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Neurotoxic effects induced by the *Drosophila* amyloid-β peptide suggest a conserved toxic function

Katia Carmine-Simmen [a], Thomas Proctor [a], Jakob Tschäpe [a], Burkhard Poeck [b], Tilman Triphan [b], Roland Strauss [b], Doris Kretzschmar [a,*]

[a] Center for Research on Occupational and Environmental Toxicology, Oregon Health and Science University, Portland, 3181 SW Sam Jackson Park Road, Portland, OR 97239, USA
[b] Lehrstuhl für Genetik und Neurobiologie, Universität Würzburg, 97074 Würzburg, Germany

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ABSTRACT

The accumulation of amyloid-β (Aβ) into plaques is a hallmark feature of Alzheimer's disease (AD). While amyloid precursor protein (APP)-related proteins are found in most organisms, only Aβ fragments from human APP have been shown to induce amyloid deposits and progressive neurodegeneration. Therefore, it was suggested that neurotoxic effects are a specific property of human Aβ. Here we show that Aβ fragments derived from the *Drosophila* orthologue APPL aggregate into intracellular fibrils, amyloid deposits, and cause age-dependent behavioral deficits and neurodegeneration. We also show that APPL can be cleaved by a novel fly β-secretase-like enzyme. This suggests that Aβ-induced neurotoxicity is a conserved function of APP proteins whereby the lack of conservation in the primary sequence indicates that secondary structural aspects determine their pathogenesis. In addition, we found that the behavioral phenotypes precede extracellular amyloid deposit formation, supporting results that intracellular Aβ plays a key role in AD.

Introduction

Hallmark features of AD include the formation of neurofibrillary tangles and amyloid plaques, the latter consisting largely of Aβ peptides derived by proteolytic cleavage of the amyloid precursor protein (APP; (Annaert and De Strooper, 2002; Selkoe, 2001; Turner et al., 2003). Strong evidence that Aβ plays a key role in AD has been provided by rare cases of familial-early onset AD, in which mutations that enhance Aβ production were identified both in APP itself and in presenilin, a component of the γ-secretase complex that processes the holoprotein (De Strooper, 2007; Goate, 2006; Levy et al., 2006). For the vast majority of AD cases, however, the cause of amyloid plaque formation and the intrinsic pathogenicity of Aβ peptides remain controversial. Clarification of these issues has been hampered by the lack of animal models that recapitulate the disease. In rodents, Aβ peptides derived from endogenous APPs do not form amyloid deposits (Link, 2005) and these models therefore rely on the analysis of transgenic animals expressing human APP. So far non-human primates appear to be the only models for which AD-like symptoms caused by the endogenous APP proteins have been described (Duff and Suleman, 2004; Price and Sisodia, 1994).

APP-related proteins have been identified in both *Caenorhabditis elegans* (apl-1; (Daigle and Li, 1993) and *Drosophila melanogaster* (APPL; (Rosen et al., 1989), as have orthologues of α-secretases (Allinson et al., 2003; Rooke et al., 1996) and components of the γ-secretase complex (Boulianne et al., 1997; Chung and Struhl, 2001; Francis et al., 2002; Hong and Koo, 1997; Levitan and Greenwald, 1995; Li and Greenwald, 1997). We and others have shown that fly γ-secretase can process human APP (Fossgreen et al., 1998; Greeve et al., 2004), suggesting that both the structural and functional features of these proteins are evolutionarily conserved. Transgenic expression of human Aβ peptides in either flies or worms leads to the production of ThioflavinS-positive amyloid plaques, neurodegeneration, and behavioral deficits (Crowther et al., 2005; Finelli et al., 2004; Iijima et al., 2004; Link, 1995). Recently, we showed that co-expression of human APP_{695} and human BACE in flies also resulted in the production of neurotoxic Aβ fragments. Surprisingly, these effects were also seen without co-expression of human BACE, suggesting that *Drosophila* expresses an endogenous BACE-like protease (Greeve et al., 2004). We have now identified a fly BACE (dBACE), and we have explored whether this enzyme is involved in the production of Aβ-related fragments from fly APPL. In addition, we have investigated

* Corresponding author. Fax: +1 503 494 6831.
E-mail address: kretzsch@ohsu.edu (D. Kretzschmar).
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whether fly Aβ peptides are capable of inducing neuropathological effects in the aging brain.

Materials and methods

Drosophila stocks

The pUAST vector and the *Drosophila Appl* cDNA (obtained from the Berkeley Drosophila Genome Project) were used to create the full-length APPL and dAβ constructs. The dAβ construct also contained an N-terminal HA-tag. A cDNA for the transcription unit GH11417 (Berkeley Drosophila Genome Project; Genebank accession number BT016133) was used to create the UAS-dBACE construct. GMR-GAL4, *elav*-GAL4, Psn^{K2}, and UAS-*kuz* were provided by the Bloomington Stock Center; Appl-Gal4 was kindly provided by L. Torroja (Universidad Autonoma de Madrid, Madrid, Spain). UAS-*psn* was kindly provided by M. Fortini (National Cancer Institute, Frederick) and UAS-psn^{D447A} by M. Guo (University of California, Los Angeles). Flies were raised under standard conditions.

Tissue sections for light and electron microscopy

Fly heads were prepared for light and electron microscopy as described in Kretzschmar et al. (1997). Paraffin sections were prepared as described in Bettencourt da Cruz et al. (2005) and stained with ThioflavinS (as described in Greeve et al. (2004)) or Congo Red (following the Congo Red Amyloid Stain Kit procedures, Diagnostic Biosystems). 2 μm serial sections and ultrathin sections for electron microscopy were prepared as described in Kretzschmar et al., (1997) and analyzed with a Jeol Jem-100CX II electron microscope (40–80 kV).

Immunohistochemistry

Cryostat sections were processed according to the protocol in Kretzschmar et al. (1997). A synthetic peptide containing aa800–813 of the APPL coding sequence (corresponding to the dAβ peptide) was used to raise the anti-dAβ antiserum in rabbits, which was applied to tissues at a 1:100 dilution in phosphate-buffered saline. The Cy2-conjugated secondary antibody was obtained from Jackson ImmunoResearch and used 1:1000. For stainings on EM sections, we followed the protocol in Kretzschmar et al. (2005), using anti-HA (Covance) at a dilution of 1:1000. Paraffin sections were prepared as described in Bettencourt da Cruz et al. (2005) and stained with the cleaved caspase-3 antibody (Cell signaling) at a dilution of 1:200. For detection we used the Vectastain kit following the manufacturer's manual.

Immunoprecipitation and Western Blots

Immunoprecipitations were performed following the protocol in Swanson et al. (2005), using 1 ml GMR-GAL4; UAS-dAβ flies and anti-HA coupled beads (Vector laboratories). Lysates were loaded on 4–12% gradient gels (Criterion, Bio-Rad) and analyzed by Western Blots (as described in Tschape et al. (2002)), using anti-dAβ at a dilution of 1:200. For the detection of N-terminal CTF fragments, an anti-AICD antiserum (kindly provided by P. Copenhaver, OHSU) was used at 1:4000. Immunoreactive bands were detected using the Visualizer Western Blot Detection Kit (Upstate).

Fast phototaxis

Five consecutive cycles in the countercurrent apparatus were used in each trial, with a time allowance of 6 s to reach the next vial. A minimum of 100 flies, in groups of 15–25 flies, were used per age and genotype. ANOVA was applied to data sets of a given age to reveal significant differences. A Student's–Keuls test was used to identify the significantly different groups. A detailed description of the conditions of this assay can be found in Kretzschmar et al. (2005).

Results

Drosophila

APPL shares about 30% overall sequence identity with human APP_{695} (Luo et al., 1990). However, the region in APPL that corresponds to the Aβ-peptide lacks significant homology with the human peptide (see Fig. 1A), which has led to the assumption that amyloidogenic peptides cannot be produced from fly APPL (Bilen and Bonini, 2005; Coulson et al., 2000; Link, 2005). Given our recent data (Greeve et al., 2004) that flies may express an endogenous secretase able to cleave human APP and to produce a neurotoxic Aβ fragment (Fossgreen et al., 1998), we explored whether *Drosophila* expresses a β-secretases-like enzyme and whether flies can produce toxic Aβ fragments from APPL.

Drosophila expresses a BACE-like secretase

Screening the fly genome against the coding domain of human BACE1, we identified a BACE-like protein (annotated as CG13095) with 25% identity to human BACE1 and 28% identity to human BACE2 (and about 50% similarity to both, e-value: $4e^{-23}$, see Supplementary Fig. 1A). In accordance with a BACE-like enzyme, several structural programs identify this protein as an aspartic peptidase (http://flybase.org/reports/FBgn0032049.html) and the regions containing the active site aspartates Asp^{87} and Asp^{270} in CG13095 showed a significantly higher conservation to human BACE including the active site D(T/S)G motif characteristic for aspartic proteases (Supplementary Fig. 1B).

For a functional analysis of this candidate *Drosophila* BACE (dBACE), we created flies co-expressing this gene with APPL in the central nervous system (CNS) using the pan-neuronal *elav*-GAL4 promoter. We could readily detect a 14.5 kDa CTF cleavage product (arrowhead) in wild type flies (Fig. 1B, lane 2) as well as in flies overexpressing APPL (Fig. 1B, lane 1). However, after co-expression of dBACE (lanes 3, 4 and Fig. 1C, lane 2) with APPL in CNS neurons, an additional 14 kDa band was visible (arrow), indicating that this novel secretase can indeed process APPL. To investigate the functional homology with vertebrate BACE, we co-expressed human BACE1 (hBACE) with APPL and these flies also exhibited an increase in the levels of the 14 kDa CTF (Fig. 1B, lane 5). This result shows that both the human and the fly secretase can cleave APPL at a similar if not identical site, suggesting that these enzymes are functionally as well as structurally conserved (a similar result was obtained co-expressing APPL and dBACE/hBACE in photoreceptors, Supplementary Fig. 2A).

That we only detected one CTF fragment in wild type suggested that APPL is predominantly processed by another secretase, similar to the preferred cleavage of human APP_{695} by α-secretase (Turner et al., 2003). To address this issue, we co-expressed APPL with *kuzbaninan*, a homologue of ADAM10 which has been shown to exhibit α-secretase activity in vertebrates (Allinson et al., 2003). This indeed enhanced the production of the 14.5 kDa fragment (Fig. 1C, lane 3, arrowhead) suggesting that KUZ has α-secretase activity. As mentioned above, a γ-secretase activity has been described in *Drosophila* and it has been shown that this activity can process human APP. However, it has not been shown that the fly γ-secretase can actually cleave APPL. To confirm that the APPL CTFs are substrates for γ-cleavage, we co-expressed presenilin (UAS-psn) and APPL. As shown in Fig. 1D (lane 2), this resulted in a decreased level of the readily detectable α-CTF (arrowhead), compared to flies without additional PSN expression (lane 1). The opposite effect was observed when we used head extracts from flies that carried one copy of a loss of function mutation of *psn* (psn^{K2}, Lukinova et al., 1999, lane 3) or expressed a dominant negative form (UAS-psn^{D447A}, http://flybase.org/reports/FBal0156036.