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

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Abstract

The accumulation of amyloid- β (A β) into plaques is a hallmark feature of Alzheimer's disease (AD). While amyloid precursor protein (APP)-related proteins are found in most organisms, only A β 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 A β . Here we show that A β 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 A β -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 A β plays a key role in AD.

Keywords

APP; APPL; *Drosophila*; Amyloid; Neurodegeneration; β -secretase; Behavioral deficits

Introduction

Hallmark features of AD include the formation of neurofibrillary tangles and amyloid plaques, the latter consisting largely of A β 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 A β plays a key role in AD has been provided by rare cases of familial-early onset AD, in which mutations that enhance A β 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

A β peptides remain controversial. Clarification of these issues has been hampered by the lack of animal models that recapitulate the disease. In rodents, A β 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 A β 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 APP₆₉₅ and human BACE in flies also resulted in the production of neurotoxic A β 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 A β -related fragments from fly APPL. In addition, we have investigated whether fly A β 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 dA β constructs. The dA β 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 Kretschmar 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 μ m 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 dA β peptide) was used to raise the anti-dA β 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-dA β 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-dA β 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 APP₆₉₅ (Luo et al., 1990). However, the region in APPL that corresponds to the A β -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 A β fragment (Fossgreen et al., 1998), we explored whether *Drosophila* expresses a β -secretases-like enzyme and whether flies can produce toxic A β 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 Asp⁸⁷ and Asp²⁷⁰ 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 APP₆₉₅ 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/FBaI0156036.html>, lane 4). In both cases, the level of the CTF was increased compared to the control flies. This shows that the fly γ -secretase, as its vertebrate homologue, does indeed cleave the CTFs (PSN overexpression also reduces the level of the β CTF, Supplementary Fig. 2B).

Co-expression of dBACE enhances APPL induced amyloid deposit formation and neurodegeneration

To test whether a *Drosophila* A β -like peptide derived from APPL can be neurotoxic, we first overexpressed full-length APPL in photoreceptors. Toluidine blue stained plastic sections through the retinae of these 30 d old flies revealed the presence of dense aggregates (Fig. 2A) that were absent at earlier ages (1 d and 5 d old flies, data not shown) or in 30 d old control animals (wild type, flies expressing GMR-GAL4 alone, and flies expressing lacZ with GMR-GAL4; see Supplementary Figs. 3A, B). To investigate whether these deposits exhibited the histological features of amyloid deposits, as in the case of human A β , we stained paraffin sections of these animals with ThioflavinS. ThioflavinS-positive deposits (0.6 ± 0.17 SEM per retina, $N = 22$) were found in the retinae of 30 d old flies expressing additional APPL (Fig. 2B, arrows) but not in age-matched controls (GMR-GAL4/lacZ flies; Supplementary Fig. 3C).

To determine whether these deposits were immuno-positive for A β -like fragments, we generated an antiserum against aa767–831 of APPL. This region corresponds to the A β region of APP₆₉₅ and was designated as *Drosophila* A β (dA β ; see Fig. 1A). With this antibody we detected immunopositive deposits in cryostat sections from 30 d old flies expressing additional APPL in photoreceptors that were comparable in size and location with those detected by toluidine and ThioflavinS staining (Fig. 2C).

To show that this effect could be recapitulated in the CNS, we expressed additional APPL in all neurons using either a GAL4 construct containing the *Appl* promoter (*Appl*-GAL4) or *elav*-GAL4. With both drivers we could detect toluidine-blue stained deposits in the brain of aged flies (data not shown), although they were quite rare. Presumably, this difference in the numbers of deposits reflects the relatively strong expression levels induced by the GMR-GAL4 driver, compared to either of the pan-neuronal promoter constructs (Greeve et al., 2004).

Co-expression of APPL with dBACE using GMR-GAL4 resulted in a marked increase in amyloid deposits. In 30 d old flies, the number of ThioflavinS-positive aggregates (Fig. 2D) increased from 0.6 ± 0.17 (SEM) per retina to 1.1 ± 0.31 (SEM) per retina ($N = 25$). Even more striking, the pan-neuronal expression of dBACE and APPL resulted in numerous toluidine blue-positive deposits in the brain (Fig. 2E, arrows). It also induced the formation of spongiform lesions scattered throughout the brain (Fig. 2F, arrowheads), a feature not seen in wild type controls or in flies of the same age overexpressing APPL alone (Supplementary Fig. 4). This suggests that processing of APPL by dBACE leads to the production of an endogenous dA β fragments with amyloidogenic potential.

dA β is sufficient to form amyloid-like deposits

To confirm that dA β can form amyloid deposits and induce spongiform lesions, we created flies that carried an HA-tagged UAS-dA β . Immunoprecipitations from fly head lysates from GMR-GAL4/UAS-dA β flies (using anti-HA) followed by a Western Blots with our anti-dA β antibody showed a weak band corresponding to the monomer (arrowhead, Fig. 3A) as well as several putative multimeric forms (arrows). Expression of the dA β peptide in the retina

also resulted in ThioflavinS-positive deposits (Fig. 3B, arrow) that were more numerous than the deposits produced by full-length APPL (2.4 ± 0.35 SEM plaques per retina versus 0.6 ± 0.17 SEM). These aggregates were also positively stained by Congo Red (Fig. 3C). Immunohistochemistry using anti-HA on cryosections from these flies confirmed that deposits contained the dA β peptide (arrow, Fig. 3D, see also Supplementary Fig. 5). Likewise, when dA β was expressed in neurons using *Appl*-GAL4, we found deposits of various sizes (Figs. 3E, F, arrows) and vacuoles in the brain (Figs. 3E, F, arrowheads), similar to the phenotypes observed after co-expression of dBACE and APPL. The deposits in the central nervous system were also immune-positive with anti-HA (Fig. 3G). These results show that the dA β peptide is by itself sufficient to induce amyloid deposits and spongiform lesions in the CNS, and they suggest that the level of dA β correlates with the severity of these phenotypes.

Age-dependent degeneration in APPL or dA β expressing flies

To demonstrate the progressive nature of the neurodegeneration induced by dA β , we performed plastic sections at various times during adult life. Young flies expressing full-length APPL in photoreceptors did not show any obvious degenerative phenotypes at 1–2 d. By 10 d, however, we could detect an increasing number of small vacuoles scattered throughout their retinæ (Fig. 4B, arrowheads), and by 30 d, these vacuoles had increased in size and number (Fig. 4C). The severity of this phenotype was exacerbated in 30 d old flies co-expressing APPL and dBACE (data not shown) and in flies expressing the dA β peptide: the retinæ of these animals had become highly disrupted and reduced in size, contained numerous dense deposits and extensive vacuolization (Fig. 4D, arrow). Thus, the severity of retinal degeneration in flies expressing either dA β or APPL plus dBACE correlates with the higher plaque load found in these animals. Control lines aged for 30 d, including GMR-GAL4/UAS-lacZ flies (Fig. 4A), did not exhibit signs of degeneration.

Expressing additional full-length APPL in the brain did not lead to detectable signs of degeneration, even after 30 d of aging (Supplementary Fig. 4). However, expressing dA β produced more dramatic results. Initially, these animals appeared indistinguishable from wild type controls (Fig. 4E, 2 d old) but in 14 d old flies scattered somata exhibiting cytoplasmic vacuolization and lysis could be detected throughout the brain (Fig. 4F, arrow). By 30 d many neurons showed signs of beginning cell death (Fig. 4G, arrows) or had died (visible as dark, shrunken cell remnants; arrowhead, Fig. 4G). Similar signs of neuronal cell death were observed in 30 d old flies co-expressing full-length APPL and dBACE (data not shown). These results show that the accumulation of dA β peptides into deposits is accompanied by the formation of spongiform lesions and neuronal cell death.

The observed shrunken nuclei suggested that these cells die via an apoptotic death pathway. To verify this, we performed immunostainings using an anti-activated caspase antibody on paraffin head sections. As shown in Fig. 5 (arrows, Figs. 5A–C) single immunopositive cells can be detected in 30 d old flies expressing dA β panneuronally while age-matched control flies do not reveal any staining (Fig. 5D). This result further supports that these neurons undergo apoptosis.

Behavioral deficits are an early sign of dA β expression

As noted above, the appearance of amyloid-like deposits and overt signs of neurodegeneration occurred relatively late in adulthood. In humans, it has been proposed that cognitive impairment can be used as an early indicator of AD, prior to the appearance of more severe neurological defects, suggesting that the accumulation of A β fragments may interfere with neuronal function before degeneration occurs (Chong and Sahadevan, 2005). To investigate this issue in *Drosophila*, we used fast phototaxis behavioral tests. This test measures a startle response with an orientation towards light, providing a performance index (PI) equivalent to the percent of successful transitions towards light (Benzer, 1967). For the behavioral tests, we only used flies expressing our constructs pan-neuronally using *elav*-GAL4 (which is only weakly expressed in the eye and does not induce photoreceptor degeneration which might impair an animal's ability to locate a light source). Control flies exhibited only a gradual decline in their performance with aging and still responded to light $91\pm1\%$ of the time when 20 d old (black squares, Fig. 6A). In contrast, flies expressing additional APPL showed a significantly lower PI by 20 d ($71\pm2\%$ stars, Fig. 5A; $p<0.001$), as did 20 d old flies expressing only dBACE (to increase the proteolytic cleavage of endogenous APPL; black dots, PI $73\pm3\%$, $p<0.001$). In both of these fly lines, their performance indices continued to decline with further aging to approximately 30% by 30 d (control flies $63\pm3\%$), despite the lack of overt signs of degeneration in the CNS of these flies (Supplementary Fig. 3). More severe deficits in performance were seen in flies co-expressing APPL and dBACE, with PIs of $59\pm3\%$ at 20 d and $11\pm2\%$ at 30 d (crosses in Fig. 6A, $p<0.005$). However, the most dramatic effect was seen in flies expressing the dA β peptide: the behavioral response of these animals was significantly reduced by as early as 6 d of adulthood (compared to controls; $p<0.001$), and continued to decline with further aging (triangles, Fig. 6B). As seen with the histological phenotypes, the level of dA β production also correlated with the severity of the observed behavioral phenotypes. However, the behavioral defects were observed much earlier, suggesting that dA β affects neuronal function before overt signs of amyloid deposit formation or neuronal degeneration are detectable.

Intracellular fibril formation precedes deposit formation and cell death

To investigate whether neurons show morphological defects before deposit formation and degeneration occurs, we performed electron microscopic studies. In 10 d old flies expressing dA β pan-neuronally, we could detect many small, electron-dense deposits and fibrils throughout the neuropil (Fig. 7A). Immunostaining 20 d old flies expressing dA β (using an anti-HA antibody) revealed substantial staining within an axon (Fig. 7B, arrows) that was localized in close proximity to these fibrillar structures (Fig. 7C), suggesting that they represent intracellular accumulations of the dA β peptide. To determine whether these structures are also formed by dA β fragments derived from the full-length protein, we expressed an extra copy of APPL and dBACE and prepared sections from 20 d old flies. In these brain sections, we could detect similar fibrillar structures (Fig. 7D, arrows) and dense bodies (which also accumulated in the dA β expressing flies, data not shown) within axons (Fig. 7E). Similar dense bodies have been described in brain sections from Alzheimer patients (Nixon et al., 2005) and mouse models of AD (Masliah et al., 1996; Perez et al.,

2005). Finally, we determined whether such abnormalities are also seen in flies expressing APPL. To detect larger deposits, we performed sections from 30 d old flies that express APPL in the retina. As shown in Fig. 7F, we could detect extracellular deposits that were localized between the retina and lamina, corresponding in to the ThioflavinS positive deposits. Such structures were not detected in 30 d old flies expressing APPL in neurons, however, we could again detect fibrils within axons (Fig. 7G). In addition, neuronal somata in the brains of these flies started to exhibit cytoplasmic shrinkage and vacuolation (Fig. 7H, arrow). These results suggest that the formation of intracellular fibrils and/or other dA β induced cellular changes could be responsible for the observed behavioral phenotypes in relatively young flies.

Discussion

The results of this study show that like human APP, fly APPL can be processed by two alternative secretase activities. Similar to vertebrates, the predominant cleavage in fly neurons appears to be α -cleavage whereas the alternative β -processing which leads to the production of dA β peptides is less frequent. Furthermore, we have identified an enzyme that is structurally similar to human BACE1 and BACE2 and is capable of cleaving APPL into a C-terminal fragment of the same size as the fragment produced by co-expression of human BACE1. We therefore propose that this fly enzyme represents an endogenous fly β -secretase and now named it dBACE (this enzyme was previously described as δ -secretase (Greeve et al., 2004) because its structural homology to vertebrate BACE was not known at that time). Intriguingly, both human BACE1 and fly dBACE induced an increase in the smaller (14 kDa) CTF which indicates that the α - and β -cleavage sites are reversed in APPL compared to human APP. In humans, the presence of the α -cleavage site within the A β sequence is thought to prevent formation of the neurotoxic peptide in the case of α -processing. In *Drosophila*, α -cleavage does result in a larger fragment than dA β which might induce a different secondary structure not able to form the β -sheaths characteristics for the toxic A β fragments.

Having identified a *Drosophila* BACE, we next investigated the possibility that neurotoxic A β -like fragments can be produced from fly APPL. Overexpression of the full-length APPL led to amyloid deposit formation, behavioral deficits, and neuronal degeneration, all of which are hallmark features of Alzheimer's disease in humans. These phenotypes were enhanced by co-expressing of dBACE, or by expressing the dA β fragment alone. Indeed, expressing dA β proved to be the most potent means of inducing these phenotypes, supporting the hypothesis that as with human APP, the deleterious effects of full-length APPL are primarily caused by the generation of dA β peptides from the holoprotein. As mentioned above, rodent models of AD rely on the ectopic expression of human APP, because their endogenous A β peptides do not form amyloid deposits (Link, 2005). Thus, our results in *Drosophila* are demonstrating for the first time that endogenous APP-related proteins and their A β fragments can induce pathological symptoms in a model system.

It was also previously assumed that the *Drosophila* APPL protein does not contain an A β -like region, due to the low degree of sequence identity between human A β ₄₂ and the *Drosophila* dA β . Consequently, it was not expected that APPL could induce amyloid

deposits (Bilen and Bonini, 2005; Link, 2005). Our data now show that overexpression of either full-length APPL or the dA β fragment can indeed lead to amyloid deposits, progressive behavioral deficits, and neurodegeneration. These results can be interpreted by two alternative hypotheses. The toxicity of A β peptides may be correlated with aspects of their secondary structure rather than their primary amino acid sequences. In this case, at least one of the three amino acids that are not conserved between mouse and human A β (asterisks, Fig. 1A) might be crucial for the formation of secondary structures with neurotoxic potential. Interestingly, one of these amino acids (His¹³ of human A β) is conserved in *Drosophila*. Alternatively, the GAL4 promoter constructs used in this study might result in higher expression levels of amyloid peptides than were achieved in mice, resulting in an enhanced aggregation rate for dA β in the fly brain. That the expression level might correlate with the severity of the induced phenotypes is supported by our results that the strong GMR-GAL4 promoter induced phenotypes earlier, compared to the weaker pan-neuronal promoters.

As already noted, while amyloid plaque loads are a defining feature of AD, neither plaque load *per se* nor the levels of insoluble A β correlate well with the severity of the disease (McLean et al., 1999). However, more recent work has suggested that the accumulation of intracellular A β prior to the formation of extracellular plaques may actually trigger its pathogenic effects (Glabe, 2001; Walsh et al., 2005; Wirths et al., 2004). Similarly, we could detect cellular changes like intracellular fibrils and dense bodies long before the appearance of deposits in the nervous system. Intriguingly, the appearance of these structures correlated with the decrease in behavioral performance seen in these animals. This suggests that fly dA β , like human A β , might form small intracellular aggregates that either directly or indirectly induce neurotoxic effects.

Together, our results show that the fly dA β is capable to induce phenotypes similar to the ones described for human A β , therefore suggesting that the neurodegenerative potential of these peptides may be better conserved among species than previously assumed. The finding that the processing and pathogenic function of A β fragments from APPL and APP is quite conserved suggests that also the function of other APP/APPL fragments might be conserved. Secreted N-terminal APP fragments have been connected with neurotrophic, neuroprotective, and proliferative effects while the C-terminal intracellular fragment (AICD), which is well conserved (52% identity) in *Drosophila*, may act as a transcriptional regulator (Annaert and De Strooper, 2002; Turner et al., 2003). However, the biological functions of secretase-derived fragments of APP are still controversial and *Drosophila* might therefore provide an excellent *in vivo* model in which the roles of these fragment can be analyzed.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.nbd.2008.10.014](https://doi.org/10.1016/j.nbd.2008.10.014).

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Fig. 1.

APPL is processed by α -, β -, and γ -secretase activities. (A) Amino acid comparison of the A β region of human and mouse APP with the equivalent region in APPL (dA β). An alignment without gaps shows seven conserved residues, including His¹³ of human A β , one of the three residues (asterisks) not conserved between mouse and human A β (upper panel). An alignment that allows gaps in the fly sequence shows 12 conserved amino acids (lower panel). The AQHAHAKEGRNVY-peptide used for immunization to create our anti-dA β antibody is indicated by the solid line. (B–D) Western Blots of fly head homogenates using an antibody against the intracellular APPL fragment. (B) Western Blot from wild type flies and flies expressing additional APPL pan-neuronally show a single CTF fragment (arrowhead) of 14.5 kDa (lanes 1, 2). After co-expression of dBACE (lanes 3, 4) or hBACE a smaller 14 kDa CTF (arrow) can be detected. 10 fly heads were used per lane (except in lane 4, where 5 heads were used). (C) Co-expression of APPL and KUZ (lane 3 arrowhead) increases the production of the larger 14.5 kDa CTF fragment, the predominant cleavage product of APPL in neurons (lane 1). In contrast co-expression of dBACE and APPL results again in the generation of the smaller 14 kDa CTF fragment (lane 2, arrow). (D) Co-expression of PSN decreased the level of the 14.5 kDa fragment (lane 2) while removing one copy of psn (lane 3) or expressing a dominant negative form of PSN (lane 4) increased the level of the CTF revealing that PSN has γ -secretase activity. Five heads were loaded in each lane in C and D. Shown below each blot is a loading control using anti-actin.

Fig. 2.

Amyloid deposits in flies overexpressing APPL. (A) Toluidine blue staining of a semi-thin plastic section through the retina of a fly expressing additional APPL using GMR-GAL4 show the formation of dark stained aggregates (arrow) between ommatidia (o). (B) Paraffin sections stained with ThioflavinS reveal an amyloid-like feature of deposits (arrows) in these retinæ. (C) Immunohistochemistry using our anti-dA β on cryosections shows that deposits are immuno-positives for dA β (arrow). (D) ThioflavinS-positive deposits (arrow) in the retina of a fly expressing dBACE and APPL using GMR-GAL4. (E, F) Co-expression of APPL and dBACE with *Appl*-Gal4 leads to toluidine-blue-stained deposits (arrows in E) and vacuoles (arrowheads in F) in the CNS. All flies were aged for 30 d. re=retina, la=lamina. Scale bar in A; 2.5 μ m, in B–D; 25 μ m, in E and F; 10 μ m.

Fig. 3. dAβ fragments accumulate in deposits. (A) Western Blot of lysates from GMR-GAL4/HA-tagged dAβ immunoprecipitated with anti-HA and labeled with anti-dAβ reveals monomeric (arrowhead) and oligomeric species (arrows) of dAβ. (B) A ThioflavinS-positive deposit (arrow) in the retina of a 30 d old fly expressing dAβ via GMR-GAL4. (C) These deposits are also stained by Congo Red (arrow) and are immune-positive with anti-HA (D). (E, F) Toluidine blue-stained semi-thin sections from a fly expressing dAβ in all neurons (using *Appl*-GAL4). Aggregates (arrows) and vacuoles (arrowheads) can be detected throughout the brain of this 30 d old fly. (G) Immunohistochemistry using anti-HA reveals an aggregation of dAb in the central nervous system of 30 d old *Appl*-GAL4/UAS-dAβ flies (arrow). re = retina, la = lamina, me = medulla, lo = lobula, lp = lobula plate. Scale bar in B–D; 25 μm, E; 2.5 μm, F and G; 12 μm.

Fig. 4.

APPL and dA β expression leads to progressive neurodegeneration and cell death. (A) A 30 d old GMR-GAL4/UAS-LacZ control fly. (B) A 10 d old fly GMR-GAL4/UAS-APPL fly shows the initial appearance of vacuoles (arrowheads). (C) The size and number of these vacuoles are markedly increased in a 30 d old fly with this genotype. (D) In a 30 d old fly expressing the dA β peptide using GMR-GAL4, the retina is severely disrupted with extensive vacuolization (arrowheads) and deposit formation (arrow). (E–G) Expression of dA β using the *Appl*-GAL4 promoter. No gross abnormalities are seen in 2 d old (E) flies. (F) In 14 d old flies some neuronal somata show cytoplasmic vacuoles (arrow), indicating the initial stages of cell lysis. (G) In 30 d old flies numerous neuronal somata exhibit signs of degeneration including cytoplasmic vacuolization and shrinkage (arrows). In addition, dark shrunken nuclei (arrowhead) and large vacuoles (asterisk) can be detected. Scale bar in A–D; 25 μ m, in E; 30 μ m, in F; 4 μ m, in G; 6 μ m.

Fig. 5. dAβ induces apoptotic cell death. (A–C) Single activated caspase positive cells are found in head sections from 30 d old flies expressing dAb pan-neuronally (*Appl-GAL4*). These cells can be found in the optic system (arrows in A and B) as well as in the central part of the brain (arrows in C). (D) An age-matched control does not reveal any immunopositive cells. la=lamina, me=medulla, lo=lobula, lp=lobula plate. Scale bar in A and D; 25 μm, in B; 7 μm, in C; 12 μm.

Fig. 6.

Behavioral deficits caused by pan-neuronal expression of APPL dBACE and dA β . (A) Control flies expressing lacZ via *elav*-GAL4 (filled squares) show only a slight decrease in their fast phototactic response with age, whereas 20 d old flies expressing additional APPL (open stars) have a significantly lower performance index (PI) than young flies or age-matched controls. A similar decline in the PI is seen in flies expressing dBACE pan-neuronally (filled dots) presumably due to increased processing of endogenous APPL. Flies co-expressing dBACE plus APPL (asterisks) show a much more severe phenotype with a PI of approximately 60 in 20 d old flies and only 20 in 30 d old flies. (B) Behavioral deficits become even more pronounced in flies expressing dA β (filled triangles) which exhibit a 25% reduction in PI by 6 d of aging (97% versus 72% $p < 0.001$). With further aging (by 15 d) their performance drops to 60%. By 20 d most of these flies have died precluding further behavioral tests.

Fig. 7.

Fibrillary aggregates and dense bodies in flies expressing additional APPL or dA β . (A) Electron microscopic section from a 10 d old fly expressing dA β pan-neuronally (via *Appl*-GAL4) reveals the formation of fibrils (arrows) within axons (a). (B) Labeling sections from these flies at 20 d with an anti-HA antibody reveals immunopositive staining within an axon (arrows), which appears to be associated with the fibrillar structures (C, arrows). (D, E) Sections from the brain of 20 d old flies co-expressing APPL and dBACE in neurons reveal similar fibrillar structures (arrows in D) and the accumulation of dense bodies (E) in axons.

(F) In 30 d old flies overexpressing APPL in the retina, a large, extracellular aggregate can be detected between the retina (re) and lamina (la). (G) In 30 d old flies overexpressing APPL in all neurons, we again occasionally see intracellular fibrils within individual axons (a). (H) Neuronal somata in these animals reveal signs of incipient cell death, including vacuole formation in the cytoplasm (arrow, n=nucleus) and shrinking nuclei. Scale bar in A, E; 0.5 μm , B; 0.2 μm , C; 50 nm, F; 1.5 μm , G; D; 0.1 μm 0.25 μm , H; 1 μm .