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 isofrm 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 via 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 (Dietzchy 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; Ladiu 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 Aβ 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 Aβ 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., 2002).
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 Aβ 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 (PacW). The exon–intron structures of the Loel to LoelVI transcripts are shown underneath. Start codons are indicated by arrows. The deletion loeD is indicated by a striped bar. B, BamHI; S, SalI; X, XhoI; C, ClaI. (B) The homology to other AMPK γ-subunits is restricted to the C-terminus (only Loel shown), including the cystathionine-β-synthase domains (light gray). The identity is given above. The N-terminal fragment of Loel (dark gray) shows homology to the rat X11α protein. (C–E) mRNA expression of loe. (C) Three transcripts are detected with a probe derived from exons 1–3 of Loel 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 LoelII 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 Loel isoform shows homology to the X11α protein which can bind to the APP protein (Borg et al., 1998); Loel and X11α 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 LoelII (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 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 LoelII transcript (Figure 2A, loeD), and these flies do not show a degeneration phenotype. This indicates that LoelII is not required for CNS integrity.

The mutation is due to an aberrant Loel transcript
Northern blot analysis of adult head mRNA fractions further supported the conclusion that the mutation is due to an effect on the Loel transcript. A probe comprised of exons 1–3 from Loel 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 Loel 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 Loel transcript (Figure 2D). As expected, other transcripts, including LoelII, are unaltered in mutant flies (Figure 2E).

To confirm the specific role of Loel, we expressed the Loel and LoelII cDNAs in different cell types using the UAS/Gal4 system (Brand and Perrimon, 1993). Lines carrying P-element vectors with either the Loel or LoelII cDNA under the control of the Gal4-binding sequence (UAS) were crossed with various Gal4 lines to induce expression of Loel 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-Loel (Figure 3D). Expression in glia using loco-Gal4 did not rescue the phenotype (data not shown) nor did expression of LoelII in neurons (Figure 3C). This finally proves that the mutation is caused by a disruption of the Loel 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 Loel is required for wild-type function
To investigate the function of the specific N-terminal region of Loel, we created N-terminally truncated Loel transgenes. Expressing a Loel 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 Loel protein. A construct deleting amino acids 1–319, removing the domain similar to X11α (amino acids 13–88), could rescue more efficiently. We could, however, still detect some holes (Figure 3F). The X11α similarity domain is therefore required for wild-type function, but other functionally important domains must reside within the N-terminus of Loel 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 Loel in neurons restored the wild-type level of cholesterol ester in the mutant (Figure 4B), confirming the role of Loel/AMPK in cholesterol homeostasis. The expression of Loel 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
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...
(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 Aβ from APP, as has statin treatment, which can dramatically decrease Aβ production (Fassbender et al., 2001). Therefore, we investigated whether loe influences APPL, the fly homolog of human APP (Rosen et al., 1989). Appd mutants, which carry a deletion in the Appl gene (Torroja et al., 1996), do not reveal any signs of neurodegeneration (Figure 6A). However, crossing Appd 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 Appd (loe/loe; Appd/+), compared with homozygous double mutants (loe/loe; Appd/Appd) 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 Appd. 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 LoeI 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 (DeStrooper and Annaert, 2000; Novotny 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 X110 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
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, Loel 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. 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<sup>112</sup>/loe, B) of the same age. (C) Control loe mutant flies (7 days) carrying only the UAS-Cib 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<sup>112</sup>/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<sup>3</sup>) is reduced in loe clb<sup>112</sup>/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 μg/ml) to the glucose solution reduces the formation of holes. (I) Feeding mevinacor to wild-type flies shows no effect.
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-soluble 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 drastically decrease Aβ 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 Aβ 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 Aβ production, and that elevated concentrations of cholesterol ester but not free cholesterol increased the generation of Aβ. 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 APP.

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* 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 effect 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 *w*1118 were used as control stocks. The *locus*-Gal4 line was kindly provided by C.Klambt, and the APPL-Gal4 line by L.Torroja. Act-Gal4 and elav-Gal4 were provided by the Bloomington stock center, and the P-element lines are from the Siegel stock center. The *Appl* mutant was kindly provided by K.White. The *clb*1:24 and *clb*1:24*gg* alleles (both strain UAS-Cib 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 μm 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, LD14124, GH28591, LD05242, LD13337 and GH08914). The plday5 (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 pBluescript KS. Reactions were performed on a Hybrid 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/acetic acid/water (50:20:10:5 by vol.) as solvents. Phospholipids were visualized on TLC plates by staining with iodine vapor, scraped off and quantified (Broekhuysen, 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 tricylglycerols, 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 (AB9522, kindly provided by K.White), diluted 1:300 and pre-adsorbed overnight against Appl° 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 mg/ml of mevinacine. Mevinacine (MSD Sharp&Doyme 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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