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Age-Dependent Neurodegeneration and Alzheimer-Amyloid Plaque Formation in Transgenic *Drosophila*

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β-Amyloid peptides that are cleaved from the amyloid precursor protein (APP) play a critical role in Alzheimer's disease (AD) pathophysiology. Here, we show that in *Drosophila*, the targeted expression of the key genes of AD, APP, the β-site APP-cleaving enzyme BACE, and the presenilins led to the generation of β-amyloid plaques and age-dependent neurodegeneration as well as to semilethality, a shortened life span, and defects in wing vein development. Genetic manipulations or pharmacological treatments with secretase inhibitors influenced the activity of the APP-processing proteases and modulated the severity of the phenotypes. This invertebrate model of amyloid plaque pathology demonstrates Aβ-induced neurodegeneration as a basic biological principle and may allow additional genetic analyses of the underlying molecular pathways.

Key words: **Alzheimer; dementia; aging; neuropathology; degeneration; *Drosophila***

Introduction

Alzheimer's disease (AD) neuropathology is characterized by the accumulation and deposition of β-amyloid (Aβ) peptides in senile plaques, by the formation of neurofibrillary tangles (NFT) composed of abnormally phosphorylated forms of the microtubule binding protein tau, and by a progressive neuronal damage and cell loss (Gomez-Isla et al., 1996; Selkoe, 1999). The interrelation of Aβ and NFT in the development of AD neuropathologic lesions is a matter of debate. However, it has been shown that in AD-linked neurodegeneration, Aβ is an essential causative pathogenic factor. Tau-related neurotoxicity was recapitulated in transgenic *Drosophila*, demonstrating that important aspects of AD-related neuropathogenesis can be studied in the fruit fly (Wittmann et al., 2001; Jackson et al., 2002). Aβ-induced neurodegeneration and plaque formation in *Drosophila* would provide powerful additional evidence for a causative role of Aβ peptide toxicity in AD.

Aβ is generated by endoproteolysis of the amyloid precursor protein (APP) by β-secretase activity of the β-site APP-cleaving enzyme (BACE) and by subsequent intramembraneous γ-secretase cleavage that depends on a complex of presenilin 1 or 2 (PS1 or PS2), nicastrin, aph-1, and pen-2 (De Strooper and Annaert, 2000; Walter et al., 2001; Esler et al., 2002; Francis et al., 2002). Autosomal dominant mutations in APP, PS1, or PS2 accelerate the onset of neurodegeneration and the progression of the disease in familial AD (FAD). These FAD mutations are associated with altered APP processing and lead to an increased generation of amyloidogenic $A\beta_{40}$ and $A\beta_{42}$ peptides or to increased ratios of $A\beta_{42}/A\beta_{total}$ (Lemere et al., 1996; Scheuner et al., 1996; Tomita et al., 1997). Previous studies of *Drosophila* suggested pro-apoptotic functions of FAD-causing *presenilin* mutations and indicated that, although the genes required for a functional γ-secretase complex appeared to be highly conserved between *Drosophila* and vertebrates, the fly is lacking a homolog of human BACE (Fossgreen et al., 1998; Ye and Fortini, 1999; Adams et al., 2000).

To model β-amyloid plaque pathology and neurodegeneration in *Drosophila*, we expressed the genes involved in the pathophysiology of AD either separately or in combination in various neuronal and non-neuronal tissues by using the GAL4/UAS (transcriptional activator Gal4/Gal4 DNA binding motif, upstream activator sequence) system (Brand and Perrimon, 1993). Transgenic flies expressing the human APP_{695} gene (UAS-APP) and three fly lines expressing *Drosophila presenilin* (UAS-DPsn) point mutations that correspond to the FAD mutants N141I, L235P, and E280A were kindly provided by R. Paro (Center for Molecular Biology, Heidelberg, Germany) and E. Fortini (Na-

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tional Cancer Institute, Molecular Genetics Section, Frederick, MD), respectively (Fossgreen et al., 1998; Ye and Fortini, 1999). We generated UAS-BACE transgenic fly lines by P-element-mediated germline transformation and expressed APP, BACE, and DPsn in photoreceptor cells of the compound eye of *Drosophila* by using the eye-specific gmr-GAL4 driver line (Rubin and Spradling, 1982; Spradling and Rubin, 1982; Brand and Perrimon, 1993).

Materials and Methods

BACE transgenic flies. A 1.9 kb *Kpn*I-*Xho*I restriction fragment of the human BACE (β-site APP-cleaving enzyme) cDNA containing the entire open reading frame was cloned into the plasmid pUAST (Brand and Perrimon, 1993). P-element-mediated germline transformation was performed as described (Rubin and Spradling, 1982; Spradling and Rubin, 1982). Three independent UAS-BACE insertion lines were used in our experiments (406, 437, 523).

APP and presenilin transgenic flies. The UAS-APP695II, UAS-APP695III (Wt-34, Wt-35) and the UAS-DPsn+14 as well as UAS-DPsn-mutants (N141I, L235P, E280A) were kindly provided by R. Paro and E. Fortini (Fossgreen et al., 1998; Ye and Fortini, 1999). *PsnC4*, *psnB3*, and the *psnK2* mutant alleles as well as the actin-GAL4 line were obtained from the Bloomington stock center. The gmr-GAL4 line from F. Pignoni was used to achieve the eye-specific expression of the transgenes.

Protein analyses. For Western blotting, fly heads and human normal brain tissue (Alzheimer Tissue Center, Northwestern University, Evanston, IL; A97–197 ITC; PMI 5 hr) were homogenized in 1× PBS, 5 mM EDTA, 0.5% Triton X-100, and a protease-inhibitor mix Complete (Roche Applied Science, Mannheim, Germany). Equal amounts of protein were separated by 10% SDS-PAGE, transferred to Immobilon membranes (Millipore, Bedford, MA), blocked in 5% low-fat milk for 2 hr at room temperature, and incubated with the monoclonal antibody (mAb) 22C11 (APP N terminal-specific; Chemicon, Temecula, CA) or polyclonal Ab (pAb) α-APP-C12 (APP C terminal-specific; kind gift from B. de Strooper, Center for Human Genetics, K.U. Leuven and Flanders Interuniversity Institute for Biotechnology, Leuven, Belgium). Bound antibodies were detected with goat anti-mouse peroxidase-conjugated (Dianova, Hamburg, Germany) or goat anti-rabbit peroxidase-conjugated (Vector Laboratories, Burlingame, CA) secondary antibodies. For immunoprecipitation, fly heads were homogenized as described above, and lysates were treated as described in the antibodies protocol guide from Clontech (Cambridge, UK). The following antibodies were used for immunoprecipitation: mAb 6E10 (α-Aβ5–10; Signet Pathology Systems, Dedham, MA), mAb 4G8 (α-Aβ17–24; Signet Pathology Systems), rabbit pAb α-APP-C12, and rabbit polyclonal antibodies Aβ1–42 and Aβ1–40 (QCB; Biosource International, Camarillo, CA). Samples were separated on 10–20% gradient Novex (Wadsworth, OH) Tris-Tricine gels (Invitrogen, San Diego, CA) and blotted onto Protran BA 79 Cellulosenitrate membranes (0.1 μm; Schleicher & Schuell, Dassel, Germany). Detection of β-amyloid or the APP C terminus was performed as described previously (Ida et al., 1996) using mAb 6E10 and goat anti-mouse peroxidase-conjugated secondary Ab (Dianova).

Immunohistochemistry and histology. For Immunostaining, adult flies were fixed in 4% paraformaldehyde for 3 hr, washed in 1× PBS, and transferred to 25% sucrose for an overnight incubation at 4°C. Flies were decapitated with a razor blade, and the heads were imbedded in Tissue Tek (Sakura, Tokyo, Japan) and snap frozen. Horizontal frozen sections (10 μm) were prepared on a cryostat. Immunostaining was done with the Vectastain Elite kit (Vector Laboratories) according to the instructions of the manufacturer. The following primary antibodies were used: 24B10 (α-chaoptin, 1:5), provided by the Developmental Studies Hybridoma Bank; mAb 4G8 (1:1000), and mAb 9G10 (αAβ42 C terminus that was generated by standard procedures; 1:500). The fly brain tissue sections were pretreated for 10 min in 70% formic acid to re-expose the epitope before 4G8 staining.

For thioflavin S staining, sections were counterstained for 5 min in Mayers Hemalum (Sigma, St. Louis, MO), rinsed for 10 min in tap water, and stained for 3 min in 1% thioflavin S (Sigma) watery solution. Slides were rinsed in several changes of distilled water, incubated for 15 min in 1% acetic acid, rinsed in tap water, and mounted in Vectashield mounting medium (Vector Laboratories). Slides were analyzed under a fluorescence microscopy (430 nm excitation, 550 nm emission).

For plastic sections, flies were fixed overnight in 5% glutaraldehyde at 4°C, decapitated with a razor blade, washed three times in PBS (5 min each), and treated with 2% osmium-tetraoxid on ice. Sections were again washed three times for 10 min each, sequentially dehydrated in 50, 70, 80, 95, and 3× 100% ethanol for 10 min each, incubated in 100% ethanol: epon 1:1 overnight, and embedded in epon. Plastic sections (1 μm) were cut on a microtome and stained with 1% toluidine blue, 1% borax. Ultra-thin sections (60 nm) were stained with aqueous uranyl acetate and lead citrate and examined using a CEM 902A (Zeiss, Thornwood, NY) electron microscope and analySIS software (SIS, Münster, Germany). Adult fly wings were removed and mounted in DPX (a mixture of distyrene, tricresyl phosphate, and xylene) mountant (Fluka, Buchs, Switzerland) for light microscopy.

Treatments with β- and γ- secretase inhibitors. The β-secretase inhibitor (Sinha et al., 1999) (Calbiochem, La Jolla, CA; 171601) or the γ-secretase inhibitor (Dovey et al., 2001) (Calbiochem; 565770) were used for treatment. The β-secretase inhibitor was added to the standard fly medium to a final concentration of 1, 5, 10, 20, and 50 nM in 0.001, 0.005, 0.01, 0.02, and 0.05% DMSO, respectively; for the γ-secretase inhibitor, a final concentration of 100 nM in 0.001% DMSO was used. Flies of the following genotypes were mated: *w;actin-GAL4/Cyo;UAS-DPsn/Tm3* x *w;UAS-APP/UAS-APP;UAS-BACE/UAS-BACE*. The eggs were developed on fly medium supplemented with the inhibitors or with the vehicle (DMSO) through the larval stage until eclosion. The eclosion rate and extra vein phenotype of flies transgenic for *w;actin-GAL4/UAS-APP;UAS-BACE523/UAS-DPsn*, *w;actin-GAL4/UAS-APP;UAS-BACE406/UAS-DPsn*, and *w;actin-GAL4/+;UAS-DPsn/+* were scored. Adult transgenic flies were continuously treated at 29°C with or without 10 nM β-secretase inhibitor in 0.01% DMSO supplied with the fly food.

Results

Amyloidogenic processing of human APP in transgenic flies

In UAS-APP-expressing flies, antibodies against the N or C termini of human APP detected two differently glycosylated full-length forms of APP that comigrated with glycosylated APP_{695} from human brain tissue at ~100 kDa (Fig. 1*A*, lanes 1, 3, 5). Transgenic coexpression of human BACE with APP resulted in the loss of higher glycosylated APP, together with the generation of a smaller 97 kDa N-terminal fragment that corresponded in molecular mass to the β-cleaved secreted ectodomain of APP (APPsβ) (Fig. 1*A*, lanes 2, 4). Thus, in *Drosophila*, human BACE appeared to cleave preferentially the higher glycosylated form of APP. In SDS protein extracts from double-transgenic flies, the monoclonal anti-Aβ Ab 6E10 (α-Aβ_{1-16}) precipitated the β-cleaved C terminus of APP [β-C-terminal fragment (CTF)] (Fig. 1*C*) as well as SDS-stable Aβ dimers and oligomers (Fig. 1*B*, lane 3, rhomb). Flies transgenic for UAS-APP without BACE generated a slightly larger form of Aβ, δ-Aβ (δ-cleaved Aβ) (Fig. 1*B*, lane 4, asterisk). Like Aβ, δ-Aβ also formed SDS-stable dimers and oligomers. Identical bands were generated by immunoprecipitation with the mAb 4G8 (α-Aβ_{17-24}) or with polyclonal $A\beta_{40}$ or $A\beta_{42}$ C terminal-specific antibodies that recognize only the free C terminus of $A\beta_{40}$ or $A\beta_{42}$, respectively (data not shown). Aβ and δ-Aβ were absent from nontransgenic w^{1118} flies and BACE transgenic flies (Fig. 1*B*, lanes 2, 9, 13) and were hardly detectable when only low levels of APP were expressed by the weak nervous system-specific elav-GAL4 line (Fig. 1*B*, lanes 5, 6). After extraction of fly proteins in the presence of 70% formic

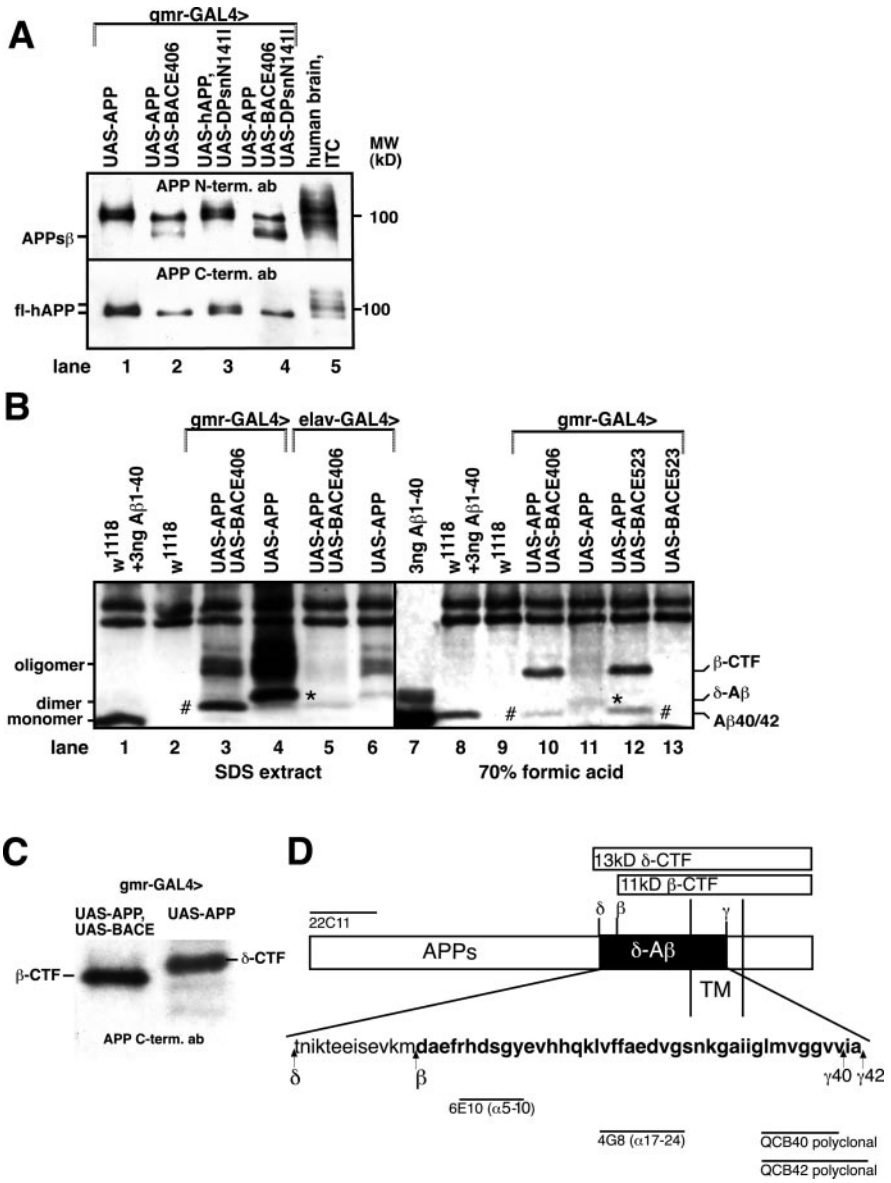

Figure 1. Amyloidogenic processing of APP in *Drosophila*. *A*, Equal amounts of protein extracts from transgenic flies at 2 d after eclosion were analyzed by Western blots, and full-length APP (fl-APP) was detected with APP-N-terminal (top panel) and APP-C-terminal (bottom panel) antibodies. Human brain extracts from the inferior temporal lobe (ITC) region were analyzed in parallel as control. *B*, Immunoprecipitation and immunoblotting of Aβ (rhomb) and the alternatively δ-cleaved δ-Aβ (asterisk) from extracts of transgenic and nontransgenic w^{1118} flies at 2 d after eclosion with and without the addition of synthetic Aβ1–40 using the Aβ-specific mAb 6E10. Fly proteins were extracted in SDS (left panel) or in 70% formic acid (right panel). *C*, Immunoprecipitation of the APP-C terminus by mAb 6E10 and detection by Western blotting with anti-APP-C12. *D*, Schematic representation of the amyloidogenic processing of APP at the secretase-cleavage sites. The amino acid sequence of the presumptive δ-Aβ peptide is indicated. The δ-secretase processing site was deduced from δ-secretase-processed APP_{695} found in rat primary neurons. Aβ sequence is indicated in bold letters. The epitopes of the antibodies that were used to characterize APP processing in transgenic flies are indicated by black bars.

acid, Aβ immunoprecipitated from UAS-APP/UAS-BACE transgenic flies comigrated exactly with the size of the synthetic Aβ (4 kDa), whereas δ-Aβ from UAS-APP transgenic flies migrated with a slightly larger molecular mass (~5 kDa) (Fig. 1*B*, right panel, rhombs and asterisk, respectively). Notably, in the presence of 70% formic acid, a β-CTF was immunoprecipitated from UAS-APP/UAS-BACE transgenic flies, whereas a δ-CTF could hardly be detected under these conditions in UAS-APP transgenic flies (Fig. 1*B*, lanes 10–12).

To further characterize the δ-secretase processing of APP in *Drosophila* in the absence of BACE, we immunoprecipitated the C terminus of APP with mAb 6E10 and used a pAb against the last 12 C-terminal amino acids of full-length APP for detection on Western blots (Selkoe et al., 1988). In UAS-APP flies, 6E10 precipitated an APP C-terminal fragment with an apparent molecular mass of 13 kDa (δ-CTF), as compared with the expected 11 kDa β-CTF in UAS-APP/UAS-BACE-expressing flies (Fig. 1*C*).

Age-dependent neurodegeneration of photoreceptor cells in transgenic *Drosophila*

The neuropathology associated with amyloidogenic APP processing was characterized in the compound eye of *Drosophila* by staining cryostat sections with the photoreceptor cell-specific mAb 24B10. At day 2 after eclosion, no structural differences between UAS-BACE and UAS-APP single or UAS-APP/UAS-BACE double transgenic flies could be detected (Fig. 2*A*). At day 18, however, a severe degeneration of the retinal photoreceptors and their axonal projections to the first and second optic ganglion, in particular the lamina, was observed in UAS-APP/UAS-BACE transgenic flies (Fig. 2*A*). This degeneration of retinal photoreceptors was more pronounced in UAS-APP transgenic flies, but completely absent in UAS-BACE-expressing control flies (Fig. 2*A*). At day 30, the neurodegeneration had progressed further as indicated by small degenerated retinas and a reduced volume of the underlying optic neuropil, the lamina. In addition, the photoreceptor cell projections to the medulla were heavily degenerated and stained only weakly.

Degeneration of photoreceptor cells is dependent on γ-secretase activity

The γ-secretase activity of the flies was modulated by coexpressing the *Drosophila* FAD-*presenilin* genes or by introducing loss of function alleles of the endogenous *presenilin* gene that reduces γ-secretase processing. *Presenilin* loss of function mutations *psnC4*, *psnK2*, *psnB3*, or one of three different human FAD mutants UAS-DPsnN141I, E280A, or L235*P* (Ye and Fortini, 1999; Ye et al., 1999) were expressed in *w; UAS-APP/+; gmr-GAL4/+* transgenic background. At 12 d of age, the heterozygous *presenilin* loss of function mutants (*w; UAS-APP/+; gmr-GAL4/psnB3*) suppressed the degeneration of photoreceptor cells of UAS-APP-expressing flies, whereas coexpression of the *presenilin* FAD mutant *L235P* (*w; UAS-APP/+; gmr-GAL4/UAS-DPsnL235P*) enhanced the degeneration (Fig. 2*B*). Similar results were obtained with two additional loss-of-function *presenilin* mutations *psnK2* and *psnC4* and with the FAD mutants UAS-DPsnN141I and UAS-DPsnE280A, and also when BACE was coexpressed (data not shown).

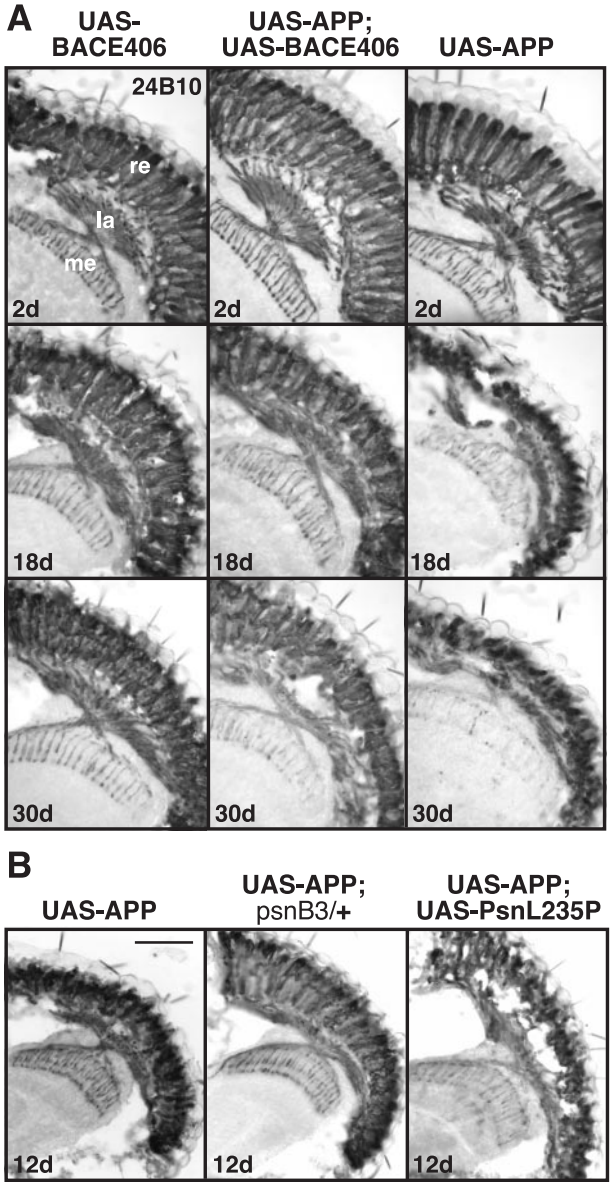

Figure 2. Age-dependent neurodegeneration in flies expressing the AD-associated transgenes under the control of the eye-specific gmr-GAL4 line. *A*, Staining of the photoreceptors in the retina (re) and their axonal projections to the lamina (la) and the medulla (me) on cryostat sections of different transgenic fly lines at days 2, 18, and 30. Photoreceptor degeneration in *w; UAS-APP/+; gmr-GAL4/+* and *w; UAS-APP/+; gmr-GAL4/UAS-BACE* transgenic flies but not in *w; gmr-GAL4/UAS-BACE* transgenic control flies depends on age. The observed age-dependent degeneration of photoreceptor cells was not gender specific. *B*, Photoreceptor staining of transgenic flies at day 12. Loss of *presenilin* function in the *w; UAS-APP/+; gmr-GAL4/psnB3* flies rescues the neurodegeneration; gain of *presenilin* function in *w; UAS-APP/+; gmr-GAL4/UAS-DPsnL235P* transgenic flies aggravates the phenotype. Scale bars: *A*, 45 μm; *B*, 50 μm.

β-amyloid staining of plaques in the retina of transgenic *Drosophila*

Cryostat sections through eyes and optic lobes of adult double or single transgenic flies with accelerated degeneration of photoreceptor cells were stained with either thioflavin S or the β-amyloid-detecting mAb 4G8. Both thioflavin S and mAb 4G8 detected large globular deposits in the retinas of UAS-APP/UAS-DPsnL235P or UAS-APP/UAS-BACE/UAS-DPsnL235P flies (Fig. 3*A–D*). These large globular deposits also could be stained with the C-terminal end-specific Aβ-42 mAb 9G10 (data not shown). The deposits occurred age dependently with an onset at day 37 after eclosion in UAS-APP/UAS-DPsnL235P and UAS-APP/UAS-BACE/UAS-DPsnL235P flies (Fig. 3*J*). In UAS-APP and in UAS-APP/UAS-BACE transgenic flies without additional DPsnL235P, the onset of plaque formation was strongly delayed and did not occur before day 68 after eclosion (Fig. 3*J*). In all of these fly lines, the neuronal degeneration preceded the occurrence of the amyloid plaques (Fig. 3*J*). Electron microscopy detected many "star-like" deposits of various sizes in the retina and neuropil regions of the first and second optic ganglion in UAS-APP/UAS-DPsnL235P and UAS-APP/UAS-BACE/UAS-DPsnL235P flies (Fig. 3*E–H*). These star-like deposits resembled the fibrillar EM ultrastructure of β-amyloid plaques in brains of human AD patients or APP-transgenic mice (Rockenstein et al., 2001) and were only found in EM sections of flies containing thioflavin S-positive plaques but not in flies without thioflavin S-positive plaques. The β-amyloid plaques that are formed by the deposition of either Aβ or δ-Aβ in UAS-APP/UAS-BACE/UAS-DPsnL235P or UAS-APP/UAS-DPsnL235P flies, respectively, shared a similar if not identical ultrastructure (Fig. 3*G*,*H*).

Ubiquitous expression of APP, BACE, and Presenilin

Next, APP, BACE, and an additional copy of the *DPsn* gene were expressed ubiquitously in neuronal and non-neuronal cells of the fly by using actin-GAL4. Flies expressing APP alone or in combination with BACE and DPsn under the control of actin-GAL4 demonstrated semilethality and ectopic wing vein formation (Table 1, lines 3, 4; Fig. 4*B–D*, arrows). Only the UAS-DPsn and UAS-BACE/UAS-DPsn-expressing flies showed a normal survival rate and presented the correct wing vein pattern consisting of five longitudinal veins (L1–L5) and two cross veins [anterior and posterior cross veins (CVs)] (Fig. 4*A*; Table 1, lines 1, 2). Both the reduced viability and the wing vein phenotype were aggravated by the introduction of an additional *presenilin* copy, and they were rescued by *presenilin* loss of function mutations (*psnC4*) (Table 1, lines 4, 5; Fig. 4*C*,*D*). Replacing the human APP by its *Drosophila* homolog APPL, which does not contain an Aβ domain, rescued the lethality completely (Table 1, line 7), which implies an Aβ-related toxicity mechanism.

β- and γ-secretase inhibitors to modulate Aβ formation

To determine whether these phenotypes are suitable for the validation of therapeutic drugs designed to reduce Aβ formation, the fly larvae were kept on a special fly medium supplemented either with BACE inhibitors or γ-secretase inhibitors (Sinha et al., 1999; Dovey et al., 2001). The eclosion rates of transgenic larvae that were raised on nonsupplemented medium and on medium supplemented with the respective inhibitors or the vehicle only were evaluated. Treatment of *Act-GAL4;UAS-APP/+; UAS-BACE/UAS-DPsn* larvae with the BACE inhibitor at concentrations ranging from 1 to 50 nM increased the survival rates in a dose-dependent manner with a maximum effectiveness at 10 nM. Although 1 nM BACE inhibitor had no effect on the survival rate of transgenic flies, 10 nM BACE inhibitor increased the survival rate by a factor of 3 (0.17 vs 0.06) (Table 1, line 8). In other words, the ratio of eclosed flies expressing the transgenes of interest to the total number of flies eclosed was 1:18 (0.06) in the group of larvae raised on the vehicle-supplemented food and 1:6 (0.17) in the 10 nM BACE inhibitor-treated group.

Prolonged treatment of adult flies with the BACE inhibitor markedly increased their life expectancy from 13 to 20 d in comparison with the vehicle-treated control flies (Fig. 5, gray and

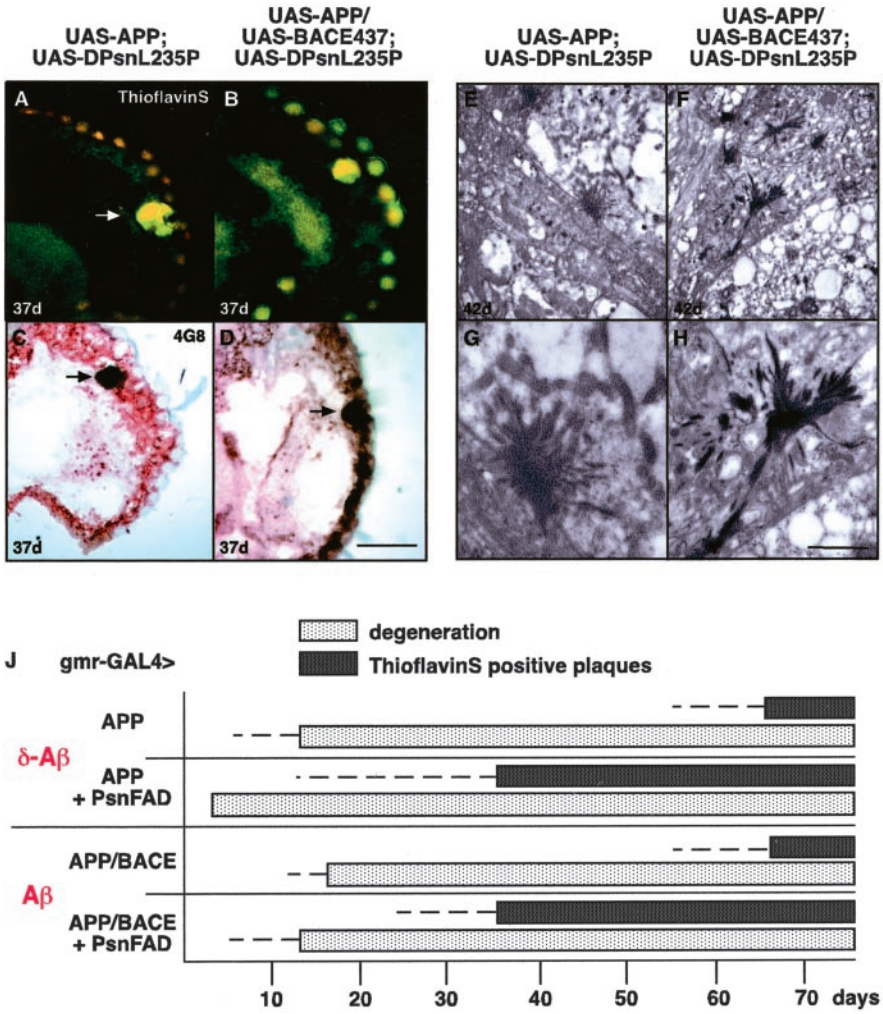

Figure 3. Amyloid-plaque deposition in the retina and neuropil region of transgenic flies expressing the indicated transgenes in the *Drosophila* compound eye. *A–D*, Thioflavin S (*A*, *B*) and 4G8 (*C*,*D*) staining of amyloid plaques deposited in the retina of *w; UAS-APP/+; UAS-DPsnL235P/gmr-GAL4* transgenic flies (*A*, *C*) and in *w; UAS-APP/UAS-BACE; UAS-DPsnL235P/gmr-GAL4* transgenic flies (*B*, *D*) from day 37 on. Large amyloid-plaque deposits were more evident in male flies, whereas female flies showed only small, more scattered, and diffuse thioflavin S-positive staining at 37 d. In *w; UAS-BACE/+; gmr-GAL4/+* - and *w; gmr-GAL4/ UAS-DPsn*-expressing control flies, no plaques could be detected by immunohistochemistry or electron microscopy at each time point investigated. Arrows indicate amyloid plaques. Scale bars: *A*, *B*, 45 μm; *C*, *D*, 50 μm. *E–H*, Ultrastructure of amyloid deposits in the retina of *w; UAS-APP/+; UAS-DPsnL235P/gmr-GAL4* transgenic flies (*E*, *G*) and in neuropil regions of the optic lobes in *w; UAS-APP/UAS-BACE; UAS-DPsnL235P/gmr-GAL4* transgenic flies (*F*, *H*). *J*, Progression of neurodegeneration and thioflavin S-positive plaque deposition. Dotted lines indicate the presence of thioflavin S-positive plaques and degenerating photoreceptor cells in <10% of the fly heads studied. Filled bars indicate detection of this phenotype in >80% of the fly heads studied. Scale bars: *E*, *F*, 15 μm; *G*, *H*, 9 μm.

black triangles). Treatment of flies that do not express APP but only an additional copy of the *Drosophila presenilin* gene with either BACE inhibitor or vehicle did not alter their life span (Fig. 5, gray and black dots). Treatment of transgenic fly larvae with the γ-secretase inhibitor at a concentration of 100 nM increased their survival rates by a factor of 4.5 [ratio of inhibitor-treated flies, 1:22 (0.045), vs ratio of vehicle-treated flies, 1:97 (0.01)] (Table 1, line 9). The eclosion rates of *Act-GAL4;UAS-DPsn/+* control flies were consistently at 0.33 and were not influenced by either the vehicle or inhibitor (Table 1, line 10). In addition, BACE inhibitor treatment reduced the ectopic wing vein phenotype in *Act-GAL4;UAS-APP/+;UAS-BACE/UAS-DPsn* transgenic flies, underscoring that it was related to aberrant APP processing during development.

Discussion

Here, we describe the generation of a *Drosophila* model for AD-related β-amyloidosis and neurodegeneration that is based on the accumulation and deposition of Aβ. In transgenic flies that express the AD-related genes *APP*, *BACE*, and *presenilin*, Aβ peptides processed from the human APP are detectable 2 d after eclosion. Most importantly, the generation of amyloidogenic Aβ peptides in the retina of transgenic flies is accompanied by an age-dependent neurodegeneration of the retinal photoreceptor cells and the deposition of β-amyloid-like plaque formations that also occur strictly dependent on aging. The degeneration of photoreceptor cells preceded the onset of β-amyloid plaque formation, indicating that β-amyloid precursors including Aβ oligomers or protofibrils mediated the toxic events. Coexpression of FAD *presenilin* mutants in UAS-APP and in UAS-APP/UAS-BACE transgenic flies accelerates Aβ-induced neurotoxicity in *Drosophila*. Age-dependent neurodegeneration and the onset of plaque deposition are prematurely induced in flies with the FAD presenilin mutants, thus resembling the effects of these mutations in human AD. This strongly suggests that the expression of human APP in flies induces analogous biochemical cascades that lead to cellular toxicity and degeneration in human AD.

The processing of human APP by BACE in transgenic flies is dependent on glycosylation. An interference of post-translational modifications with APP processing has been reported previously (Tomita et al., 1998; Galbete et al., 2000; Georgopoulou et al., 2001). The processing of human APP in transgenic flies lacking BACE leads to the generation of a slightly larger peptide, δ-Aβ, by cleavage aminoterminally to the usual β-cleavage site. A similar form of δ-cleavage of APP_{695} at Thr 584 was found previously in cultured rat neurons (Simons et al., 1996). However, the responsible secretase has not been characterized thus far (Simons et al., 1996).

Both δ-Aβ and Aβ generated through either δ- or β-cleavage aggregate to SDS-stable oligomers, suggesting that both cleavage events generate amyloidogenic products in *Drosophila*. Aβ oligomers are present in brains of AD patients, and there is growing evidence that Aβ oligomers are neurotoxic *in vivo* (Walsh et al., 2002; Wang et al., 2002; Gong et al., 2003). In human patients with AD, the density of β-amyloid plaques in the brain cortex is only weakly linked to the severity of dementia, whereas secreted monomeric and oligomeric forms of Aβ match the process of cell loss and impaired cognitive function more closely (Scheuner et al., 1996; Lue et al., 1999; McLean et al., 1999; Walsh et al., 2002). Therefore, the loss of photoreceptor cells in our *Drosophila* model could be attributable to toxic effects of the oligomeric

Table 1. Ubiquitous expression of APP, BACE, and DPsn as a model system to identify genetic modifiers and validate pharmacologically active agents

Actin-GAL4>	Ratio of flies expected	Ratio of flies scored		Wing phenotype	
(1) UAS-DPsn/+	1:4 (0.25)	1:4 (0.25)		Wild type	
(2) UAS-BACE/UAS-DPsn	1:4 (0.25)	1:4 (0.25)		Wild type	
(3) UAS-APP;UAS-BACE/+	1:4 (0.25)	1:40 (0.025)		Ectopic veins (75%)	
(4) UAS-APP;UAS-BACE/UAS-DPsn	1:4 (0.25)	1:97 (0.01)		Ectopic veins (100%)	
(5) UAS-APP;UAS-BACE, *psnC4*/+	1:4 (0.25)	1:13 (0.077)		Weak ectopic veins (70%)	
(6) UAS-APP	1:4 (0.25)	1:127 (0.008)		Wild type	
(7) UAS-APPL1;UAS-DPsn/+	1:4 (0.25)	1:4 (0.25)		Wild type	
	Nonsupplemented	Vehicle supplemented	Inhibitor supplemented	Wing phenotype	Treatment
(8) UAS-APP;UAS-BACE523/UAS-DPsn	1:17 (0.06)	1:18 (0.06)	1:6 (0.17)	Weak ectopic veins (100%)	10 nM BACE inhibitor
(9) UAS-APP;UAS-BACE406/UAS-DPsn	1:100 (0.01)	1:97 (0.01)	1:22 (0.045)	Ectopic veins (100%)	100 nM γ-secretase inhibitor
(10) UAS-DPsn/+	1:2.6 (0.38)	1:3 (0.33)	1:2.7 (0.37)	No ectopic veins	10 nM BACE or 100 nM γ-secretase inhibitor

Ratios of flies expressing the indicated transgenes under the control of the actin-GAL4 driver to nonexpressing flies. A comparison of expected and scored ratios indicates increased lethality during development of the fly. Lethality rate and wing phenotypes of transgenic flies (lines 1–7) and of transgenic larvae raised on nonsupplemented and vehicle supplemented versus BACE- or γ-secretase inhibitor supplemented medium (lines 8–10) are shown.

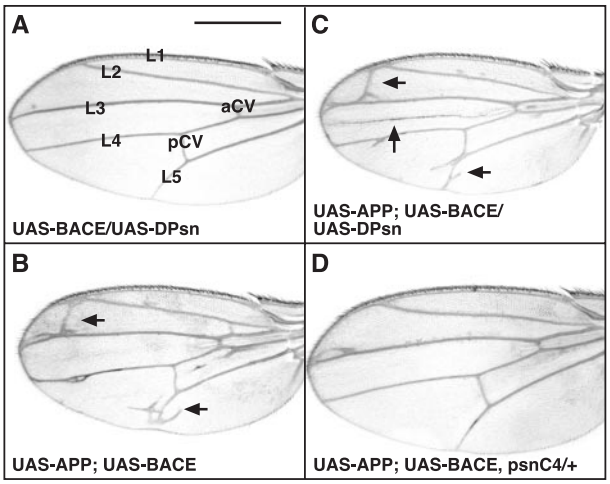

Figure 4. Ectopic veins in wings of transgenic flies expressing APP, BACE, and DPsn ubiquitously under the control of the actin-GAL4 driver. *A–C*, *w; actin-GAL4/+; UAS-BACE/UAS-DPsn* transgenic flies presented the normal wing vein pattern (*A*), whereas *w; actin-GAL4/UAS-APP; UAS-BACE/+* and *w; actin-GAL4/UAS-APP; UAS-BACE/UAS-DPsn* transgenic flies developed ectopic longitudinal and CVs (*B*, *C*, arrows). *D*, Introducing a loss-of-function *presenilin* allele (*psn*C4/+) rescued the wing vein phenotype.

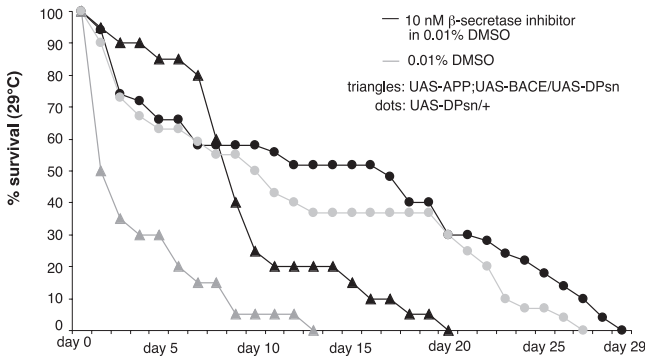

Figure 5. Increased median life span of BACE inhibitor-treated flies. Survival curves of *w;act-GAL4/UAS-APP;UAS-BACE/UAS-DPsn* and of *w;act-GAL4/+;UAS-DPsn/+* transgenic adult flies after prolonged treatment with the BACE inhibitor (black triangles and circles, respectively) and of nontreated flies of the same genotypes (gray triangles and circles, respectively).

forms of Aβ. Recent findings implicate an involvement of intracellular Aβ accumulation in the pathogenesis of AD disease (Oddo et al., 2003). Although we have no evidence for intracellular Aβ accumulation in our fly model of Alzheimer's disease, we cannot exclude the possibility that intracellular Aβ participates in the neurodegenerative process in our transgenic fly model. β-amyloid deposits localized outside the retina, despite expression of the transgenes in photoreceptor cells, suggest Aβ secretion by photoreceptor cells into their projection areas in addition to local secretion within the retina.

The accelerated degeneration of photoreceptor cells in flies generating the variant δ-Aβ peptide may indicate a more toxic effect of this peptide. Alternatively, it could be related to the absence of the N-terminal secreted part of APP (APPs) in these flies, because Western blots did not reveal a δ-cleaved APP-ectodomain analogous to APPsβ. APPs may act as a neuroprotective factor (Sisodia and Gallagher, 1998), and the absence of stable APPs in flies with δ-cleavage of APP may promote neurotoxic effects of δ-Aβ. In addition, a disruption of axonal transport mechanisms leading to axonal vesicle stalling may also contribute to the degenerative phenotypes in flies expressing full length APP_{695} (Gunawardena and Goldstein, 2001). However, the observation that coexpression of an additional copy of FAD *presenilin* mutants in either combination, UAS-APP or UAS-APP/UAS-BACE, caused more severe degenerative phenotypes along with earlier β-amyloid plaque deposition supports an Aβ-related toxicity mechanism.

The reason for the sexual dimorphism observed with respect to the occurrence of larger and more extensive β-amyloid plaques in male flies as compared with females is unknown but may be related to the smaller body weight and size of male flies.

Ubiquitous expression of APP, BACE, and DPsn resulted in semilethality and a visible wing phenotype. This phenotype could be used to screen for genes, drugs, or metabolites that modulate APP processing and have the potential to decrease Aβ-induced cellular degeneration. Both, γ- and β-secretase inhibitor treatment increased the survival rates of the UAS-APP/UAS-BACE/UAS-DPSn transgenic flies. γ-secretase cleavage is required not only for APP processing but also for cleavage of other proteins such as Notch in vertebrates and invertebrates (Fortini, 2002; De Strooper, 2003). The γ-secretase inhibitors available are not specific for the cleavage of APP. In *Drosophila* as well as in mice, loss-of-function mutants of the endogenous PS gene cause embryonic lethal Notch phenotypes (Shen et al., 1997; Ye et al., 1999). The absence of Notch phenotypes in our experiments indicates that the concentrations of the γ-secretase inhibitor used did not lead to a complete inhibition of γ-secretase cleavage and allowed sufficient endogenous DPsn activity required for normal

development of the fly. However, this inhibition clearly prolonged the survival of the transgenic flies as compared with nontreated flies, suggesting that toxic β-amyloid accumulation could be prevented.

The targeted expression of human disease genes in *Drosophila* has been used previously as a model system to study the complex pathophysiology and potential treatment forms of human neurodegenerative diseases such as Parkinson's disease and amyotrophic lateral sclerosis as well as polyglutamine-repeat diseases (Feany, 2000; Fortini and Bonini, 2000). Our model closely mimics main characteristics of the human AD neuropathogenesis in the eye of the fly and demonstrates that Aβ-induced neurodegeneration is a conserved principle that acts even in flies. In particular, this system may be useful in the search of inhibitors of β- or γ-secretases that are currently regarded as a valid option for the treatment and prevention of AD.

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