AKT-sensitive or insensitive pathways of toxicity in glial cells and neurons in *Drosophila* models of Huntington’s disease

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Huntington’s disease (HD) is caused by an extended polyglutamine (polyQ) tract in the Huntingtin protein. Neuronal and glial dysfunction precedes the neurodegeneration and appears to be the primary cause for the early symptoms in HD. In recent years, development of *Drosophila* models of polyQ-related diseases facilitated research of candidate rescuer genes. In most cases, analysis in *Drosophila* was performed by assessing toxicity on retinal and/or brain neurons. However, none of the potential rescuers were evaluated on glial alterations. Here we used a genetic approach in *Drosophila* to characterize the phenotypic effects of mutant Huntingtin (mHtt) expressed in neurons or different glia subsets and we established a sensitive assay for evaluating modifiers of glial alterations. We determined the level of cell protection ensured by activation of the AKT and ERK anti-apoptotic kinases in the retina as well as in neurons and glia of the fly brain, compared with the rescuing effects of the HSP70 chaperone. We found that both AKT and HSP70 alleviated mHtt-induced toxicity in the retina. In contrast, their protective effects differed in the brain. HSP70 rescued neurodegeneration, locomotor defects and early lethality of flies expressing mHtt in neurons or glia. AKT failed to prevent brain neuronal death and lethality of flies, but significantly improved their locomotor performance when co-expressed with mHtt in glia. ERK had no beneficial effects in the retina or brain. These results indicate that mHtt activates distinct pathways of toxicity in *Drosophila*, either sensitive to AKT in retinal photoreceptors and glia, or independent in brain neurons.

INTRODUCTION

Huntington’s disease (HD) is an inherited neurodegenerative disorder caused by the expansion of a CAG triplet repeat in the first exon of the IT15 gene, resulting in increased length of a polyglutamine (polyQ) stretch in the Huntingtin protein (Htt). Pathological symptoms appear with polyQ repeat >37 glutamine residues (1,2). At least eight additional neurodegenerative diseases including spinocerebellar ataxia (SCA) 1, 2, 3, 6, 7 and 17, dentatorubral pallidoluysian atrophy and bulbospinal muscular atrophy (SBMA) are also caused by expanded polyQ tract in specific proteins. Htt is ubiquitously expressed in brain, highly in neurons but yet significantly in glia. Moderate levels are also present in peripheral tissues such as testes, liver, heart and lungs (3–8). Despite such a widespread distribution, mutant Huntingtin (mHtt) induces late-onset neuronal loss preferentially in the striatum and cerebral cortex (9). There is evidence that cell brain function impairments preceed neurodegeneration and appears to be the primary cause for the early phases of HD pathogenesis (10,11).

Over the last decade, neuronal dysfunction and death were the focus of many studies. Only recently, it was established that expression of mHtt in glia directly compromises the functions of these cells, such as glutamate removal from synaptic cleft (7,12). In post-mortem brains from HD patients, glial expression of glutamate and glucose transporters was found

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to be significantly reduced in the striatum (13,14), although astrocytes proliferate in this cerebral structure. Moreover, activated astrocytes express high levels of the apoptosis-related markers caspase-3 and poly(ADP-ribose) polymerase (15,16). Thus, disruption of glia functions and neuron–glia interaction might further challenge neurons already altered by intrinsic dysfunction. Testing therapeutic candidates on both neuronal and glial pathologies could prove invaluable to define novel reasoned strategies to treat HD.

The fruit fly Drosophila melanogaster is a powerful model organism to identify pathways involved in HD pathogenesis. Fly models replicate HD pathological features including axonal and glial dysfunctions, neuronal loss restricted to some cerebral structures and shortened adult lifespan (12,17–21). In recent years, a number of candidate rescuer genes were discovered from genetic analysis of polyQ diseases in Drosophila. The most effective modifiers were generally molecular chaperones and apoptosis- or prosurvival-related proteins. For instance, they include chaperone proteins such as HSP40, HSP70, HSC70 and Tetratricopeptide repeat protein 2 (TPR2) (22–28). Genetic manipulation of genes regulating apoptosis such as dIAP1, Apaf-I and p53 (28–30) was also found to modulate polyQ toxicity. In most cases, rescuer gene analysis was performed by expressing expanded polyQ peptides in the fly retina and some of them were further tested on neuronal pathology. However, none of them were evaluated on glial pathology.

Epidermal growth factor receptor (EGFR) signalling is known to play major roles in cell development, differentiation and survival and is a key actor in the induction and maintenance of the astroglial reactivity in pathological situations (31). We and others reported that mHtt antagonizes the activation of AKT and ERK kinases by EGFR in cell-based and Drosophila HD models (12,32–34). Here we used a genetic approach in Drosophila to characterize the phenotypic effects of mHtt expression in neurons or different glia subtypes. We first developed a sensitive assay for evaluating modifiers of glial alterations. Then we evaluated the potential benefits of anti-apoptotic AKT and ERK kinases and HSP70 chaperone when co-expressed with mHtt in the eye photoreceptors and brain neurons or glia. Although both AKT and HSP70 alleviated the mHtt-induced cell toxicity in the retina, they did not share similar protective effects in the brain. HSP70 widely rescued neuronal degeneration in the brain, locomotor defects and early lethality of flies expressing mHtt in neurons or glia. AKT failed to prevent brain neuronal death and lethality of adult HD flies, but significantly improved their locomotor performance when co-expressed with mHtt in glia. Finally, active ERK expression did not improve HD phenotype in any cell type. Thus we provided in vivo evidence that increasing AKT delayed cell-specific HD phenotypes, whereas HSP70 suppressed mHtt-induced cell dysfunction and death.

RESULTS

HSP70 and AKT similarly suppress cell toxicity in the HD Drosophila retina

The UAS-GAL4 system (35) was used to target expression of the first exon of human Htt with 93 CAG repeats (Httex1p Q93) to cell subtypes in the Drosophila nervous system. Identification of genetic modifiers in the Drosophila models of neurodegenerative disorders has been mostly performed in the retina to harness the power of rapid genetic testing. Previous studies showed that expression of various peptides containing an expanded polyQ tract in all cell types of the retina with the GMR-GAL4 driver resulted in progressive pigment cell degeneration (17,36,37). We similarly observed that Httex1p Q93 expression with GMR-GAL4 leads to late-onset eye depigmentation (Fig. 1A5). Numerous cells with pigment loss were observed beyond 1 month of adult flies. The Drosophila AKT gene was co-expressed with mHtt in the retina using two independent UAS-AKT1 insertion lines. In both cases, expression of AKT did not change the external appearance of the eye but strongly suppressed Httex1 Q93-mediated pigment cell degeneration (Fig. 1A8). In contrast, co-expression of the constitutively active Drosophila ERK (UAS-rolledSEM) did not prevent eye depigmentation (Fig. 1A7). ERK was by itself deleterious for eye formation inducing a rough phenotype (Fig. 1A3). As a control for non-specific suppression effects, green fluorescent protein (GFP) was co-expressed with Httex1p Q93 in the eye using UAS-GFP-65T with no detected change in eye mHtt-induced depigmentation (Fig. 1A6).

Expression of Httex1p Q93 with the neuronal-specific elav-GAL4 driver disrupted the regular arrangement of the retina decreasing the number of photoreceptors in each ommatidium as observed by pseudopupil analysis (Fig. 1B). Co-expression of AKT markedly slowed down photoreceptor degeneration. Figure 1C shows the distribution of photoreceptors per ommatidium 1 or 9 days after eclosion from pupae. The number of ommatidia that remained intact with seven photoreceptors was only 36% at day 1 and fell to 18% at day 9 in flies expressing Httex1p Q93 alone. AKT overexpression by itself had no effect on the photoreceptors (data not shown). Co-expression of AKT with Httex1p Q93 significantly rescued photoreceptor degeneration: the number of remaining intact ommatidia was doubled compared with flies expressing Httex1p Q93 alone at 1 and 9 days (Fig. 1C). ERK expression in photoreceptors had deleterious effects; as previously reported (38) eyes showed a rough phenotype with additional ectopic photoreceptors. Quantitative pseudopupil analysis could not be performed because of the strong eye malformation in the presence of ERK.

Since chaperone proteins are described as potent modifiers of expanded polyQ proteins in Drosophila models of SCA1 and SCA3 (22,28) or SBMA (39), we evaluated the effects of HSP70 in the eye of HD Drosophila model. We found that expression of HSP70 reduced retinal depigmentation (Fig. 2A) and photoreceptor loss (Fig. 2B). As observed with AKT, the number of ommatidia that remained intact was doubled at day 9 (Fig. 2C).

Neuronal and glial expression of mHtt causes reduced lifespan and locomotor deficits

Previous studies described that the presence of Httex1p Q93 in fly neurons resulted in cell death, locomotor impairments and shortened lifespan (18,19). We further reported that expression of Httex1p Q93 in a subset of glial cells that contain the
glutamate transporter, dEAAT1, was detrimental for adult Drosophila survival (12). Here, we more precisely compared the effects of mHtt selectively expressed in a large range of neurons or glia under the control of the elav-GAL4 or repo-GAL4 drivers, respectively. In both cases, adult flies died between days 8 and 28 of adult age with a similar lifespan curve, whereas control flies heterozygous for each driver lived 28 days at 25°C (Fig. 3A). Next, we used selective drivers to evaluate whether expression of mHtt in different glia subtypes has different effects on fly lifespan (Table 1). Based on morphological criteria and location in the nervous system, glial cells can be placed into three major subtypes in the Drosophila brain (40). Cortex glia extend cellular processes to the synapse and thus modulate synaptic neurotransmission, whereas neuropil glia ensheath and isolate axons. Surface glia cover the central nervous system and forms the blood-brain barrier. In the peripheral nervous system, peripheral glia enwrap and insulate axons, maintain the nerve-blood barrier and regulate transmission at the neuromuscular junction. Selective targeting of Httex1p Q93 into surface glia under control of moody-GAL4 driver (41) or into peripheral glia with gliotactin-GAL4 driver (42) did not affect fly behaviour nor the survival rate until 28 days after eclosion (Table 1). In adult Drosophila, glia that produce the unique glutamate transporter dEAAT1 correspond to cortex glia subsets in the brain and peripheral glia at the neuromuscular junction that cap the axon terminals and regulate glutamate transmission (43,44). Expression of Httex1p Q93 in dEAAT1-positive glia with dEAAT1-GAL4 driver induced lethality as early as the age of 12 days (Fig. 3A). These data indicate that the reduced lifespan we
observed in the presence of mHtt in repo-GAL4-positive glia is not due to a breakdown of the blood-brain barrier but rather to alterations of perisynaptic glia positive for the glutamate transporter dEAAT1. However, since we have no specific driver to evaluate effects in neuropil glial cells, we cannot exclude a role for this glia subset.

Analysis of locomotor defects was assessed by measuring the climbing ability of flies using the negative geotaxis test as previously described (43). Figure 3B shows the percent of 12 day old flies that reached the top or remained at the bottom of the column 30 s after startle. Almost all control flies did not show behavioural defects and were able to reach the top of the column within 30 s (>70% of flies). In contrast, only 43 and 10% of flies expressing Httex1p Q93 under control of either elav-GAL4 and repo-GAL4 drivers, respectively, were able to reach the top of the column. Climbing scores (see section Materials and Methods) at 4, 8 and 12 days are presented in Figure 3C. Neuronal expression of Httex1p Q93 induced abnormal locomotor performance as early as 4 days and the defect remained stable at 8 and 12 days. In contrast, flies expressing Httex1p Q93 in Repo-positive glia showed a more progressive decline in locomotor ability between 4 and 12 days. More precisely, flies with either the elav or repo-driver displayed a similar reduced speed when they walk, whereas flies expressing Httex1p Q93 in Repo-positive glia also exhibited a temporary paralysis and/or seizure after being startled. This ‘bang-sensitive’ phenotype increased with the age and at 12 days, some flies remained paralyzed for up to 20 s after tapping. This explains why more flies remained at the bottom for the pan-glial driver repo-GAL4 (74%) when compared with the pan-neuronal driver elav-GAL4 (21%) at 12 days. Surprisingly, flies expressing Httex1p Q93 in dEAAT1-positive glia did not show any locomotor dysfunction, paralysis or seizure even at 12 days (Fig. 3B and C). This suggests that the locomotor deficits observed with the repo-GAL4 driver result from mHtt-induced defects in distinct glia subsets that do not express dEAAT1 such as the neuropil glia.

Effects of AKT and ERK on HD pathology in the fly nervous system

We then examined the protective effect of AKT and ERK on mHtt-induced brain alterations and the ensuing locomotor deficits and reduced survival. The effect of Httex1p Q93 was assessed by immunostaining of Fasciclin II to visualize the mushroom bodies, a central brain structure involved in locomotion, learning and memory (45). We observed that pan-neuronal expression of Httex1p Q93 with elav-GAL4 resulted in a decreased Fasciclin II immunostaining in the mushroom body γ-lobe at 10 days (Fig. 4A). Quantitative analysis (Fig. 4B) showed that in the mHtt-expressing flies, the fluorescence intensity fell to 65% in the γ-lobe, whereas Fasciclin II levels remained unchanged in the α and β lobes as compared to wild-type (WT) flies (fluorescence levels: 159 ± 7 versus 144 ± 5 in control and Httex1p Q93 flies, respectively). These results indicate that the decrease in fluorescence intensity did not result from a mHtt-induced down-regulation of Fasciclin II expression, but rather, was due to a selective degeneration of the Kenyon’s cells that compose the γ-lobe, as previously described in Drosophila models of polyQ disorders (18,28). We found that the co-expression of AKT or ERK did not modify the mHtt-induced decrease in the γ-lobe fibre density. Therefore, AKT is able to prevent neuronal cell toxicity in the retina, but not in mushroom bodies.

Next, we observed that co-expression of AKT or ERK did not ameliorate the survival time of flies that expressed Httex1p Q93 either in neurons (Fig. 5A) or glia (Fig. 5B).
and C). As a control, we found that both protein kinases did not decrease lifespan when expressed alone, except for flies expressing ERK in dEAAT1-positive glia that died beyond 24 days. AKT was unable to prevent locomotor defects at 12 days caused by the neuronal expression of Httex1p Q93 (Fig. 6A). In contrast, AKT had a significant beneficial effect on the locomotor ability of the 12-day old Drosophila expressing mHtt in glia (Fig. 6A). In the presence of AKT, 62% of flies reached the top of the column, whereas only 22% of flies succeeded when Httex1p Q93 was expressed

Table 1. Comparison of mutant Huntingtin toxicity in Drosophila neurons and glia

<table>
<thead>
<tr>
<th>Driver</th>
<th>Cell types</th>
<th>Drosophila survival</th>
<th>Locomotor performance</th>
<th>Bang-sensitivity</th>
</tr>
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<tbody>
<tr>
<td>elav-GAL4</td>
<td>All neurons</td>
<td>&lt;28 days</td>
<td>Decreased</td>
<td>No</td>
</tr>
<tr>
<td>repo-GAL4</td>
<td>All glial cells</td>
<td>&lt;28 days</td>
<td>Decreased</td>
<td>Yes</td>
</tr>
<tr>
<td>dEAAT1-GAL4</td>
<td>Cortex glia subset in CNS and perisynaptic glia in PNS</td>
<td>&lt;28 days</td>
<td>No change</td>
<td>No</td>
</tr>
<tr>
<td>moody-GAL4</td>
<td>Surface glia in CNS</td>
<td>No change</td>
<td>No change</td>
<td>No</td>
</tr>
<tr>
<td>gliotactin-GAL4</td>
<td>Peripheral glia</td>
<td>No change</td>
<td>No change</td>
<td>No</td>
</tr>
</tbody>
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Figure 3. Expression of Httex1p Q93 in neurons or glia shortens Drosophila lifespan and induces locomotor deficits. (A) Survival rates of adult flies expressing either no transgene (control) or Httex1p Q93 either in neurons (elav-GAL4 driver), a large range of glia (repo-GAL4 driver) or glutamate transporter-expressing glia subsets (dEAAT1-GAL4 driver). Data were analyzed by the Kaplan–Meier method and statistical significance was assessed by logrank test (**P < 0.001 versus control). (B) Locomotor impairments evaluated by negative geotaxis test on 12-day old flies expressing either no transgene or Httex1p Q93 under control of the pan-neuronal driver elav-GAL4 and the glial drivers, repo-GAL4 and dEAAT1-GAL4. Bars indicate the proportion of flies that climbed to the top of the column or that remained at the bottom after 30 s. (C) Climbing scores of flies expressing Httex1p Q93 in neurons or glia at 4, 8 and 12 days. Climbing scores are defined as \[ \frac{1}{2}(n_{\text{total}} + n_{\text{top}} - n_{\text{bottom}})/n_{\text{total}} \]. Statistical significance was assessed by Student’s t-test (*P < 0.05; **P < 0.01; ***P < 0.001).
alone under regulation of repo-GAL4 driver. Thus, co-expression of AKT with mHtt in glia doubled the climbing score of flies (Fig. 6B). Actually, the flies remained paralyzed for a shortened period of time after tapping the column, indicating that AKT mostly ameliorated the bang-sensitive phenotype. Therefore, although AKT expression did not protect against mHtt-induced decrease in lifespan, it did partly restore neuron–glia interaction when co-expressed with mHtt in glia.

Chaperone proteins suppress mHtt-induced toxicity on neuronal and glial pathologies in the fly nervous system

We investigated whether or not putative modifiers of HD phenotypes may also display a selective rescue of the neuronal and glial pathologies. We focused our screen on chaperone proteins that were proved to be the most effective modulators for neuronal dysfunction/death in various Drosophila models of polyQ disorders (22–28). Life expectancy of flies expressing mHtt in glia under control of the dEAAT1-GAL4 driver was used to assess the effect of modifiers (Table 2). We found that overexpression of human HSP70 chaperone or Drosophila homologs of HSP40 co-chaperones HDJ1 (dHDJ1) and MRJ (dMRJ) significantly delayed the early lethality of adult flies expressing mHtt in glia. Chaperones increased by >30% the life expectancy of flies expressing mHtt in dEAAT1-positive glia. TPR2 that regulates the multi-chaperone system (46) also improved survival rate of flies.

Next, we selected one of these modifiers, HSP70, to analyze more precisely its effects on neuronal and glial pathologies in the fly nervous system. We found that HSP70 abolished the loss of Fasciclin II immunostaining in the γ-lobe of mushroom bodies (Fig. 4C and D), indicating that HSP70 suppressed mHtt-induced neuronal toxicity in the eye as well as in the central brain.

We showed that co-expression of HSP70 significantly ameliorated lifespan of flies expressing Httex1p Q93 in neurons (Fig. 7A) or glia (Fig. 7B and C). Whereas almost all flies
expressing Httex1p Q93 alone did not survive beyond 24 days, >57% of flies co-expressing HSP70 together with Httex1p Q93 remained alive at 24 days with any one of the drivers. The climbing ability of Httex1p Q93-expressing flies was also improved by HSP70 as late as 12 days of adult age (Fig. 7D). Accordingly, climbing scores were twice and 3-fold increased when HSP70 was co-expressed with Httex1p Q93. Expression of ERK alone (without Httex1p Q93) under control of dEAAT1-GAL4 driver shortened fly lifespan.

Finally, we further analyzed whether HSP70 may also rescue the mHtt-induced glial alterations. We previously showed that Httex1p Q93 expression in glia resulted in down-regulation of the glial glutamate transporter dEAAT1 (12). Protein levels of dEAAT1 were assessed in flies expressing GFP-tagged-dEAAT1 (UAS-dEAAT1-GFP) in the absence and presence of Httex1p Q93 alone or plus HSP70. Figure 8A and B shows representative views of adult brains at 10 days of age and quantitative analysis of the GFP fluorescence, respectively. The presence of Httex1p Q93 diminished the level of GFP-tagged dEAAT1 by 74% in both the optic lobes and central brain. Co-expression of HSP70 significantly prevented the decline in GFP-tagged dEAAT1. Fluorescence levels of GFP-tagged dEAAT1 in Httex1p Q93-expressing flies were doubled by the presence of HSP70. Therefore our data indicate that HSP70 strongly reduced the mHtt-induced pathology in neurons as well as in glia.

DISCUSSION

The overall aim of this study was to evaluate the rescuing potency of AKT and ERK anti-apoptotic kinases and HSP70 chaperone towards mHtt toxicity in different cell types of the Drosophila nervous system. We compared protection against retina degeneration with rescue of fly locomotor behaviour and early death when mHtt is expressed in brain neurons and glial cells, which is more the hallmark of HD than photoreceptor cell loss. Previously, genes identified in fly models as
suppressors of expanded polyQ toxicity were generally found by screening for modifiers of the Drosophila eye phenotypes. Many of retinal rescuers were also neuroprotective in the fly brain (22–28). However, in a few cases, effects in the retina did not mirror protective effects in brain neurons (28). Among the modifiers, HSP70 chaperone protected against toxicity of polyQ proteins in both the retina and postmitotic brain neurons in Drosophila models (22,28,39) and thus it was used as a standard rescuer in the present study. We found that AKT and HSP70 had similar benefits on photoreceptor neuron loss in the fly eye. In contrast, their effects differed in the brain. Although HSP70 widely suppressed neuronal and glial phenotypes, we detected no beneficial effects of AKT on neuronal degeneration in mushroom bodies, locomotor deficits and lifespan when mHtt was expressed in neurons. Another level of complexity is that mHtt is also expressed in non-neuronal brain cells (7,47,48). Here we provided further evidence that the accumulation of mHtt in glial cell subsets may compromise neuron functioning and contribute to HD pathogenesis. Candidate modifiers should be tested on brain neurons but also on glia to enhance relevance to human diseases. Interestingly, increasing AKT signalling improved locomotor performance when mHtt was selectively expressed in glia but not in neurons, indicating that mHtt can induce AKT-sensitive or insensitive pathways of toxicity in distinct cell types.

Neuron and glia pathologies in HD Drosophila model

Drosophila HD models have been developed so far by expressing N-terminal fragments of human mHtt, whose total length is 3144 amino acids in its native form: the first 81 amino acids corresponding to exon 1 (19) as we used here, the first 548 amino acids (20) and recently the first 336 amino acids (49). In all cases, pan-neuronal expression replicates key HD features such as late onset and progressive neuronal dysfunction and degeneration, leading to decline in locomotor performance and premature death. Neuronal loss appears to be restricted to a few cerebral structures in Drosophila as it is the case in HD patient brains. Accordingly, when all neurons were immunostained with anti-Elav antibodies in HD flies, we did not observe massive neuronal loss in the brain (data not shown). As previously described

Figure 8. Expression of HSP70 decreases Httex1p Q93-induced glial cell alterations. (A) dEAAT1-GFP fluorescence levels in whole-mount brains from 10 day-old adult flies. The dEAAT1-GFP fusion protein was expressed in glia subset under control of the dEAAT1-GAL4 driver either with no transgene (control), Httex1p Q93 alone, or Httex1p Q93 together with HSP70. Magnification: ×20. HSP70 prevented the decline in GFP-tagged dEAAT1 that was observed in the presence of Httex1p Q93 in the optic lobe (OL) and the central brain (CB). (B) Quantitative analysis of fluorescence intensity in the optic lobe and the central brain. Data are expressed as the mean ± SEM of values. Statistical significance: ***P < 0.001 (Student’s t-test).
by Agrawal et al. (18), we detected neuronal degeneration selectively in the mushroom body γ-lobes, whereas the α and β lobes remained intact.

Neuron–glia interactions are crucial to ensure optimal brain function and accumulation of mHtt may compromise crosstalk between both cell types (7,12). Gial-specific expression of polyQ-expanded proteins was found to have adverse effects on nervous system function in Drosophila (12,50). Expression of polyQ-expanded human ataxin-3 in neurons or glia led to behavioural changes and reduced lifespan of flies (50). Here, we showed that pan-neuronal and pan-glial expressions of mHtt also triggered abnormal locomotor behaviour and shortened lifespan. Whereas in the both cases locomotor performance was compromised using the negative geotaxis test, only flies expressing mHtt in Repo-positive glia suffered from ‘bang-sensitivity’, i.e. temporary paralysis and/or seizure following a mechanical shock, such as after tapping a column to make the flies fall to the bottom. The bang-sensitive phenotype likely reflects the disruption of glial-specific functions by expanded polyQ proteins. The targeting of mHtt in surface and peripheral glia, which are implicated in maintenance of the blood-nervous system barrier, did not result in locomotor defects and shortened lifespan. Since disruption of the blood-brain barrier leads to paralytic or shaking phenotypes and reduced larval or adult survival time (41,51,52), our results suggest that mHtt does not strongly challenge this glial function. In contrast, when we expressed mHtt in dEAAT1-positive glia, flies exhibited early adult lethality, comparable to that observed when mHtt is expressed in Repo-positive glial cells, but surprisingly did not show reduced climbing ability. This suggests that locomotor defects are not related to alteration in perisynaptic glia that regulates glutamate transmission in the brain and at the neuromuscular junction in Drosophila. We previously reported that expression of mHtt does not produce a massive loss of glia (12). Similarly, overexpression of mHtt in cultured astrocytes, the mammalian counterpart of Drosophila cortex glia, did not cause obvious cell death. In HD mouse brain, degenerating glial cells have been observed but they were scarce (7). Therefore, the phenotype we observed is likely mediated by cell dysfunction rather than glia degeneration and strikingly differs according to the type of glial cells targeted.

Chaperone proteins modulate glia alterations in HD

Expression of chaperones was previously found to protect against toxicity of polyQ proteins in the retina and in brain neurons of Drosophila models (22–28). Here we provide additional evidence that several chaperones display glioprotective effects and thereby stand as worthwhile therapeutic targets in HD. Chaperones likely reduce toxicity at early stage of HD pathogenesis by directly acting on mHtt protein. However results from HD mouse model had disappointing outcome at this respect. HSP70 overexpression in R6/2 mouse model resulted in only modest effects on disease progression (53) or no improvement of the phenotype except for a delay in aggregate formation (54). Similarly, R6/2 mice transgenic for HSP27 chaperone did not display a phenotypic improvement (55). Overexpression of yeast HSP104 in another HD mouse model prolonged the lifespan, but did not rescue the motor deficits and weight loss of mice (56). One explanation is that molecular chaperones generally function in cooperation and thus the decreased levels of HSP70 and HSP40 in HD mouse models (54,56) might compromise the beneficial effects of one particular chaperone. Accordingly, co-expressed Drosophila Hdj1 (dHdj1) and HSP70 act synergistically to suppress toxicity in SCA3 fly model (57). Therefore, a more effective approach might be to induce stress response, thereby increasing expression of many chaperones. In support of this, expression of the active form of heat shock transcription factor 1, which regulates transcription of stress-inducible genes, reduces body weight loss and increases lifespan of R6/2 HD mice (58). The recent development of geldanamycin derivatives that cross the blood brain barrier and induce heat-shock response (59) could well lead to new therapeutic strategies.

AKT/ERK cell signalling and cell degeneration in HD pathology

There is strong support for an involvement of the protein kinases AKT or ERK in HD pathology. AKT is degraded in HD patient brain and its activity is decreased or increased, respectively, in the brain of mHtt-transfected rat or knock-in mouse models of HD (34,60). Altered levels of phosphorylated ERK are measured in mHtt-expressing cells and in neurons of R6/2 HD mice (61,62). Although ERK activation was found to be protective against mHtt-mediated cell toxicity in PC12 and cultured striatal cells (61), we observed no benefit of ERK activation on HD phenotype in Drosophila. Similarly, overexpression of MEK, the kinase upstream of ERK cascade, did not rescue polyQ-induced lethality in flies (25). One possible explanation is that ERK activation is sustained during pre-adult and adult life stages under our in vivo conditions, whereas ERK activation was transient in transfected cultured cells. Flies expressing active ERK displayed abnormal rough appearance and ectopic photoreceptors in the eye. Such deleterious effects on eye development were previously described after expression of gain of function alleles of components acting upstream of ERK such as EGFRI, Ras or Raf (12,38,63,64). In contrast, expression of active ERK alone in the brain had no effects on mushroom body structure and on organisinal lifespan, except for flies expressing ERK in dEAAT1-positive glia that died well beyond 24 days. Thus, the lack of protective effects of ERK on mHtt toxicity into the brain was likely not due to toxic effects of ERK.

In vitro studies previously showed that AKT abolished death of cultured striatal neurons expressing N-terminal fragments of expanded polyQ Htt (65,66). Here, we provide in vivo evidence that AKT rescued mHtt-dependent cell degeneration in the fly retina but not in brain neurons. Neuronal degeneration seems to be highly restricted in the brain since mHtt-mediated death was only detected in a specific subtype of Kenyon’s cell giving rise to the axonal fibres of the γ-lobes. Although AKT expression protected retinal cells from mHtt-mediated death, we did not detect beneficial effects on neuronal loss in mushroom bodies. Moreover, AKT failed to rescue early lethality when co-expressed with mHtt in all neurons or large subsets of glia. Therefore, the cellular pathways of mHtt-mediated...
cell death are likely different in the eye and brain cells, being respectively AKT-sensitive or insensitive.

There are a number of possible mechanisms by which AKT could lead to a prosurvival response. AKT was demonstrated to induce the phosphorylation of mHtt at Ser421, thereby reducing its toxicity (66). However, we expressed a shorter N-terminal fragment that does not contain this phosphorylation site. Prosurvival effect of AKT likely results from its anti-apoptotic function. For instance, AKT controls p53 levels via Mdm2 phosphorylation, the E3 ubiquitin ligase that triggers degradation of p53 (67,68) and could directly inactivate apoptosis pathway by phosphorylating the pro-apoptotic Bcl-2-related protein, BAD and Caspase 9/Apaf-1 complex (69). Accordingly, deletion of p53 was found to ameliorate HD pathologies in transgenic Drosophila and mouse models (30) and genetic inactivation of Apaf-1 suppressed mHtt-induced cell death in the fly retina (29). Alternatively, AKT could increase survival by inhibiting the stress-activated protein kinase pathways, JNK and p38 (69). Expression of mHtt causes increased activation of JNK in HD in vitro models (61,70,71). Pharmacological or genetic inhibition of JNK pathway protected against cultured cell apoptosis induced by mHtt and against neurodegeneration in a Drosophila HD model (33,61,71). Finally, AKT may also improve cell survival by direct effects on glycolysis and mitochondrial functions (reviewed in 72,73).

AKT signalling and behavioural deficits in HD models

Interestingly, we found that AKT ameliorated the locomotor performance of HD flies when selectively co-expressed with mHtt in glia but not in neurons. This was obviously due to a decrease in the duration of the bang-sensitive paralysis/seizure behaviour, suggesting that neuron–glia interactions were partly restored. Rescue was likely not caused by an improvement of glutamate transmission because (i) expression of mHtt in glia that contains the glutamate transporter dEAAT1 did not result in bang-sensitivity and (ii) dEAAT1 expression levels are not regulated by PI3K/AKT signalling pathway (12). Bang-sensitivity was previously described in mutants affecting sodium/potassium asymmetric distribution across the plasma membrane, ATP levels and mitochondrial energy production (74,75). Two particular functions of neuron-pil and cortex glia are to supply neurons with energy metabolites and to maintain energy-dependent ionic composition of the extracellular environment. Thus, abnormal energy metabolism in glia may explain the ensuing effects on neuron function. AKT is known to regulate glucose uptake, glycolysis and glycogen synthesis in a cell-intrinsic and cell type-specific manner (72,73). Therefore, we propose that AKT may rescue fly behaviour in HD model by stimulating glycolytic metabolism in glia. This view is supported by data from positron emission tomography studies in brain of early HD patients showing a selective impairment of cerebral glycolytic, not oxidative, metabolism that is predominantly a glial metabolic process (76). More recently, inhibitors of metabolism such as rotenone were found to rescue cell death in cellular and Drosophila eye models of HD (77). However, rescue by these inhibitors was not due to changes in ATP, reactive oxygen species or NADH levels but rather due to the activation of ERK or AKT (77).

In conclusion, we provided in vivo evidence that increasing AKT has beneficial effects on HD Drosophila phenotypes in a cell-type specific manner. AKT prevents some cell-intrinsic mechanisms leading to late neuronal death in the retina. Our data further suggest that increasing AKT signalling pathway may improve glial energy metabolism and dependent neuron functioning. Further works are required to understand the role and importance of energy metabolism in the alternation of neuron–glia interaction in HD pathogenesis.

MATERIALS AND METHODS

Drosophila strains and culture

Flies were grown at 25°C on a standard cornmeal/agar/yeast medium. The following fly stocks were used: dEAAT1-GAL4 and UAS-dEAAT1-GFP (43), moody-GAL4 (41), gliotactin-GAL4 (42), UAS-Httx1p Q93 (19), UAS-rolledSEM (ERK; (38)), UAS-dJpr2 (24), UAS-dHDJ1 (24) and UAS-dMRJ (26). All other strains: GMR-GAL4, repo-GAL4, elav-GAL4 (line c155), UAS-AKT (2 insertions) and UAS-HspA1L (HSP70; (22)) were obtained from the Bloomington Drosophila Stock Center.

Lifespan analysis

Female progeny were collected within 24 h of emergence. For each desired genotype, 10–20 flies per vial in 4–8 vials were incubated at 25°C. They were transferred into fresh vials every 2 days and the number of surviving flies was recorded. Cumulative survival curves were generated by the Kaplan–Meier method and statistical analysis was performed by logrank test (GraphPad Prism software).

Locomotion assay

Locomotor ability was assessed with a negative geotaxis assay as described previously (43). Female flies were anesthetized with CO2 and placed in a plastic column (length: 18 cm × diameter: 1.3 cm). After a recovery of 30 min, columns were disposed vertically and flies were gently tapped to the bottom of the column. WT flies quickly recovered and started climbing up the column. Flies that have reached the top or remained at the bottom of the column were counted after 30 s. Three trials were performed at 1 min of intervals. The data are presented as the number of flies at the top ($n^{\text{top}}$) or at the bottom ($n^{\text{bottom}}$) and are expressed as percentage ± SEM of the total number of flies ($n^{\text{total}}$). Climbing scores are defined as $rac{1}{2}(n^{\text{total}} - n^{\text{bottom}})$. Statistical significance was assessed by using the Student’s t-test.

In situ fluorescence

Adult Drosophila brains expressing UAS-dEAAT1-GFP were dissected in phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde, rinsed in PBS and mounted in Mowiol (Calbiochem, La Jolla, CA).
Immunocytochemistry

Reagents were prepared in PBS. Adult fly brains were dissected, fixed in 4% paraformaldehyde for 30 min, permeabilized with 0.3% Triton X-100 for 20 min, saturated in 0.1% Triton X-100 with 5 mg/ml bovine serum albumin for 2 h. Brains were incubated in the latter solution containing primary antibodies overnight, rinsed and incubated with fluorochrome-conjugated secondary antibodies. After rinsing, brains were mounted in Mowiol (Calbiochem).

The primary monoclonal antibodies against Fasciculin II (1/100 dilution, Developmental Studies Hybridoma Bank, Iowa) was used to visualize the fly mushroom bodies. Alexa-488-anti mouse antibodies (Molecular probes, Eugene, OR) were used as secondary antibodies at 1/400 dilution.

Fluorescence detection and quantification

For quantitative analysis of dEAAT1-GFP or Fasciculin II, microscopic examination was performed using a Nikon E800 fluorescent microscope (Champigny sur Marne, France) equipped with a Hamamatsu Photonics ORCA-ER digital camera (Massy, France). Quantification of the signal was done with the Image J software. Fluorescent intensities were expressed as percent of the mean of control flies. Data from at least six 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. Each experiment was performed twice.

NOTE ADDED IN PROOF

Since acceptance of this manuscript, Branco et al. have reported with another Drosophila model of HD that AKT overexpression reduced retinal phenotype, which is in agreement with our results. Conversely in a SCA1 fly model, AKT overexpression behaved as an enhancer of the phenotype in the retina. (Branco, J., Al-Ramahi, I., Ukanli, L., Perez, A.M., Fernandez-Funez, P., Rincón-Limas, D. and Botas, A.J. Comparative analysis of genetic modifiers in Drosophila points to common and distinct mechanisms of pathogenesis among polyglutamine diseases. Hum. Mol. Genet., in press).

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