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Expanded polyglutamine peptides disrupt EGF receptor signaling and glutamate transporter expression in *Drosophila*

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Huntington's disease (HD) is a late onset heritable neurodegenerative disorder caused by expansion of a polyglutamine (polyQ) sequence in the protein huntingtin (Htt). Transgenic models in mice have suggested that the motor and cognitive deficits associated to this disease are triggered by extended neuronal and possibly glial dysfunction, whereas neuronal death occurs late and selectively. Here, we provide *in vivo* evidence that expanded polyQ peptides antagonize epidermal growth factor receptor (EGFR) signaling in *Drosophila* glia. We targeted the expression of the polyQ-containing domain of Htt or an extended polyQ peptide alone in a subset of *Drosophila* glial cells, where the only fly glutamate transporter, dEAAT1, is detected. This resulted in formation of nuclear inclusions, progressive decrease in *dEAAT1* transcription and shortened adult lifespan, but no significant glial cell death. We observed that brain expression of *dEAAT1* is normally sustained by the EGFR-Ras-extracellular signal-regulated kinase (ERK) signaling pathway, suggesting that polyQ could act by antagonizing this pathway. We found that the presence of polyQ peptides indeed abolished *dEAAT1* upregulation by constitutively active EGFR and potently inhibited EGFR-mediated ERK activation in fly glial cells. Long polyQ also limited the effect of activated EGFR on *Drosophila* eye development. Our results further indicate that the polyQ acts at an upstream step in the pathway, situated between EGFR and ERK activation. This suggests that disruption of EGFR signaling and ensuing glial cell dysfunction could play a direct role in the pathogenesis of HD and other polyQ diseases in humans.

INTRODUCTION

Nine progressive neurodegenerative diseases are caused by the expansion of a CAG repeat sequence in specific genes, leading to abnormally long polyglutamine (polyQ) tracts in the corresponding proteins. These disorders include Huntington's disease (HD), spinocerebellar ataxia 1 (SCA1), SCA2, SCA3 (Machado–Joseph disease), SCA6, SCA7 and SCA17, as well as dentatorubral pallidoluysian atrophy (DRPLA) and spinal bulbar muscular atrophy (SMA, Kennedy's disease) (reviewed in 1,2). All these diseases show, as common features, the progressive degeneration of neuronal subsets in distinct brain areas and the formation of polyQ-containing protein aggregates forming characteristic nuclear or cytoplasmic inclusions. The age at which the first symptoms appear correlates with the length of the glutamine repeat. For instance, HD symptoms are observed when patients carry more than 36 CAG repeats in the first exon of the huntingtin (Htt) gene (3,4), and juvenile-onset HD patients present more than 65 CAG repeats (5). It is now assumed that brain cell dysfunction precedes the late and selective neuronal loss in these diseases (2,6). Although the role of expanded polyQ tract expression in the pathogenesis is well established, the contribution of aggregates remains unclear (7).

The different polyQ disorders share common deleterious gain-of-function mechanisms that could trigger neuronal dysfunction. PolyQ disease proteins interact with molecular chaperones and proteasome subunits and this would inhibit the degradation of misfolded proteins and increase their accumulation (8). The mutant proteins could also interfere with gene expression by binding directly to transcriptional regulatory proteins (9–14). Neurotransmitter signaling pathways are

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affected by the HD mutation in transgenic mouse models (15–18), as well as the expression of brain-derived neurotrophic factor (BDNF), a neuromodulator and survival factor (19). Finally, recent evidence indicates that both HD and Kennedy's disease proteins disrupt axonal transport (20,21).

Some data suggest that glial cell function is also compromised in polyQ pathologies. In mouse HD models, glial glutamate uptake is reduced (22,23). This is because of selective downregulation of the glutamate transporter GLT1/EAAT2, which is mainly expressed in astrocytes. The expression of glutamine synthetase, an enzyme that converts glutamate to glutamine in glia, was also found to be altered (22,23). However, the potential implication of glial cells in HD pathogenesis has not been thoroughly investigated. Whether glial dysfunction is secondary to neuronal impairment or results from a direct mechanism remains to be clarified.

The fruit fly *Drosophila melanogaster* recently proved to be a powerful model organism to study human neurodegenerative diseases and in particular polyQ-mediated diseases (reviewed in 6,24–28). Here, we used *Drosophila* models to investigate the potential roles of glial cell dysfunction and alteration in epidermal growth factor receptor (EGFR) signaling in HD pathogenesis. In *Drosophila*, a single glutamate transporter (dEAAT1) is present, which is expressed at all developmental stages in a glia subset (29–32) and it is required for glutamate buffering and adult fly survival (33). Using the UAS–GAL4 bipartite system (34), we expressed selectively the polyQ-containing domain of human Htt (Httex1p) or an expanded polyQ alone (13) in the *dEAAT1*-expressing glial cells. This led to a progressive decrease in glutamate transporter expression, as in mouse HD models, and a marked shortening of adult fly lifespan. We provide evidence that the polyQ peptides potently antagonize EGFR signaling in *Drosophila* glia, as well as in the fly eye, by acting at a step upstream of extracellular signal-regulated kinase (ERK) activation and that this can at least in part account for glutamate transporter downregulation. Therefore, our results suggest that glial cell dysfunction directly contributes to the pathogenesis in HD and results from a disruption of the EGFR signaling pathway induced by expanded polyQ peptide accumulation.

RESULTS

Expression of expanded polyQ peptides in *Drosophila* glia

The transgenes we used to express polyQ peptides in *Drosophila* contained either the first exon of human Htt with 20 or 93 CAG repeats (Httex1p Q20 or Httex1p Q93) or 48 CAG repeats only with a myc/flag tag (Q48-myc) (13). To activate expression, the corresponding UAS strains were crossed with *dEAAT1-GAL4* flies, which express GAL4 in a subset of glia under regulation of the glutamate transporter promoter (33). The somas of these cells lie in the cortex at the periphery of the brain (Fig. 1B), and they send cytoplasmic projections in the central neuropil, where synaptic contacts occur and glutamate transporter is selectively addressed (33). Control flies heterozygous for the *dEAAT1-GAL4* insertion or *dEAAT1-GAL4*, *UAS-Httex1p Q20* flies showed no behavioral deficits and lived $\geq$60 days at 25°C. In contrast, flies expressing Q48-myc or Httex1p Q93 in *dEAAT1-GAL4*-positive glia subsets died much earlier (Fig. 1A) and were found to be lethargic, a few days before dying. The mean lifespan was ~21 days for Q48-myc and Httex1p Q93-expressing flies. Few of them survived >30 days. The Httex1p Q93- and Q48-expressing flies exhibited no significant loss of *dEAAT1*-expressing cells in the brain and optic lobes when compared with wild-type flies as late as at 21 days (Fig. 1C). This indicates that the early lethality of these flies most likely resulted from glial dysfunction rather than cell death.

We observed Htt-containing aggregates all over the adult brain and optic lobes in glial cells expressing Httex1p Q93, but not Httex1p Q20 (Fig. 2A, panels 1 and 2). Aggregate formation also occurred in Q48-myc-expressing flies as assessed by c-Myc immunostaining (Fig. 2A, panels 3 and 4). These inclusions were found to be located in the nucleus and to grow with the age (Fig. 2B). At 2 days of adult age, Hoescht-stained nuclei showed one or several small aggregates (panels 1 and 3), whereas at 10 days they generally presented a single large inclusion (panels 2 and 4).

Expanded polyQ peptides decrease glutamate transporter level and expression

Protein levels of the glutamate transporter dEAAT1 were assessed in flies expressing GFP tagged-dEAAT1 (*UAS-dEAAT1–GFP*) (33) in the absence (control) or presence of polyQ peptides under control of the *dEAAT1-GAL4* driver. Figure 3A shows representative views of adult brains at 2, 5 and 14 days of age. Note that the whole CNS appears fluorescent, because the transporter is ubiquitously addressed to the different neuropil regions and excluded from the glial cell bodies (33). Quantitative analysis of fluorescence in the optic lobes is presented in Figure 3C. No age-related change in dEAAT1–GFP level was detected in the presence of Httex1p Q20 or in control flies. In contrast, the presence of expanded polyQ tracts led to a progressive decrease in fluorescence intensity. The intensity decreased to 43 and 22% at 14 days for Httex1p Q93- and Q48-expressing flies, respectively. In wild-type flies, western blot experiments similarly demonstrated a significant decrease of endogenous dEAAT1 protein level in the presence of expanded polyQ peptides, which could be demonstrated as early as at 7 days of adult age (Fig. 3D).

This decrease in expression level could result from either a degradation of the dEAAT1 protein or an inactivation of the *dEAAT1* promoter. We tested the efficiency of the *dEAAT1* promoter by using *UAS-mCD8-GFP*, a non-specific membrane marker, instead of *dEAAT1-GFP*, as a reporter gene. Expression of expanded polyQ peptides induced a marked reduction (~70%) in mCD8-GFP signal at 14 days (Fig. 3B and C), indicating that *dEAAT1* downregulation is at least in part caused by inhibition of gene expression. This was confirmed by direct analysis of mRNA levels by RT–PCR. The levels of *dEAAT1* mRNA were markedly reduced in 7-day-old (data not shown) and 14-day-old (Fig. 3D) Httex1p Q93- and Q48-expressing flies compared with Httex1p Q20 and control flies, whereas actin mRNA levels remained unchanged. Therefore, the presence of expanded polyQ peptides in *Drosophila* glia potently inhibits glutamate transporter transcription.

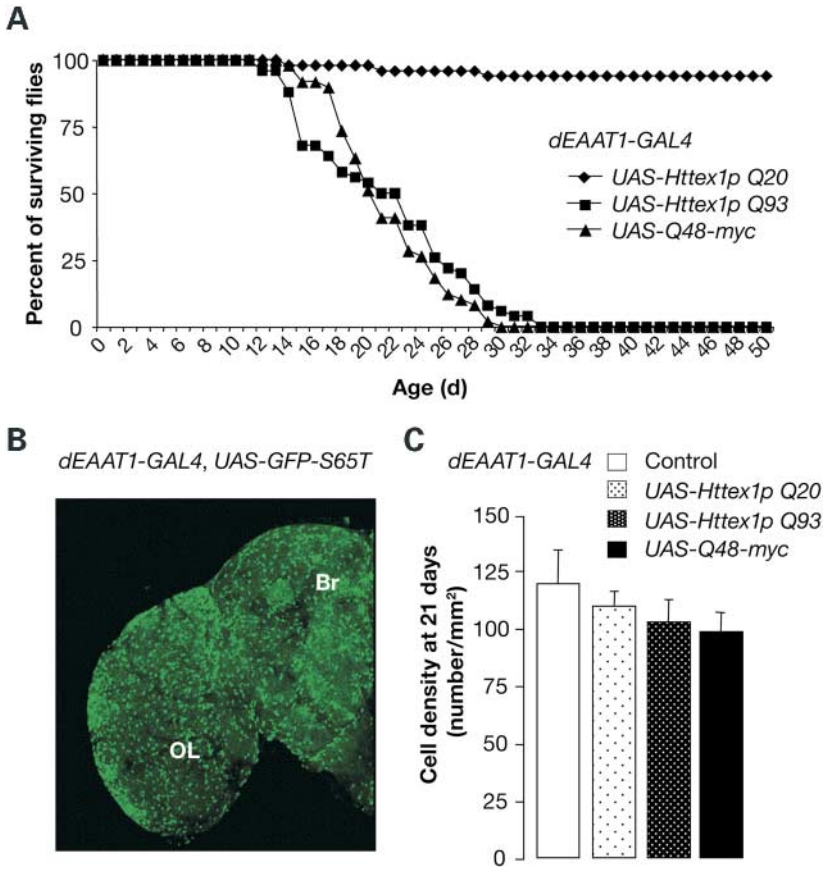

Figure 1. Expression of expanded polyQ peptides in *Drosophila* glial cells reduces adult lifespan. (**A**) Lifespan analysis of adult flies expressing the polyQ-containing peptides Httex1p Q20, Httex1p Q93 or Q48-myc in a subset of glial cells, which express the glutamate transporter dEAAT1. (**B**) Whole-mount adult *Drosophila* CNS showing ubiquitous distribution of the dEAAT1-expressing glial cell bodies in brain (Br) and optic lobe (OL). Glial cell bodies were revealed by autofluorescence of the cytoplasmic GFP-S65T marker expressed with the *dEAAT1-GAL4* driver. (**C**) Density of *dEAAT1*-expressing cells determined in multifocal confocal views of whole brains of 21-day-old adult flies expressing either no transgene (control) or polyQ-containing peptides Httex1p Q20, Httex1p Q93 or Q48-myc in glial cell subsets under control of the *dEAAT1-GAL4* driver. Data are the mean ± SEM of values obtained in three to six flies per condition. The differences between the cell numbers counted in control and polyQ-expressing flies were not statistically significant.

dEAAT1 expression is regulated by the EGFR signaling and the Ras/ERK pathway

In vitro studies in mammalian cells have suggested that glial glutamate transporter expression can be independently upregulated by the activation of EGFR signaling and the p42/p44 MAP kinase/ERK or phosphatidylinositol 3-kinase (PI3K) pathway (35–38). We investigated the potential role of these regulators on *Drosophila* glutamate transporter expression *in vivo* by a transgenic approach. The dEAAT1–GFP fusion protein was co-expressed with constitutively active *Drosophila* Egfr (λtorpedo), Ras (Ras85D.V12) or Erk (rolled^{SEM}) under regulation of *dEAAT1-GAL4*. This generally led to increased GFP fluorescence intensity in both the optic lobes and brain of 7- and 14-day-old flies, compared with control flies (Fig. 4A and B). Active Ras seems to be more efficient at stimulating dEAAT1 expression in 7-day-old flies than in 14-day-old flies, but this is most certainly explained by the deleterious effects of active Ras on fly survival after 2 weeks when it is expressed in glial cells (data not shown). Overall, these results indicate that activation of both the EGFR and Ras/ERK pathway upregulates *dEAAT1* expression in living flies.

In normal conditions, ERK is activated by phosphorylation in the cytoplasm and then translocated into the nucleus. This nuclear translocation can be prevented by overexpression of a cytoplasmic ERK-docking molecule, the inactive MAP kinase phosphatase 3 (MKP3-C/S) (39,40). Expression of MKP3-C/S significantly decreased dEAAT1–GFP levels when compared with control (Fig. 4A and B). Expression of dominant negative Ras (Ras85D.N17) also significantly inhibited dEAAT1 at 14 days, but not as much as at 7 days, suggesting that its effect is progressive and becomes obvious only in older flies. These results suggest that the Ras/ERK pathway is required for normal level of *dEAAT1* expression during the adult life. In contrast, overexpression of *Drosophila PI3K* (*Dp110*) under the control of *dEAAT1-GAL4* did not alter the dEAAT1–GFP fluorescence level (Fig. 4C and D). This indicates that regulation of glutamate transporter expression by EGFR signaling does not involve the PI3K pathway in the *Drosophila* brain.

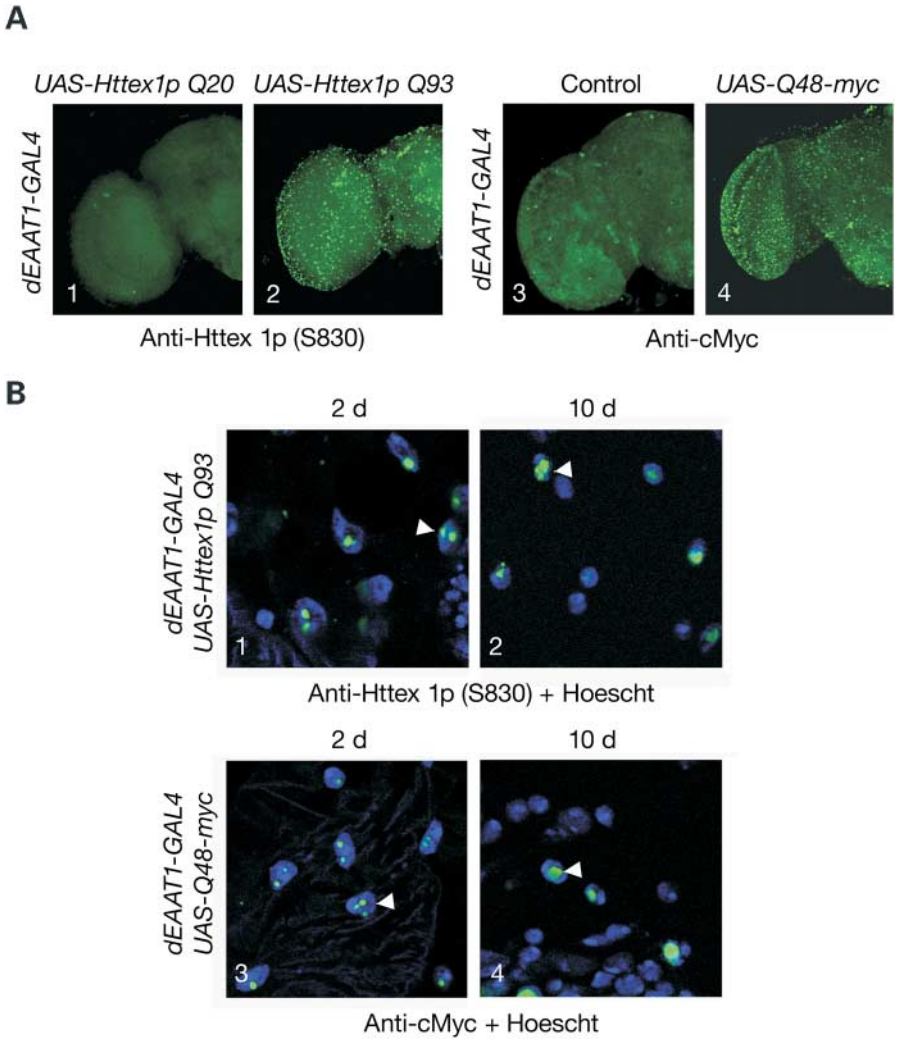

Figure 2. Expanded polyQ forms intranuclear inclusions in *Drosophila* glial cells. (**A**) Htt exon 1 (S830 antibody) (panels 1 and 2) or c-Myc (panels 3 and 4) immunostaining in whole-mount brains of 7 day-old adult flies showing the presence of numerous aggregates in glial cells expressing the expanded polyQ peptides only. Note that the background labeling in panel 3 does not correspond to aggregates but to non-specific staining of nerve cells by the c-Myc antibody. Magnification of photomicrographs: x60. (**B**) Co-staining with Hoescht 33258 marker and Htt exon 1 (panels 1 and 2) or c-Myc (panels 3 and 4) antibodies at 2 and 10 days of adult age, indicating the nuclear localization of the aggregates. Several small aggregates were found in the nucleus at 2 days of age, whereas they formed a single large aggregate at 10 days of age. Arrowheads point out characteristic inclusions. Magnification: 450x.

Expanded polyQ prevents EGFR- but not Ras/ERK-induced dEAAT1 upregulation

The previous results suggested to us that downregulation of *dEAAT1* by the expanded polyQ peptides (Fig. 3) could result from an inhibition of EGFR signaling. As shown in Figure 5A and B, the presence of Httex1p Q93 or Q48-myc peptides in glial cells indeed abolished the upregulation of *dEAAT1-GFP* expression induced by constitutively active *Drosophila Egfr* in the brain and optic lobes of 10- and 15-day-old flies. In contrast, we observed that active *Erk* was still able to increase dEAAT1–GFP expression in the presence of Httex1p Q93 (Fig. 5C) and the same result was obtained with active Ras and Q48-myc (Fig. 5D). Although we did not test all the possible combinations, these results suggest that expanded polyQ inhibits EGFR signaling, and consequently dEAAT1 expression, at a step upstream of Ras/ERK activation. Alternatively, co-expression of active Erk or Ras could be more potent than that of active EGFR and strong enough to overcome polyQ-induced inhibition of the pathway.

Expanded polyQ peptides prevent EGFR-induced ERK activation

We, therefore, directly examined the effect of polyQ peptides on the activation state of ERK with a specific antibody against the phosphorylated form of this protein. Western blot experiments were performed on protein extracts from adult fly heads. First, ERK phosphorylation was found to be significantly increased by the expression of constitutively active *Egfr* in *dEAAT1*-expressing glial cells (Fig. 6). Secondly, we observed that co-expression of either Httex1p Q93 or Q48-myc strongly inhibited EGFR-mediated ERK phosphorylation.

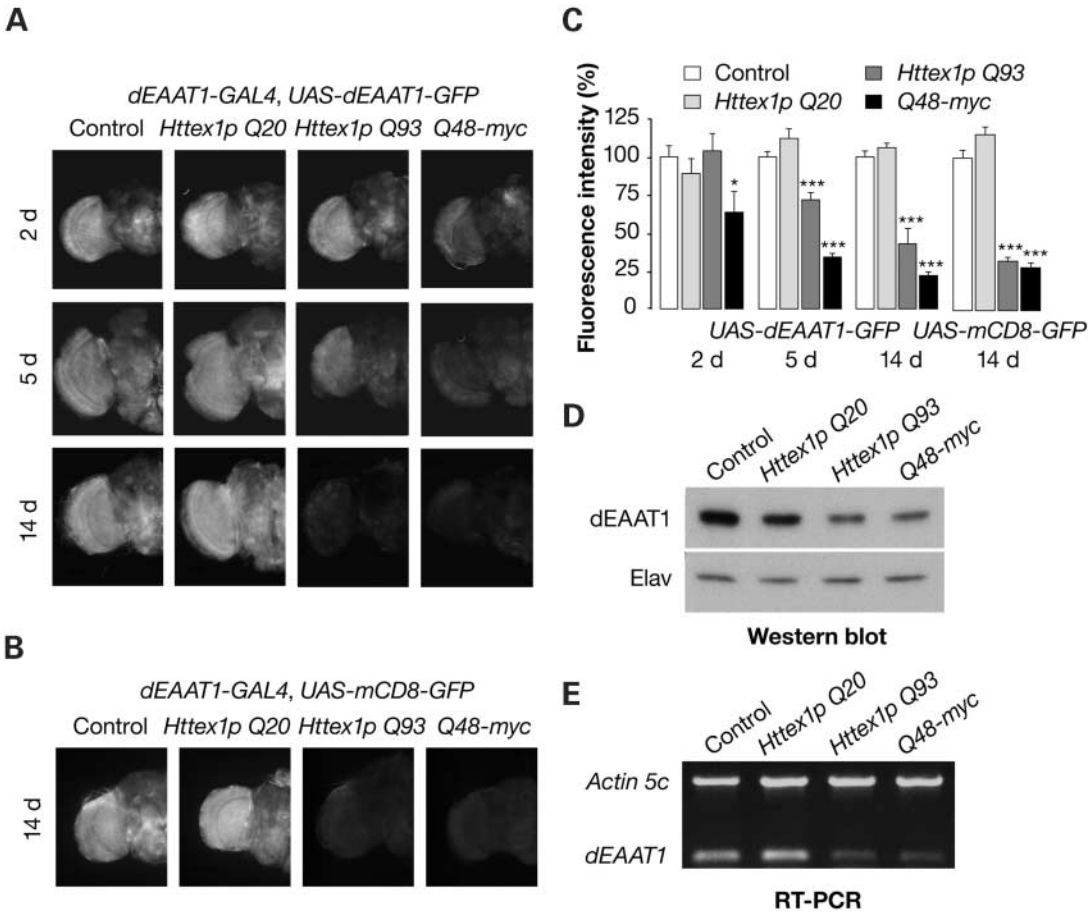

Figure 3. Expression of the *Drosophila* glutamate transporter dEAAT1 is progressively decreased in the presence of polyQ peptides. (**A**) Photomicrographs showing autofluorescence levels in whole-mount brains from 2-, 5- and 14-day-old adult flies. The dEAAT1–GFP fusion protein was expressed in glial cell subsets with the *dEAAT1-GAL4* driver either alone (control) or together with the polyQ-containing peptides Httex1p Q20, Httex1p Q93 or Q48-myc. A progressive decline in the expression of GFP-tagged dEAAT1 was observed in the presence of Httex1p Q93 and Q48-myc only. Magnification: 50x. (**B**) Similar experiments performed by replacing dEAAT1–GFP with the generic membrane marker mCD8-GFP. The autofluorescence of mCD8-GFP was also reduced at 14 days in the presence of expanded polyQ. (**C**) Quantitative analysis of fluorescence intensity in the optic lobes. Data are the mean ± SEM of values obtained in six to nine flies per condition. Statistical significance: $^{*}P > 0.05$ and $^{***}P > 0.001$. (**D**) Western blot analysis of dEAAT1 and Elav proteins in head extracts of 7-day-old flies expressing no transgene (control), Httex1p Q20, Httex1p Q93 or Q48-myc under control of *dEAAT1-GAL4*. The presence of expanded polyQ tracts in glial cells decreased endogenous dEAAT1 levels 1 week before start of the lethality effect. (**E**) RT-PCR analysis of *dEAAT1* and *Actin5C* mRNA levels in *Drosophila* heads at 14 days of age. Same transgenic backgrounds as in (D). A marked and selective decrease in glutamate transporter mRNA levels was observed when peptides containing expanded polyQ tracts were expressed in *dEAAT1*-expressing glial cells.

This result confirmed that the presence of expanded polyQ peptides in *Drosophila* glia disrupts the EGFR signaling pathway at a step upstream of ERK activation.

Expanded polyQ alters the EGFR pathway in the *Drosophila* eye

To attest further that expanded polyQ peptides antagonize EGFR signaling *in vivo*, we expressed the Q48-myc peptide in the eye with the *GMR-GAL4* driver. The *Drosophila* eye depends on regulated EGFR pathway for its development (41,42). The eye structure of Q48-expressing flies was apparently normal but presented a characteristic depigmentation (Fig. 7A, panel 2), which increased with the age, as previously described (43). We observed that expression of constitutively active *Egfr* with *GMR-GAL4* is pupal lethal (panel 3). However, when active *Egfr* was co-expressed with Q48-myc, a few adult escapers survived, which showed rough and severely collapsed eyes (panel 4). Thus, polyQ expression can in part rescue the deleterious effects of constitutively active *Egfr*. In contrast, the rough eye phenotype induced by expression of active *Erk* (panel 5) was not rescued by the polyQ peptide. When active *Erk* was combined with Q48-myc, fly eyes appeared similarly rough and also depigmented (panel 6). This indicates again that expanded polyQ peptides do not interact with components of the EGFR signaling pathway downstream of ERK activation.

Because GAL4 is less effective at lower temperature, flies were grown at 18°C instead of 25°C and the eyes were examined by both light and scanning electron microscopy (SEM). At 5 days of age, Q48-expressing flies exhibited a less pronounced eye depigmentation than at 25°C (compare Fig. 7B, panel 1 to Fig. 7A, panel 2). Active *Egfr*-expressing flies survived at this lower temperature, but their eyes were

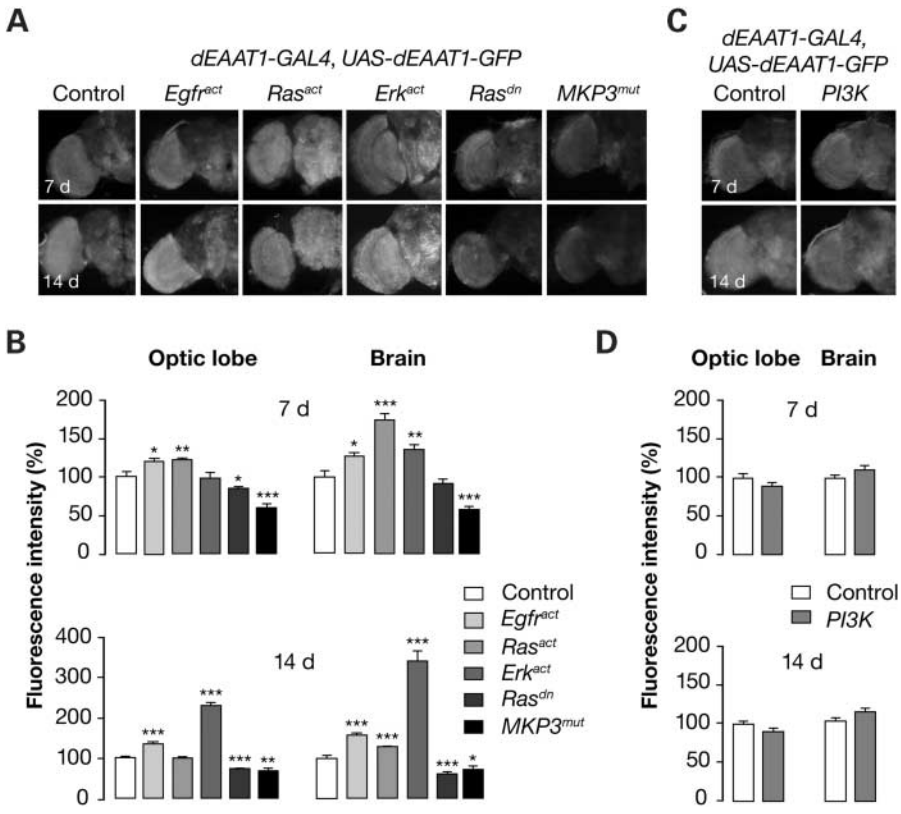

Figure 4. *dEAAT1* expression is regulated by the EGFR and Ras/ERK but not PI3K pathway. (**A** and **C**) Levels of fluorescence in whole-mount brains of 7- and 14-day-old flies co-expressing *dEAAT1–GFP* and no other transgene (control), constitutively active *Drosophila Egfr* ($Egfr^{act}$), *Ras* (Ras^{act}) or *Erk* (Erk^{act}), dominant negative *Ras* (Ras^{dn}), mammalian inactive *MKP3-C/S* ($MKP3^{mut}$) or *Drosophila* PI3K under control of *dEAAT1-GAL4*. Magnification: 50x. (**B** and **D**). Quantitative analysis of fluorescence intensity in the optic lobes and brains. Data are the mean of values obtained in six to nine flies per condition. Statistical significance: $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$. *dEAAT1–GFP* expression is significantly increased by activated EGFR, Ras and ERK and decreased by dominant negative Ras and mutant MKP3. In contrast, overexpression of *PI3K* is ineffective on dEAAT1 expression.

rough and reduced in size with fused ommatidia (middle panels). In these conditions, we observed that co-expression of Q48-myc significantly decreased the eye phenotype induced by active *Egfr* (right panels). Therefore, in the eye, as in the brain glial cells, expanded polyQ antagonizes EGFR signaling in *Drosophila*.

DISCUSSION

Possible role of glia in HD and other polyQ-mediated diseases

Despite the crucial role of glial cells in brain development and function, few data are currently available on their involvement in polyQ-mediated neurodegenerative diseases. Current hypotheses concerning HD pathogenesis were often focused on the putative neurotoxicity of neuronal nuclear aggregates. However, Htt is an ubiquitous protein, which is also expressed in astrocytes (44–46). Here, we expressed different polyQ peptides in a subset of *Drosophila* glial cells that produce the only fly glutamate transporter *dEAAT1* and account for ~10% of total brain glia. The expression of only 48 CAG repeats (Q48-myc) or the first exon of Htt containing 93 CAG repeats (Httex1p Q93) resulted in early adult lethality without triggering a massive degeneration of these cells (Fig. 1). Therefore, lethality is likely mediated by glial dysfunction rather than cell death. This conclusion is supported by the striking progressive decrease in *dEAAT1* expression observed in the whole brain and optic lobes in the presence of expanded polyQ (Fig. 3).

Expression of polyQ in *Drosophila* glia induced the appearance of numerous intranuclear inclusions (Fig. 2). Glial nuclear inclusions have been detected in mouse HD models, but their occurrence was found to be relatively rare when compared with neuronal inclusions (47). This could be related to the fact that nuclear localization of mutant Htt does not occur in dividing glial cells (48). Similarly, in the larval *Drosophila* brain, polyQ aggregates were found to be exclusively cytosolic in proliferating neuroblasts, whereas they were located in the nucleus in non-dividing neurons (49). Because nerve cell lineages are fixed and glial cells do not divide actively in the insect brain, mutant Htt is expected to accumulate in the nucleus in *Drosophila* glia. This could explain the higher occurrence of intranuclear polyQ-containing aggregates in fly glial cells.

However, the lack of intranuclear inclusions does not imply that the mammalian glial cells function normally in the presence of expanded polyQ proteins. In contrast, a selective

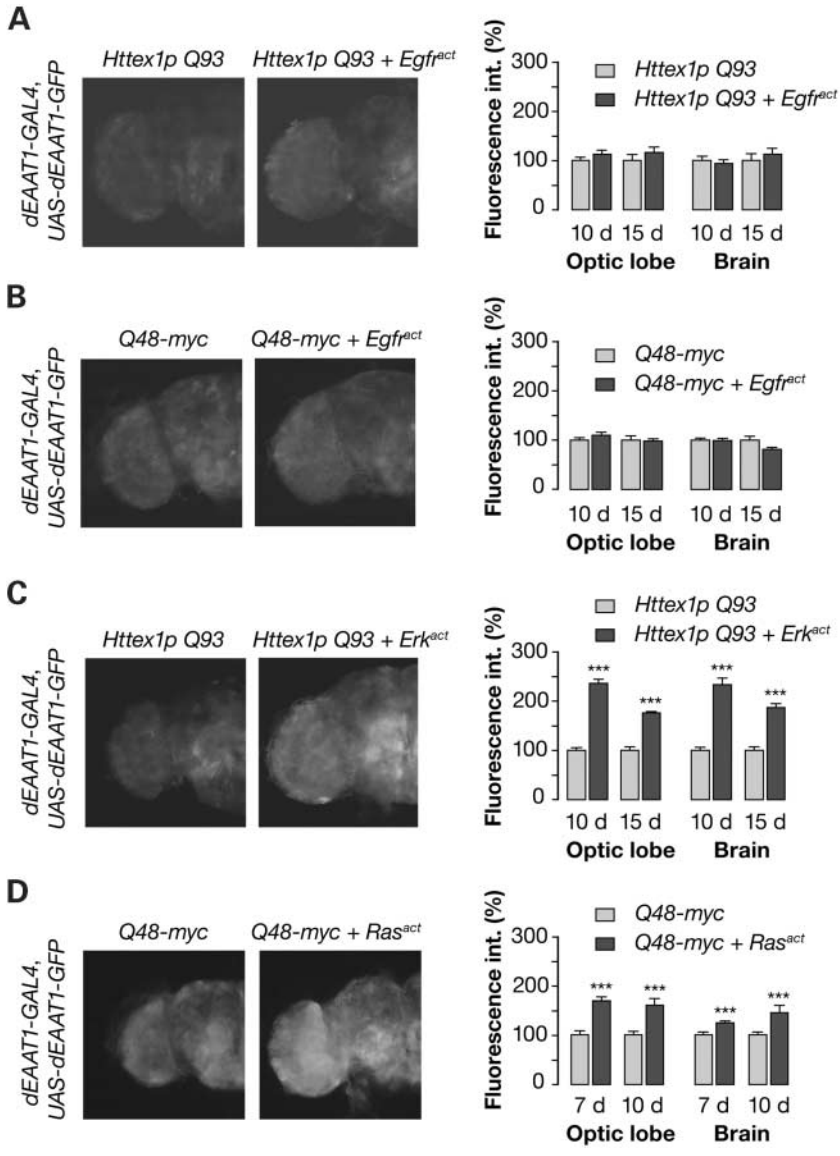

Figure 5. Expanded polyQ blocks EGFR- but not Ras/ERK-mediated dEAAT1 upregulation. Photomicrographs showing the autofluorescence of GFP-tagged dEAAT1 co-expressed with either Httex1p Q93 (**A** and **C**) or Q48-myc (**B** and **D**) in the absence (left panels) or presence (right panels) of constitutively active *Drosophila Egfr* (A and B), *Erk* (C) or *Ras* (D), under the control of *dEAAT1-GAL4.* Whole brains where dissected from 10-day-old flies. Magnification: 60x. Quantitative analysis of fluorescence levels was performed at 10 and 15 (A–C) or 7 and 10 (D) days of age in the optic lobes and brains of six to nine flies per condition. In D, experiments were conducted at 7 and 10 days because the expression of active Ras induced lethality at older ages. Statistical significance: $^{***}P < 0.001$. PolyQ peptides abolish EGFR-induced, but not Ras- or Erk-induced, increase in *dEAAT1* expression.

downregulation of the glial glutamate transporter GLT1/EAAT2 has been described in a transgenic mouse model of HD (22,23). Similarly, in post-mortem brains from HD patients, levels of glutamate transporter were found to be significantly and selectively reduced in the striatum, although astrocytes proliferate in this cerebral region (50,51). This is also principally because of lower cellular expression of the glial transporter GLT1/EAAT2 (51). These data are fully in agreement with the progressive decrease in dEAAT1 levels we observed when expanded polyQ peptides were expressed in *Drosophila* glial cells. Therefore, downregulation of glial glutamate transport is a constant feature of HD in human patients, as well as in the mouse and fly models. Assuming that comparable molecular mechanisms occur in vertebrate and *Drosophila* glia, we could infer from these observations that the formation of intranuclear inclusions is not involved in glutamate transporter downregulation.

It has long been proposed that glutamate excitotoxicity could be involved in disease progression in HD (52–55). In mouse HD models, NMDA glutamate receptors were found to be hypersensitive to agonist action (56–59). The presence of mutant Htt was shown to confer to medium-sized spiny striatal neurons, a higher vulnerability to NMDA receptor activation, which may explain their selective degeneration (60). We recently reported that inactivation of the glutamate transporter *dEAAT1* by RNA interference promotes neurite

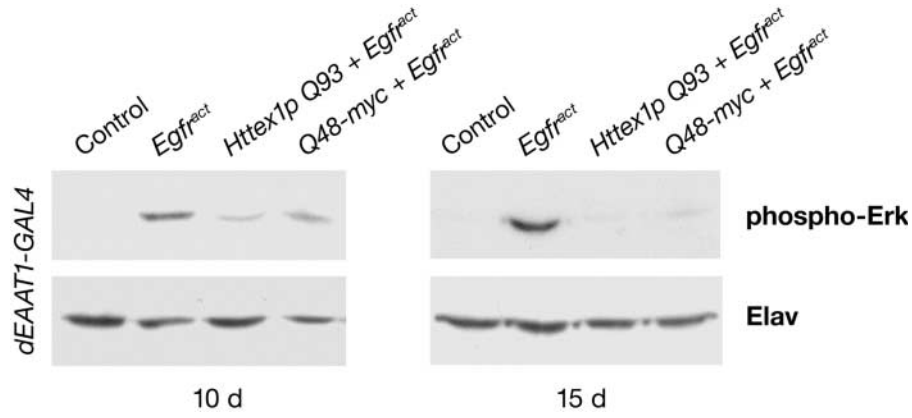

Figure 6. Expanded polyQ blocks EGFR-mediated ERK activation. Western blot analysis of phosphorylated ERK and Elav proteins in head extracts of 10- and 15-day-old flies expressing no *UAS* transgene (control), activated *Drosophila Egfr* alone or activated *Egfr* plus Httex1p Q93 or Q48-myc under control of *dEAAT1-GAL4*. The presence of expanded polyQ peptides prevented EGFR-induced ERK activation. Experiments were repeated three times at each time point with similar results.

degeneration in the fly brain and shortens adult lifespan (33). Our present results suggest that the expression of expanded polyQ proteins in glial cells perturbates their normal function, leading to reduced glutamate buffering capacity in brain and higher challenge to specific neurons that are already predisposed to excitotoxic damage. The pathological phenotypes described here may result in part from *dEAAT1* downregulation, but the lethality could result from decreased expression of other vital glial genes. Overall, this provides compelling evidence that the accumulation of expanded polyQ peptides in glial cells may directly contribute to disease progression in HD and other polyQ diseases.

Expanded polyQ inhibits EGFR signaling *in vivo*

Activation of EGFR signaling is started by receptor dimerization and tyrosine autophosphorylation and can induce activation of the Ras/ERK, phospholipase C or phosphatidylinositol 3-kinase (PI3K) pathway (61). In mammals, expression of GLT1/EAAT2 in cultured astrocytes is upregulated by the EGFR, Ras/ERK and PI3K pathways (35–38). Here, we present *in vivo* evidence that the fly glutamate transporter *dEAAT1* is upregulated when EGFR signaling and Ras/ERK pathway are activated in adult brain glial cells (Fig. 4). EGFR activation plays a major role in glial cell survival during embryonic development in *Drosophila* (62). Therefore, *dEAAT1* regulation could result from a direct effect on gene transcription or a better glial cell survival or both. In contrast, PI3K overexpression did not affect dEAAT1 expression, indicating that PI3K is not a regulator of glutamate transporter expression in *Drosophila*.

In vitro studies in PC12 cells have previously suggested that mutant Htt antagonizes EGFR activity (63,64). Here, we show that EGFR-mediated glutamate transporter upregulation is abolished in *Drosophila* glial cells in the presence of the polyQ peptides Httex1p Q93 or Q48-myc (Fig. 5). We further confirmed that expanded polyQ peptides antagonize EGFR action *in vivo* by co-expressing Q48-myc and *Egfr* in the *Drosophila* eye during development (Fig. 7). These results suggest that expanded polyQ peptides downregulate *dEAAT1* expression by disrupting EGFR signaling in glia. In contrast, expression of the polyQ peptides did not hamper stimulation of *dEAAT1* expression by active Ras or ERK (Fig. 5). Therefore, although the effect of EGFR on *dEAAT1* expression is likely mediated by ERK activation, polyQ appears to act on EGFR signaling at a step upstream of Ras/ERK. This is supported by the potent inhibitory effect of polyQ peptides on EGFR-induced ERK activation (Fig. 6). If the polyQ peptides acted downstream of ERK, they would not be expected to alter the level of ERK phosphorylation.

There are a number of possible mechanisms by which expanded polyQ repeat could lead to inhibition of EGFR signaling. Htt is associated to activated EGFR through SH3 domain-containing molecules such as Grb2 (growth factor receptor-binding protein 2) and RasGAP (65). It has been recently reported that the expression of full-length mutant Htt reduces the stability of the interaction between native Htt and Grb2 (63). As another possibility, mutant Htt could sequester Htt-associated protein-1 (HAP1) (66), which is required for EGFR signaling (64). Therefore, further work is required to understand precisely the effects of polyQ on EGFR action.

In conclusion, we provide *in vivo* evidence that polyQ expansion inhibits EGFR signaling in *Drosophila* by acting upstream of ERK activation. This effect can at least in part account for the progressive decrease in glutamate transporter expression observed when expanded polyQ peptides are present in glial cells. Such an inhibition of EGFR signaling could play a role in the disruption of essential glial and neuronal functions and later to the neurodegeneration in patients with HD or other polyQ-mediated diseases.

MATERIALS AND METHODS

Drosophila strains, culture and transformation

Unless otherwise specified, flies were grown at 25°C on a standard cornmeal–agar medium. The following fly stocks were used: *dEAAT1-GAL4* and *UAS-dEAAT1-GFP* (33); *UAS-Httex1p-Q20*, *UAS-Httex1p-Q93* and *UAS-Q48-myc/flag*

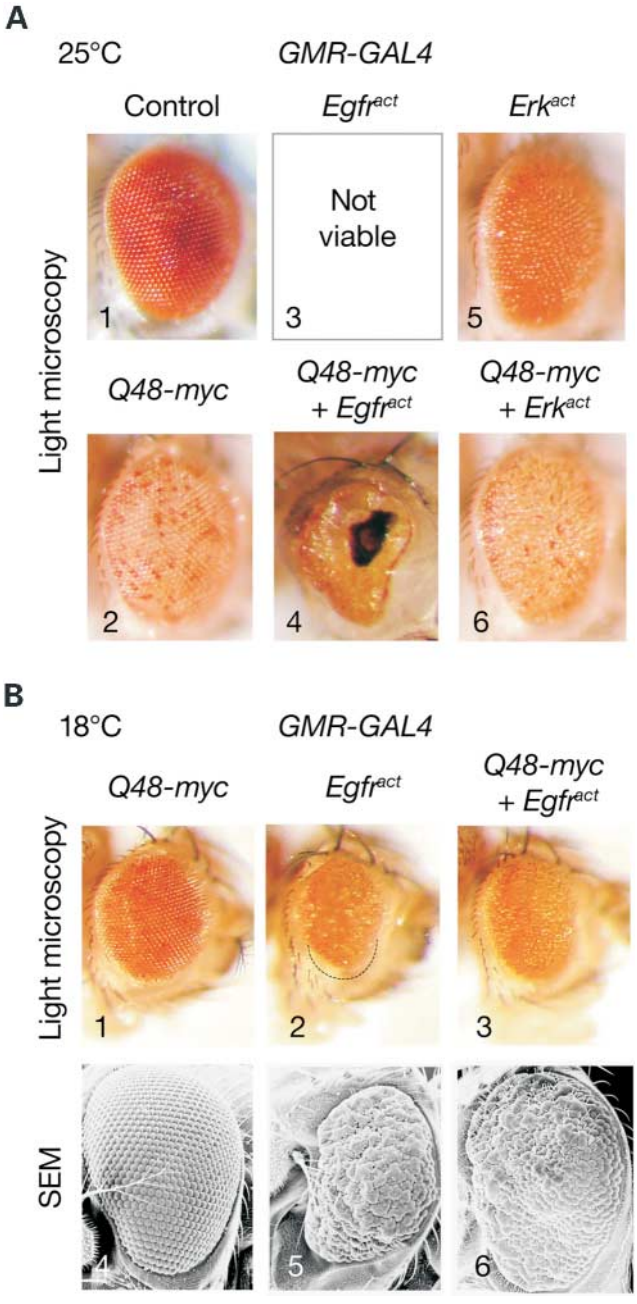

Figure 7. Expanded polyQ antagonizes EGFR but not ERK signaling in the *Drosophila* eye. External appearance of eyes from 5-day-old flies raised at 25°C (**A**) or 18°C (**B**) expressing, as indicated, no transgene (control), the Q48-myc peptide, activated *Drosophila Egfr*, Q48-myc plus activated *Egfr*, activated *Drosophila Erk* or Q48-myc plus activated *Erk*, under control of the *GMR-GAL4* driver. Note that no adults were recovered when $Egfr^{act}$ was expressed alone at 25°C. Light and SEM views. Presence of the polyQ peptide antagonized the deleterious effects of *Egfr* overexpression on development and eye formation.

(13), gifts of Dr L.M. Thompson (Irvine, CA, USA); *UAS-λtorpedo* ($Egfr^{act}$) (67), gift of Dr B. Charroux (Marseille, France); *UAS-rolled*^{SEM} (Erk^{act}) (68), gift of Dr D. Bohmann (New York, USA) and *UAS-Dp110 (PI3K)* (69), gift of Dr S.J. Leevers (London, UK). *UAS-MKP3-C/S* was constructed by insertion of the 1.7 kb *Eco* RI–*Xba* I DNA fragment from the recombinant PSG5 vector containing the inactive C/S mutant of the human MAP kinase phosphatase 3 (*MKP3*) gene (39) (gift of Dr S.M. Keyse, Dundee, UK) into the pUAST plasmid (34). Germ-line transformants were generated by standard procedure (70). All other strains (*GMR-GAL4*, *UAS-GFP-S65T*, *UAS-mCD8-GFP*, *UAS-Ras85D.N17* and *UAS-Ras85D.V12*) were obtained from the Bloomington *Drosophila* Stock Center.

Lifespan analysis

Newly eclosed flies were placed in vials (20–25 flies per vial) and incubated at 25°C. They were transferred into fresh vials every 4 days, and the number of surviving flies was recorded daily. Data correspond to the percentage of surviving flies as a function of time.

Reverse transcription–PCR

Total RNA was extracted from 15 adult fly heads, using the Nucleospin RNA II kit (Macherey Nagel). Reverse transcription was done with Superscript II reverse transcriptase (Invitrogen), and PCR was performed with the PCR Master Mix (MBI Fermentas). The *dEAAT1* and *Actin 5C* primers were added together in the same PCR tube. Cycling conditions were 35 cycles for 1 min at 94°C, 1 min at 65°C and 30 s at 72°C. The *dEAAT1* primers (5′-TCGACGGCTATGATGAGG and 5′-CACAGCCTCATAGAGAGCC) and *Actin 5C* primers (5′CGACAACGGCTCTGGCATGT and 5′-TCCATTGTGC ACCGCAAGTG) amplify a 212 and 1094 bp cDNA fragment, respectively. Control experiments were carried out in the absence of reverse transcriptase. PCR products were separated on 1% agarose gels and visualized by ethidium bromide staining. Each experiment was repeated three times with similar results.

In situ fluorescence

Whole *Drosophila* brains expressing GFP were dissected in 1 × phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde, rinsed in PBS and mounted in Vectashield (Vector). Microscopic examination was performed using a Zeiss Axioplan 2 fluorescent microscope equipped with a Nikon digital camera (DXM 1200). Quantification of the signal was done with the ImageJ software. Fluorescent intensities were expressed as percent of the mean of control flies. Data from six to nine flies per condition were averaged and are presented as mean ± SEM. Statistical analysis was performed using a Student's *t*-test. A significance of $P < 0.05$ was required for rejection of the null hypothesis. Confocal micrographs were produced using a Zeiss confocal LSM 510 microscope and Zeiss LSM 5 image browser software. Density of *dEAAT1*-expressing cells was determined on multifocal confocal views of whole brains expressing *GFP-S65T* with the *dEAAT1-GAL4* driver to visualize glial cell bodies (33). Cells were counted in two 0.4 mm^2 areas in the brain and optic lobes of three to six flies. Data were averaged and are presented as mean ±SEM.

Immunocytochemistry

Whole fly brains were dissected, fixed in paraformaldehyde and incubated in primary and secondary antibodies as described (71). Detection of aggregates in UAS-Httex1p-Q93 expressing flies was performed using the polyclonal sheep S830 antibody (1/1000 dilution), which was raised against a Htt GST exon 1 fusion protein containing 51 glutamines (72,73). Detection of aggregates in *UAS-Q48-myc/flag* expressing *Drosophila* was done using a polyclonal rabbit anti-cMyc A14 antibody (Santa Cruz Biotechnology; 1/1000 dilution). TRITC-labeled anti-sheep or anti-rabbit antibodies (Jackson immunoResearch) were used as secondary antibodies at 1/200 dilution. The nuclear stain Hoescht 33258 (Sigma) was used at 1:200 dilution. Mounting was performed in Vectashield (Vector).

Western blot analysis

Twenty *Drosophila* heads were thawed and then homogenized in 20 mM NaPi (15.5 mM Na_2HPO_4, 4.5 mM NaH_2PO_4, pH 7.4) containing 1 mM phenylmethylsulfonyl fluoride and a protease inhibitor cocktail (Boehringer Mannheim, Germany). After 30 s sonication and 2 min centrifugation at 4°C (15 000*g*), 1/5 (v/v) sample Laemmli buffer was added to the supernatant. Total protein was electrophoresed through a 10% polyacrylamide resolving gel and electroblotted onto Hybond-P membrane (Amersham Pharmacia Biotech). Before immunodetection, blots were stained with Ponceau-S to visualize transfer efficiency. Blots were blocked in PBST (1 × PBS, 0.1% Tween-20) containing 5% dry milk for 1 h and incubated with purified rabbit polyclonal anti-dEAAT1 antibodies (1/300 dilution) or monoclonal anti-phosphorylated ERK antibodies (1 μg/ml, clone MAPK-YT, Sigma) overnight at 4°C. Blots were washed in PBST, incubated with peroxidase-labeled anti-rabbit or mouse secondary antibodies (1/5000; Jackson ImmunoResearch) for 2 h and rinsed in PBST. The signal was visualized on Kodak MR films with enhanced chemiluminescence (ECL, Pierce). The blots were then washed in stripping buffer (62.5 mM Tris–HCl pH 6.8, 2% SDS, 100 mM β-mercaptoethanol) for 15 min at room temperature and reprobed using rat anti-Elav antibodies (1/1000; Hybridoma Bank) and anti-rat secondary antibodies (1/5000; Jackson ImmunoResearch). Each experiment was repeated twice or three times.

Scanning electron microscopy

Flies were washed in 1 × PBS, fixed in 2.5% glutaraldehyde for 4 h, rinsed in 1 × PBS and post-fixed in 1% osmium tetroxide for 1–2 h. They were dehydrated once in 30, 50, 70, 95 and 100% ethanol for 30–60 min, twice in hexamethyldisilidane for 1 h and finally once overnight. Fly heads were then mounted on pin mounts and covered with gold on S150B Edwards. Observation was performed on MEB Leica 440.

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NOTE ADDED IN PROOF

In agreement with the results we show in Figures 1 and 2, it has been lately reported that expression of a polyQ-containing fragment of the SCA3 protein in *Drosophila* glia induces formation of nuclear aggregates and leads to early adult lethality (74).

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