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Deregulation of the Egfr/Ras Signaling Pathway Induces Age-related Brain Degeneration in the *Drosophila* Mutant *vap*

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Ras signaling has been shown to play an important role in promoting cell survival in many different tissues. Here we show that upregulation of Ras activity in adult *Drosophila* neurons induces neuronal cell death, as evident from the phenotype of *vacuolar peduncle* (*vap*) mutants defective in the *Drosophila RasGAP* gene, which encodes a Ras GTPase-activating protein. These mutants show age-related brain degeneration that is dependent on activation of the EGF receptor signaling pathway in adult neurons, leading to autophagic cell death (cell death type 2). These results provide the first evidence for a requirement of Egf receptor activity in differentiated adult *Drosophila* neurons and show that a delicate balance of Ras activity is essential for the survival of adult neurons.

INTRODUCTION

Forward and reverse genetics has been successfully used in *Drosophila* to identify new genes involved in neuronal degeneration and in the study of human genes linked to neurodegenerative diseases. Recently, a number of articles have corroborated the use of *Drosophila* as a powerful model organism to investigate the process of age-related neuronal cell death (reviewed in Fortini and Bonini, 2000). It is well known that neurons need a constant supply of growth factors for their survival, and in cell culture, the withdrawal of these factors or blocking their signal transduction pathways leads to cell death (Barres *et al.*, 1993; Raff *et al.*, 1993; Xia *et al.*, 1995; Le-Niculescu *et al.*, 1999). A variety of factors including fibroblast growth factor (FGF) and epidermal growth factor (EGF) promote cell survival by binding and activating receptor tyrosine kinases (RTKs), which stimulate the activation of the Ras proto-oncogene products (Gardner and Johnson, 1996; Yamada *et al.*, 1997). The involvement of Ras-dependent pathways in the process of neuronal cell survival has been studied in cell culture (Bonni *et al.*, 1999; Mazzoni *et al.*, 1999) and in vivo using *Drosophila*. Perhaps the most interesting data linking Ras activation with cell survival arise from the studies in photoreceptor apoptosis in *Drosophila*: Ras promotes cell survival in the eye by downregulating the expression of the apoptotic gene *hid* during development (Bergmann *et al.*, 1998; Kurada and White, 1998). On the other hand, the overexpression of *argos*, an inhibitor of the Egfr/Ras signaling pathway, causes extensive cell death in developing *Drosophila* eyes (Sawamoto *et al.*, 1998).

Conversely, some reports exist that associate an activated Ras cascade with enhanced cell death. The ectopic expression, for instance, of an active form of Ras leads to hyperplastic growth and induces widespread cell death in *Drosophila* imaginal discs (Karim and Rubin, 1998), and the expression of oncogenic mutated Ras in human cancer cells leads to cell death that shares features of autophagic degeneration (cell death type 2) (Chi *et al.*, 1999; Kitanaka and Kuchino, 1999). The expression of oncogenic Ras has been also implicated in senescence in cultured human fibroblasts. In primary cells, Ras is initially mitogenic but eventually induces senescence, suggesting the existence of a protective mechanism in the prevention of Ras-induced neoplasia (Lin *et al.*, 1998; Lee *et al.*, 1999).

GTPase activating proteins (GAP) proteins act as direct negative regulators of Ras signaling by accelerating the intrinsic Ras GTPase activity. The *Drosophila* RasGAP has been shown to be required for the negative regulation of the

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Torso signaling pathway, which specifies the embryonic terminal structures (Cleghon *et al.*, 1998). *Drosophila* RasGAP stimulates the GTPase activity of the mammalian H-Ras, and its overexpression suppresses the phenotypes induced by hyperactivation of several receptor tyrosine kinases, suggesting that it can function as an inhibitor of signaling pathways mediated by Ras in vivo (Feldmann *et al.*, 1999).

Several reports implicate RasGAP in controlling a cell death mechanism that could be dependent on deregulation of Ras. Mice lacking p120RasGAP show a variety of developmental defects including extensive neuronal cell death, and fibroblasts from RasGAP$^{-/-}$ embryos show aberrant regulation of Ras and MAPK after activation (Henkemeyer *et al.*, 1995; van der Geer *et al.*, 1997). Other reports also point to the same direction, indicating that RasGAP might play a key role in inhibition of cell death: The inhibition of p120RasGAP induces apoptosis in tumor cells, suggesting a specific role for RasGAP in tumor cell survival (Leblanc *et al.*, 1999), and it has been shown that RasGAP is cleaved by caspases in some apoptotic paradigms (Wen *et al.*, 1998).

To investigate the physiological importance of RasGAP in the process of neuronal cell survival, we have isolated and characterized the first mutant alleles of the *Drosophila RasGAP* gene. Total and partial loss-of-function mutations induce age-related neuronal degeneration with a morphology resembling that of cell death type 2 described for human cancer cells expressing oncogenic Ras. This phenotype can be enhanced and suppressed by using different elements of the Egf receptor/Ras pathway already identified in *Drosophila*. Our results provide the first evidence on the effects of an aberrant regulation of the Egfr/Ras pathway on the adult fly brain, and new insights into the role of this important signal transduction cascade in the maintenance of the adult nervous system.

MATERIALS AND METHODS

Drosophila Stocks

All stocks were maintained and raised under standard conditions. For heat shock experiments, the flies were kept in a water bath with cycles of 5 h at 28°C and 1 h at 35°C. For the temperature-shift experiment flies were raised at 18° and then moved to 28° for aging. The *vap*^{1} allele was induced by EMS in the Berlin wild-type and was isolated in screens for structural brain defects (de Belle and Heisenberg, 1996). The alleles *vap*^{2} and *vap*^{3} were identified in a histological screen from a collection of P-element lines provided by U. Schäfer and H. Jäckle (Melzig *et al.*, 1998). *vap*^{2} is caused by a deletion of 239 base pairs (from position 999 base pairs in the *RasGAP* cDNA, accession number AJ012609) and deletes the splice donor site at position 1099 base pairs. This creates an aberrant transcript using a donor splice site at 907 base pairs, and the resulting protein is shortened by 64 aa (aa 206–269). *vap*^{3} is due to an insertion of the P-element in the first intron. Jump-out experiment as described in Grigliatti (1998) reverts the *vap*^{3} phenotype. The following mutant and transgenic fly strains were used in this study: *Egfr*^{top1P02} (Clifford and Schupbach, 1989), *Egfr*^{ts1a} (Kumar *et al.*, 1998), *sty*^{S73} (Casci *et al.*, 1999), *drk*^{k02401} (Roch *et al.*, 1998), *Ras*^{DeltaC40B} (Hou *et al.*, 1995), P[hsp-*rho*] (Sturtevant *et al.*, 1993), P[hsp-*DER*] (Schweitzer *et al.*, 1995), P[*UAS-Ras1*], P[*UAS-Ras1*^{V12}], P[*UAS-Ras1*^{V12S35}], P[*UAS-Ras1*^{V12G37}], P[*UAS-Ras1*^{V12C40}] (Karim and Rubin, 1998), P[*UAS-RasGAP*] (Feldmann *et al.*, 1999), *Gap1*^{EP45} (Rørth, 1996), P[*elav-AL4*] (Robinow and White, 1988), and P[*appl-GAL4*] (Torroja *et al.*, 1999).

Immunohistochemistry

Mass histology of adult heads was performed following the protocol for paraffin sections and immunohistochemistry described in Jäger and Fischbach (1989) and Buchner *et al.* (1989). ab49 is a mAb against the synaptic cysteine-string protein (csp, kindly provided by A. Hofbauer) and was used in a dilution of 1:500 in PBS. Anti–β galactosidase (Sigma, Munich, Germany) and anti-ELAV (Robinow and White, 1991) antibodies were used on cryosections after fixation with 4% PFA. For detection and staining the Vectastain mouse IgG ABC kit from Vector Laboratories (Burlingame, CA) was used.

Plastic Sections for Light and Electron Microscopy

Adult brains were prepared, cut, and stained as described in Kretzschmar *et al.* (1997). Ultrathin Epon plastic sections were poststained with 2% uranyl acetate, followed by Reynolds' lead citrate (Reynolds, 1963) and stabilized for transmission electron microscopy by carbon coating. Examination was done with a Zeiss EM10C/VR (Oberkochen, Germany) electron microscope at 40–80 kV. Glial cell material was clearly identified by its characteristically higher electron density (Saint Marie and Carlson, 1983a, 1983b).

Phenotype Quantification

An average of 10 optic lobes was used for each genotype analyzed. The percentage of vacuolizated area from medulla and lobula complex of three consecutive 5-μm horizontal paraffin sections was calculated using analySIS 2.1 (Soft-Imaging Software GmbH, Munich, Germany).

In Situ Hybridization

Frozen head sections of adult wild-type flies were fixed and hybridized with a sense and antisense RNA probes derived from a RasGAP cDNA as described in Kretzschmar *et al.* (1997).

Sequencing of the Mutant Alleles

Oligos of 17 and 20 bp, complementary to base pairs 443–460 and 3195–3215 of the *RasGAP* cDNA, were synthesized. From *vap*^{1} flies genomic DNA was isolated using standard conditions (Ashburner, 1989), from *vap*^{2} total RNA was isolated with the Qiagen (Hilden, Germany) RNeasy Mini Kit following the kit protocol, and the cDNA was synthesized according to Sambrook *et al.* (1989). PCR reactions were performed using the Expand High Fidelity PCR system (Boehringer, Ingelheim, Germany). The amplification product was visualized on a gel, purified with the Qiagen QIAquick Gel Extraction kit, and digested with different restriction endonucleases. The resulting fragments were subcloned into pBluescript KS. The position of the P-element in *vap*^{3} was determined by isolating a rescue plasmid (Wilson *et al.*, 1989). Sequencing of the plasmid DNAs was performed using the Thermo Sequenase fluorescent labeled primer cycle sequencing kit from Amersham (Freiburg, Germany) Reactions were done on a Hybaid Omn-E (MWG) thermocycler according to the instruction manual for the sequencing kit. Sequence analysis followed with the ALFexpress sequencing system (Pharmacia, Freiburg, Germany) using Hydrolink Long Ranger gels (FMC BioProducts, Rockland, ME).

Western Blots

Third instar larvae or adult heads of different genotypes were homogenized in extraction buffer (Suri *et al.*, 1999). Equal amounts of soluble protein lysates were separated on SDS-PAGE gels (Sambrook, 1989) and transferred by wet-blotting onto nylon membranes (Hybond C, Amersham). Detection and staining were performed using a rat anti-RasGAP antibody (Feldmann *et al.*, 1999) in a 1:3 dilution and detected with a secondary antibody from Pierce (Rockford, IL) and the ECL chemiluminescence reaction kit (Amersham)

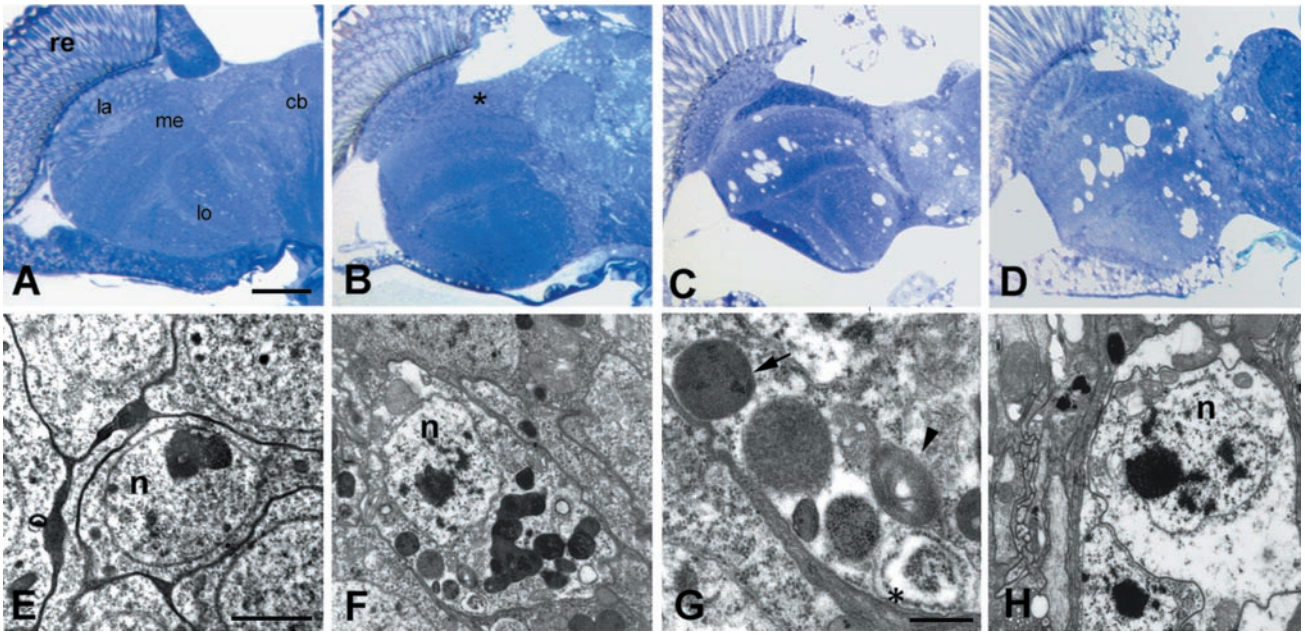


Figure 1. Age-related brain degeneration in *vap* mutants. Toluidin blue staining of horizontal semithin plastic sections from adult flies (A-D). In 1-day-old *vap*^{1} flies (B) no apparent difference can be found when compared with wild type (A). Seven-day-old *vap*^{1} flies (C) show vacuolization in the optic lobe (ol) and central brain (cb). In 14-day-old *vap*^{1} flies (D), the stronger spongiform appearance of the brain reveals that the degenerative phenotype in *vap* flies is age related. The ultrastructural analysis (E–H; G is a magnification from F) shows that neurons of 1-day-old *vap*^{1} flies accumulate autolysosomes (F and arrow in G), vacuoles containing whorls of membranous material (F and arrowhead in G), empty vacuoles or vacuoles still containing some material (F and asterisk in G); all signs of autophagic cell death that are absent in wild-type neurons (E). In 7-day-old *vap* flies dying neurons show a digested cytoplasm, whereas the nucleus (n) remains intact (H). The asterisk in B indicates the medula cortex, the region where the EM pictures were taken from. Scale bars, 50 μm in (A), 2 μm in (E) and 0.5 μm in (G). re (retina), la (lamina), me (medula), lo (lobula complex), cb (central brain).

following the manufacturer's manual. To study activation of the MAPK a mouse antibody, in a 1:2000 dilution, reacting against phosphorylated MAPK (Sigma) was used. The cysteine-string protein (csp) was used as loading control detected by ab49 (dilution 1:1000). Three different blots were analyzed for quantification with the NIH image 1.60 software.

RESULTS

Age-related Neurodegeneration in vap *Mutants*

In a screen to identify X-chromosome–linked genes involved in age-related brain degeneration we have found a complementation group of two P-element–induced alleles that fail to complement the neurodegenerative mutant *vap* (*vacuolar peduncle*) (Heisenberg and Bohl, 1979; de Belle and Heisenberg, 1996). All alleles show brain degeneration with 100% penetrance (Figures 1, A–D), however, with an allele-specific time course. The spongiform appearance of both, central brain and optic lobes, increases with age and correlates with the neuronal cell death that can be assessed by electron microscopy (Figures 1, E–H). Interestingly, in *vap* mutants the retina remains unaffected despite the extensive optic lobe degeneration. All mutants are homozygous viable and fertile. In addition, *vap*^{1} and *vap*^{2} show a reduction of the maximum life span (25 and 55 d, respectively) when compared with the parental line (90 d). Complementation tests with deficiencies and several duplications in the region map the affected gene to the cytological position 14A1–14B1.

The GTPase-activating Protein RasGAP Is Affected in vap *Mutants*

Molecular analysis has revealed that the gene affected in the *vap* mutants encodes the *Drosophila* GTPase-activating protein *RasGAP*. The *Drosophila* RasGAP protein consists of an amino-terminal region containing SH2-SH3-SH2 domains involved in interaction with other proteins, a central part containing PH and C2 domains involved in phospholipid binding, and a GAP catalytic domain in the carboxy-terminal part of the protein. In the EMS induced allele *vap*^{1}, a G-to-A transition creates a stop codon terminating the protein before the GAP catalytic domain (Figure 2A). The corresponding protein is, therefore, a null in terms of GAP activity and so low in abundance that it cannot be detected in Western blots (Figure 2B). This suggests that RasGAP is not essential for the development of *Drosophila*. *vap*^{2} carries a deletion of 239 base pairs that removes a splice donor site and, if no splicing occurs, it causes a frame shift in the sequence, and a stop codon would generate a nondetectable truncated protein shorter than in *vap*^{1} (see MATERIAL AND METHODS). The deletion creates also an aberrant splicing event that generates a new and shorter transcript, as detected by RT-PCR. This encodes a protein in which the SH3 and the second SH2 domains are affected but with an intact GAP catalytic domain (Figure 2, A and B). The levels of the mutant protein detected by Southern blots suggest that the new aberrant splicing event might be inefficient (Figure 2B).

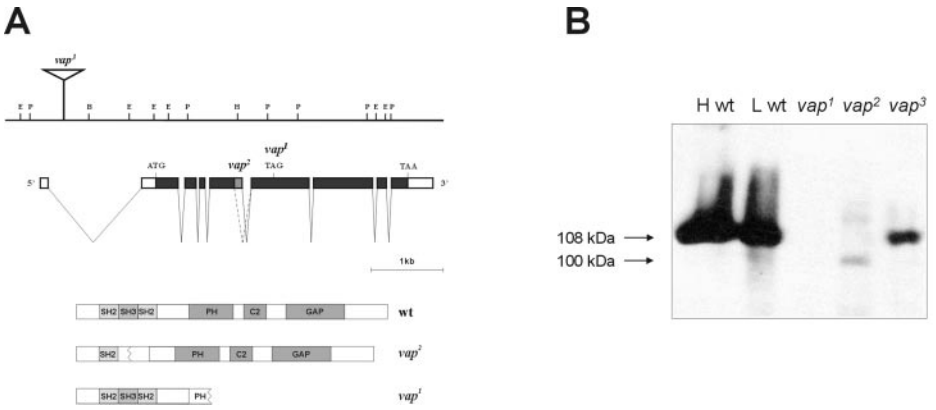


Figure 2. Molecular characterization of the *vap* mutant alleles. (A) Molecular map of the *RasGAP* gene showing the mutations and the expected mutant gene products of the different *vap* alleles. In vap^2, a deletion generates the aberrant splicing event shown with a dashed line. P, *Pst*I; E, *Eco*RI; H, *Hin*dIII; B, *Bam*HI. (B) Western blot analysis of the *vap* mutants. Equal amounts of protein were loaded in all lanes. Hwt, wild-type adult heads; Lwt, 3rd instar wild-type larvae. For the analysis of the mutants, protein extracts from 3rd instar larvae were used. In vap^1 no signal could be detected. In vap^2 a 100-kDa mutant protein results from the aberrant splicing induced by the deletion. The transposon insertion in vap^3 causes a reduction in the expression of RasGAP protein.

As expected, flies carrying the vap^2 allele show a weaker phenotype characterized by a delayed onset of vacuolization (day 15) and extended life span (see above) when compared with those carrying vap^1 (day 7). vap^3 is a P-element insertion within an intron that splits the 5′UTR of the gene and flies carrying this allele show reduced levels of RasGAP protein compared with wild type (Figure 2, A and B). vap^3 do not show a reduced life span, and the onset of vacuolization (day 30) is delayed when compared with vap^1 and vap^2 flies. The data above suggest that vap^2 and vap^3 are hypomorphic alleles of *RasGAP*.

Neuronal Cell Death in the vap *Mutant Is Due to Autophagic Degeneration*

The brain degeneration in *vap* mutants is due to neuronal cell death; glia cells remain unaffected even in the oldest flies tested (as revealed by ultrastructural analysis and using different molecular markers; unpublished data). The vacuolization observed in neuropils of the adult brain can also be found at the ultrastructural level and correlates with the extensive occurrence of neuronal cell death in the corresponding cortex regions (Figure 1). Independent experiments show that dying neurons do not undergo apoptosis. First, apoptotic nuclei were not detected by TUNEL labeling (unpublished data). Second, dosage reduction of the apoptotic genes reaper, grim, and hid does not modify the vap phenotype, and the neuronal cell death cannot be blocked by the pan-neuronal expression of the antiapoptotic protein p35, suggesting a caspase-independent mechanism for death execution (unpublished data). Third, morphological analysis did not reveal the typical characteristics of apoptotic cells but did reveal features of an alternative mechanism called cell death type 2 or autophagic cell death (Clarke, 1990). The nuclei remain well preserved even in the latest stages of the death process, whereas the cytoplasm shows signs of autophagy (compare Figure 1F and 1H). Although in 3rd instar larvae mutant brains seem unaffected, many autophagic features at different stages can be found in the imago, including vacuoles containing whorls of membranous material, autolysosomes, and empty vacuoles from the first day after eclosion (Figure 1G). In later phases of this process autophagic vacuoles leave a digested empty cytoplasm (Figure 1H). Strikingly, all these features are observed in dying human cancer cells after the expression of an oncogenic form of Ras (Chi *et al.*, 1999; Kitanaka and Kuchino, 1999). Therefore, a parallelism could be established between this experimental paradigm and the situation in *vap* mutants where a negative regulator of Ras is affected and the same Ras-dependent signaling pathways might be deregulated.

Functional Specificity of the Drosophila *RasGAP*

Analysis of *RasGAP* expression by in situ hybridization showed that the gene is expressed in the whole cortex of the adult *Drosophila* head (Figure 3). No expression was observed in neuropil glial cells (Figure 3C). In addition, vap^3 acts as an enhancer-trap line that shows a β-galactosidase expression pattern resembling the distribution of the neuronal marker ELAV in adult heads (Figure 3, E and F). Altogether, these data indicate that, in the adult brain, *RasGAP* is expressed in a panneuronal pattern. Consequently, the GAL4-UAS system (Brand and Perrimon, 1993) was used to express the *UAS-RasGAP* wild-type cDNA under the control of the neuron-specific drivers appl- (amyloid precursor protein-like) or elav-GAL4. The pan-neuronal expression of *RasGAP* rescued the neurodegenerative phenotype of the different *vap* alleles (Figure 4B). We could not detect expression of *RasGAP* in neuropil glial cells (Figure 3C), and the expression of RasGAP with different glial GAL4 drivers was not able to rescue the vap phenotype. Altogether, these data show that the function of *RasGAP* is required in neurons and not in glial cells.

In *Drosophila*, apart from RasGAP, there are two more GAP proteins that have been well characterized: Gap1 and NF1. Null mutants for these genes do not show brain degeneration and were not able to modify the phenotype of vap^2 (unpublished data). Gap1 acts as a negative regulator of

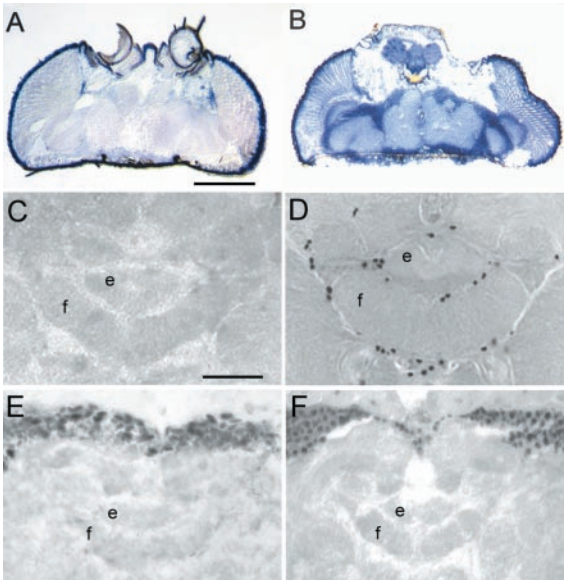


Figure 3. Neuronal expression of *RasGAP*. In situ hybridization of *RasGAP* RNA on cryosections from wild-type flies. (A) Control, using a sense RNA probe. (B) *RasGAP* is expressed in the entire brain cortex, suggesting widespread expression in cortical cell bodies as detected by using an antisense RNA probe. (C) Magnification of the neuropil areas of the central complex shows no expression of *vap* in neuropil glial cells. (D) Localization of the neuropil glia cells distribution around the neuropil area of the central complex visualized by autofluorescence. (E) β-Galactosidase detection in the enhancer-trap line vap^3. (F) Anti-ELAV stainings of wild-type heads of the same cortical area as shown in E. Scale bar: (A) 100 μm; and (C) 10 μm. (f): fan shaped body; (e) ellipsoid body.

RTK signaling in the eye, and its function is required within the cone cell precursor to downregulate Ras (Buckles *et al.*, 1992; Gaul *et al.*, 1992). The structure of the *Drosophila* Gap1 is quite different from that of RasGAP. Apart from the catalytic domain required for stimulating Ras GTPase activity, Gap1 shows two N-terminal C2 domains and a PH domain in the carboxy part of the protein, and it lacks the amino-terminal adapter-like region of the RasGAP proteins. Gap1 is, to date, the best characterized *Drosophila* GAP protein studied that acts in the Egfr pathway. We show below that *vap* function is required to modulate Egfr signal. To test, therefore, whether or not Gap1 was able to substitute the function of RasGAP in the central brain, we ectopically expressed a fully functional Gap1 cDNA ($Gap1^{EP45}$, Rørth, 1996) using specific neuronal GAL4 drivers (appl- and elav-GAL4). The ectopic expression of *Drosophila* Gap1 was not able to rescue the *vap* phenotype (Figure 4C). This reveals that the function of RasGAP is rather specific in the adult *Drosophila* brain and that Gap1 cannot complement the lack of RasGAP function.

vap *Interacts with the EGF Receptor Pathway*

To verify the role of *vap* in the RTK/Ras signal transduction cascade, we have analyzed several genetic interactions with members of this pathway. For this purpose we have used the allele vap^2 because it provides a more sensitized background for genetic interactions compared with vap^1. Enhancement or suppression of the vap phenotype was quantified by calculating the percentage of vacuolization in the optic lobes (Figure 5J; see MATERIAL AND METHODS). Genetic tests provide clear evidence that *vap* interacts with the EGF receptor (Egfr) and Ras pathway in the brain (Figure 5). We have found that in aged vap^2 flies heterozygous for a null allele of the *Egfr* there is a strong suppression of the phenotype (compare Figure 5H and 5B). vap^2 flies carrying heat shock constructs for the misexpression of the *Egfr* (hs-DER) or *rhomboid* (hs-Rho), a specific activator of Egfr signaling (Bier *et al.*, 1990; Golembo *et al.*, 1996) exhibit strongly enhanced phenotypes (Figure 5, D, E, and J). When the hs-Rho construct was used we observed this enhancement at 25°C, even without heat shock induction. In the case of hs-DER, we observed a strong degeneration of the central brain (arrow in Figure 5E), but the optic lobe remained intact. The lack of optic lobe degeneration could be due to a heterogeneous misexpression of the *Egfr* using the hs-DER construct. Moreover, the overexpression of *Egfr* using the hs-DER construct in the RasGAP null mutant background vap^1 resulted in lethality. The misexpression of *rho* and *Egfr* in wild-type flies did not cause brain degeneration (unpublished results), indicating that the ectopic activation of the *Egfr* pathway can be effectively downregulated in wild-type flies. Other genetic interactions also support the hypothesis that the Egfr pathway is deregulated in *vap* flies. First, reducing the dose of *Grb2/drk* (downstream of receptor kinase), a SH2/SH3 adapter protein required for the signaling between RTKs and Ras (Gale *et al.*, 1993; Simon *et al.*, 1993), suppresses the neurodegenerative phenotype of vap^2 mutants (Figure 5, G and J) and, second, reducing the dose of *sprouty* (*sty*), an intracellular inhibitor of the Egfr/Ras signaling (Casci *et al.*, 1999), enhances the phenotype of vap^2 (Figure 5, C and J). All these results confirmed our hypothesis of the role of RasGAP as a negative regulator of the Egfr pathway: the overexpression of *Egfr* or *Rho* as well as the reduction of the

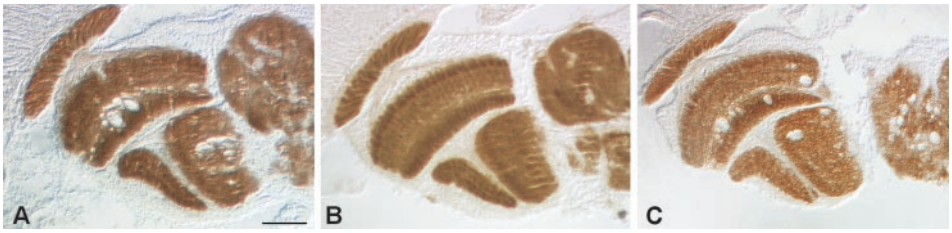
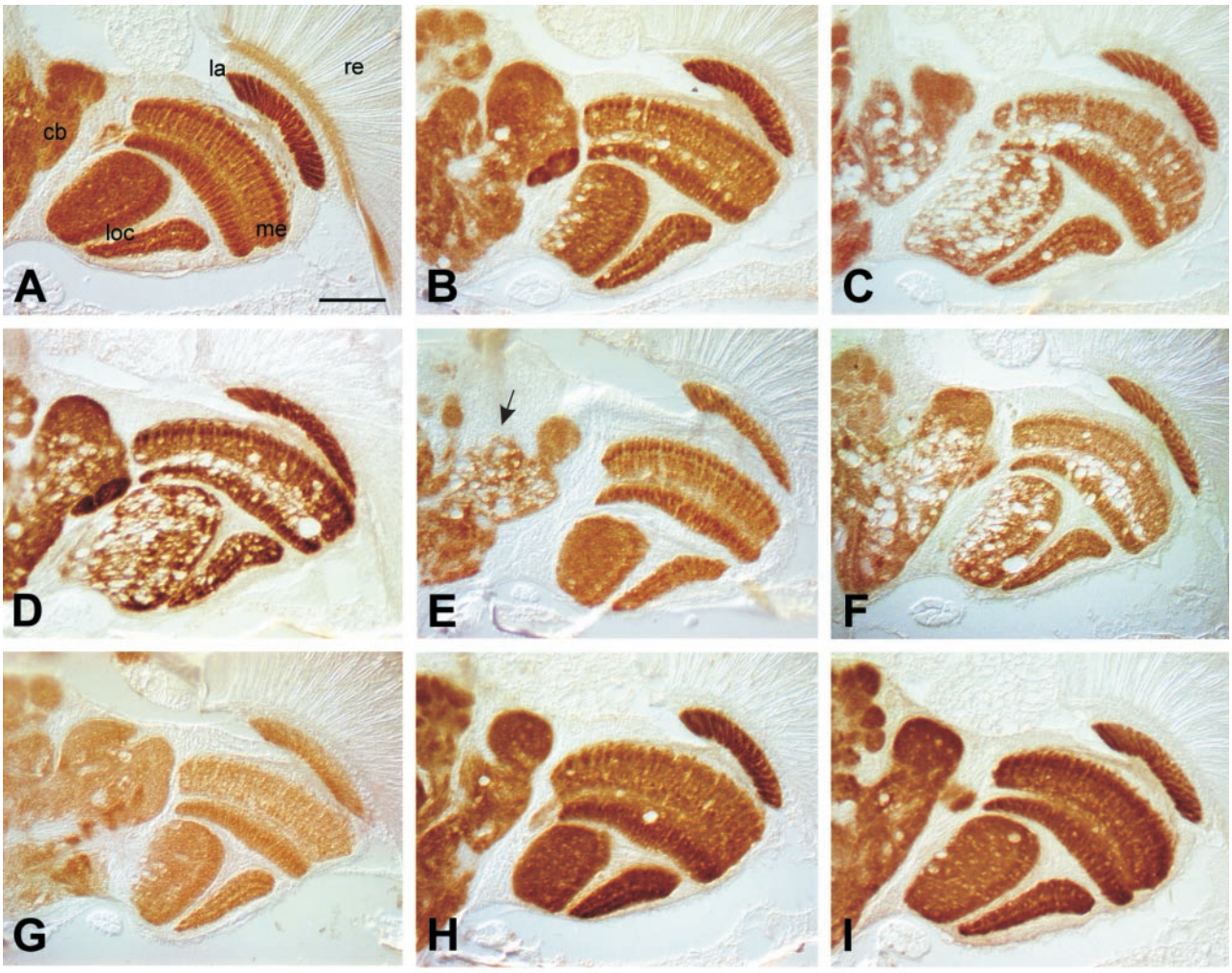


Figure 4. Functional specificity of *RasGAP*. Horizontal paraffin sections of adult fly heads were stained with an antibody (ab49) against Csp to visualize the neuropiles. The degenerative phenotype of 7-day-old vap^1 flies (A) can be rescued by expressing the *RasGAP* cDNA in neurons (B). This phenotype cannot be rescued by the pan-neural expression of the GTPase–activating protein Gap1 (C). The genotypes are vap^1 (A), vap^1;*UAS-RasGAP/+*;*elav-GAL4/+* (B) and vap^1;*UAS-Gap1/+*;*elav-GAL4/+* (C). Scale bar is 50 μm.



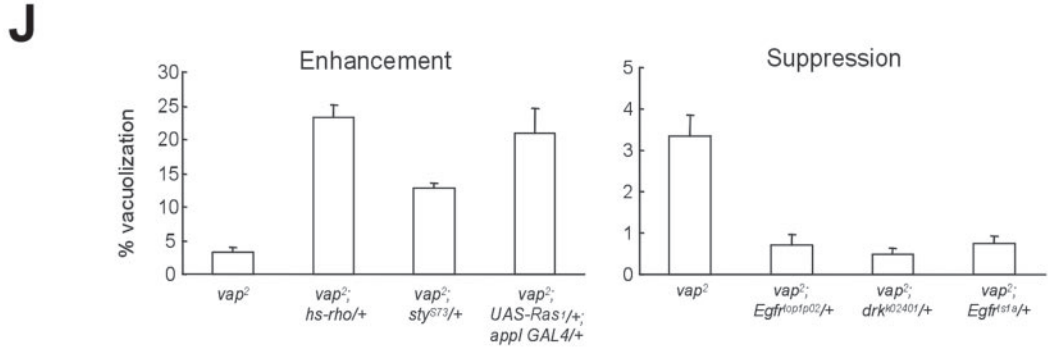


Figure 5. vap interacts with members of the Egfr/Ras pathway. In all panels flies were 18-day-old except in E and F, which were aged for 6 days. (A) Wild-type. The degenerative phenotype of 18-day-old *vap*^{2} flies (B) can be enhanced by reducing the dose of *sprouty* (*vap*^{2};*sty*^{S73}/+) (C and J). The overexpression of *rhomboid* (*vap*^{2};*hs-rho*/+) (D and J), Egfr (*vap*^{2};*hs-DER*/+) (E, the arrow shows the area of degeneration) or the pan-neural expression of *Ras* (*vap*^{2};*UAS-Ras1*/+;*appl-GAL4*/+) (F and J) also enhances the phenotype. Reduction in the dose of *drk* (*vap*^{2};*drk*^{k02401}/+) (G and J) or *Egfr* (*vap*^{2};*Egfr*^{top1p02}/+) (H and J) suppresses the phenotype. The suppression of the phenotype can be also achieved by reducing the dose of Egfr in adulthood (*vap*^{2}; *Egfr*^{ts1a}/+) (I, J). re: retina, la: lamina, me: medula, loc: lobula complex, cb: central brain. Scale bar is 50 μm.

levels of *sty* enhances the *vap* phenotype, whereas decreasing the signal by reducing the dose of *Egfr* or *drk* suppresses it.

Egfr Activity in the Adult Brain Is Necessary to Develop the vap *Phenotype*

To investigate whether the phenotype observed in *vap* flies is due to a deregulation of the Egfr signaling in adult neurons or a developmental defect, vap^2 control flies and vap^2 flies carrying a temperature-sensitive allele of the *Egfr* ($Egfr^{ts1a}$) were raised at 18°C and transferred to 28°C for aging (see MATERIALS AND METHODS). In Figure 5, I and J, we show that the temperature-sensitive allele of the *Egfr* was able to suppress the brain degeneration, whereas no difference in the phenotype was observed between those flies kept at 18°C (unpublished data). Although this result does not rule out the possibility that the *vap* phenotype could be partially caused by a deregulation of the Egf/Ras pathway during development, it shows that there is Egfr activity in the brain of adult *vap* flies and that this activity contributes to the neuronal cell death observed in the *vap* mutant.

Pan-neural Expression of Ras1 *Enhances the* vap *Phenotype*

Although the results above show an interaction of *vap* with different genes involved in the regulation of the Egfr/Ras pathway, no interaction was found using vap^2 flies heterozygous for a null allele of *Ras1* ($Ras^{deltaC40B}$), indicating that the amount of Ras in this case might be above the critical threshold necessary for genetic interactions. Alternatively, other GTPases, different from *Ras1*, might be involved in the phenotype of *vap* flies. To assess the role of Ras1 in the *vap* phenotype, we have used the GAL4-UAS system to express a wild-type copy of the *Drosophila Ras1* in neurons of *vap* and wild-type flies. In *vap* flies, the pan-neural expression of *Ras* induces, already in 6-day-old flies, a strong enhancement of the phenotype (Figure 5, F and J), whereas control flies carrying only the appl-GAL4 driver show no sign of degeneration at this age. The expression in wild-type flies did not result in a neurodegenerative phenotype. Altogether, these data show that the phenotype observed in *vap* mutants is related to a hyperactivation of Ras and suggest an aberrant regulation of Ras and Ras-dependent pathways in the brain of *vap* mutants.

Downstream Ras-dependent Signaling Is Upregulated in vap

To verify the hypothesis of an aberrant regulation of Ras in *vap* flies, we have analyzed the activation of the *Drosophila* MAPK *rolled*, a downstream element of this pathway. By Western blot analysis of larval or adult head protein extracts, we were not able to detect significant differences in MAPK activation between *vap* and wild-type flies. An explanation for this negative result is that the level of signaling to MAPK might not be high enough to detect changes in MAPK activation. Alternatively, the upregulation of the pathway might be cell specific and, therefore, undetectable by analyzing protein extracts of whole larvae or adult heads. To test whether or not vap was able to downregulate the Egfr pathway, we have set up an experimental paradigm to switch on the Egfr pathway in vivo and analyze the activation of MAPK, via the Ras-Raf-Mek pathway. For this, 3rd instar wild-type and mutant vap^1 larvae carrying one copy of hs-DER were given a heat shock treatment of 1 h at 37°C. Samples from different time points after the pulse of Egfr expression were analyzed by Western blots using an antibody against the activated form of rolled. As can be seen in Figure 6, the heat shock induces an increase in the signal of active MAPK in wild-type larvae after 2 h of the treatment, and the levels decrease after 3 h. In *vap* mutant larvae, there is, already after 1 h of heat shock, a stronger increase of MAPK activation, and the level of activation is overall higher than in wild type. The same results were obtained using protein extracts from heads of adult flies. These results show that, as expected, the function of RasGAP is not required for activation of the MAPK. In addition, the level but not the duration of MAPK activation is seriously compromised during development and in adult *vap* flies after stimulation of the Egfr/Ras pathway. This indicates that *vap* is able to downregulate the Egfr/Ras/MAPK signaling pathway and, in agreement with the genetic interaction experiment with elements of this signaling cascade, suggests an aberrant regulation of the pathway in *vap* mutants.

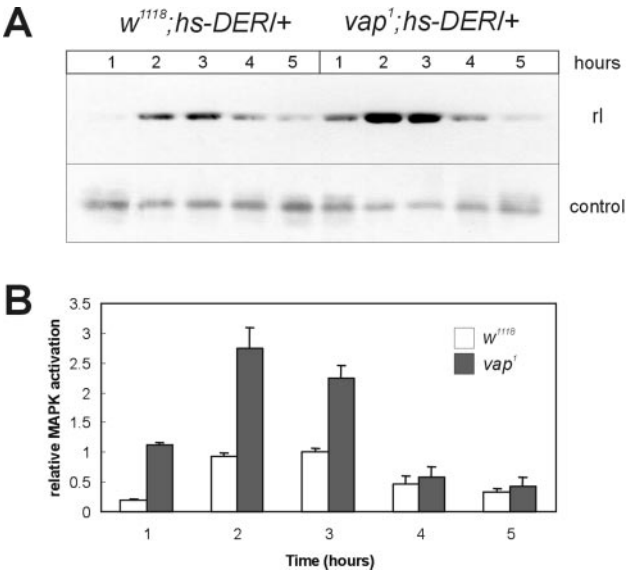


Figure 6. Deregulation of MAPK in *vap* mutants. (A) Western blot analysis of larval (3rd instar) protein extracts of wild-type and vap^1 flies carrying a heat shock–inducible *Egfr* construct. After 1-h heat shock, the level but not the duration of the MAPK activation (rl) is deregulated in *vap* mutants compared with wild-type flies (w^{1118}). Detection of the cysteine-string protein (csp) was used as loading control. (B) Quantification of three independent experiments.

Surprisingly, we do not find genetic interaction between *vap* and different alleles of Raf or the MAPK Rolled (unpublished data), suggesting that other than the Raf/MAPK pathway might be involved in the phenotype of *vap* flies. In mammals, apart from the Raf/MAPK pathway, Ras proteins activate multiple effectors the best characterized of these being RalGDS and related proteins (Rgl and Rlf), which are guanine nucleotide exchange factors for the small GTPase Ral, and class IA phosphoinositide 3-kinases (PI3Ks; Reuther and Der, 2000). Homologues of these Ras effectors are present in *Drosophila*, although thus far only Raf has been shown to function downstream of Ras1 in this organism.

Therefore, we decided to study the effect of the activation of different Ras-dependent pathways in *vap* flies. We have used the GAL4-UAS system to express different Ras effector loop mutants that allow the activation of particular downstream pathways: $Ras1^{V12S35}$ for the activation of the Raf/MAPK pathway, $Ras1^{V12G37}$, which activates the RalGDS pathway, and $Ras1^{V12C40}$, which signals to the PI3K pathway (White *et al.*, 1995; Rørth, 1996; Karim and Rubin, 1998). Wild-type and vap^2 flies carrying the UAS-$Ras1^{V12S35}$/*elav-GAL4* transgenes resulted in lethality, preventing us from assessing the contribution of the Ras/Raf/MAPK pathway to the degenerative phenotype. Neuronal expression of $Ras1^{V12G37}$ or $Ras1^{V12C40}$ neither affects viability nor induced brain degeneration in the wild-type background. Overexpression of these mutant forms of Ras did not modify the vap phenotype (unpublished data), which indicates that the RalGDS and PI3K pathways are not involved in the neurodegeneration observed in *vap* flies. The results above seem to indicate that a novel Ras pathway, different from those studied by us, might be involved in the vap phenotype. It is also possible that the *vap* mutant background might not be suitable to detect genetic interactions between *vap* and elements of the Raf/MAPK pathway. Alternatively, the synergistic upregulation of more than one pathway might account for the neuropathological defects observed in *vap* flies.

DISCUSSION

In *Drosophila*, many studies have been done to assess the role of the Egfr/Ras signal transduction cascade in cell proliferation, differentiation, and developmental cell survival but little is known about the function of the Egfr/Ras pathway in differentiated mature neurons. The tight regulation of Ras activity seems to be a central point for cell survival as the inhibition or the overactivation of the Ras signaling pathway leads to developmental cell death (see Introduction). The deregulation of Ras activity might also represent a challenging situation in differentiated neurons. We have found different mutant alleles of a gene that directly regulates Ras and that is required in differentiated neurons to prevent brain degeneration: the *Drosophila* GTPase-activating protein *RasGAP*. The analysis of flies carrying a null mutation in *RasGAP* shows that its function is not essential for *Drosophila* development and that the effects of mutations in this gene are apparent in adult flies that develop an age-related brain degenerative phenotype. The lack of neurodegeneration in *Gap1* and *NF1* mutants together with the fact that no rescue of the vap phenotype was achieved by overexpressing Gap1 suggests a specific function of RasGAP to prevent neurodegeneration in *Drosophila*.

Neurons of adult *vap* brains do not undergo apoptosis but show signs of autophagic degeneration. This type of cell death has been described, together with apoptosis, as an important mechanism involved in cell loss in some neurodegenerative diseases such as Huntington's disease, Parkinson's disease, and Alzheimer's disease (Jellinger, 2001). In the early stages of this process, neurons accumulate autolysosomes that disappear in the later phases, leaving an empty cytoplasm, whereas the nuclei do not seem to be affected. This is consistent with the lack of positive TUNEL labeling observed in adult mutant brains. Moreover, neurons of adult *vap* flies undergo a type of cell death that is caspase independent as judged by the failure of the rescue experiment using the antiapoptotic protein p35. Interestingly, this caspase-independent autophagic degeneration is the same type of cell death found in human cancer cells expressing an oncogenic form of Ras (Chi *et al.*, 1999; Kitanaka and Kuchino, 1999). The lack of a negative regulator of Ras, such as RasGAP might generate the same sort of effects as those elicited by a deregulation of Ras. In fact, RasGAP has also been found in mice to be essential for the downregulation of Ras, and *RasGAP* null mutants show, apart from other phenotypes, extensive brain degeneration. We have found that the neurodegenerative phenotype in *vap* flies is due to an aberrant regulation of the Egfr/Ras signal transduction cascade. The phenotype can be modified by overexpression or by the use of mutant alleles of different elements of the pathway. Reducing the dose of *Egfr* during development of *vap* mutant flies suppresses the neurodegenerative phenotype.

The same suppression effect can be observed using a temperature-sensitive allele of the *Egfr* and aging the adult flies at the restrictive temperature. This experiment shows that a deregulation of the *Egfr* signaling pathway in adult *vap* flies is sufficient to cause degeneration of mature neurons. Strikingly, strong expression of the *Egfr* and *Ras* genes can be found in the brain of adult flies (Schejter *et al.*, 1986; Segal and Shilo, 1986), and although nothing is known about their possible function in the adult fly brain, the suppression of the phenotype in adult *vap* mutants by reducing the Egfr activity shows that the Egfr is active in *Drosophila* adult neurons. This is consistent with the onset of the phenotype that takes place only in the adult fly. Signaling through the Egfr seems to induce, therefore, high levels of active Ras, which might be the cause of the phenotype. Genetic interactions with *drk* and *sprouty*, members of the Egfr/Ras pathway, also support this idea. Drk is a signaling protein that has been shown to bind in vitro the C terminal tail of the SOS protein, thereby linking receptor tyrosine kinase to Ras activation (Olivier *et al.*, 1993). It has been also found that Drk signals to the Ras protein in vivo (Raabe *et al.*, 1995). Therefore, reducing the levels of Drk might lead to a decrease of the levels of activated Ras in the *vap* mutant and to the subsequent suppression of the phenotype.

We have shown that *sprouty*, a negative regulator of the Egrfr/Ras pathway, also interacts with *vap*, enhancing the neurodegenerative phenotype. To further test this idea, we asked whether the artificial activation of the pathway could modify the phenotype of the *vap* mutant. We have induced the activation of the Egfr/Ras transduction cascade using the inducible constructs hs-DER and hs-Rho. In both cases the ectopic activation of the pathway enhanced the *vap* phenotype. We also observed an enhancement of the *vap* phenotype when a wild-type allele of *Ras* was ectopically expressed in neurons using the GAL4-UAS system. Altogether these data indicate that in the brain of *vap* mutants there is an aberrant regulation of the Egfr/Ras pathway that is responsible for age-related brain degeneration and that this deregulation also takes place in the brain of adult flies, as judged by the result of the experiment with the temperature-sensitive allele of the *Egfr*.

Inhibitory proteins such as RasGAP that inactivate Ras are important to set both the right levels of Ras activation and the duration of the Ras downstream signal. An aberrant regulation of Ras might, therefore, lead to the subsequent deregulation of Ras-dependent pathways that are switched on after growth factor stimulation. We have found a deregulation of the MAPK activation in *vap* larvae and adult flies

after stimulation by inducing a pulse of *Egfr* expression. The result also shows that, as expected, in *Drosophila*, RasGAP is not required for the activation of MAPK. We could not find a genetic interaction between *vap* and a null allele of *Raf* or with the Drosophila MAPK *rolled*. These results suggest that upregulation of the Raf/MAPK pathway might not be responsible for the phenotype in *vap* flies. Alternatively, *vap* does not provide a sensitive mutant background for genetic interaction with downstream elements of Ras. The expression in adult *vap* flies of $Ras1^{V12S35}$, an activated form of Ras that specifically signals to the Raf/MAPK pathway, leads to lethality, preventing us from assessing the contribution of the Raf/MAPK pathway to the neurodegenerative phenotype. The above results, together with the fact that no interaction was found with overactivated Ras/PI3K or Ras/RalGDS pathways in *vap* flies suggest that other downstream elements of Ras, acting alone or in conjunction with MAPK, might be necessary to induce neurodegeneration. Alternatively, the lethality and brain degeneration phenotypes observed in *vap* flies could be a consequence of the simultaneous deregulation of different Ras-dependent pathways.

Our results provide the first evidence that RasGAP acts as a negative regulator of the Egfr/Ras signaling cascade in *Drosophila* neurons and that the aberrant regulation of this pathway leads to an extensive age-related degeneration of the adult brain. The *vap* mutant offers an invaluable opportunity to assess the role of this important signaling pathway in differentiated neurons and a new model to study the process of autophagic cell death in the context of neurodegenerative diseases.

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