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