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Amyloid precursor proteins are protective in *Drosophila* models of progressive neurodegeneration

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ABSTRACT

The processing of Amyloid Precursor Proteins (APPs) results in several fragments, including soluble N-terminal ectodomains (sAPPs) and C-terminal intracellular domains (AICD). sAPPs have been ascribed neurotrophic or neuroprotective functions in cell culture, although β-cleaved sAPPs can have deleterious effects and trigger neuronal cell death. Here we describe a neuroprotective function of APP and fly APPL (Amyloid Precursor Protein-like) in vivo in several *Drosophila* mutants with progressive neurodegeneration. We show that expression of the N-terminal ectodomain is sufficient to suppress the progressive degeneration in these mutants and that the secretion of the ectodomain is required for this function. In addition, a protective effect is achieved by expressing *kuzbanian* (which has α-secretase activity) whereas expression of fly and human BACE aggravates the phenotypes, suggesting that the protective function is specifically mediated by the α-cleaved ectodomain. Furthermore, genetic and molecular studies suggest that the N-terminal fragments interact with full-length APPL activating a downstream signaling pathway via the AICD. Because we show protective effects in mutants that affect different genes (AMP-activated protein kinase, MAP1b, rasGAP), we propose that the protective effect is not due to a genetic interaction between APPL and these genes but a more general aspect of APP proteins. The result that APP proteins and specifically their soluble α-cleaved ectodomains can protect against progressive neurodegeneration in vivo provides support for the hypothesis that a disruption of the physiological function of APP could play a role in the pathogenesis of Alzheimer's Disease.

Introduction

APP proteins are type-one, membrane-spanning proteins which are subject to proteolytic processing resulting in several distinct fragments (De Strooper and Annaert, 2000; Turner et al., 2003). Cleavage of human APP_{695}, the predominant isoform in the brain (Tanaka et al., 1989), by the β- and γ-secretase generates the Aβ peptides which accumulate in the plaques characteristic for Alzheimer's Disease (Selkoe, 2000). However, the processing also produces large extracellular fragments, sAPPα after α-processing and sAPPβ after β-processing, in addition to cytoplasmic fragments called AICDs. Both, the sAPP fragments and the AICD contain a number of interaction motifs (De Strooper and Annaert, 2000; Turner et al., 2003) which are shared by APPL (Amyloid Precursor Protein-like), the *Drosophila* orthologue of APP. APPL is approximately 30% identical to human APP_{695}, with significantly higher homology in specific extracellular domains and in the AICD (Martin-Morris and White, 1990). We recently showed that APPL can be processed by secretases resembling the α-, β-, and γ-secretases (Carmine-Simmen et al., 2009), resulting in proteolytic fragments that are comparable to APP fragments. Also similar to APP_{695}, APPL is expressed in all neurons and during embryogenesis it is especially abundant in growing axons and in areas of synapse formation (Luo et al., 1990). Most strikingly, flies lacking APPL exhibit behavioral phenotypes that can be partially rescued by expression of human APP_{695} (Luo et al., 1992).

Although this structural and functional conservation from fly to human suggests an important function, relatively little is known about the physiological roles of APP proteins and their fragments. While the AICD of APP_{695} has been connected with transcriptional regulation (Kimberly et al., 2001), the soluble fragments have been shown to interact with the extracellular matrix (Beher et al., 1996) and they can promote substrate binding and cell adhesion (Turner et al., 2003). They have also been shown to have proliferative, neurotrophic, or synaptotrophic effects in cell culture, whereby the potency of sAPPα might be different from that of sAPPβ (Araki et al., 1991; Mucke et al., 1994; Mattson, 1997). In some studies, only sAPPα

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was protective while sAPPβ was shown to have deleterious effects (Li et al., 1997) and recently, β-secretase processed APP was shown to trigger neuronal cell death after trophic factor deprivation (Nikolaev et al., 2009). APP proteins have also been shown to have a protective effect against acute and chronic excitotoxicity in vivo (Masliah et al., 1997) and this also appears to be mediated by sAPPα because overexpression of ADAM10, a metalloprotease with α-secretase activity, had a similar effect (Clement et al., 2008).

Here we show that APP_{695} and its fly orthologue APPL can protect against progressive neurodegeneration in several *Drosophila* models. This function is mediated by the extracellular ectodomains and, as overexpression of secretases suggest, specifically by the α-cleaved form. In addition, our experiments show that these fragments have to be secreted and that the presence of full-length APP proteins is required for the protective effect, suggesting a model in which the α-cleaved N-terminus acts as a ligand and the full-length APP as a receptor.

Materials and methods

Drosophila stocks

loe has been described in Tschape et al. (2002), UAS-dBACE and UAS-APPL in Carmine-Simmen et al. (2009), human UAS-BACE1 in Greeve et al. (2004), *vap* in Botella et al. (2003), *sws* in Kretzschmar et al. (1997), and *futsch*^{olk} in Bettencourt da Cruz et al. (2005). UAS-APP_{695}, UAS-*kuz*, and *elav*-GAL4 were provided by the Bloomington Stock Center, *Appl*-GAL4 by L. Torroja (Universidad Autonoma de Madrid, Spain) and UAS-APPL^{sd} by V. Budnik (University of Massachusetts, Worcester). The *Appl*^{d} mutant allele was kindly provided by K. White. Stocks were maintained and raised under standard conditions.

Constructs

The APPL constructs were created using the GH04413 cDNA obtained from the Drosophila Genomics Resource Center. The N-terminal sAPPL fragment contains the start codon, followed by the signal sequence and up to aa758, the start of the deletion in APPL^{sd} which removes the N-terminal cleavage sites (Torroja et al., 1999). The APPL-AICD consists of aa835-887 with a start codon added. The sAPPα and sAPPβ constructs include the start codon, signal sequence and up to aa596 for sAPPβ and aa612 for sAPPα. All constructs contain C-terminal HA-tags and were cloned into pUAST for P-element transformation.

Paraffin sections and determination of vacuole size

To analyze the neurodegenerative phenotype, we performed paraffin serial sections as described in Bettencourt da Cruz et al. (2005) and took photographs from the section that contained the brain region of interest and displayed the most severe degeneration. For *vap* and *loe* the area of all vacuoles in the optic lobes was determined. For *sws* the analysis was restricted to the deutocerebral neuropil as described in Bettencourt da Cruz et al. (2008) and for *futsch*^{olk} pictures were taken from the antennal lobes and measured as described in Bettencourt da Cruz et al. (2005). For double blind analyses, pictures were taken and numbered and the area of vacuoles was then measured in pixels in Photoshop and subsequently converted into μm^2 (Bettencourt da Cruz et al., 2005). The statistical analysis was done using one-way ANOVA.

Survival assay

Male and female flies were separated and kept on standard food vials at 25 °C in groups of 15–25 flies. Vials were exchanged after 1 week. At least ten independent tests were performed for each genotype with 171–508 flies total for each sex and genotype. The *Appl*^{d} line had been outcrossed against wild type three times to control for background effects. P-values were obtained by comparing the percentage of dead flies at day 8 using two-way ANOVA.

Western Blots

Western Blots were performed as described in Tschape et al. (2002), using an anti-APPL AICD antiserum kindly provided by P. Copenhaver (OHSU) at 1:4000 and peroxidase conjugated donkey anti-chicken secondary antibodies from Jackson at 1:10,000. Head lysates from 1 to 2 d old flies were loaded on 4–12% gradient gels (Criterion, Bio-Rad) and bands were detected using the Visualizer Western Blot Detection Kit (Upstate).

Secretion assay

Four brains each were dissected from male larvae expressing sAPPL, sAPPα, or sAPPβ via *Appl*-GAL4 and gently ripped apart into a few pieces. The brain fragments were collected with a 6 min centrifugation at 6000 rpm and washed before being incubated in 1 ml modified Schneider's media in a tube for 48 h at 25 °C with constant rocking. To collect lysates and media for Western Blot analysis the tubes were spun for 4 min at 6000 rpm, the media removed and the pellet spun for an additional hour at 14,000 rpm at 4 °C. The pellet containing the harvested cells was then lysed with cold RIPA buffer containing protease inhibitors (Sigma P8340) on ice for 5 min. Both media and lysates were mixed with Laemmli buffer and Western Blots performed as described above. The constructs were detected using an anti-HA antibody (Covance). Modified Schneider's media 50 μg/ml insulin (Gibco #12585-014), 10% heat inactivated Fetal Bovine Serum, 1% penstrep (Gibco 15240–062), 0.5% 20 Hydroxy Ecdysone (Sigma H-5142) in Schneider's media (Gibco 11720–034).

Co-immunoprecipitation

Co-immunoprecipitations were performed using lysates from transfected Kc cells. Using CellfectinII reagent (Invitrogen) cells were co-transfected with 1 ng each of pUAST-APPL, pACT-GAL4, and pUAST-sAPPL, pUAST-sAPPα, or pUAST-sAPPβ, respectively. As a control we used a HA-tagged mitochondrial protein (CG4495) cloned into pACT (kindly provided by M. Forte, OHSU). Transfected cells were grown for 48 h on 100 mm dishes and harvested by rinsing them off in PBS and then lysing with NP40 buffer on ice for 5 min. Protein A/G (Santa Cruz) were blocked in 0.2% BSA in PBS overnight at 4 °C. Cell lysates were incubated with 1 μg of anti-HA antibodies (Covance) overnight at 4 °C before being combined with blocked beads for 4–6 h at room temperature. After three washes (with NP40 buffer), the eluate was obtained using 2× Laemmli buffer and boiling the sample for 1 min. Western Blots were performed as described above and the full-length APPL detected with an antisera against the C-terminus (kindly provided by P. Copenhaver).

Results

We previously described that *loechrig* (*loe*) mutant flies show increasing vacuolization of the adult brain with aging followed by early death after about two weeks of adult life (Tschape et al., 2002). *loe* also shows degeneration of 3rd instar larval brains, but not during developmental stages including pupa in which the adult brain develops. *loe* is caused by a P-element insertion that disrupts one transcript for the γ-subunit of the AMP-activated protein kinase (AMPK). The insertion affects only one of the three identified protein isoforms and as we have shown this isoform is expressed and required in neurons. Interestingly, we also found that the degenerative phenotype of *loe* is severely enhanced by removing the *Appl* gene

which encodes the sole APP protein in flies. This observation suggested that APPL or one of its proteolytic fragments might protect against the progressive degeneration observed in *loe* mutants.

Additional expression of APPL delays neurodegeneration in loe

To determine whether APPL can serve a neuroprotective function in *loe*, we used flies expressing APPL under the control of the UAS sequence. To induce this construct in a pattern corresponding to endogenous APPL expression, we employed a GAL4 construct containing the *Appl* promoter (*Appl*-GAL4, kindly provided by L. Torroja, Universidad Autonoma de Madrid). These lines were then crossed to express APPL pan-neuronaly in *loe* mutant flies. After eclosion from the pupal case, female flies were aged for five days before performing paraffin head sections to determine the extent of degeneration. As controls, we used *loe* flies that came out of the same cross (for a comparable genetic background) but only carried the UAS-APPL construct and therefore did not express additional APPL (Fig. 1A). Determining the average area of vacuoles in the optic lobes of these control flies showed that the vacuolization was not significantly changed compared to the original *loe* stock ($231 \pm 23\ \mu m^2$ versus $253 \pm 23\ \mu m^2$). However, flies that expressed the full-length APPL protein (Fig. 1B) showed substantially less vacuolization with $137 \pm 11\ \mu m^2$ (Fig. 1C, $p \ll 0.001$). This confirmed that APPL indeed has a neuroprotective function in *loe* mutant flies.

The soluble N-terminus of APPL is sufficient for the neuroprotective function

Next, we investigated whether the protective function can be attributed to specific fragments of APPL. For this purpose, we created constructs encoding the soluble N-terminal fragment of APPL (sAPPL, Fig. 1D) or the intracellular domain of APPL (dAICD) and induced them with *Appl*-GAL4. Western blot analyses confirmed that the expression levels of the sAPPL construct were similar to the sAPPL produced from the APPL construct (Supplementary Fig. 1A). We also confirmed expression of the dAICD fragment by immunohistochemistry because due to its small size we were not able to detect it in Western blots (Supplementary Fig. 2). Whereas sAPPL reduced the degeneration to the same extent as the full-length protein (Fig. 1C; $125 \pm 14\ \mu m^2$, $p \ll 0.001$), expression of the dAICD did not affect the *loe* phenotype ($222 \pm 23\ \mu m^2$). Control flies from these crosses yielded the same values as the previously used controls ($230 \pm 23\ \mu m^2$ and $235 \pm 28\ \mu m^2$).

Although these results showed that the protective function is mediated by the N-terminus, which can be secreted, they did not determine whether its secretion is actually required. To address this issue, we used a secretion-deficient form of APPL ($APPL^{sd}$, Fig. 1D), which lacks the N-terminal cleavage sites, but still contains the region corresponding to sAPPL. As shown in Fig. 1C, this construct did not improve the degenerative phenotype ($287 \pm 18\ \mu m^2$), but even significantly enhanced the vacuolization compared to controls ($p < 0.05$). The lack of protection is not due to lower expression levels of this construct because this construct is at least as strong expressed as the wild type APPL (Supplementary Fig. 1A). We also tested whether expression of the $APPL^{sd}$ construct alone can have a deleterious function and found that in about half of these flies (55%) we can detect single vacuoles, which were restricted to the lamina, at 5 d of age (Supplementary Fig. 3A). Together these experiments show that the ectodomain of APPL does have to be secreted to fulfill its neuroprotective function and that preventing the cleavage might even have deleterious consequences.

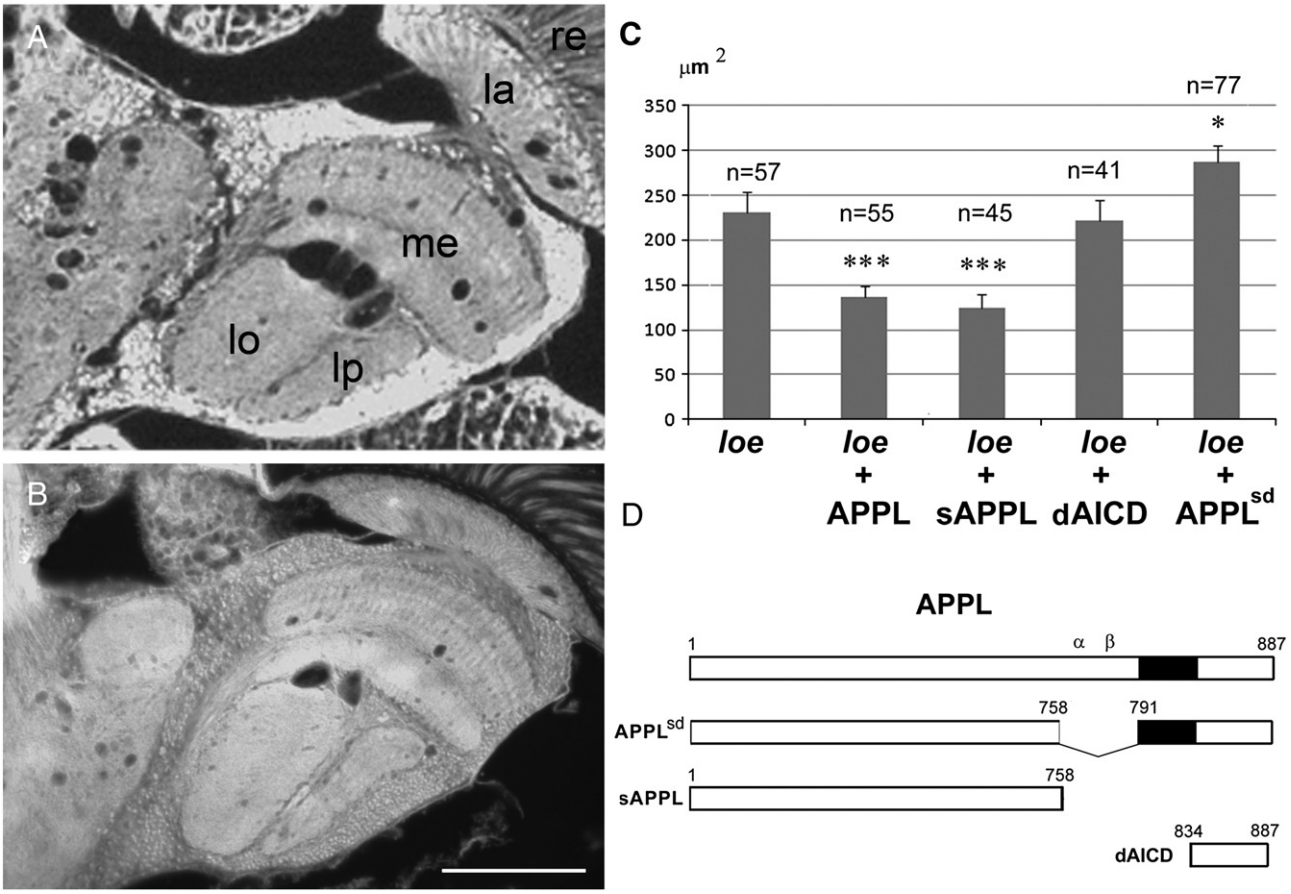

Fig. 1. Neuroprotective effects of APPL. (A) Paraffin head section of a 5 d old *loe* control fly, carrying the UAS-APPL construct, but not *Appl*-GAL4. The vacuole formation characteristic for *loe* mutants of this age is easily detectable. (B) In contrast, a *loe* fly expressing full-length APPL via Appl-GAL4 shows much less vacuolization. (C) Mean area of vacuoles in μm^2 in the different genotypes tested. SEMs and the number of brain hemispheres analyzed are indicated, *<0.05, **<0.01, ***<<0.001. All flies were 5 d old females. (D) Schematic of the different APPL constructs used. Scale bar in A, B = 50 µm. re = retina, la = lamina, me = medulla, lo = lobula, lb = lobula plate.

Increasing α-processing is protective while β-processing enhances the degeneration

We recently showed that APPL, like APP, can be processed into two alternative N-terminal fragments (Carmine-Simmen et al., 2009). Although both N-terminal cleavage sites reside within the region deleted in APPL^{sd} the exact locations have not been determined preventing us from creating the adequate sAPPLα and sAPPLβ construct. However, we also showed that *kuzbanian (kuz)*, an ADAM10 homolog, can act as α-secretase, and we identified a BACE-like enzyme (dBACE) that promoted β-cleavage of APPL (Carmine-Simmen et al., 2009). We therefore investigated whether increasing the levels of KUZ or dBACE, and thereby the production of the α- or β-cleaved N-terminus from the endogenous APPL present in *loe* flies, has a beneficial effect. As shown in Fig. 2A, expression of dBACE via *Appl*-GAL4 enhanced vacuole formation in *loe* mutants ($316 \pm 25\ \mu m^2$, $p<0.01$) whereas expression of KUZ reduced vacuolization ($136 \pm 11\ \mu m^2$, $p \ll 0.001$) compared to controls ($209 \pm 28\ \mu m^2$). The decrease in vacuolization when the α-secretase KUZ is expressed, strongly suggest that the protective function of APPL is specifically provided by the α-cleaved ectodomain. In contrast, dBACE increases the production of the β-cleaved ectodomain from endogenous APPL and even aggravates the degeneration in *loe*. This enhancement does not seem to be due to an additive effect caused by increased Aβ production because expression of dBACE via *Appl*-GAL4 in 5 d old wild type flies does not result in degeneration (data not shown).

The protective function is conserved in human APP$_{695}$

To determine whether the neuroprotective function is a conserved property of APP proteins, we expressed human APP$_{695}$ in *loe* mutant flies. As shown previously, human APP can be processed by the endogenous fly secretases, including α-processing by KUZ (Greeve et al., 2004; Loewer et al., 2004). Indeed APP expression resulted in a dramatic reduction of the vacuolization ($82 \pm 5\ \mu m^2$ versus $223 \pm 22\ \mu m^2$, $p \ll 0.001$, Fig. 2B), possibly by the production of a protective sAPPα fragment. As with the fly version, expression of only the AICD of APP$_{695}$ had no effect ($215 \pm 17\ \mu m^2$, data not shown). To test whether it is specifically the α-cleaved secreted N-termini of APP, we created constructs encoding sAPPα and sAPPβ, respectively and induced them again with *Appl*-GAL4. We first tested whether these constructs were expressed and secreted at levels comparable to sAPPL by dissecting brains and keeping them in culture for two days before harvesting the media and lysates. sAPPα and sAPPβ were detected at similar levels as sAPPL in lysates and the media (Fig. 2C), confirming that both human fragments are expressed at similar levels as sAPPL and can be secreted efficiently. Surprisingly however, neither of the sAPPs reduced vacuolization when expressed in *loe* ($244 \pm 24\ \mu m^2$ and $222 \pm 21\ \mu m^2$ compared to $223 \pm 22\ \mu m^2$). As an alternative, we tested whether co-expression of human BACE1 with APP$_{695}$, which should increase the production of the non-protective sAPPβ at the expense of sAPPα, reduced the protective function of full-length APP$_{695}$. As shown in Fig. 2B this indeed increased the vacuolization to $119 \pm 13\ \mu m^2$ compared to $82 \pm 5\ \mu m^2$ after expression of APP$_{695}$ alone ($p<0.05$), supporting our results obtained with APPL that increased β-cleavage is deleterious and that the protective function is probably mediated by the α-cleaved sAPP.

The protective function of the ectodomain is mediated by an interaction with full-length APPL

It has been shown that vertebrate APP proteins can form homo- and heterodimers (Scheuermann et al., 2001) and we therefore investigated whether an interaction between the secreted ectodomain and endogenous full-length APPL could play a role for the protective function. To address this issue, we created flies that were double mutants for *loe* and the *Appl^d* allele, which lacks APPL (see Fig. 5), and then expressed our UAS-APPL construct in these flies using *Appl*-GAL4. In this set of experiments, we analyzed four days old males, which due to the X-chromosomal location of *Appl*, were hemizygous for *Appl^d* and homozygous for the *loe* mutation. Because *loe* males show a more severe phenotype than *loe* females, the mean value of vacuole area was $348 \pm 36\ \mu m^2$ in these males (Fig. 3A) and as described before (Tschape et al., 2002), the double mutant showed a significantly more severe degenerative phenotype with $543 \pm 48\ \mu m^2$ of vacuole area than *loe* alone (Fig. 3A). This enhancement is not due to an additive degenerative effect of the two mutations because 4 d old *Appl^d* flies do not show any vacuole formation (Supplementary Fig. 3B) and we therefore assume that it is caused by the loss of the beneficial function normally provided by endogenous APPL. As expected, expression of full-length APPL rescued the vacuolization decreasing it to approximately the level observed when endogenous APPL is present ($384 \pm 37\ \mu m^2$, $p<0.05$). In contrast, expressing sAPPL did not result in a reduction of the vacuolization in the double mutant ($627 \pm 43\ \mu m^2$) although this construct was equally as beneficial as full-length APPL in the *loe* mutation alone (see Fig. 1C). This shows that sAPPL expression

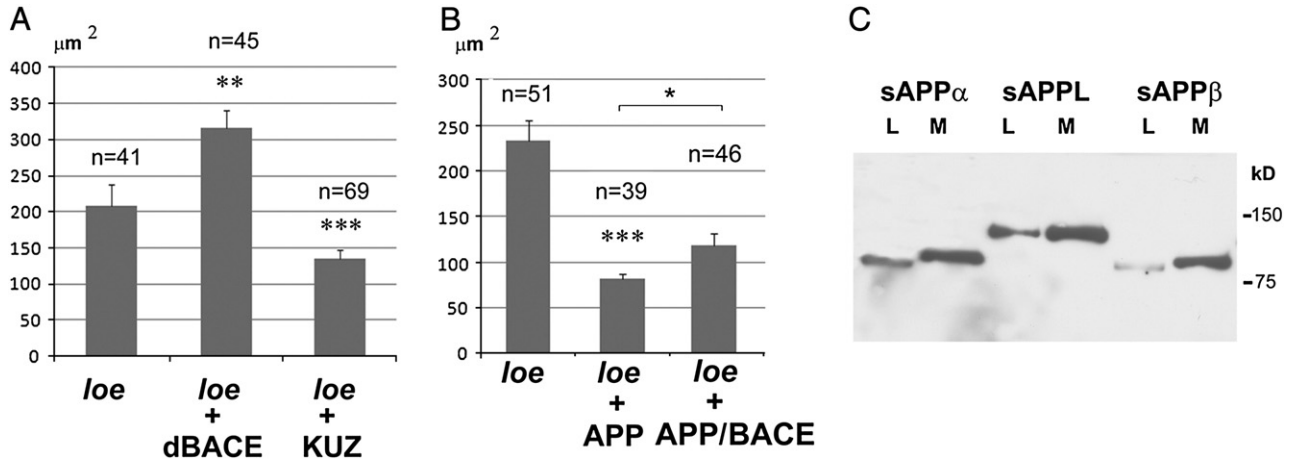

Fig. 2. Expression of *kuzbanian* or human APP$_{695}$ ameliorates the *loe* phenotype. (A) Effects of altering the processing of endogenous APPL in *loe* flies. The mean area of vacuoles in μm^2 in *loe* flies overexpressing dBACE is increased whereas KUZ overexpression suppresses vacuole formation. (B) Expressing full-length APP$_{695}$ also reduces the mean area of vacuoles in *loe* flies. Co-expression of human BACE1 with APP$_{695}$ significantly reduced the suppressing effect compared to expression of APP$_{695}$ alone. SEMs are indicated, *<0.05, **<0.01, ***<<0.001. (C) A Western Blot using anti-HA reveals that all soluble fragments are expressed when induced via *Appl*-GAL4 and secreted into the media (M) after the brains were kept in culture. L = lysate from cultured brains.

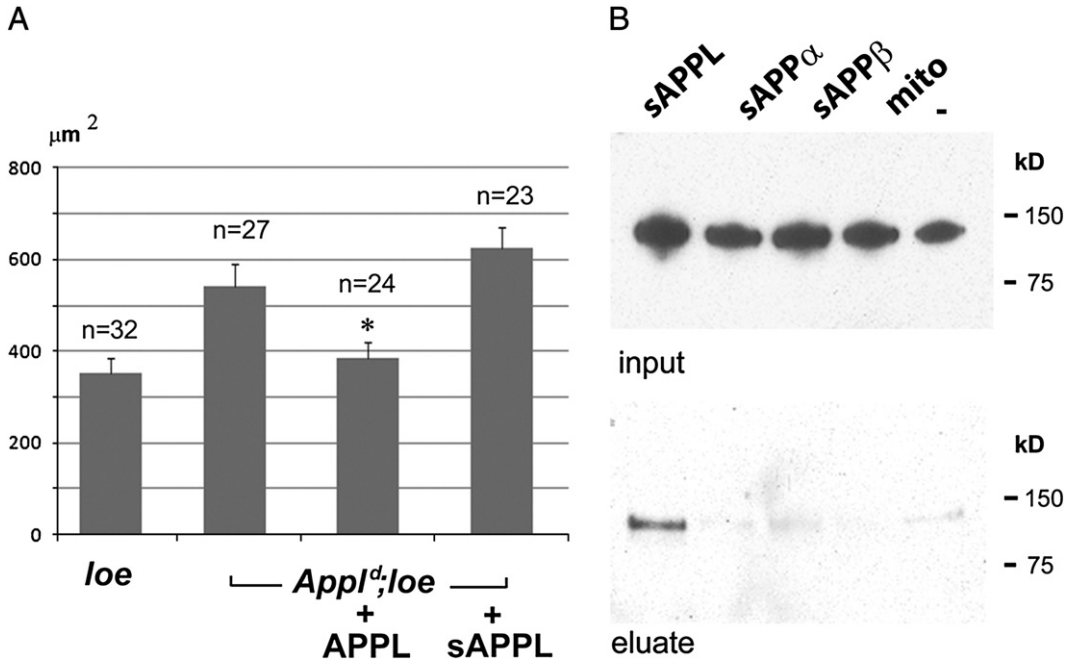

Fig. 3. The protective function is mediated by full-length APPL. (A) Loss of APPL enhances the degeneration in *Appl^d;loe* double mutants. Expression of full-length APPL in *Appl^d;loe* double mutants can rescue the effects of this loss and decreases the vacuolization. In contrast, expression of sAPPL has no effect in the in *Appl^d;loe* double mutant. Flies were 4 d old males. SEMs are indicated. (B) Western Blot using α-APPL-AICD on immunoprecipitates from Kc cells expressing HA-tagged soluble fragments and untagged full-length APPL. Similar amounts of full-length APPL in the input (upper panel) indicate comparable transfection efficiencies in these samples. Whereas a significant amount of full-length APPL can be found in the eluate (lower panel) from sAPPL expressing cells (lane 1), showing that it can bind to full-length APPL. In contrast, only marginal amounts of full-length APPL are detectable in the co-immunoprecipitations using the human sAPPα or sAPPβ fragment. A control that does not contain an HA-tagged construct also reveals marginal amounts of full-length APPL.

can only ameliorate the degenerative effects in *loe* when full-length APPL is also present. To investigate whether the ectodomain can directly bind, or at least form a complex, with the full-length protein, we performed co-immunoprecipitation experiments in *Drosophila* Kc cells. Cells were co-transfected with pACt-GAL4, the HA-tagged sAPPL, and full-length APPL (not tagged), the HA-tagged constructs were precipitated using HA antibodies and Western Blots performed using an antiserum against the AICD to detect full-length APPL. For controls, we used an HA-tagged mitochondrial protein (CG4495) instead of sAPPL and Kc cells in which the sAPPL construct was omitted. As shown in Fig. 3B (upper panel), we detected similar amounts of full-length APPL in the inputs showing that the cells had been transfected with a comparable efficiency. However, after precipitation and eluation significant amounts of APPL were only detectable in lysates from sAPPL expressing cells (lane 1) suggesting that the ectodomain can bind to the full-length protein. A weak band is also detectable in the control lanes with the HA-tagged mitochondrial protein (mito, lane 4) and the control without an HA-tagged construct (lane 5) indicating that a small amount of residual APPL is present in the eluate. Interestingly, we found that APPL was not co-precipitated with human sAPPα (lane 2) or sAPPβ (lane 3), suggesting that these fragments cannot interact with fly APPL. We therefore assume that human sAPPα cannot improve the degenerative phenotype in *loe* because it cannot interact with the full-length fly APPL protein needed to activate downstream protective events.

APPL is protective in several neurodegenerative mutants

The observed effect of APPL/APP on the *loe* phenotype could either be due to a specific interaction between these genes and therefore a suppression of the phenotype or to a more general protective function of APP proteins. To test this, we used several other mutants that show an age-dependent degeneration of the central nervous system, including *swiss-cheese (sws)*, *vacuolar peduncule (vap)*, and *futsch^{olk}*. *futsch^{olk1}* is a mutation in the *Drosophila* MAP1b orthologue that affects mostly the olfactory system (Bettencourt da Cruz et al., 2005). After about 6 d small vacuoles are detectable in the mechanosensory region of the antennal lobes which dramatically increase with further aging, resulting in the formation of large vacuoles that take up an area of $50.8 \pm 6.9\ \mu m^2$ in four week old flies (Figs. 4A, B). Expression of sAPPL in *futsch^{olk1}* via the pan-neuronal *elav*-GAL4 promoter (which was chosen instead of the X-chromosomal *Appl*-GAL4 due to *futsch^{olk}* also residing on the X-chromosome) resulted in a reduction of the vacuolization to $18.1 \pm 1.0\ \mu m^2$ ($p < 0.05$, Fig. 4B).

vacuolar peduncule (vap) encodes a rasGAP protein, a component of the EGF signaling pathway (Botella et al., 2003). *vap* shows degeneration in all parts of the brain (Fig. 4C) with a vacuole area of $96 \pm 12.2\ \mu m^2$ in the optic lobes of 14 d old male flies carrying the *vap^1* allele. Inducing sAPPL with *elav*-GAL4 in hemizygous *vap^1* mutants also decreased the vacuolization to $63 \pm 9.5\ \mu m^2$ (Fig. 4D).

Finally, we used *sws^1* which is caused by a mutation in a gene with phospholipase and Protein Kinase A regulatory activity (Muhlig-Versen et al., 2005; Bettencourt da Cruz et al., 2008) and, like *vap* and *loe*, *sws* leads to a progressive degeneration in all parts of the adult central nervous system (Fig. 4E). Focusing on the vacuoles in the deutocerebral neuropil, as previously described (Bettencourt da Cruz et al., 2008), we detected a vacuole area of $41 \pm 4\ \mu m^2$ in 14 d old *sws^1* males (Fig. 4F). However, in contrast to the mutants described above, sAPPL expression via *Appl*-GAL4 had no significant effect on the degeneration observed in *sws^1* after 14 d ($41 \pm 10\ \mu m^2$, Fig. 4F). To confirm this result, we also tested the full-length APPL construct in these flies which also failed to show a significant protective effect ($34 \pm 4.5\ \mu m^2$). This negative result reveals that although APPL can be protective in several genetically induced conditions of neurodegeneration, it is not always beneficial.

The protective effect does not correlate with changes in the processing pattern of APPL

We previously showed that *loe* interferes with APPL processing because the levels of full-length APPL protein were increased in *loe*

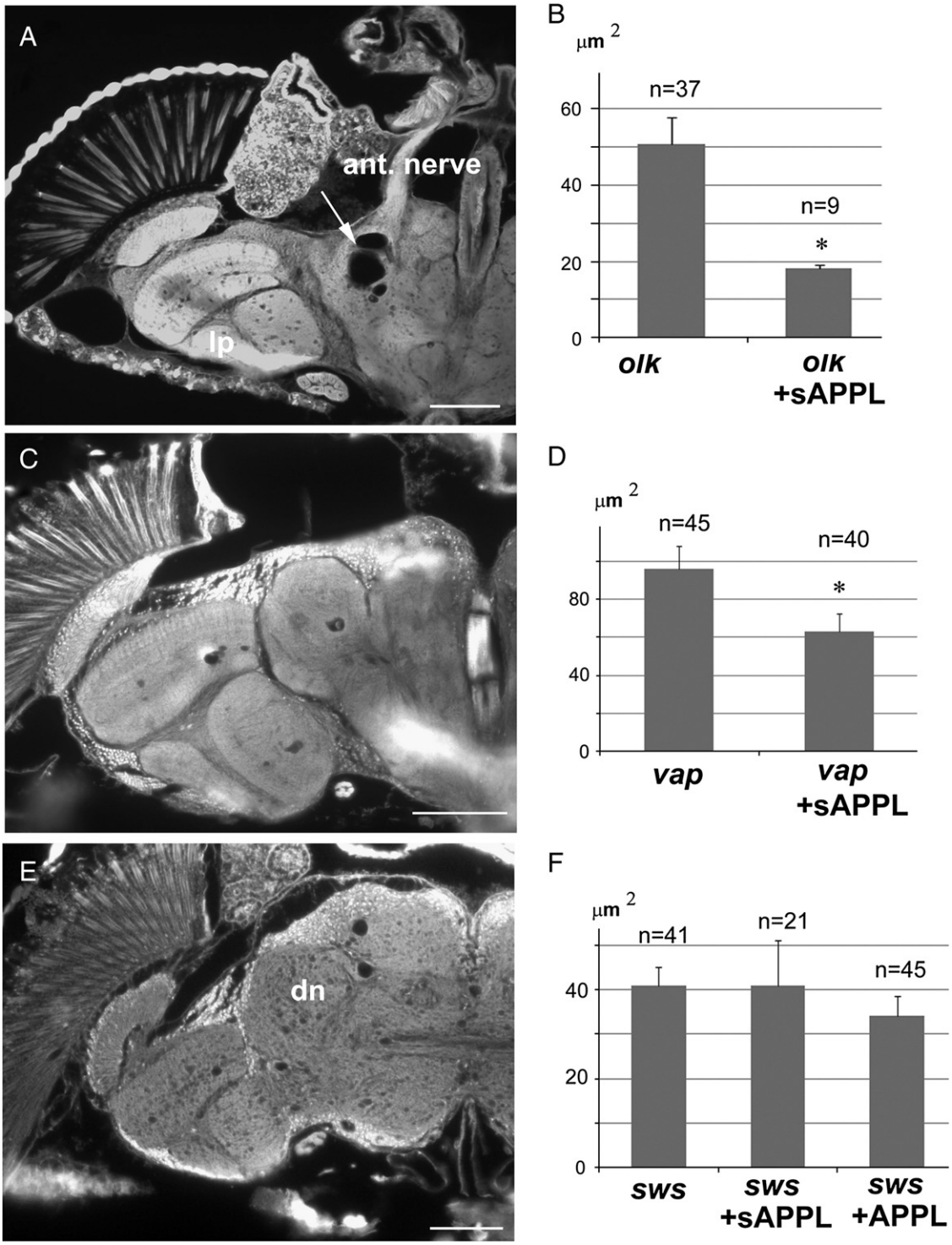

Fig. 4. APPL is protective in several neurodegenerative mutants. (A) A four week old *futsch*^{olk1} fly shows the characteristic vacuolization in the mechanosensory area of the antennal lobe. (B) Mean area of vacuoles in µm^2 in 4 week old *futsch*^{olk1} flies with or without sAPPL expression. (C) *vap*^{1} mutants show vacuoles scattered throughout the brain after two weeks of aging. (D) Also in this mutant the mean area of vacuoles is significantly reduced by expression of the secreted APPL ectodomain. (E) A section from a 14 d old *sws*^{1} fly reveals vacuoles in all brain areas. (F) In contrast to *vap*^{1} and *futsch*^{olk1}, this phenotype is not significantly altered after induction of sAPPL or full-length APPL. All flies were males. *<0.05, **<0.01and the bars indicate SEMs. Scale bar in A, C, E = 50 µm. ant. nerve = antennal nerve, dn = deutocerebral neuropil.

mutant flies, while the levels of soluble ectodomains were decreased (Tschape et al., 2002). In contrast, flies expressing additional LOE in neurons showed reduced amounts of the full-length protein and an increase in the soluble fragments (we could not differentiate between sAPPPLα and sAPPLβ in these experiments). We therefore tested whether mutants that benefited from the expression of additional APPL act through a common mechanism by interfering with the production of the protective α-cleaved ectodomain. To address this, we analyzed the levels of C-terminal fragments (CTFs) of APPL using an antiserum against the AICD. In head lysates from control flies, we could readily detect the full-length protein (Fig. 5A, lane 1, arrow) and the α-cleaved CTF of 14.5kD (arrowhead), which are both missing in flies lacking APPL (*Appl*^{d}, lane 4). In agreement with our previous results, we found reduced levels of the α-CTF in head lysates from *loe* mutant flies (lane 2,

arrowhead) whereas flies expressing additional LOE showed increased amounts of the α-CTF (lane 3). However, we did not detect a reduction in the levels of the α-CTF in head lysates from *vap* or *futsch*^{olk} mutant flies (Fig. 5B) suggesting that these mutants do not interfere with the production of α-cleaved soluble fragments. In addition, there was no detectable change in the amounts of full-length APPL indicating that theses mutants do also not affect its expression or degradation.

Flies lacking APPL show a neurodegenerative phenotype and reduced survival

The experiments described above clearly demonstrated that APPL ectodomains can be neuroprotective when flies are challenged by mutations. To determine whether we can also detect a protective function of APPL in an otherwise wild type background, we performed head sections from *Appl*^{d} mutant flies. Whereas we could not detect signs of degeneration in these flies when they were relatively young (1 d, 5 d, or 14 d, data not shown), we did detect some vacuoles scattered throughout the brain in 4 week old flies (Fig. 6B). Although this is a relatively weak phenotype it occurred with 100% penetrance and none of the age-matched control flies showed vacuolization. In addition, we found that the lack of APPL significantly reduced life span; whereas wild type females lived up to a maximum of 92 d, female *Appl*^{d} flies survived only up to 54 d (Fig. 6C). Males showed an even shorter life span, with a maximum of 40 d compared to wild type with 102 d (Fig. 6D), whereby a significant difference in survival was already detectable in young flies. After 8 d of adulthood already 7% of the *Appl*^{d} flies (males and females) were dead whereas only 1% of the wild type females and 2% of the wild type males had died ($p \ll 0.001$). This suggests that the lack of APPL reduces the life span by increasing the likelihood to die at any given age and not by causing death at a certain age. The lethality can be rescued by pan-neuronal expression of APPL (Supplementary Fig. 5) confirming that it is due to the lack of APPL.

Discussion

Vertebrate APP ectodomains have been connected with a variety of functions, including substrate binding, cell adhesion, and neurotrophic effects (Mattson et al., 1993; Mucke et al., 1996; Turner et al., 2003; Copanaki et al., 2010). Concerning the latter, several cell culture studies have suggested that the beneficial function is only mediated by the α-cleaved form, whereas the β-cleaved sAPP can even be deleterious and trigger neuronal death (Li et al., 1997; Nikolaev et al., 2009; Copanaki et al., 2010). The structure and processing of the *Drosophila* APPL protein is similar to the vertebrate protein, also resulting in the production of secreted ectodomains; either produced by an α-secretase or a β-secretase (Jacobsen and Iverfeldt, 2009; Iijima-Ando and Iijima, 2010). In this study, we show that both APP and APPL can protect against progressive neurodegeneration of the adult central nervous system in several fly models and that the ectodomain is sufficient for this function. Our experiments also support the previous observation in cell culture that the protective function is specific to the α-cleaved ectodomain. Expressing KUZ, an orthologue of vertebrate ADAM10 (Sapir et al., 2005) which we previously showed to increase α-processing of APPL (Carmine-Simmen et al., 2009), ameliorates the degenerative phenotype, whereas additional expression of dBACE, the fly BACE-like enzyme (Carmine-Simmen et al., 2009), enhances the degenerative phenotype. This enhancing effect indicates that the β-cleaved ectodomains are not only lacking the protective capabilities but can actually promote cell death. That the β-cleaved ectodomains have deleterious effects on neuronal survival is consistent with the recent findings in vertebrate neurons showing that β-cleaved fragments of APP induce axonal degeneration and cell death by activating death receptor 6 after trophic factor deprivation (Nikolaev et al., 2009). In contrast, it has been shown that mice overexpressing ADAM-10 display an increased number of synaptic boutons and that infusion of mice with sAPPα had a synaptotrophic effect (Bell et al., 2008).

We also show that the soluble ectodomain of APPL has to be secreted to fulfill the neuroprotective function, because a secretion-deficient

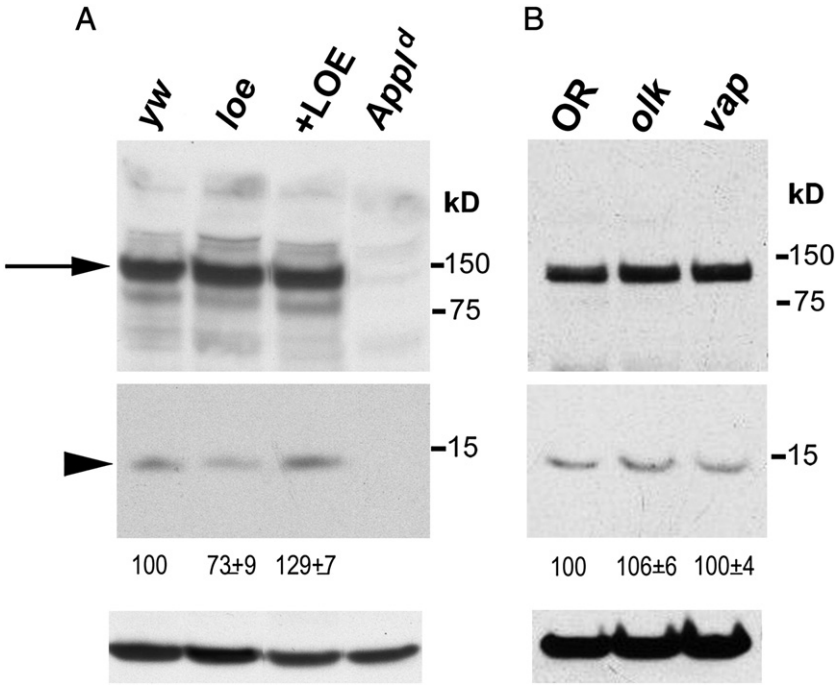

Fig. 5. LOE interferes with APPL processing whereas *vap* or *futsch*^{olk} do not. (A) A Western Blot using anti-APPL-AICD reveals a decrease in the α-cleaved CTF (arrowhead) in *loe* mutants compared to *y w* (the genetic background of *loe*) whereas the production of this fragment is increased after additional LOE expression. Full-length APPL is indicated by the arrow. (B) In contrast, neither *vap*^{1} nor *futsch*^{olk1} seem to interfere with the production of the α-CTF or the levels of full-length APPL when compared to their appropriate background Oregon R (OR). A quantification of the α-cleaved APPL CTF (±SEM) obtained from at least three independent Western blots, is shown underneath the blot. Loading controls using anti-actin are shown below.

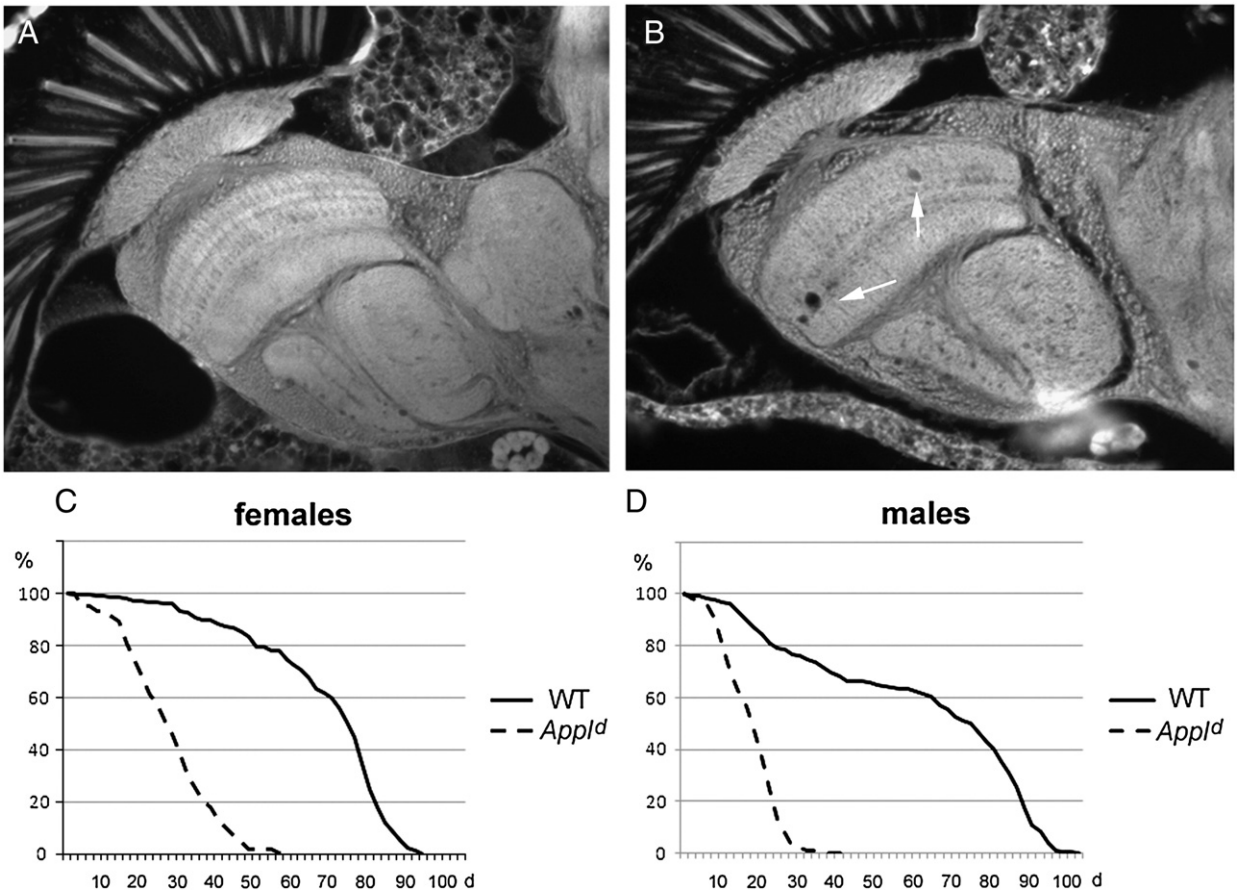

Fig. 6. Flies lacking APPL show degeneration and reduced survival. (A) A four week old female wild type fly does not show obvious signs of neurodegeneration whereas a few vacuoles have formed in the CNS of an age-matched *Appl*^{d} female (B, arrows). (C, D) Measuring the survival rate of *Appl*^{d} flies revealed a dramatically reduced life span to 59% of the life span of wild type in females and 39% in male flies. At least 10 independent experiments were performed for each genotype and gender.

form of APPL was not protective but aggrevated the degeneration. This suggests that the secreted N-terminus acts as a soluble ligand and not by cell–cell contact in the context of the membrane-bound full-length protein. Therefore, the observed enhancement of the degenerative phenotype after expression of the secretion-deficient form of APPL could be due to a competition of this form with the secreted ectodomain for a receptor.

Our co-immunoprecipitation experiments suggest that this receptor is full-length APPL.

Indeed APPL and APP have the structural features reminiscent to membrane spanning receptors. Both contain domains in their AICD that have been shown to interact with various binding partners, thereby affecting various cellular pathways ranging from regulating cytoskelatal dynamics to apoptosis (Okamoto et al., 1995; Torroja et al., 1999; Chang and Suh, 2010). It has also been postulated that APP_{695} could act like the Notch receptor, a type 1 glycoprotein receptor that is also a substrate of secretase processing (Kadesch, 2000). In the case of Notch, γ-secretase processing is induced by interactions with extracellular ligands, resulting in the release of an intracellular fragment (NICD) that then translocates to the nucleus and acts as a transcriptional regulator. A transcriptional function of the AICD is supported by the observation that AICDs can be transported to the nucleus, although only under certain conditions (Kimberly et al., 2001; von Arnim et al., 2005). Although we propose a model in which the secreted α-cleaved N-terminus acts as a ligand for the full-length APPL, our results indicate that the release of the dAICD is at least not sufficient for promoting survival. This is based on the observation that the expression of the dAICD (or the human, AICD, data not shown) does not seem to have a neuroprotective effect, suggesting that the dAICD interacts with other partners in the context of the full-length protein, thereby activating downstream neuroprotective effectors. However, although we have shown by immunohistochemistry that the dAICD construct is expressed and can be detected in the nucleus (Supplementary Fig. 2), it could still be to weakly expressed to have an effect. The importance of full-length APPL for the protective function is further supported by the result that sAPPL expression is not protective in mutants lacking endogenous APPL whereas it is protective in the wild type background, although these results do not provide evidence for a direct role of full-length APPL as the receptor. As our co-immunoprecipitation experiments reveal, the sAPP constructs cannot or only weakly bind APPL which is probably the reason why the failed to be protective. Nevertheless, the protective function is conserved because full-length APP_{695} is quite effective, presumably because it provides the sAPPα as well as the full-length APP receptor which would then interact with the appropriate downstream factors through the highly conserved AICD.

Interestingly, although APPL is protective in three of our neurodegenerative mutants it is not beneficial in the fourth one tested, the *sws* mutant. Therefore APPL could either specifically interact with the three genes affected in these mutants or it may be protective against certain kinds of damages. We think the former is unlikely for several reasons; these mutations affect different genetic pathways and the lack of APPL is also detrimental in the wild type background, in which genetic interaction between mutants should not play a role. Based on our observation in the *loe* mutant, which does affect processing of APPL, we also tested whether these mutants might share a common mechanism by interfering with the processing or production of APPL. However, neither *vap* nor *olk* seemed to affect the processing or the levels of APPL

So can all these genes be connected with a specific cellular function? FUTSCH is the fly orthologue of MAP1b (Hummel et al., 2000), which plays a role in regulating microtubule stability and dynamics (Gordon-Weeks and Fischer, 2000; Riederer, 2007). In addition, it has been shown that MAP1b can coordinate actin remodeling, either directly or indirectly by cross-talk between the microtubule and actin network (Bouquet et al., 2007; Montenegro-Venegas et al., 2010). *loe* is caused by a mutation in the regulatory subunit of the AMPK complex (Tschape et al., 2002) and AMPK has also been connected with cytoskeleton dynamics by regulating microtubule polymerization (Nakano et al., 2010) and actin reorganization (Miranda et al., 2010). However, it should be mentioned that AMPK is involved in a plethora of pathways, including apoptosis, cell growth and polarity, as well as lipid and carbohydrate metabolism (Kemp et al., 1999; Steinberg and Kemp, 2009). *vap* encodes a rasGAP protein, which is part of the EGF signaling pathway (Botella et al., 2003), and EGF has been shown to induce actin reorganization via effects on ras and Rho (Boonstra et al., 1995; Chang et al., 1995; Sharma, 1998). In contrast, the SWS protein has phospholipase activity and interferes with the phospholipid content of cells (Muhlig-Versen et al., 2005) and has not been connected to the cytoskeleton. Interestingly, it has been suggested that APP and specifically the AICD could have effects on the actin cytoskeleton (Muller et al., 2008) and pathological changes in the cytoskeleton, including changes in actin, have been observed in Alzheimer's Disease and other neurodegenerative diseases (Bamburg and Bloom, 2009; Bamburg et al., 2010). Hopefully, future experiments will identify the signaling pathways involved in the protective function of APP and whether effects on cytoskeletal dynamics play a role in this function.

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