

The neurodegeneration mutant *löchrig* interferes with cholesterol homeostasis and *Appl* processing

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The novel *Drosophila* mutant *löchrig* (*loe*) shows progressive neurodegeneration and neuronal cell death, in addition to a low level of cholesterol ester. *loe* affects a specific isoform of the γ -subunit of AMP-activated protein kinase (AMPK), a negative regulator of hydroxymethylglutaryl (HMG)-CoA reductase and cholesterol synthesis in vertebrates. Although *Drosophila* cannot synthesize cholesterol *de novo*, the regulatory role of fly AMPK on HMG-CoA reductase is conserved. The *loe* phenotype is modified by the level of HMG-CoA reductase and suppressed by the inhibition of this enzyme by statin, which has been used for the treatment of Alzheimer patients. In addition, the degenerative phenotype of *loe* is enhanced by a mutation in amyloid precursor protein-like (APPL), the fly homolog of the human amyloid precursor protein involved in Alzheimer's disease. Western analysis revealed that the *loe* mutation reduces APPL processing, whereas overexpression of *Loe* increases it. These results describe a novel function of AMPK in neurodegeneration and APPL/APP processing which could be mediated through HMG-CoA reductase and cholesterol ester.

Keywords: amyloid precursor protein-like/cholesterol/*Drosophila*/neurodegeneration

Introduction

Cholesterol metabolism has been investigated for a long time in peripheral cells, yet relatively little is known about it in brain cells. This is all the more surprising as the brain is the organ richest in cholesterol (Dietschy and Turley, 2001). Most cells in the body take up the required amount of cholesterol via the LDL or VLDL (low- and very low-density lipoprotein) receptor pathway (Fisher *et al.*, 1999; Simons *et al.*, 2001). After uptake, the lipoproteins are degraded and the cholesterol released within the cell where it can be either used as free cholesterol or stored in the form of cholesterol ester (Poirier, 1994; Weisgraber and Mahley, 1996). This transport mechanism is highly

conserved in vertebrates and invertebrates (Fisher *et al.*, 1999). In addition, vertebrate cells can produce cholesterol by *de novo* synthesis in the endoplasmic reticulum (Simons *et al.*, 2001). Due to the blood–brain barrier, brain cells are unable to receive their supply of lipoproteins from the plasma and it has been suggested that only very little is supplied by uptake (Kabara, 1973). At least oligodendrocytes seem to meet their demand for cholesterol by *de novo* synthesis (Morell and Jurevics, 1996). Nevertheless, the cerebrospinal fluid contains special lipoproteins, the apolipoproteins apoE and apoAI (Roheim *et al.*, 1979; Ladu *et al.*, 2000), and most probably these brain lipoproteins are not involved in the transport of cholesterol to and from the brain but in the redistribution of cholesterol within the brain (Mahley, 1988).

Cholesterol regulates the physical properties of the cell membrane, and its level is therefore tightly controlled. Recent work has shown that cholesterol plays a role in membrane compartmentalization and in the formation of lipid rafts (Simons and Ikonen, 1997). This important function might be the reason for the connection between cholesterol and neurodegeneration. Studies have shown that the cholesterol level influences the production of the pathogenic A β peptide, which is produced from the amyloid precursor protein (APP) by cleavage through β - and γ -secretase (Refolo *et al.*, 2000; DeStrooper and Annaert, 2000). It has been suggested that A β processing occurs within rafts, whereas the non-amyloidogenic α -processing occurs outside (Lee *et al.*, 1998; Simons *et al.*, 1998; Kojro *et al.*, 2001). Cholesterol synthesis in neurons is regulated by hydroxymethylglutaryl-CoA (HMG-CoA) reductase, which again has been connected to Alzheimer's disease. Inhibition of this enzyme by statins not only reduces cholesterol synthesis but also inhibits β -secretase cleavage of APP (Frears *et al.*, 1999). In addition, clinical studies indicate that patients treated with statins have a decreased prevalence of Alzheimer's disease (Wolozin *et al.*, 2000). HMG-CoA reductase activity is negatively regulated via phosphorylation through the AMP-activated protein kinase (AMPK), a heterotrimeric complex, consisting of the catalytic α -subunit and β - and γ -subunits, found in all eukaryotes (Hardie *et al.*, 1998; Kemp *et al.*, 1999).

Here we show that the *Drosophila* mutant *löchrig* (*loe*) disrupts a specific isoform of the AMPK γ -subunit, which leads to a low level of cholesterol ester together with a strong neurodegenerative phenotype. *loe* interacts genetically with HMG-CoA reductase and influences processing of the β -amyloid protein precursor-like (APPL) gene. Although the regulation and most downstream targets of HMG-CoA reductase are conserved, this enzyme is not involved in cholesterol synthesis in insects, because they cannot synthesize cholesterol *de novo* (Gertler *et al.*,

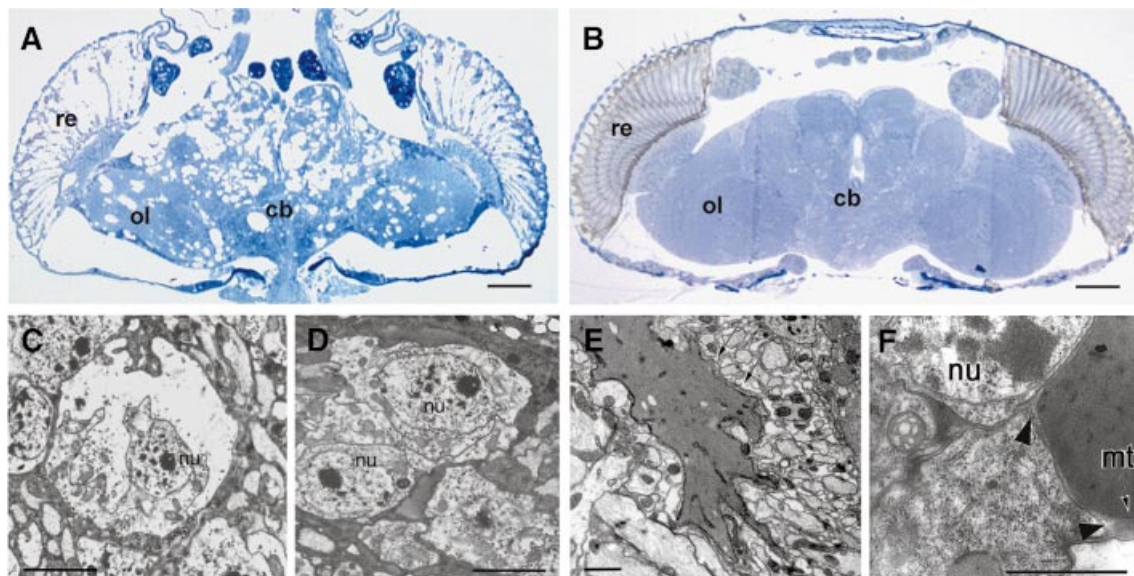


Fig. 1. Necrotic cell death, accumulation of fatty acids and neurodegeneration in *loe*. (A and B) Horizontal plastic sections of the brain stained with toluidine blue. (A) A 10-day-old *loe* fly shows massive degeneration compared with wild-type (B). (C–F) Electron microscopy on adult heads. (C) Dying neurons, in this case a monopolar cell of the optic system, in 7-day-old *loe* flies show the characteristic swelling and lysis of necrotic cell death, while the nucleus (nu) remains intact. (D) Wild-type monopolar cells. (E) EM brain sections from 7-day-old *loe* flies reveal fatty inclusions (arrows). (F) The unsaturated fatty acids seem to originate from within the cell because they are surrounded by residual cell cytoplasm (arrowheads), including a mitochondrion (mt). re, retina; cb, central part of the brain; ol, optic lobes. Bars: (A) and (B) 50 μ m; (C–F) 2 μ m.

1988). Our mutant now shows that HMG-CoA reductase and its regulator AMPK are also involved in neurodegeneration in insects. The low level of cholesterol ester suggests that the mediator could be cholesterol ester rather than cholesterol, which might be important in the context of Alzheimer's disease because the level of cholesterol ester has been directly correlated with A β production in cell culture experiments (Puglielli *et al.*, 2001).

Results

Progressive degeneration and necrosis of neurons in *loe*

loe was isolated from a collection of P-element insertion lines from Deak *et al.* (1997). About 800 lines which have a shortened adult life span were aged and screened histologically for signs of neurodegeneration. Two of these lines showed severe vacuolization of the central nervous system (CNS) which increased with aging, and one of them was named *löchrig* (the German word for full of holes). The vacuolar pathology is most prominent around the central complex and in the central parts of the brain, while the optic lobes are less affected (Figure 1A). Previous developmental studies suggested that the vacuolization and degeneration in *loe* are confined to differentiated, probably synaptically active neurons, whereas neuroblasts and developing neurons are unaffected (Tschäpe *et al.*, 2002).

To assess whether dying cells undergo apoptotic or necrotic cell death, we performed TUNEL stainings (Gavrieli *et al.*, 1992) and electron microscopic (EM) studies. The observed swelling and lysis of cell bodies, while the nucleus stays intact, are characteristic features of necrotic cell death (Figure 1C), which is supported by the negative TUNEL staining on head cryosections (data not

shown). The EM sections confirmed that the dying cells are neurons because glial cells appeared morphologically normal.

In addition, the electron microscopic analysis revealed the accumulation of a substance (Figure 1E and F), which presumably is unsaturated fatty acids due to the stabilization by osmium in the fixative (Ruthmann, 1966). The accumulations are sometimes still embedded in the cell cytoplasm (Figure 1F).

***loe* encodes a subunit of the AMP-dependent protein kinase complex**

To verify that the mutation is caused by the insertion of the P-element, we remobilized the P-element (O'Kane, 1998) to restore the wild-type phenotype. We established two lines which showed a reversion of the vacuolization phenotype in paraffin head sections (data not shown) and a precise excision of the P-element. These confirm the mutagenic effect of the P-element, which consequently was used to isolate neighboring genes via plasmid rescue (O'Kane, 1998).

We isolated ~20 kb of genomic DNA adjacent to the P-element insertion site. Within this region, we found homology to a cDNA fragment from the Berkley Sequencing Project and to genomic clones from the *Drosophila* Genome Project. Various other cDNAs were isolated by their homology to either of these clones. Their further characterization revealed that they represent at least six alternatively spliced transcripts for the *Drosophila* γ -subunit of AMPK (Figure 2A). The different mRNAs encode at least three different protein isoforms, all sharing the same C-terminus while varying in their N-terminal part. The C-terminus includes the so-called CBS (cystathionine- β -synthase) domains which are highly conserved between yeast, mammals (Hardie *et al.*, 1998)

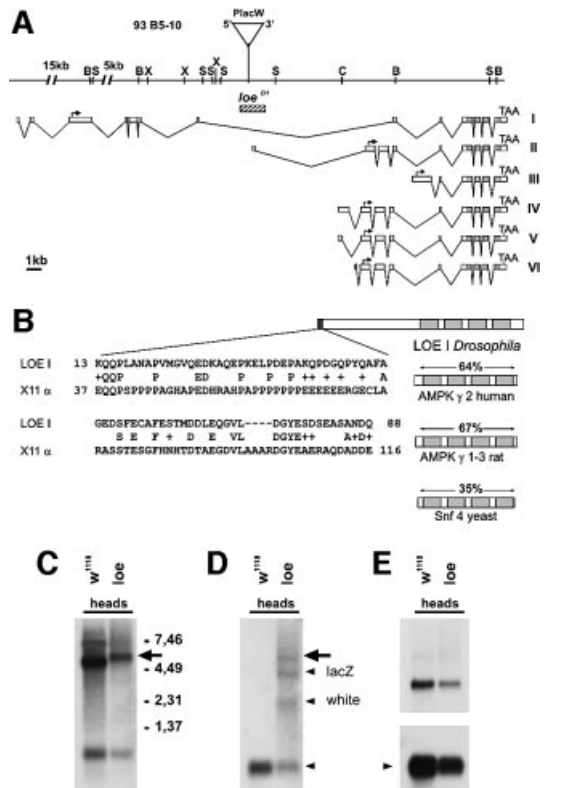


Fig. 2. Analysis of the *loe* gene. (A) Genomic region adjacent to the P-element (PlacW). The exon–intron structures of the LoeI to LoeVI transcripts are shown underneath. Start codons are indicated by arrows. The deletion *loe^{D1}* is indicated by a striped bar. B, *Bam*HI; S, *Sst*I; X, *Xba*I; C, *Cla*I. (B) The homology to other AMPK γ -subunits is restricted to the C-terminus (only LoeI shown), including the cystathionine- β -synthase domains (light gray). The identity is given above. The N-terminal fragment of LoeI (dark gray) shows homology to the rat X11 α protein. (C–E) mRNA expression of *loe*. (C) Three transcripts are detected with a probe derived from exons 1–3 of LoeI in the heads of *w¹¹¹⁸* flies. Analysis of these transcripts reveals a larger fusion transcript for the strongly expressed 4.7 kb form (arrow in C) in the *loe* mutant, which is also recognized by a P-element-specific probe (D) (lacZ and white are transcripts encoded by the P-element, arrowheads). (E) The expression of LoeII is unaltered. *w¹¹¹⁸* was used as control because this line provides the same genetic background as the mutant. rp49 was used as loading control.

and *Drosophila* (Figure 2B). Interestingly, a region in the unique N-terminus of the LoeI isoform shows homology to the X11 α protein which can bind to the APP protein (Borg *et al.*, 1998); LoeI and X11 α are 28% identical and 41% similar over a stretch of 80 amino acids (Figure 2B). The P-element is inserted in the seventh intron of this transcript and 38 bp upstream of the transcription start site of LoeII (Figure 2A), suggesting that one or two transcripts are affected by the insertion (all other transcripts are >10 kb downstream of the insertion site and therefore most probably are not affected by the P-element). We created a small deletion of 1.3 kb around the insertion site, removing exon 1 of the LoeII transcript (Figure 2A, *loe^{D1}*), and these flies do not show a degeneration phenotype. This indicates that LoeII is not required for CNS integrity.

The mutation is due to an aberrant LoeI transcript

Northern blot analysis of adult head mRNA fractions further supported the conclusion that the mutation is due to

an effect on the LoeI transcript. A probe comprised of exons 1–3 from LoeI detected three transcripts (Figure 2C), with a size of 7.6, 4.7 and 0.7 kb in *w¹¹¹⁸* flies. Comparing transcripts in head homogenates from *w¹¹¹⁸* and *loe* mutant flies revealed a change of the 4.7 kb LoeI transcript only, increasing it in size to ~5.5 kb in the mutant (Figure 2C, arrow). The hybridization of this aberrant transcript with a P-element-specific probe proves that it is due to splicing parts of the P-element to the LoeI transcript (Figure 2D). As expected, other transcripts, including LoeII, are unaltered in mutant flies (Figure 2E).

To confirm the specific role of LoeI, we expressed the LoeI and LoeII cDNAs in different cell types using the UAS/Gal4 system (Brand and Perrimon, 1993). Lines carrying P-element vectors with either the LoeI or LoeII cDNA under the control of the Gal4-binding sequence (UAS) were crossed with various Gal4 lines to induce expression of Loe in different cell types. A rescue of the *loe* phenotype could only be achieved by using the neuron-specific elav-Gal4 line (Luo *et al.*, 1994) in combination with UAS-LoeI (Figure 3D). Expression in glia using loco-Gal4 did not rescue the phenotype (data not shown) nor did expression of LoeII in neurons (Figure 3C). This finally proves that the mutation is caused by a disruption of the LoeI transcript. In addition, these experiments reveal a requirement for this transcript in neurons, because glial expression cannot rescue the phenotype.

The unique N-terminus of LoeI is required for wild-type function

To investigate the function of the specific N-terminal region of LoeI, we created N-terminally truncated LoeI transgenes. Expressing a LoeI transgene deleting amino acids 1–738, leaving the conserved C-terminus intact, in neurons, could only partially improve the degeneration phenotype (Figure 3E). This indicates the importance of the unique N-terminus for the function of the LoeI protein. A construct deleting amino acids 1–319, removing the domain similar to X11 α (amino acids 13–88), could rescue more efficiently. We could, however, still detect some holes (Figure 3F). The X11 α similarity domain is therefore required for wild-type function, but other functionally important domains must reside within the N-terminus of LoeI because longer deletions result in a more incomplete rescue.

Loe is involved in cholesterol homeostasis

To assess whether the *loe* mutation influences cholesterol metabolism, a role described for AMPK (Kemp *et al.*, 1999; Figure 4C), we measured the lipid composition of fly heads. The analysis of phospholipids, triglycerides and free cholesterol (Figure 4A) did not reveal any significant differences between 1- to 5-day-old wild-type and mutant flies. The amount of cholesterol ester, however, was reduced by ~40% (Figure 4B). Expressing LoeI in neurons restored the wild-type level of cholesterol ester in the mutant (Figure 4B), confirming the role of Loe/AMPK in cholesterol homeostasis. The expression of LoeI restores the cholesterol ester level as well as the neurodegenerative phenotype, directly connecting cholesterol ester and neurodegeneration in the *loe* mutant. These results reveal an involvement of AMPK in cholesterol ester levels in the brain independent of *de novo* cholesterol synthesis. In

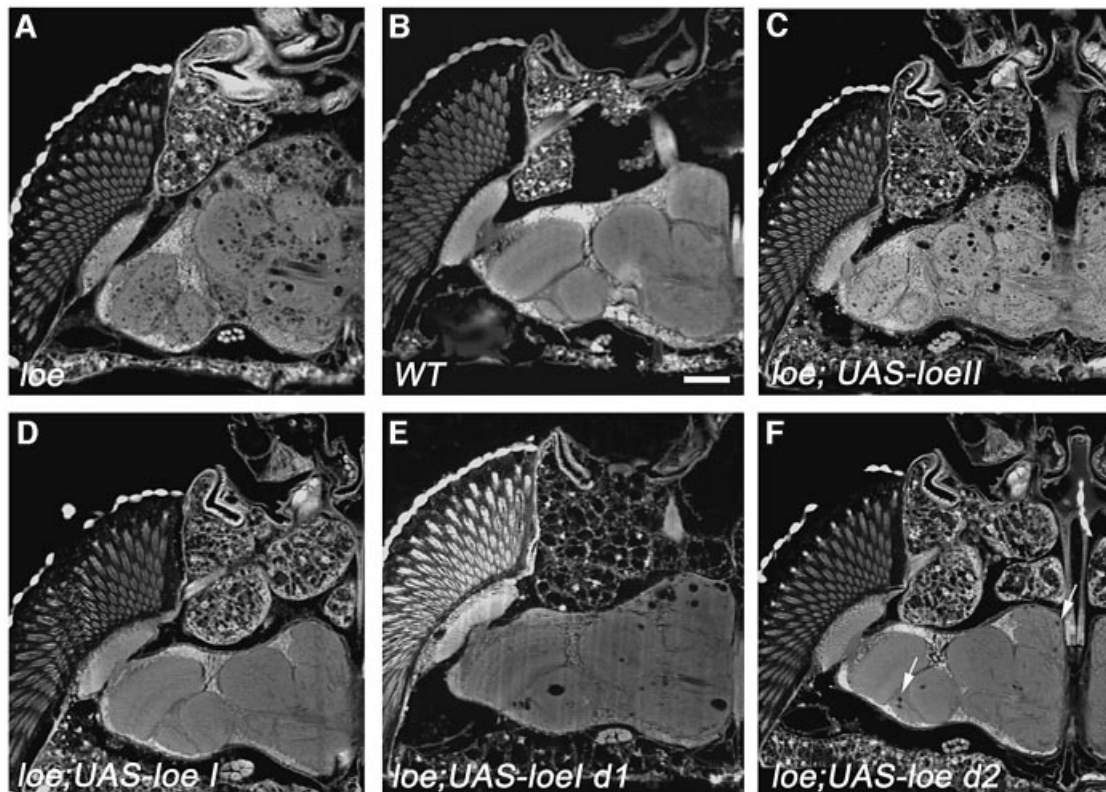


Fig. 3. LoeI expression rescues the phenotype. Paraffin sections from 14-day-old flies reveal the characteristic *loe* phenotype. (A) A control *loe* fly carrying only the UAS-LoeI construct without the Gal4 driver construct. (B) Wild-type. (C) Expressing LoeII in neurons with elav-Gal4 does not rescue the phenotype. (D) In contrast, expression of LoeI in neurons completely restores the wild-type phenotype in *loe*. (E) A construct deleting the first 738 amino acids of LoeI, while leaving the conserved C-terminus intact, shows only partial rescue ability. (F) The construct without the domain similar to X11 α (deleting amino acids 1–319) reveals a better but still incomplete rescue, because some holes are forming (arrows). Bar: 50 μ m.

peripheral tissues, vertebrate AMPK inhibits the activation of a hormone-sensitive lipase, an enzyme involved in the breakdown of cholesterol ester (Garton *et al.*, 1989). A conserved regulatory pathway in the brain could account for the decreased amount of cholesterol ester.

A functional homology to the mammalian AMPK is supported further by the accumulation of fatty acids in the mutant, another pathway regulated by AMPK (Figure 4C).

***loe* interacts with *columbus*, the fly homolog of HMG-CoA reductase**

AMPK negatively regulates HMG-CoA reductase, a key enzyme in cholesterol synthesis in vertebrates. In *Drosophila*, this protein is encoded by the *columbus* (*clb*) gene (Van Doren *et al.*, 1998). To assess whether *loe* interacts with the *clb* mutation, we created flies homozygous for *loe* and heterozygous for two strong, embryonic lethal alleles of *clb* (which both had the same effect). *clb*^{+/+}; *loe* showed a weak but significant suppression of vacuolization (Figure 5B) compared with *loe* mutant flies (Figure 5A). To confirm an interaction, we used lines expressing Clb in the *loe* background. In contrast to the *clb* mutation, Clb overexpression enhanced the phenotype (Figure 5D). Control flies, containing only the UAS-Clb construct but no neuronal promoter construct (Figure 5C), did not differ from the original *loe* mutants. The interaction was quantified by counting holes in the different genotypes (Figure 5E) and measuring their total volume (Figure 5F).

The enhancement by Clb overexpression and suppression by the *clb*^{+/+} mutant suggests that HMG-CoA reductase is negatively regulated by AMPK as in other organisms. In addition, we investigated an influence on the cholesterol ester level of *loe*. Overexpression of Clb slightly reduced, and introduction of one mutant copy of *clb* slightly increased, the cholesterol ester level in *loe* (Figure 4B); however, the differences are not significant. Nevertheless, they are in agreement with the results on the neurodegenerative phenotype because the *clb* mutation suppresses and additional Clb enhances the phenotype. Interestingly, the function of HMG-CoA reductase in cholesterol synthesis is not conserved because insects cannot synthesize cholesterol *de novo*. However, many other downstream genes and regulatory feedback mechanisms are conserved (Gertler *et al.*, 1988), and one of them might connect HMG-CoA reductase and cholesterol ester.

Treatment with statin suppresses the *loe* phenotype

As mentioned in the Introduction, HMG-CoA reductase can be inhibited pharmacologically by a class of drugs called statins, which have also been shown to decrease the prevalence of Alzheimer's disease (Wolozin *et al.*, 2000). To assess whether treatment with statins influences the neurodegeneration in *loe*, we compared flies fed on glucose with or without the drug lovastatin. Flies kept on lovastatin showed a suppression of the vacuolization

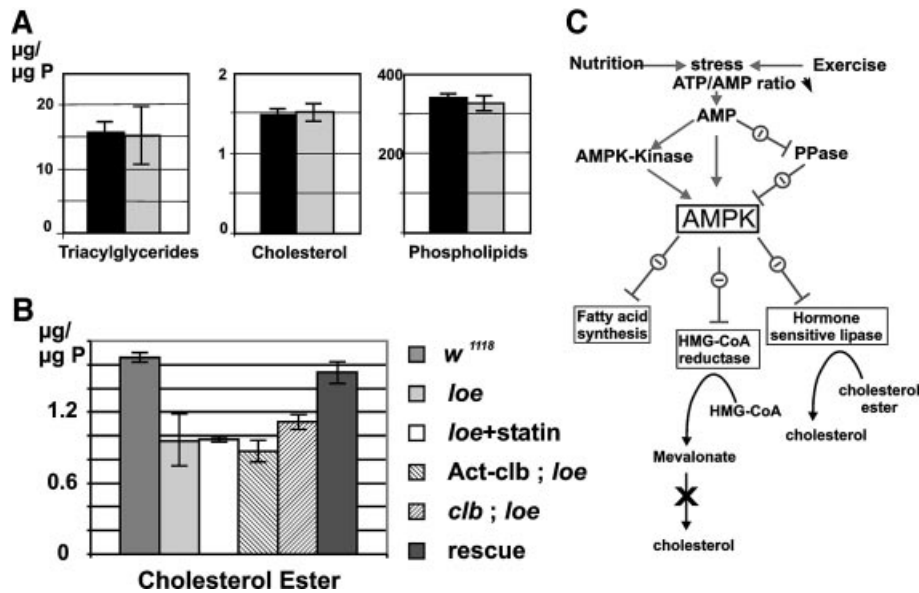


Fig. 4. Cholesterol ester is reduced in *loe*. **(A)** The amount of triglycerides, phospholipids and free cholesterol is unchanged in the *loe* mutant (black bar) compared with *w¹¹¹⁸* (the genetic background of *loe*; light gray bar). **(B)** The level of cholesterol ester is reduced to ~60% (mean value from nine measurements) in *loe*. It can be restored to wild-type levels by expressing the *Loel* transcript in neurons using elav-Gal4 (light gray bar). Expression of *Clb* (striped bar, -45°) in *loe* slightly increases and one copy of mutant *clb* in *loe* slightly lowers the amount of cholesterol ester (striped bar, 45°), although not significantly. Statin treatment (white bar) does not cause a difference in cholesterol ester. The SEM is indicated. **(C)** AMPK is involved in several regulatory pathways, including fatty acid synthesis and the breakdown of cholesterol ester. AMPK, AMPK kinase; PPase, protein phosphatase. Modified from Kemp *et al.* (1999).

(Figure 5H) compared with control animals (Figure 5G). Treatment of wild-type flies with lovastatin revealed no adverse effects (Figure 5I). These results show that the progressive neurodegeneration in *loe* can be slowed successfully by treatment with statins. We also tested the level of cholesterol ester in *loe* flies treated with statins, but could not find a significant difference (Figure 4B).

***Loe* interacts with amyloid precursor protein-like**

Cholesterol homeostasis has been implicated in the processing of A β from APP, as has statin treatment, which can dramatically decrease A β production (Fassbender *et al.*, 2001). Therefore, we investigated whether *loe* influences APPL, the fly homolog of human APP (Rosen *et al.*, 1989). *App^ld* mutants, which carry a deletion in the *App^l* gene (Torroja *et al.*, 1996), do not reveal any signs of neurodegeneration (Figure 6A). However, crossing *App^ld* with *loe* flies shows an enhancement of the *loe* vacuolization (Figure 6C and D). The effect is weaker in *loe* flies carrying one copy of *App^ld* (*loe/loe; App^ld/+*) compared with homozygous double mutants (*loe/loe; App^ld/App^ld*) and can be detected in the central brain as well as the optic system (Figure 6D).

To determine whether *loe* might influence the APPL protein, we performed western blot analysis of brain extracts. Using an anti-APPL polyclonal antibody (Torroja *et al.*, 1996), we detect two bands in *w¹¹¹⁸* flies, representing the genetic background used to induce the *loe* mutation (Figure 7A). The bands correspond to the membrane-associated 145 kDa precursor and the 130 kDa secreted form (Luo *et al.*, 1990), which are absent in *App^ld*. In the *loe* mutant, we find similar amounts of APPL precursor protein; however, the level of the processed secreted form is reduced. Conversely, we find more of the

secreted form when additional *Loel* is expressed in neurons. This reveals a role for *loe* in APPL processing or stabilization of the processed form. To assess whether this effect is specific for APPL, we investigated the processing of Notch, which is cleaved by a mechanism similar to that of APP (De Strooper and Annaert, 2000; Nowotny *et al.*, 2000). We could not detect any differences in the processing of Notch (Figure 7B), suggesting a specific function of *loe* in APPL processing, possibly mediated by the X11 α similarity domain. In addition, we investigated whether *Clb* or statin treatment influences APPL processing in *loe*. Additional expression of *Clb*, which enhanced the neurodegenerative phenotype of *loe*, also enhanced the processing effect, causing a slight further reduction of APPL processing (Figure 7D). On the contrary, one copy of mutant *clb* or statin treatment slightly increased processing. This suggests that the neurodegenerative phenotype is correlated with the processing of APPL.

Supplementary data for this paper are available at *The EMBO Journal* Online.

Discussion

In this report, we showed that a mutation in the AMPK γ -subunit causes progressive neurodegeneration in *Drosophila*. AMPK is a central component of a protein kinase cascade conserved in eukaryotes (Hardie *et al.*, 1998; Kemp *et al.*, 1999; Winder and Hardie, 1999) that acts as a metabolic sensor to monitor the cellular AMP and ATP levels. In cases of ATP depletion, its major function described so far is to activate energy-providing mechanisms while inactivating energy-consuming processes (Hardie *et al.*, 1998; Kemp *et al.*, 1999). AMPK is a

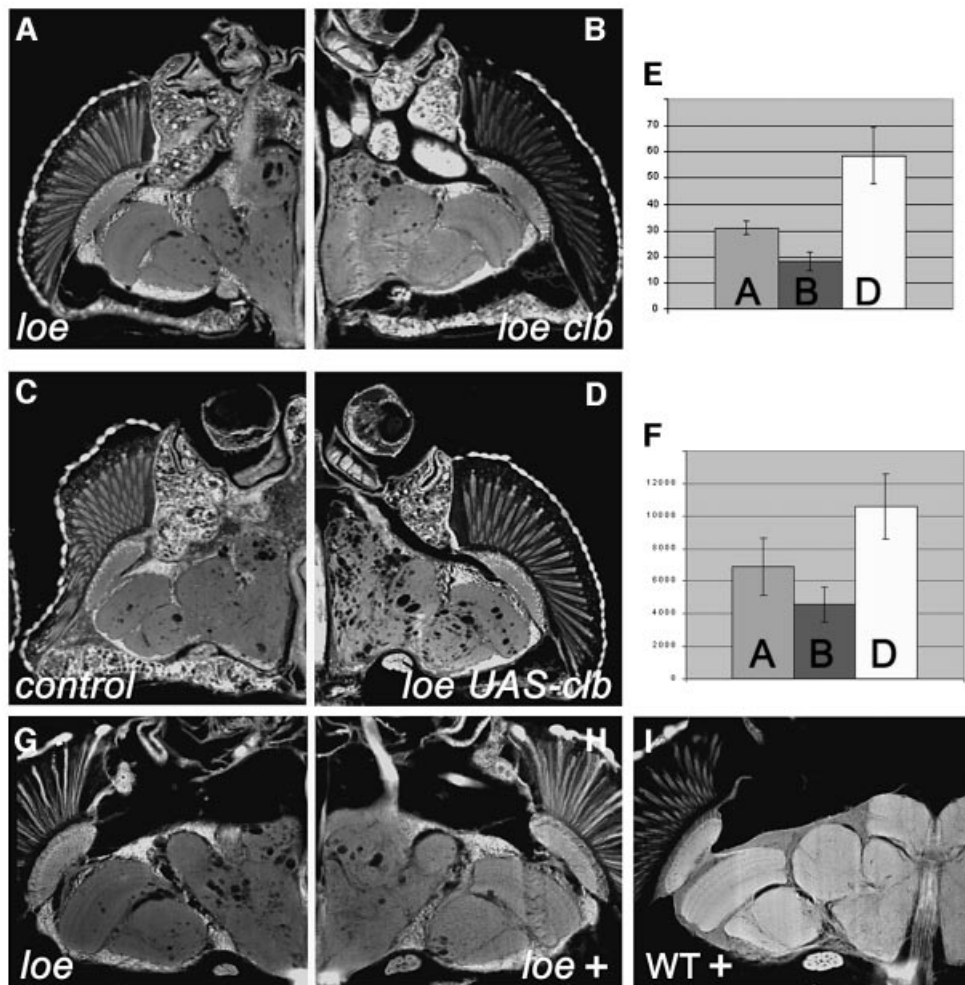


Fig. 5. Interaction of *loe* with *clb*. (A) 7-day-old *loe* flies reveal the characteristic vacuolization, which is reduced in flies additionally heterozygous for a mutation in the HMG-CoA reductase homolog *clb* (*loe clb^{11.54}/loe*, B) of the same age. (C) Control *loe* mutant flies (7 days) carrying only the UAS-Clb construct, without a promoter construct, are not distinguishable from the original *loe* strain. (D) In contrast, flies expressing additional *clb* in neurons (Appl-Gal; UAS-*clb*; *loe* 7 days) show an enhancement of the neurodegenerative phenotype. To quantify the enhancement, we counted the holes in these flies. (E) The number of holes is reduced to ~59% in *loe clb^{11.54}/loe* flies (dark gray bar) and almost doubled (1.9-fold) in flies expressing *clb* in neurons (white bar). (F) The size of the forming holes is not changed significantly because the total volume of holes (in μm^3) is reduced in *loe clb^{11.54}/loe* to 66% (dark gray bar), whereas it is increased to 150% in Appl-Gal; UAS-*clb*; *loe* flies (white bar). *loe*, light gray bar. The SD is indicated. (G) A *loe* fly kept for 8 days on glucose. (H) Adding mevinacor (1 ng/ml) to the glucose solution reduces the formation of holes. (I) Feeding mevinacor to wild-type flies shows no effect.

heterotrimer, consisting of the catalytic α -subunit and the β - and γ -subunits which are required for stabilization of the complex and kinase activity. The activity of the complex is regulated by phosphorylation through an upstream kinase, and both phosphorylation and dephosphorylation are sensitive to AMP levels (Davies *et al.*, 1995). For all three subunits, different isoforms have been identified that assemble into specific AMPK complexes with distinguishable tissue distribution in peripheral tissues in vertebrates (Stapleton *et al.*, 1996; Thornton *et al.*, 1998). Whereas most tissues predominantly express one γ isoform, the human brain expresses three different isoforms (Cheung *et al.*, 2000). Interestingly, two of them have extended N-termini with no significant homology to each other, LoelI or any other protein (Cheung *et al.*, 2000). The *loe* mutation shows, for the first time, that such a brain-specific isoform has a unique function in brain maintenance, which cannot be substituted by other

isoforms. This function probably goes beyond the basic role in energy regulation because all isoforms share the C-terminus which is sufficient for a functional γ -subunit and, therefore, a functional AMPK complex. It will be interesting to discover whether one of the human isoforms is also required specifically for neuronal survival.

AMPK has a central role in cholesterol metabolism by regulating HMG-CoA reductase and hormone-sensitive lipase, which is involved in the breakdown of cholesterol ester in vertebrates (Garton *et al.*, 1989). Although hormone-sensitive lipase has not been found in the brain, a cholesterol ester hydrolase activity is described for the brain (Gosh and Grogan, 1990); however, nothing is known about the potential regulation of this enzyme by AMPK. An inhibitory function of AMPK in the brain would lead to an overactivity of this hydrolase and, therefore, to a reduced level of cholesterol ester. A *Drosophila* protein with homology to hormone-sensitive

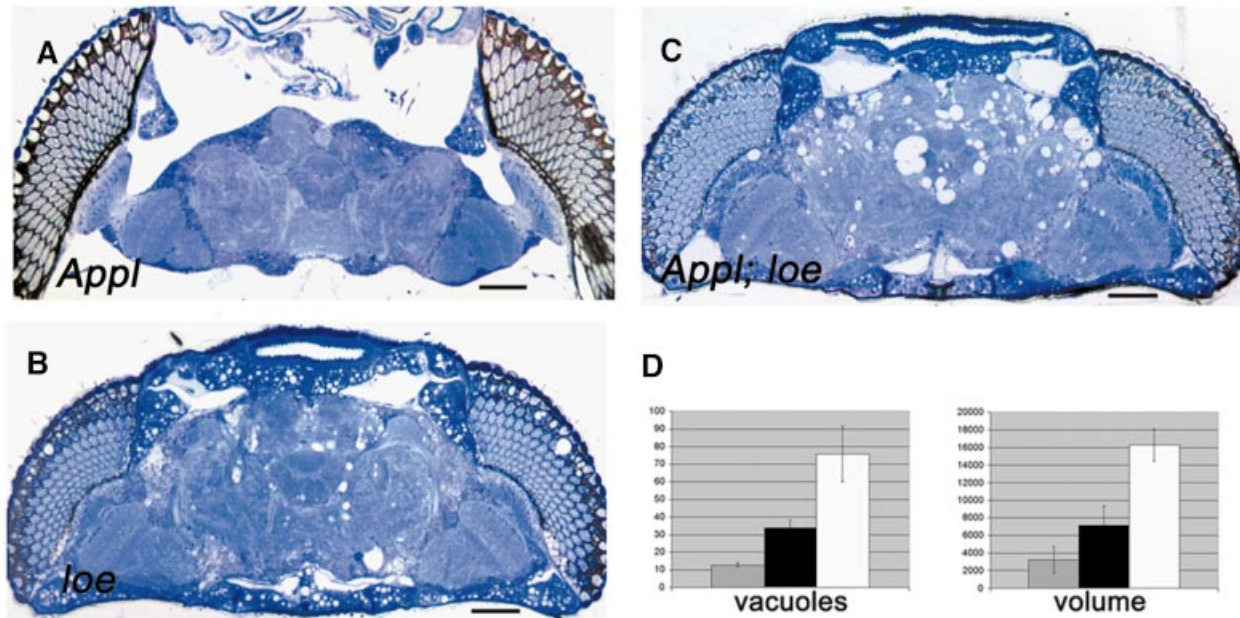


Fig. 6. Interaction between *loe* and *Appl*. (A) A 4-day-old *Appl*^Δ mutant reveals no vacuolization, whereas a *loe* fly at the same age displays many holes (B). (C) In a 4-day-old *Appl*^Δ; *loe* double mutant, this phenotype is enhanced and more and larger holes are formed. Bar: 50 μm. (D) To quantify the enhancement, we counted the holes in 7-day-old *loe* flies (gray bar) and flies which also carry, besides *loe*, a heterozygous (black bar) or homozygous (white bar) *Appl* mutation. The number of holes is approximately doubled in heterozygous *Appl*^{Δ/+} flies and >4-fold increased in homozygous *Appl*^{Δ/Δ} mutants. Whereas the number increases, the size of the forming holes does not increase significantly because the total volume of holes (in μm³) also increases by two or four, respectively. The SD is indicated.

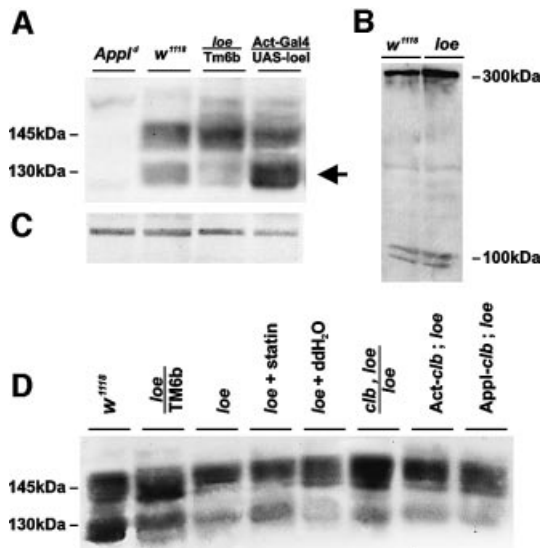


Fig. 7. Aberrant processing of APPL in *loe*. (A) The APPL antiserum reveals in western blots two APPL bands, which are missing in the *Appl*^Δ mutant. Whereas the amount of the APPL precursor protein of 145 kDa and its different post-translational modifications is similar in *loe* flies compared with the control line *w*¹¹¹⁸, the amount of the secreted form of 135 kDa and its modifications is decreased (arrow). The opposite effect can be found in flies overexpressing *loe* in neurons where the amount of the secreted form is increased. (B) Western blot using an α -Notch antibody (DSHB, University of Iowa). The full-length Notch of 300 kDa and the processed species of 100 kDa (Pan and Rubin, 1997) are detected in equal amounts in wild-type and *loe*. (C) Load control. (D) The processing in *loe* is influenced by Clb because flies carrying one mutant copy of *clb* or treated with statin have slightly more of the processed form (lane 5 is the control for lane 4 kept under the same conditions as treated flies). Additional expression of Clb using *Appl*-Gal 4 or *Act*-Gal4 seems to decrease processing compared with *loe* alone. Flies were 1 day old.

lipase can be found in the *Drosophila* Sequencing Project, but unfortunately no mutant has been described so far. However, a deficiency deleting this enzyme was tested for genetic interactions with *loe*. Because this deficiency had no influence on the *loe* phenotype (data not shown), we assume that it is not involved in the neurodegenerative phenotype. In contrast, we could show a genetic as well as a pharmacologically induced interaction of *loe* with HMG-CoA reductase (*clb*). The interaction reveals that, as in vertebrates, AMPK acts upstream of HMG-CoA reductase. Because a mutation in *clb* suppresses and overexpression enhances the neurodegenerative *loe* phenotype, the inhibitory function of AMPK on HMG-CoA reductase seems to be conserved. Interestingly, the function of HMG-CoA reductase is not completely conserved between vertebrates and insects, because arthropods cannot synthesize cholesterol *de novo*. Rather, HMG-CoA reductase is involved in the production of non-sterol isoprenoids from mevalonate (Gertler *et al.*, 1988; Duportets *et al.*, 2000). The effect of HMG-CoA reductase on neurodegeneration cannot, therefore, be mediated through cholesterol synthesis and, as our measurements show, the cholesterol level is unaltered in *loe*. However, the amount of cholesterol ester is lowered in *loe* and adding or removing Clb has a slight influence on it, and APPL processing in *loe* is influenced by Clb. In this context, it is worth mentioning that statins dramatically decrease A β production before a reduction in cholesterol can be detected (Fassbender *et al.*, 2001). This suggests that other members of the cholesterol pathway might regulate APP processing (Wolozin, 2001), possibly cholesterol ester.

The *loe* mutation reveals a connection between cholesterol ester and progressive neurodegeneration in the model

system *Drosophila*. In vertebrates, such a link has been established by the finding that accumulation of A β can decrease cholesterol esterification in neurons (Koudinova *et al.*, 1996; Liu *et al.*, 1998). Puglielli *et al.* (2001) found that the level of cholesterol ester directly correlates with A β production, and that elevated concentrations of cholesterol ester but not free cholesterol increased the generation of A β . On the other hand, it has been shown that lowering the cholesterol concentration inhibits APP cleavage by secretases and interferes with the localization of APP in membrane rafts (Simons *et al.*, 1998; Frears *et al.*, 1999). These are membrane microdomains consisting of lipids, proteins and cholesterol, and their correct composition seems to be required for normal APP processing (DeStrooper and Annaert, 2000; Drouet *et al.*, 2000). Our results strengthen the likelihood of a role for cholesterol ester because the *loe* mutant links a reduced level of cholesterol ester, leaving free cholesterol unaltered, with decreased processing of APPL.

Our results clearly reveal a function of AMPK in APPL processing. On the other hand, the *Appl* mutant enhances the neurodegenerative phenotype of *loe*. Like knock-outs of APP in mice, the *Appl^d* null mutation displays only subtle neurological deficits (Luo *et al.*, 1992; Müller *et al.*, 1994; Zheng *et al.*, 1995). In the *loe* mutant background, however, *Appl* can be connected to progressive neurodegeneration, which might help to understand the function of APP proteins. Because the lack of APPL enhances the phenotype, this hints at a neuroprotective function, perhaps specifically of the soluble form, of APPL which was also suggested by cell culture studies of APP (Perez *et al.*, 1997). In our model, neurons would be more vulnerable to the effect of the *loe* mutation when APPL and its soluble form are missing. The *Appl* mutant itself might not show degeneration because the damaging event is absent.

With the isolation of the *loe* mutant, we have connected AMPK, a second enzyme besides HMG-CoA reductase involved in cholesterol homeostasis, to neurodegeneration and APPL processing. This underlines the importance of the cholesterol biosynthesis pathway for the maintenance of the nervous system and for understanding of neurodegenerative diseases such as Alzheimer's. With the *Drosophila loe* mutant available, we can now study the role of this pathway in neurodegeneration in an easily accessible model organism.

Materials and methods

Drosophila stocks

All stocks were maintained and raised under standard conditions. Canton S wild-type and *w¹¹¹⁸* were used as control stocks. The *loco-Gal4* line was kindly provided by C. Klämbt, and the APPL-Gal4 line by L. Torroya. Act-Gal4 and *elav-Gal4* were provided by the Bloomington stock center, and the P-element lines are from the Szeged stock center. The *Appl^d* mutant was kindly provided by K. White. The *clb^{11.54}* and *clb^{26.31}* alleles (both strand UAS-Clb carrying stocks) were kindly provided by R. Lehmann. Flies were raised and aged at 25°C.

Tissue sections for light and electron microscopy

Adult heads were prepared for light and electron microscopy as described in Kretzschmar *et al.* (1997). For light microscopy, 1 μ m serial sections were cut and stained with 1% toluidine blue, 1% borax. Ultrathin Epon plastic sections were post-fixed with osmium and stained with 2% uranyl acetate, followed by Reynolds' lead citrate (Reynolds, 1963), and

stabilized for transmission EM by carbon coating. Examination was performed with a Zeiss EM10C/VR electron microscope at 40–80 kV. Paraffin mass histology was performed as described by Jäger and Fischbach (1989).

Cloning and sequencing

The cDNA clones for the various *loe* transcripts and genomic clones were isolated from the *Drosophila* Genome Project (cDNAs: SD02114, LD45665, LD28468, SD02088, GH16589, LD19285, LD41424, GH28591, LD05242, LD13337 and GH08914). The pIndy5 (kindly provided by L. Seroude) and pCaSpeR3-UAS (pUAST, Flybase) vectors were used for the pUAS-*Loe* constructs.

Sequencing was performed using the Thermo Sequenase fluorescent-labeled primer cycle sequencing kit from Amersham Pharmacia after subcloning cDNA fragments into pBluscript KS. Reactions were performed on a Hybaid Omn-E (MWG) thermocycler according to the instruction manual for the sequencing kit. Sequence analysis was performed with the ALFexpress sequencing system (Pharmacia) using Hydrolink Long Ranger gels (FMC Bio Products).

Northern blots

Total RNA was isolated using the Trizol method described in Goodwin *et al.* (1997), and poly mRNA was selected with the Promega PolyAtract system. Northern blots were performed following the protocol of Ausubel *et al.* (1996).

Lipid and sterol measurements

A 20 mg aliquot of heads from 1- to 5-day-old flies was homogenized mechanically and chloroform/methanol extracted as described in Folch *et al.* (1957). Phospholipids were separated by two-dimensional thin-layer chromatography on Silica gel 60 plates (Merck) using chloroform/methanol/25% NH₃ (65:35:5 by vol.) and chloroform/acetone/methanol/acetic acid/water (50:20:10:10:5 by vol.) as solvents. Phospholipids were visualized on TLC plates by staining with iodine vapor, scraped off and quantified (Broekhuysse, 1968).

For the analysis of neutral lipids, extracts were applied to silica gel 60 plates with a sample applicator (Linomat IV; CAMAG), and chromatograms developed in an ascending manner using the solvent system light petroleum/diethyl ether/acetic acid (70:30:2 by vol.). Quantitation of sterol and sterol ester was carried out by densitometric scanning at 275 nm with ergosterol as a standard. Neutral lipids were visualized by post-chromatographic staining using a chromatogram immersion device (CAMAG). Quantification of triacylglycerols, sterol and sterol ester was carried out by densitometric scanning at 400 nm with triolein (NuCheck, Inc.). As for the phospholipid measurements, at least six measurements from three independent samples were performed for each genotype.

Western blot analysis

Fly heads were homogenized as described in Torroja *et al.* (1996) and loaded on 7.5% SDS-polyacrylamide gels using standard methods (Laemmli, 1970). Proteins were transferred onto nitrocellulose membranes (Towbin *et al.*, 1979). Immunoreactions with anti-APPL (Ab952, kindly provided by K. White), diluted 1:300 and pre-adsorbed overnight against *Appl^d* embryos, were carried out according to the manufacturer's protocol for the ECL Western Blot Detection System (Amersham). The anti-Notch supernatant (Developmental Studies Hybridoma Bank, University of Iowa) was used in a 1:100 dilution. Flies of the different genotypes were used at the same age in one set of experiments. Different ages (1 and 5 days) were tested and showed the same result.

Measurement of the vacuolar pathology

The computer set-up described in Heisenberg *et al.* (1995) was used to count the holes and measure their volume. Measurements were performed at two distinct levels of the brain and in two different brain areas. At least four flies of the same age and processed on one slide, using paraffin mass histology, were used for quantification.

Statin treatment

Late third instar larvae were transferred to vials containing a 5% glucose solution with or without 1 ng/ml of mevinacor. Mevinacor (MSD Sharp&Dohme GMBH) contains the cholesterol-lowering drug lovastatin.

Accession numbers

The DDBJ/EMBL/GenBank accession Nos for *loe* transcripts 1–7 are AY166752–AY166758.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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