanied by vacuolation (arrows). The histological changes were further accelerated in flies expressing a single copy of the Arctic $A\beta_{1-42}$ transgene. On day 0 (q, r) there was widespread, intense intracellular accumulation (arrowhead). Intracellular staining was still present at day 5 (s, t); however, extracellular deposits (arrow) were now visible. By day 9 there was widespread vacuolation (arrows) with concomitant reduction in staining intensity (u, v). The same features are evident at earlier time points in flies expressing two copies of the $A\beta_{1-42}$ transgene (*Alz42.2*+*Alz42.3*).

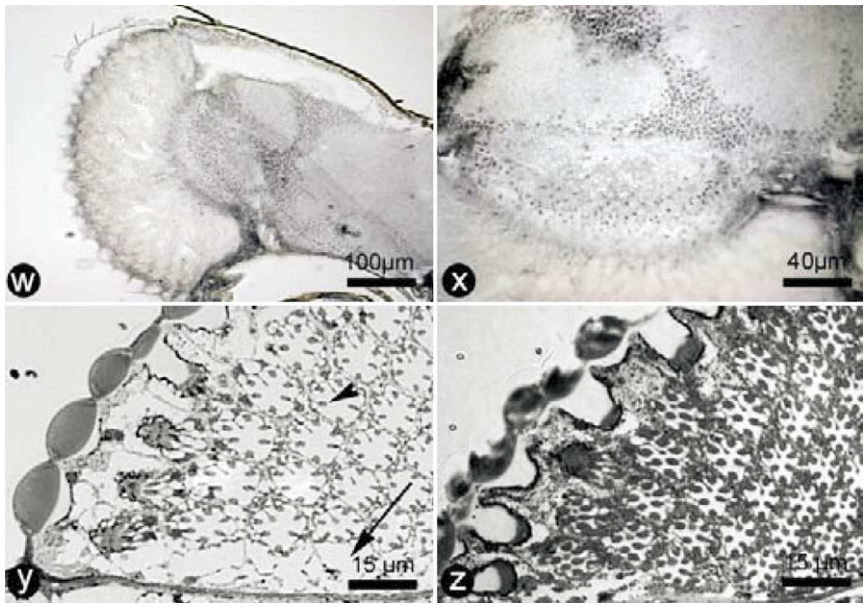

Fig. 1. Expression of a single copy of the $A\beta_{1-40}$ transgene gave clear intracellular staining; however, plaques and vacuolation were absent at day 21 (w, x). Detailed examination of the structure of the brain and retina using resin-embedded tissue demonstrated vacuolation and atrophy of neuronal cells. These changes were seen most clearly in sections of the retina. Panel y shows the atrophy (arrowhead) and vacuolation (arrow) in the retina of a 21 day old fly expressing a single copy of the $A\beta_{1-42}$ transgene (*Alz42.1*), as compared with a 21 day old control *Gal4-elav*^{c155} fly (z). DAB staining was enhanced with nickel in e, g, i, k, m, o, q, s, u, w and x.

derived in which the coding sequences for the $A\beta_{1-42}$ peptide (*Alz42.1*, *Alz42.2*, *Alz42.3*), the $A\beta_{1-40}$ peptide (*Alz40.1*, *Alz40.2*, *Alz40.3*), or the $A\beta_{1-42}$ peptide containing the Arctic mutation (Glu22Gly; *AlzArc1*, *AlzArc2*, *AlzArc3*) were placed downstream of multiple binding sites for the yeast transcriptional activator *Gal4*. The Glu22Gly amino acid substitution causes more rapid protofibril formation with increased neurotoxicity and results in early onset Alzheimer's disease (Nilsberth et al., 2001; Murakami et al., 2002). In contrast the $A\beta_{1-40}$ peptide aggregates less readily than $A\beta_{1-42}$ and provides a powerful control for the non-specific effects of peptide expression. Lines of transgenic flies carrying the *Gal4*-responsive constructs were then crossed with *Gal4-elav*^{c155} flies in order to drive expression of the Aβ peptides in the nervous system and retina (Brand and Perrimon, 1993). Parental flies were cultured for 5 days at 25 °C to promote efficient egg laying. The developing offspring were subsequently incubated at 29 °C to enhance expression of the Alzheimer's transgene.

Histological phenotype of flies expressing Aβ

Initial observations of the Alzheimer's flies revealed a commonly described mild developmental abnormality of the corneal lens of the compound eye. The resulting rough eye phenotype contrasts with the smooth appearance of the eye in flies expressing only the Gal4-*elav*^{c155} driver (Fig. 1a). This effect was gene dose dependent, such that flies expressing one copy of the $A\beta_{1-42}$ transgene had a mild rough eye (Fig. 1b) whereas flies expressing two copies of the $A\beta_{1-42}$ transgene (Fig. 1c) had a moderate rough eye. A single copy of Glu22Gly transgene (Fig. 1d) was sufficient to cause a marked rough eye at hatching (eclosion). In contrast flies expressing the $A\beta_{1-40}$ peptide, the form of the peptide that causes less oligomer and fibril formation (Jarrett and Lansbury, 1993), had normal eyes (data not shown). Despite the rough eye phenotype, microscopic inspection of glutaraldehyde-fixed, araldite-embedded sections demonstrated that the architecture of the underlying retina and brain was normal at eclosion in all transgenic lines expressing the Aβ peptides (data not shown).

Age-related changes in the brain and retina of the transgenic flies were visualized by staining tissue sections with the 4G8 monoclonal antibody that detects all Aβ peptides. In all fly strains we found Aβ to be most intense in regions of the brain that contain neuronal cell bodies. Flies expressing one copy of the $A\beta_{1-42}$ transgene (*Alz42.1*) did not have detectable levels of $A\beta_{1-42}$ expression on the day of eclosion (day 0; Fig. 1e, f); however, by day 10 (Fig. 1g, h) intracellular accumulation was apparent (arrowheads). By day 15 (Fig. 1i, j) the intracellular accumulation was more marked (arrowheads) and there were large accumulations of peptide (arrow) where neuronal bodies predominate, particularly bordering the lamina and medulla.

In flies possessing two copies of the $A\beta_{1-42}$ transgene (*Alz42.2*+*Alz42.3*) intracellular accumulation of Aβ was apparent at day 0 (Fig. 1k, l). By day 5 (Fig. 1m, n) the intracellular staining was more marked (arrowhead) and numerous larger foci of staining were apparent (arrows) with a distribution similar to the *Alz42.1* flies at day 15. At day 10 (arrowhead, Fig. 1o, p) the intracellular staining remained; however, the larger foci of Aβ appeared to be extracellular and were associated with vacuolation (arrows), a sign of neurodegeneration. The same pathological changes were more marked and occurred earlier in flies

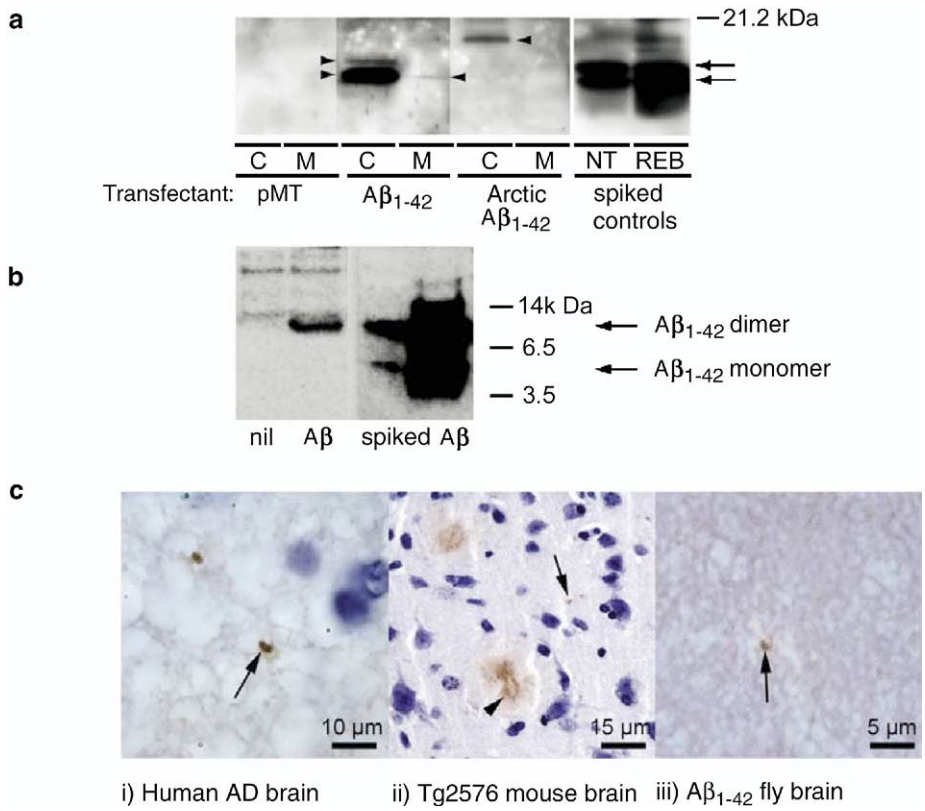

Fig. 2. Detection of Aβ in transfected S2 *Drosophila* cells, brain tissue from transgenic flies and mice and an Alzheimer's disease patient. (a) Cell lysates (C) and culture media (M) from S2 cultures transfected with the empty pMT vector, $A\beta_{1-42}$ and Arctic $A\beta_{1-42}$ were immunoprecipitated with 1 μg of the 4G8 monoclonal antibody bound to sepharose-protein A. Immunocomplexes were resolved on an 18% w/v Tris–glycine SDS-PAGE gel and detected by Western blotting with 4G8 (0.2 ng/μl). Control cell lysates from non-transfected cells (NT) and RIPA extraction buffer (REB) were spiked with 100 pmol of pure $A\beta_{1-42}$ and processed in parallel. $A\beta_{1-42}$ was correctly processed and was found predominantly as an intracellular monomer ($A\beta_{1-42}$, C, lower arrowhead) with some SDS-resistant dimer ($A\beta_{1-42}$, C, upper arrowhead). A small amount of $A\beta_{1-42}$ was secreted into the medium ($A\beta_{1-42}$, M, arrowhead). Arctic $A\beta_{1-42}$ ran as SDS-stable oligomers, predominantly tetramers, in the cell lysates only (Arctic $A\beta_{1-42}$, C, arrowhead). (b) Protein extracts from 100 fly heads were immunoprecipitated with 1 μg of the 4G8 monoclonal antibody bound to sepharose-protein A. Immunocomplexes were resolved on an 18% Tris–glycine SDS-PAGE gel and detected by Western blotting with 4G8 (0.2 ng/μl). *Alz42.2+Alz42.3* flies expressing two copies of the $A\beta_{1-42}$ transgene (Aβ) were compared with control flies (nil) or control fly extract spiked with pure synthetic Aβ peptide (spiked Aβ). (c) Immunostaining of human Alzheimer's disease brain section (i), brain section of a Tg2576 transgenic mouse (ii) and *Alz42.2+Alz42.3* flies expressing $A\beta_{1-42}$ (iii) with Aβ-oligomer-specific antiserum. All three tissues showed small spherical or bullet-shaped structures in the neuropil (arrows) on immunostaining. The murine tissue was unique in that the amyloid plaques exhibited diffuse staining (arrowhead).

expressing a single copy of the Glu22Gly transgene (*AlzArc2*). Arctic $A\beta_{1-42}$ expression was most intense at eclosion (Fig. 1q, r) with staining being intense within cells (arrowhead). Intracellular staining was still present at day 5 (arrowhead, Fig. 1s, t); however, extracellular deposits of Aβ were now apparent in higher power images (arrow). The few flies surviving to day 9 (Figs. 1u, v) showed reduced $A\beta_{1-42}$ staining with widespread vacuolation of the brain and retina (arrows). Confirmation that these appearances represent a specific model of Alzheimer's disease in humans came from the expression of $A\beta_{1-40}$. Flies that expressed one copy of the $A\beta_{1-40}$ transgene showed peptide expression without plaque formation or vacuolation for up to 21 days after eclosion (Fig. 1w, x).

Greater resolution of the structure of the eye was possible with glutaraldehyde-fixation and resin-embedding of tissue. Flies expressing one copy of the $A\beta_{1-42}$ transgene (*Alz42.1*) demonstrated normal retinal and brain structure at days 0, 5 and 15 (data not shown) but by day 21 (Fig. 1y) there was marked vacuolation of the retina (arrow) with atrophy of photoreceptors (arrowhead) in marked contrast with control flies at day 21 which retained normal retinal structure (Fig. 1z).

Handling of the $A\beta_{1-42}$ transgene product by *Drosophila* cells

We demonstrated that processing of the $A\beta_{1-42}$ peptide occurs correctly by using the *Drosophila* S2 cell culture system. *Drosophila* S2 cells were transfected with plasmids expressing the same signal peptide-Aβ construct that was used to generate the corresponding transgenic flies. The $A\beta_{1-42}$ peptide was visualized as a 4 kDa band in the cells and culture media by immunoprecipitation and sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis followed by Western blot analysis with the 4G8 monoclonal antibody (Fig. 2a) indicating the correct removal of the secretion signal peptide. The wild type $A\beta_{1-42}$ peptide was predominantly retained

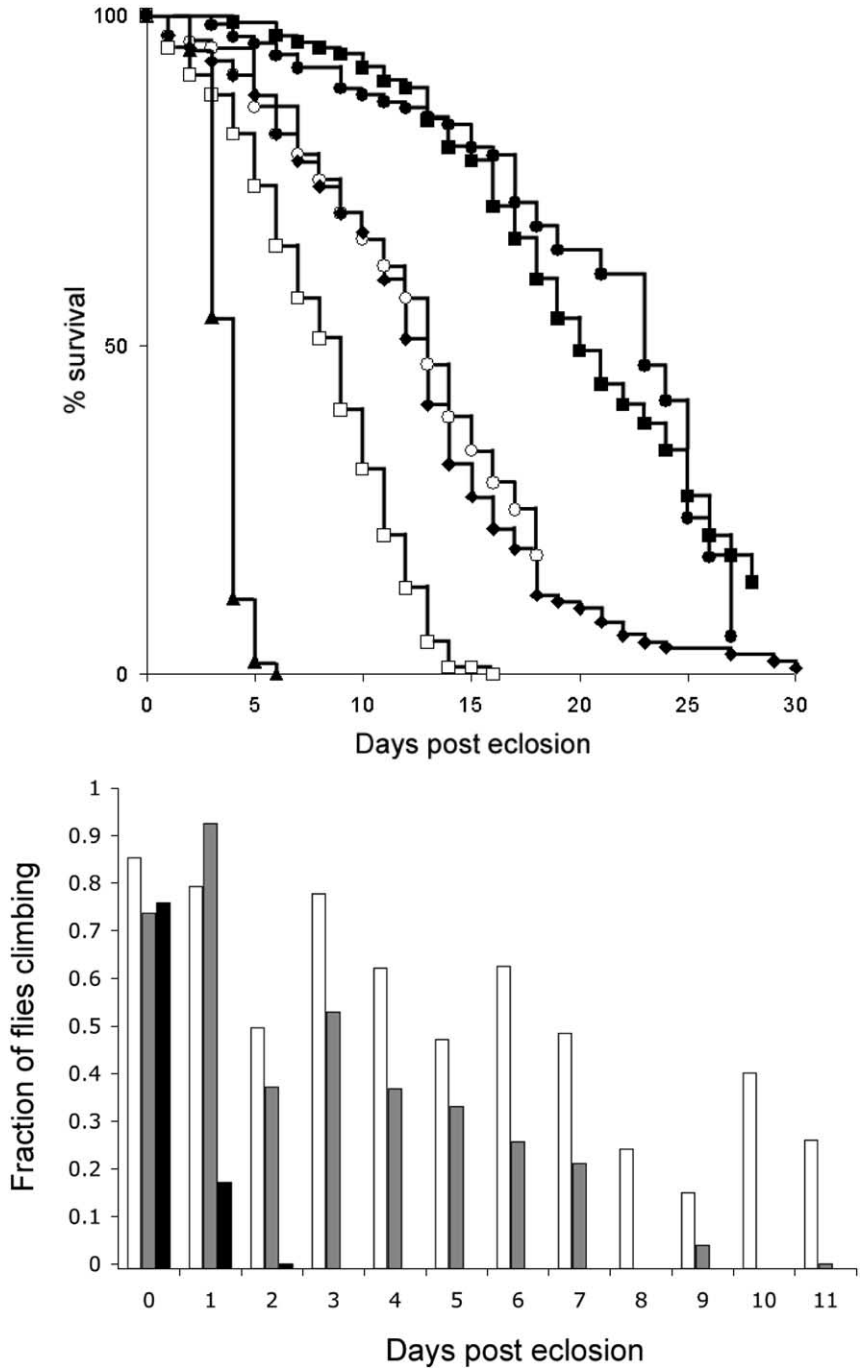

Fig. 3. Characterization of flies expressing Aβ peptides. (a) The longevity of control w^{1118} flies (filled squares) was compared with flies expressing one (*Alz42.2*, diamonds; and *Alz42.3*, empty circles) or two copies (*Alz42.2*+*Alz42.3*; empty squares) of the $A\beta_{1-42}$ transgene, one copy (*AlzArc2* shown, representative of *AlzArc1* and *AlzArc3*; triangles) of the $A\beta_{1-42}$ peptide with the Arctic mutation or one copy (*Alz40.1* shown, representative of *Alz40.3* and *Alz40.2*; filled circles) of $A\beta_{1-40}$ under the control of the *Gal4-elav*^{c155} driver. The flies were cultured at 29 °C and transferred to fresh food every 2–3 days. Expression of the $A\beta_{1-42}$ peptide in the fly's nervous tissue resulted in a dose-dependent reduction in survival. (b) The ratio of Alzheimer's flies that climbed to the top tube was compared with age-matched control flies. Control flies (the *Gal4-elav*^{c155} driver line) incubated at 29 °C climb less with age becoming immobile by day 17 (empty bars; 1244 fly observations in 55 locomotor assays). Alzheimer's flies that express the $A\beta_{1-42}$ transgene (*Alz42.1*; gray bars; 1036 fly observations in 44 locomotor assays) show an accelerated decline in climbing behavior as compared with controls, a phenotype that is further accelerated by expression of the transgene containing the Arctic mutation (*AlzArc1*; black bars; 319 fly observations in 13 locomotor assays).

within the cells, mostly as a monomer but with some SDS-resistant dimeric peptide present (Fig. 2a, $A\beta_{1-42}$, lane C, arrowheads). A small amount of wild type $A\beta_{1-42}$ was secreted into the media (Fig. 2a, $A\beta_{1-42}$, lane M, arrowhead). $A\beta_{1-42}$ with the Glu22Gly showed higher molecular mass bands within the cells that represent SDS-resistant oli-

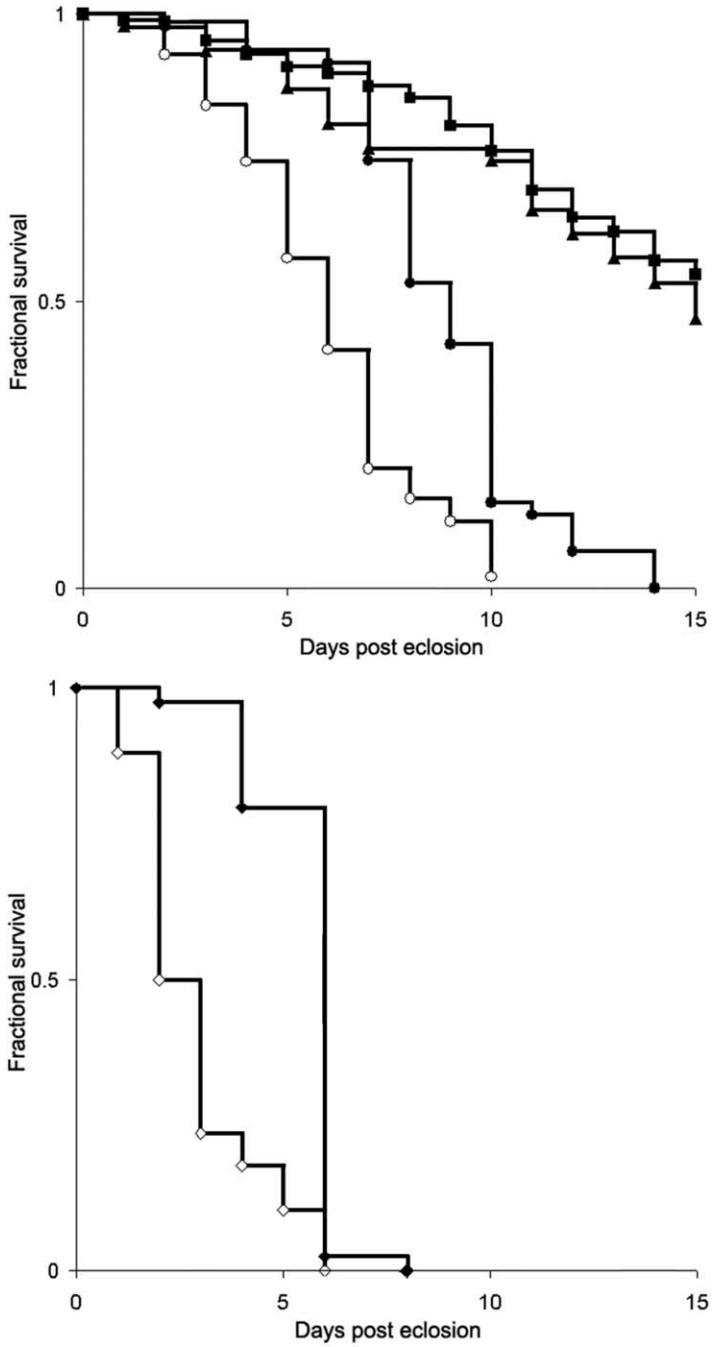

Fig. 4. Effect of MK-801 on Alzheimer's flies. (a) Three micromolar MK-801 prolonged the survival of flies expressing two copies of the $A\beta_{1-42}$ transgene (*Alz42.2+Alz42.3*; filled circles; $n=47$, $P<0.0001$) as compared with untreated flies (empty circles; $n=113$). The effect of MK-801 was a specific rescue of the Alzheimer's phenotype because the survival of control w^{1118} flies (filled squares; $n=356$) was not prolonged when treated with 3 μM MK-801 (filled triangles; $n=47$, $P>0.2$). (b) Treatment of Arctic flies (*Arc2E*; empty diamonds; $n=183$) with 3 μM MK-801 also prolonged survival (filled diamonds; $n=47$, $P<0.0001$).

gomers, predominantly tetramers, of the Arctic $A\beta_{1-42}$ peptide (Fig. 2a, Arctic $A\beta_{1-42}$, lane C, arrowhead). We could not detect secreted Arctic $A\beta_{1-42}$ (Fig. 2a, Arctic $A\beta_{1-42}$, lane M). Specific bands were not observed in samples from control cells lacking the $A\beta_{1-42}$ plasmid (data not shown) or in cells transfected with the blank pMT plasmid (Fig. 2a, pMT, lanes C and M). Immunoprecipitation of $A\beta_{1-42}$ from transgenic fly heads demonstrated the presence of a species consistent with a correctly processed dimer (Fig. 2b, Aβ), using control extract spiked with pure synthetic $A\beta_{1-42}$ peptide as the molecular weight marker (Fig. 2b, spiked Aβ). A similar band was not present in control fly extract (Fig. 2b, nil). Thus *Drosophila* cells are able to process the transgene product to generate a 4 kDa peptide that

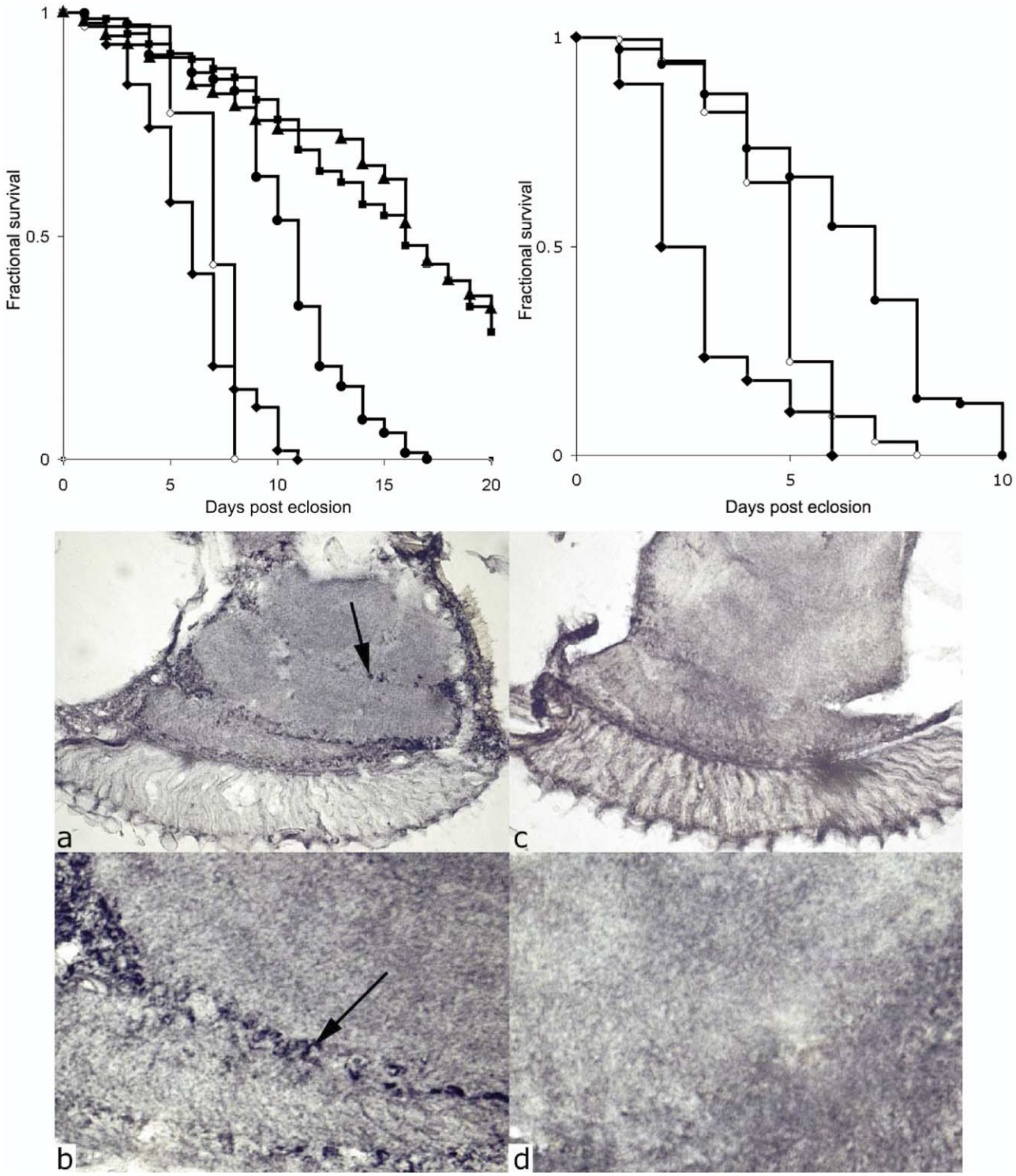

Fig. 5. Effect of Congo Red on Alzheimer's flies. (a) Five percent w/v Congo Red markedly prolonged the survival of flies expressing two copies of the $A\beta_{1-42}$ transgene (*Alz42.2*+*Alz42.3*; filled circles; $n=73$, $P<0.0001$) as compared with untreated flies (filled diamonds; $n=113$). However 1% w/v Congo Red did not significantly prolong the survival of Alzheimer's flies (empty circles; $n=60$, $P>0.1$). Ten percent and 15% w/v Congo Red gave similar results to 5% w/v Congo Red. The Congo Red effect was specific because there was no prolongation in survival when control w^{1118} flies (filled squares; $n=356$) were treated with 5% w/v Congo Red (filled triangles; $n=100$, $P>0.05$). (b) Treatment of Arctic flies (*Arc2E*; filled diamonds; $n=183$) with either 1 or 5% w/v Congo Red significantly prolonged survival as compared with controls (1% w/v Congo Red, empty circles, $n=160$, $P<0.0001$; and 5% w/v Congo Red, filled circles, $n=408$, $P<0.0001$). Treatment with 10% and 15% w/v Congo Red gave similar results to 5% w/v Congo Red. (c) Treatment with Congo Red improved the histological appearances in 9 day old flies expressing two copies of the $A\beta_{1-42}$ transgene (*Alz42.2*+*Alz42.3*). The burden of Aβ deposits (arrows) was markedly higher in untreated Alzheimer's flies (a, b) as compared with flies treated with 5% w/v Congo Red (c, d) stained with the 4G8 monoclonal antibody. Magnification was 40× for a and c and 100× for b and d.

corresponds to the $A\beta_{1-42}$ peptide that accumulates in the brains of humans with Alzheimer's disease. Despite being correctly processed into the secretory pathway, the majority of the Aβ peptide is not secreted but is retained within the cell as aggregates, at least a fraction of which are SDS-stable on gel electrophoresis.

$A\beta_{1-42}$ oligomers are present in *Drosophila* brain

The brains of flies expressing $A\beta_{1-42}$ were stained with an antiserum that recognizes only the oligomeric conformer of the Alzheimer's peptide (Kayed et al., 2003). We found that the pattern of staining achieved with this antiserum was dependent on the handling of tissue, in particular the degree of antigen retrieval. However, using identical immunochemical conditions for sections from human Alzheimer's disease brain (Fig. 2 ci), murine Alzheimer's model brain sections (Tg2576 mice; Fig. 2 cii) or flies expressing $A\beta_{1-42}$ in their brains (Fig. 2 ciii) we could demonstrate similarities in the pattern of staining. In all three tissues we could observe small, immunoreactive spheroid- or bullet-shaped bodies in the neuropil. The murine tissue also showed staining of the amyloid plaques that characterize this model system. There was no clear time course in the appearance of these bodies in the fly models of Alzheimer's disease. No staining was seen in flies that expressed only the *elav* driver (data not shown).

Neurodegeneration occurred in the Alzheimer's flies in the absence of β-amyloid formation as evidenced by the lack of staining with the dye Congo Red and the absence of apple-green birefringence in sections of flies expressing $A\beta_{1-42}$ or Arctic $A\beta_{1-42}$ at any age (data not shown). This was in contrast to the positive staining of control amyloid-containing tissue. Moreover, examination of neural tissue from flies expressing the $A\beta_{1-42}$ transgene with transmission electron microscopy did not reveal any amyloid fibrils (data not shown). Our data suggest that intracellular $A\beta_{1-42}$ and/or subsequent non-β-amyloid aggregates can cause neurodegeneration.

Alzheimer's flies have reduced longevity

The effect on the whole fly of expressing the $A\beta_{1-42}$ peptide in fly neural tissue was then assessed. We performed longevity assays on flies expressing Aβ peptides and found that expression of the $A\beta_{1-42}$ peptide caused a marked reduction in survival (Fig. 3a) as compared with flies without a transgene. Survival assays were repeated in excess of three times for all lines of flies and the SPSS 11 Kaplan-Meier software package was used to analyze the significance of the data. The three lines of flies that expressed a single Alzheimer's transgene (*Alz42.1*, *Alz42.2* and *Alz42.3*) had a median survival of 13 days (*Alz42.1*, $n=100$; *Alz42.2*, $n=100$; *Alz42.3*, $n=100$), compared with 20 days for the control flies (w^{1118}, $n=100$; $P<0.0001$ w^{1118} vs. *Alz42.2*). Viability was further reduced if two copies of the $A\beta_{1-42}$ transgene (*Alz42.2*+*Alz42.3*; $n=100$) were expressed in neural tissue, such that the median survival was 9 days ($P<0.0001$, *Alz42.2* vs. *Alz42.2*+*Alz42.3*). The Glu22Gly had a striking effect such that flies expressing one copy of this transgene having a median survival of only 4 days ($n=94$, $P<0.0001$ *AlzArc2* vs. *Alz42.2*+*Alz42.3*). At 29 °C there was no transgene-dosage effect for the Arctic flies such that the double transgenic flies *AlzArc1*+*AlzArc2* also had a median survival of 4 days; when the expression of the Arctic $A\beta_{1-42}$ peptide was reduced by culturing the flies at 25 °C a transgene-dosage effect was apparent. In this case the median survival for flies expressing one (*AlzArc2*, $n=212$) or two copies (*AlzArc1*+*AlzArc2*, $n=89$) of the Arctic $A\beta_{1-42}$ transgene was 10 and 6 days respectively ($P<0.0001$).

Expressing one copy of the $A\beta_{1-40}$ transgene at 29 °C had no effect on survival (median survival of *Alz40.1* was 18 days, $n=107$, $P>0.4$ vs. w^{1118}, $n=100$), demonstrating that the less aggregatory Aβ peptide alone was not toxic.

Behavioral phenotype of Alzheimer's flies

Initial observations indicated that the Alzheimer's flies behaved normally at eclosion and subsequently developed locomotor deficits. Locomotor activity was assessed with a climbing assay that has been used to monitor aging-related changes in *Drosophila* (Ganetzky and Flanagan, 1978; Le Bourg and Lints, 1992) and to characterize transgenic Parkinson's flies with locomotor dysfunction (Feany and Bender, 2000). Flies expressing one $A\beta_{1-42}$ transgene showed accelerated decline in climbing behavior relative to control flies, becoming immobile by day 10 (Fig. 3b). This locomotor dysfunction occurs in association with intracellular Aβ but prior to the development of extracellular plaques (Fig. 1g, h) and prior to signs of neurodegeneration. The locomotor phenotype was enhanced by the expression the Arctic $A\beta_{1-42}$ transgene (immobile by day 2).

By expressing Aβ in the brain of the flies we are modeling the final common pathway of neurodegeneration that, in Alzheimer's disease, is triggered by a wide range of upstream factors. We show clearly that the locomotor, neurodegenerative and survival phenotypes were more severe under conditions that favored the $A\beta_{1-42}$ aggregation (raising the temperature or increasing the number of copies of the $A\beta_{1-42}$ transgene) or expressing the Arctic mutation that favors oligomer formation. This is in keeping with the overproduction of $A\beta_{1-42}$ in the sporadic and most of the genetic forms of Alzheimer's disease (Selkoe, 2001).

Validation of the Alzheimer's fly for human drug screening

Having achieved a biologically faithful model of Alzheimer's disease we validated the fly as a platform for drug discovery by testing the therapeutic efficacy of MK-801, an inhibitor of the excitatory action of glutamate on the NMDA receptor. The excitotoxic action of glutamate in Alzheimer's disease is supported clinically by the finding that Memantine, a non-competitive glutamate antagonist, is the only therapy that slows the progression of Alzheimer's disease (Reisberg et al., 2003). *Drosophila* have closely homologous NMDA receptors expressed in the brain (Ultsch et al., 1993) and consequently we administered 3 μM MK-801 in the fly food from the embryonic stage onwards. Treatment with MK-801 markedly prolonged the survival of Alzheimer's flies express-

ing two copies of the $A\beta_{1-42}$ transgene (*Alz42.2+Alz42.3*; Fig. 4a; $P<0.0001$) and flies expressing the Glu22Gly mutant of $A\beta_{1-42}$ (*AlzArc1*, Fig. 4b, $P<0.0001$). There was no additional rescue with 30 and 150 μM MK-801. Therefore the Alzheimer's fly can be rescued by a therapeutic intervention that is effective in the treatment of human Alzheimer's disease.

Congo Red, an inhibitor of $A\beta_{1-42}$ aggregation, rescues the Alzheimer's fly

We then assessed whether blocking oligomerization of the $A\beta_{1-42}$ peptide would rescue the Alzheimer's phenotype. The azo-dye Congo Red binds to β-amyloid (Klunk et al., 1989) and inhibits fibrillization of $A\beta_{1-42}$ *in vitro* (Lorenzo and Yanker, 1994; Podlisny et al., 1998). Indeed Congo Red has recently been shown to inhibit oligomerization of polyglutamine repeats in association with a striking improvement in motor function and survival in a mouse model of Huntington's disease (Sánchez et al., 2003). It also rescues neurodegeneration and improves motor function in a *Drosophila* model of polyglutamine repeat disease (Apostol et al., 2003). The double $A\beta_{1-42}$ transgenic Alzheimer's flies (*Alz42.2+Alz42.3*) were therefore fed on a 30% w/v mix of Congo Red dye with yeast paste from the time of hatching but this had no effect on survival. However, feeding the double transgenic (*Alz42.2+Alz42.3*; Fig. 5a) and the Arctic (*AlzArc1*; Fig. 5b) flies on 5% w/v Congo Red from the embryonic stage resulted in a significant increase in longevity ($P<0.0001$ for both lines). The specific rescue by Congo Red of the Alzheimer's phenotype was demonstrated by the absence of any effect on the survival of control flies (Fig. 5a). Immunohistological sections from double $A\beta_{1-42}$ transgenic flies (*Alz42.2+Alz42.3*) fed on 5% w/v Congo Red showed a clear reduction in plaque formation and delay in vacuolation with preservation of the architecture of the brain and retinal tissues (Fig. 5c). We were not able to determine the effect of Congo Red on the climbing phenotype because of non-specific toxicity of Congo Red on control flies (treated flies were immobile by day 7 vs. day 17 for untreated flies). Thus Congo Red is able to rescue models of both the sporadic and familial forms of Alzheimer's disease by inhibiting Aβ aggregation *in vivo*.

DISCUSSION

The evidence that pro-aggregatory Aβ peptides underlie the neuronal dysfunction and death seen in Alzheimer's disease is robust (Hardy and Selkoe, 2002). However it is not clear which species is directly neurotoxic and whether the toxic effect is mediated from within the cell or from the extracellular space. We have developed a *Drosophila* model of Alzheimer's disease based on the secretion of Aβ peptides to gain insight into these questions. Initially Aβ was expressed in a *Drosophila* cell line to demonstrate that the secretion signal peptide was being removed and that $A\beta_{1-42}$ was being correctly generated. Although Aβ correctly entered the secretory pathway our data showed that much of the $A\beta_{1-42}$ and all of the Arctic $A\beta_{1-42}$ was retained within the cell as monomer or SDS-resistant oligomers. Similarly in brain sections from young transgenic flies, Aβ is initially intracellular and only later are extracellular deposits and vacuolation observed. Given our observations in the fly it is interesting to note the evidence that intracellular Aβ peptide accumulation is an early step in both Alzheimer's disease (LaFerla et al., 1997; Yang et al., 1999; Gouras et al., 2000; Glabe, 2001) and the neurodegeneration associated with Down syndrome (Gyure et al., 2001; Mori et al., 2002). There is further evidence that Aβ neurotoxicity is mediated primarily from an intracellular location (Zhang et al., 2002; Casas et al., 2004). In our fly models the process of intracellular accumulation and neurodegeneration was accelerated by expressing $A\beta_{1-42}$ with the Arctic mutation that favors protofibril formation and increases neurotoxicity (Nilsberth et al., 2001; Lashuel et al., 2003). Furthermore the survival of the flies was prolonged and the accumulation of Aβ was reduced by feeding flies with Congo Red, an azo-dye that reduces $A\beta_{1-42}$ oligomerization *in vitro* (Podlisny et al., 1998). Flies expressing the less aggregatory $A\beta_{1-40}$ peptide acted as controls for the non-specific effects of expressing a foreign hydrophobic peptide in the *Drosophila* brain. The $A\beta_{1-40}$ peptide was clearly visualized within cell bodies of neurones in a similar distribution to the $A\beta_{1-42}$ peptides; however, extracellular deposition and vacuolation did not occur.

These transgenic fly models also allow dissection of the temporal relationship between the detection of intracellular Aβ accumulation and the observed locomotor phenotype. Flies expressing the $A\beta_{1-42}$ peptide had a progressive decline in locomotor function. This was apparent at a time when only intracellular Aβ was seen within the brain. A likely conclusion from this observation is that non-amyloid aggregates of Aβ, probably peptide oligomers, induce neuronal dysfunction that in turn is manifest as a behavioral phenotype. We have performed immunohistochemistry with an oligomer-specific antiserum on sections from human Alzheimer's disease brain, mouse model brain and fly model brain. A similar pattern of staining in all three tissues suggests that the role of oligomeric Aβ in the fly model is representative of its role in the human disease. The presence of oligomer staining when Aβ accumulation is exclusively intracellular suggests that the toxic oligomers of Aβ exert their neurotoxic effects from an intracellular location. Taken together these data show that intracellular $A\beta_{1-42}$ is able to cause locomotor deficits and may play an important role in neuronal dysfunction in the early stages of Alzheimer's disease.

The focal accumulations of $A\beta_{1-42}$ that form in the brains of transgenic *Drosophila* did not stain with Congo Red or display fibrils on electron microscopy. These Aβ aggregates are similar to the diffuse, or pre-amyloid plaques, that are thought to be a precursor of the mature neuritic plaques seen in Alzheimer's disease (Iwatsubo et al., 1994, 1995). This is supported by previous reports that the early plaques are composed exclusively of the $A\beta_{1-42}$ peptide with the more mature plaques being a mixture of $A\beta_{1-42}$ and $A\beta_{1-40}$ (Hirayama et al., 2003). Remarkably the Alzheimer's fly resembles a family that carries a mutation at the γ-secretase cleavage site in the

APP that results in almost exclusive $A\beta_{1-42}$ production (Kumar-Singh et al., 2000). This kindred suffers an aggressive form of Alzheimer's disease characterized by the deposition of abundant, predominantly non-fibrillar, diffuse plaques. Our data from transgenic *Drosophila* provide strong supportive evidence that intracellular $A\beta_{1-42}$ and/or non-amyloid extracellular plaques are sufficient to cause neuronal dysfunction and death in Alzheimer's disease in the absence of mature β-amyloid plaques.

One of the aims of this project was to develop a model that could be used for testing novel agents that may be effective in slowing the progression of Alzheimer's disease. We validated the fly as a platform for human drug discovery by demonstrating that the glutamate receptor antagonist MK-801 significantly rescued the Alzheimer's phenotype, reproducing the beneficial effect of the drug memantine. The further demonstration that treatment with Congo Red can ameliorate the Alzheimer's phenotype shows not only that blocking β-strand linkage and $A\beta_{1-42}$ aggregation reduces the toxicity of the $A\beta_{1-42}$ peptide, but also that the fly is useful for screening and testing anti-aggregation agents. There is no suggestion from previous work that either MK-801 or Congo Red non-specifically reduces protein expression. Furthermore Congo Red is not toxic to control flies as would be expected if protein synthesis were impaired. For these reasons it is unlikely that the beneficial effects of Congo Red and MK-801 can be explained by a non-specific reduction in expression of the Aβ transgenes. Therefore our *Drosophila* model will be useful in assessing therapies that attenuate aggregation of the $A\beta_{1-42}$ peptide as well as those that block the excitotoxic effects of glutamate. The fly has many advantages over existing mouse models of Alzheimer's disease as a platform for drug discovery. These include the clarity of the phenotype, the rapid progression of the disease, the low cost of fly culture and the power of genetic manipulations and screens in the fly.

In summary, our *Drosophila* model of Alzheimer's disease provides a clear demonstration of the temporal relationship between the accumulation of intracellular $A\beta_{1-42}$, extracellular non-amyloid plaques and neurodegeneration. Locomotor dysfunction is observed prior to plaque formation and neurodegeneration and may be caused by intracellular $A\beta_{1-42}$. The time of onset of neurodegeneration is inversely proportional to the propensity of the Aβ peptide to form oligomers and is rescued by feeding Alzheimer flies with Congo Red that blocks Aβ oligomerization. These data show that intracellular Aβ and extracellular non-amyloid plaques cause neurodegeneration in our fly model of Alzheimer's disease. Our data provide strong support for the development of therapeutic agents that block oligomer formation *in vivo*.

Acknowledgments—This work was supported by the Wellcome Trust, the Medical Research Council (UK) and Papworth NHS Trust. D.C.C. is a Wellcome Trust Advanced Clinical Fellow, K.J.K. is an MB/PhD student and R.P. is a PhD student, both funded by Merck, Sharpe and Dohme. Work by D.C.G. was supported by a MRC Programme Grant to M. Ashburner, D.C. Gubb and S. Russell. We are grateful to Matthew Savoian, Department of Genetics, University of Cambridge, for his help with microscopy; Neil Wilkie and David Smith, The Neuroscience Research Centre, Merck Sharp & Dohme, and Steve Russell and Iain Robinson, Department of Genetics, University of Cambridge, for helpful discussions; Matthew Adam, Department of Genetics, University of Cambridge, for assistance with fly culture; and Robin Carrell, Department of Hematology, University of Cambridge, for critical review of the manuscript.

REFERENCES

Apostol BL, Kazantsev A, Raffioni S, Illes K, Pallos J, Bodai L, Slepko N, Bear JE, Gertler FB, Hersch S, Housman DE, Marsh JL, Thompson LM (2003) A cell-based assay for aggregation inhibitors as therapeutics of polyglutamine-repeat disease and validation in *Drosophila*. Proc Natl Acad Sci USA 100:5950–5955.

Brand AH, Perrimon N (1993) Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development 118:401–405.

Casas C, Sergeant N, Itier JM, Blanchard V, Wirths O, van der Kolk N, Vingtdeux V, van de Steeg E, Ret G, Canton T, Drobecq H, Clark A, Bonici B, Delacourte A, Benavides J, Schmitz C, Tremp G, Bayer TA, Benoit P, Pradier L (2004) Massive CA1/2 neuronal loss with intraneuronal and N-terminal truncated Abeta42 accumulation in a novel Alzheimer transgenic model. Am J Pathol 165:1289–1300.

Crowther DC (2002) Familial conformational diseases and dementias. Hum Mutat 20:1–14.

Dahlgren KN, Manelli AM, Stine WB Jr, Baker LK, Krafft GA, LaDu MJ (2002) Oligomeric and fibrillar species of amyloid-beta peptides differentially affect neuronal viability. J Biol Chem 277:32046–32053.

Davis S, Laroche S (2003) What can rodent models tell us about cognitive decline in Alzheimer's disease? Mol Neurobiol 27:249–276.

Dickson DW (1997) The pathogenesis of senile plaques. J Neuropathol Exp Neurol 56:321–339.

Feany MB, Bender WW (2000) A *Drosophila* model of Parkinson's disease. Nature 404:394–398.

Ganetzky B, Flanagan JR (1978) On the relationship between senescence and age related changes in two wild-type strains of *Drosophila melanogaster*. Exp Gerontol 13:189–196.

Glabe C (2001) Intracellular mechanisms of amyloid accumulation and pathogenesis in Alzheimer's disease. J Mol Neurosci 17:137–145.

Gouras GK, Tsai J, Naslund J, Vincent B, Edgar M, Checler F, Greenfield JP, Haroutunian V, Buxbaum JD, Xu H, Greengard P, Relkin NR (2000) Intraneuronal Abeta42 accumulation in human brain. Am J Pathol 156:15–20.

Green C, Levashina E, McKimmie C, Dafforn T, Reichhart J-M, 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.

Greeve I, Kretzschmar D, Tschape JA, Beyn A, Brellinger C, Schweizer M, Nitsch RM, Reifegerste R (2004) Age-dependent neurodegeneration and Alzheimer-amyloid plaque formation in transgenic *Drosophila*. J Neurosci 24:3899–3906.

Gyure KA, Durham R, Stewart WF, Smialek JE, Troncoso JC (2001) Intraneuronal abeta-amyloid precedes development of amyloid plaques in Down syndrome. Arch Pathol Lab Med 125:489–492.

Hardy J, Selkoe DJ (2002) The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. Science 297:353–356.

Hirayama A, Horikoshi Y, Maeda M, Ito M, Takashima S (2003) Characteristic developmental expression of amyloid beta40, 42 and 43 in patients with Down syndrome. Brain Dev 25:180–185.

Hsiao K, Chapman P, Nilsen S, Eckman C, Harigaya Y, Younkin S, Yang F, Cole G (1996) Correlative memory deficits, Abeta elevation, and amyloid plaques in transgenic mice. Science 274:99–102.

Iijima K, Liu HP, Chiang AS, Hearn SA, Konsolaki M, Zhong Y (2004) Dissecting the pathological effects of human A{beta}40 and A{beta}42 in *Drosophila*: a potential model for Alzheimer's disease. Proc Natl Acad Sci USA 101:6623–6628.

Iwatsubo T, Mann DM, Odaka A, Suzuki N, Ihara Y (1995) Amyloid beta protein (A beta) deposition: A beta 42(43) precedes A beta 40 in Down syndrome. Ann Neurol 37:294–299.

Iwatsubo T, Odaka A, Suzuki N, Mizusawa H, Nukina N, 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.

Jarrett JT, Lansbury PT Jr (1993) Seeding 'one-dimensional crystallization' of amyloid: a pathogenic mechanism in Alzheimer's disease and scrapie. Cell 73:1055–1058.

Kayed R, Head E, Thompson JL, McIntire TM, Milton SC, Cotman CW, Glabe CG (2003) Common structure of soluble amyloid oligomers implies common mechanism of pathogenesis. Science 300:486–489.

Klunk WE, Pettegrew JW, Abraham DJ (1989) Quantitative evaluation of Congo red binding to amyloid-like proteins with a β-pleated sheet conformation. J Histochem Cytochem 37:1273–1281.

Kumar-Singh S, De Jonghe C, Cruts M, Kleinert R, Wang R, Mercken M, De Strooper B, Vanderstichele H, Lofgren A, Vanderhoeven I, Backhovens H, Vanmechelen E, Kroisel PM, Van Broeckhoven C (2000) Nonfibrillar diffuse amyloid deposition due to a gamma(42)-secretase site mutation points to an essential role for N-truncated A beta(42) in Alzheimer's disease. Hum Mol Genet 9:2589–2598.

LaFerla FM, Troncoso JC, Strickland DK, Kawas CH, Jay G (1997) Neuronal cell death in Alzheimer's disease correlates with apoE uptake and intracellular Abeta stabilization. J Clin Invest 100:310–320.

Lai MT, Chen E, Crouthamel MC, DiMuzio-Mower J, Xu M, Huang Q, Price E, Register RB, Shi XP, Donoviel DB, Bernstein A, Hazuda D, Gardell SJ, Li YM (2003) Presenilin-1 and presenilin-2 exhibit distinct yet overlapping gamma-secretase activities. J Biol Chem 278:22475–22481.

Lambert MP, Barlow AK, Chromy BA, Edwards C, Freed R, Liosatos M, Morgan TE, Rozovsky I, Trommer B, Viola KL, Wals P, Zhang C, Finch CE, Krafft GA, Klein WL (1998) Diffusible, nonfibrillar ligands derived from Abeta1–42 are potent central nervous system neurotoxins. Proc Natl Acad Sci USA 95:6448–6453.

Lashuel HA, Hartley D, Petre BM, Walz T, Lansbury PT Jr (2002) Neurodegenerative disease: amyloid pores from pathogenic mutations. Nature 418:291.

Lashuel HA, Hartley DM, Petre BM, Wall JS, Simon MN, Walz T, Lansbury PT Jr (2003). Mixtures of wild-type and a pathogenic (E22G) form of Abeta40 in vitro accumulate protofibrils, including amyloid pores. J Mol Biol 332:795–808.

Le Bourg E, Lints FA (1992) Hypergravity and aging in *Drosophila melanogaster*: 4. Climbing activity. Gerontology 38:59–64.

Lorenzo A, Yanker BA (1994) β-Amyloid neurotoxicity requires fibril formation and is inhibited by Congo red. Proc Natl Acad Sci USA 91:12243–12247.

Mori C, Spooner ET, Wisniewsk KE, Wisniewski TM, Yamaguch H, Saido TC, Tolan DR, Selkoe DJ, Lemere CA (2002) Intraneuronal Abeta42 accumulation in Down syndrome brain. Amyloid 9:88–102.

Murakami K, Irie K, Morimoto A, Ohigashi H, Shindo M, Nagao M, Shimizu T, Shirasawa T (2002) Synthesis, aggregation, neurotoxicity, and secondary structure of various A beta 1–42 mutants of familial Alzheimer's disease at positions 21–23. Biochem Biophys Res Commun 294:5–10.

Naslund J, Haroutunian V, Mohs R, Davis KL, Davies P, Greengard P, Buxbaum JD (2000) Correlation between elevated levels of amyloid beta-peptide in the brain and cognitive decline. JAMA 283: 1571–1577.

Nilsberth C, Westlind-Danielsson A, Eckman CB, Condron MM, Axelman K, Forsell C, Stenh C, Luthman J, Teplow DB, Younkin SG, Naslund J, Lannfelt L (2001) The 'Arctic' APP mutation (E693G) causes Alzheimer's disease by enhanced Abeta protofibril formation. Nat Neurosci 4:887–893.

Podlisny MB, Walsh DM, Amarante P, Ostaszewski BL, Stimson ER, Maggio JE, Teplow DB, Selkoe DJ (1998) Oligomerization of endogenous and synthetic amyloid β-protein at nanomolar levels in cell culture and stabilization of monomer by Congo Red. Biochemistry 37:3602–3611.

Puchtler H, Sweat F (1965) Congo red as a stain for fluorescence microscopy of amyloid. J Histochem Cytochem 13:693–694.

Puglielli L, Tanzi RE, Kovacs DM (2003) Alzheimer's disease: the cholesterol connection. Nat Neurosci 6:345–351.

Reisberg B, Doody R, Stöffler A, Schmitt F, Ferris S, Möbius HJ (2003) Memantine in moderate-to-severe Alzheimer's disease. N Engl J Med 348:1333–1341.

Sánchez I, Mahlke C, Yuan J (2003) Pivotal role of oligomerization in expanded polyglutamine neurodegenerative disorders. Nature 421:373–379.

Selkoe DJ (2001) Alzheimer's disease: genes, proteins, and therapy. Physiol Rev 81:741–766.

Ultsch A, Schuster CM, Laube B, Betz H, Schmitt B (1993) Glutamate receptors of *Drosophila melanogaster*: primary structure of a putative NMDA receptor protein expressed in the head of the adult fly. FEBS Lett 324:171–177.

Walsh DM, Klyubin I, Fadeeva JV, Cullen WK, Anwyl R, Wolfe MS, Rowan MJ, Selkoe DJ (2002) Naturally secreted oligomers of amyloid beta protein potently inhibit hippocampal long-term potentiation in vivo. Nature 416:535–539.

Westerman MA, Cooper-Blacketer D, Mariash A, Kotilinek L, Kawarabayashi T, Younkin LH, Carlson GA, Younkin SG, Ashe KH (2002) The relationship between Abeta and memory in the Tg2576 mouse model of Alzheimer's disease. J Neurosci 22:1858–1867.

Wilcock GK, Esiri MM (1982) Plaques, tangles and dementia: a quantitative study. J Neurol Sci 56:343–356.

Yang AJ, Chandswangbhuvana D, Shu T, Henschen A, Glabe CG (1999) Intracellular accumulation of insoluble, newly synthesized abetan-42 in amyloid precursor protein-transfected cells that have been treated with Abeta1–42. J Biol Chem 274:20650–20656.

Zhang Y, McLaughlin R, Goodyer C, LeBlanc A (2002) Selective cytotoxicity of intracellular amyloid beta peptide1–42 through p53 and Bax in cultured primary human neurons. J Cell Biol 156:519–529.

(Accepted 17 December 2004)