Protective role of Engrailed in a Drosophila model of Huntington’s disease

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Huntington’s disease (HD) is caused by the expansion of the polyglutamine (polyQ) tract in the human Huntingtin (hHtt) protein (polyQ-hHtt). Although this mutation behaves dominantly, htt loss of function may also contribute to HD pathogenesis. Using a Drosophila model of HD, we found that Engrailed (EN), a transcriptional activator of endogenous Drosophila htt (d htt), is able to prevent aggregation of polyQ-hHtt. To interpret these findings, we tested and identified a protective role of N-terminal fragments of both Drosophila and Human wild-type Htt onto polyQ-hHtt-induced cellular defects. In addition, N-terminal parts of normal hHtt were also able to rescue eye degeneration due to the loss of Drosophila endogenous d htt function. Thus, our data indicate that Drosophila and Human Htt share biological properties, and confirm a model whereby EN activates endogenous d htt, which in turn prevents polyQ-hHtt-induced phenotypes. The protective role of wild-type hHtt N-terminal parts, specifically onto polyQ-hHtt-induced cellular toxicity suggests that the HD may be considered as a dominant negative disease rather than solely dominant.

INTRODUCTION

Engrailed (EN) encodes a homeodomain transcription factor highly conserved from flies to humans (1,2). In Drosophila, it is involved in embryonic epidermal patterning, giving a posterior identity to cells expressing EN within each segment (3). This gene is also expressed in a subset of neuroblasts and neurons and is involved in the development of the nervous system (1), where it plays an important role in axonal growth and pathfinding (4), (5). Interestingly, EN homologs in vertebrates were also found to be important in axonal guidance (6). In addition, EN mis-expression was associated to several neurodegenerative diseases, including Parkinson’s disease or autism (7), (8), (9).

Identification of direct target genes of transcription factors is a key issue in the developmental biology, in order to better understand how these factors play their roles. Using chromatin immunoprecipitation (ChIP) approach from Drosophila embryos, we previously identified direct target genes of EN at a genomic scale (10). Among the 203 targets identified, around 40 are known to be involved in neurogenesis. In this screen, the Drosophila huntingtin (d htt) gene has been identified as a potential direct target of EN regulation (10).

Drosophila Huntingtin (dHtt) was first isolated because of its homologies with human Huntingtin (hHtt) (11). In humans, abnormal expansions of a polyglutamine (polyQ) stretch within the hHtt protein were found to be responsible for Huntington’s disease (HD), a progressive dominant degenerative disorder. When the number of repeats exceeds 35, the resulting mutant protein (polyQ-hHtt) forms intracellular aggregates and causes neuronal degeneration, by mechanisms that are still not fully understood. These mechanisms involve both the gain of new toxic function as well as the loss of the protective properties of wild-type Huntingtin. Even though several reports indicate an influence of the normal hHtt allele on the disease (12–16 and reviewed in 17), others rather favour a mild requirement of wild-type hHtt on striatal neuropathology induced by polyQ-hHtt (18–21), making this question still opened (22).
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Models for HD often include transgenic animals expressing small N-terminal portion of the hHtt protein that recapitulate landmarks of HD, including both formation of aggregates and neurodegeneration (23–26). Aggregates are indeed formed by small N-terminal polyQ-hHtt fragments obtained by proteolysis (27–28), and cleavage of full-length hHtt was recently found to appear at late stages of pathogenesis (26), suggesting that this cleavage is part of normal regulated hHtt catabolism (27). N-terminal cleaved fragments of polyQ-hHtt were also shown to be more toxic than full-length polyQ-hHtt (22,26,29,30). Therefore, important advances in the study of HD came from the development of transgenic models in mice or Drosophila, where different N-terminal portions of hHtt carrying different lengths of CAG have been expressed.

Another important question is whether a model is suitable to learn about the functions of proteins involved in a human disease. Whereas both mouse and Drosophila models show neurodegeneration and axonal pathology in the absence of endogenous Htt (12,31), the biological properties shared by vertebrate Htt and their putative Drosophila homolog are still unclear. We approach this question in this report.

Here, we present several arguments for a direct regulation of Drosophila d htt by the EN transcription factor. We first verified that EN binding sites identified by ChIP experiments within d htt gene (10) bind EN specifically. We then demonstrated that the EN expression was able to interfere on mutant human polyQ-hHtt aggregation, in a Drosophila model of HD. Therefore, one hypothesis that has been tested in this report is that EN might activate endogenous d htt, which in turn could prevent human polyQ-hHtt aggregation. This suggested (i) that the ratio between wild-type and mutant polyQ-hHtt might be important in the onset of polyQ-hHtt-induced phenotypes, and ii) that human and Drosophila Htt might share biological properties.

By using in vivo controlled level of expressions, we provide in this report data that fit with a dominant negative hypothesis for HD. We indeed show that the ratio between wild-type hHtt N-terminal fragment and mutant polyQ-hHtt is critical for the onset of polyQ-hHtt-associated defects, such as aggregation and eye toxicity. This has been verified both in flies and in cultured mammalian cells. We also show that both human and Drosophila wild-type Htt are able to rescue phenotypes induced by polyQ-hHtt, and that loss of function of Drosophila d htt can be rescued by wild-type hHtt, confirming that the human and Drosophila Htt share biological properties.

Altogether these results show that EN protective role on polyQ-hHtt aggregation results from an activation of the endogenous d htt. Therefore, studying transcriptional regulation of htt in Drosophila might provide important leads to further understand HD and the transcriptional regulation of hHtt.

RESULTS

Properties of different Drosophila models of Huntington’s disease

In this report, we used a Drosophila model of HD, where the N-terminal part of human Huntingtin (hHtt) covering 171 aa, with different lengths of polyQ domains (18Q or 138Q), has been inserted into the genome. This model is using the human Htt transgenes that have been tested in mice models and that were found to recapitulate different hallmarks of HD (24). Our transgenes (HA-hHtt171aa-18Q; HA-hHtt171aa-138Q) contain a HA-tag, so that the expression of hHtt can be easily analysed. In addition, the coding sequences for all the transgenes are placed under the control of upstream activator sequences (UAS) that will be recognized by the Gal4 transcription activator (32). Using this UAS/Gal4 system, we could express these constructs in different tissues and at different developmental stages, in order to follow either the formation of aggregates or eye degeneration, two phenotypes resulting from polyQ-hHtt accumulation. Interestingly, increasing or decreasing the number of UAS transgenes can modulate the level of Gal4 induced expression (32).

Phenotypes have first been analysed in larval salivary glands, using MS1096-Gal4 driver (Fig. 1A). Because of the large size of the cells, the nucleo-cytoplasmatic repartition of the proteins could easily be followed. As shown on Figure 1, HA-hHtt171aa-18Q is localized in both the nucleus and the cytoplasm (Fig. 1A1 and A3). In the cytoplasm, this wild-type protein colocalizes with the microtubule network (Fig. 1A1–A3). These observations are in accordance with previous data reporting that wild-type hHtt is present in the nucleus, but is predominantly found in the cytoplasm where it is associated with microtubules (15,31,33,34). In contrast, overexpression of HA-hHtt171aa-138Q (corresponding to mutant polyQ-hHtt) leads to formation of large aggregates only localized in the cytoplasm. In this case, there is no HA-hHtt171aa-138Q protein colocalizing with the microtubule network (Fig. 1A4–A6). This observation fits with the hypothesis of an alteration of cellular transport in the disease models (15).

Using a gmr-Gal4 line, driving expression in the eyes (Fig. 1B), we found that the expression of wild-type HA-hHtt171aa-18Q does not lead to any adult eye phenotype (Fig. 1B1 and B2), whereas the expression of mutant polyQ-hHtt (HA-hHtt171aa-138Q) in developing eyes leads to adults with a loss of eye pigmentation (Fig. 1B3), and a rough eye phenotype correlated with abnormal ommatidial array as detected by scanning electron microscopy (Fig. 1B4). This phenotype is related to progressive neurodegeneration of eye retina with a loss of photoreceptors (data not shown), as previously described in other Drosophila models of HD (35,36). Indeed transgenic flies that are carrying different lengths of N-terminal hHtt protein already exist. These are covering either exon1 corresponding to the first 67 amino acids (23,35) or the N-terminal 548 amino acids (36). In the presence of expanded polyQ domain (>35), all constructs behave as mutant polyQ-hHtt, forming aggregates and leading to eye degeneration whatever is the length of N-terminal hHtt retained (67, 171 or 548 aa). However whereas mutant hHtt171aa-138Q or hHtt548aa-128Q induces aggregates that are localized in the cytoplasm, mutant hHtt171aa-93Q expression leads to aggregates found mainly in nuclei (Supplementary Material, Fig. S1). This shows that although cleaved hHtt proteins always produce toxic fragments when the polyQ repeat exceeds 35, their behaviours can be slightly different according to the Htt sequence.
Figure 1. The Drosophila model of HD. Expression of normal (UAS-HA-hHtt\textsuperscript{171aa-18Q}) or mutant (UAS-HA-hHtt\textsuperscript{171aa-138Q}) human Huntingtin. (A) In salivary glands, using MS1096-Gal4 driver. Expression of normal hHtt is shown in 1–3, and of mutant polyQ-hHtt in 4–6. Anti-HA immunostaining is in red (1 and 4), anti β-tubulin immunostaining is in green (2 and 5). Panels 3 and 6 correspond to the merged images. (B) In the adult eyes, using gmr-Gal4 driver. Expression of normal hHtt is shown in 1 and 2, and of mutant polyQ-hHtt is shown in 3 and 4. Panels 2 and 4 correspond to ommatidial arrays of the different eye phenotypes, as visualized using scanned electronic microscopy.
length present. We found the hHtt$^{171\text{aa}-138\text{Q}}$ protein was suitable to follow aggregation behaviour, since it recapitulates the different hallmarks of HD, in the same way as the larger hHtt$^{54\text{aa}-128\text{Q}}$ protein.

**Protective role of EN on polyQ-hHtt aggregation**

*Drosophila d htt* has been previously identified as a potential direct target of EN regulation (10). The genomic fragment isolated by *Drosophila* embryonic chromatin immunoprecipitation (ChIP) corresponds to a 108 bp DNA fragment (named 4A8) that is lying within intron 15 of the *d htt* gene (Fig. 2A), suggesting that regulatory elements for *d htt* must be contained in intronic regions. This fragment contains EN consensus binding sites (C8), and is able to specifically bind EN in vitro as shown by gel shift assays, where complexes are formed when adding increasing amounts of EN protein (Fig. 2B). These complexes are not formed in the presence of cold D2 polyhomeotic fragment that strongly binds EN (37), or in the presence of cold C8 EN binding consensus (10), whereas complexes still exist in the presence of either N polyhomeotic fragment (that does not bind EN) (38), or mutated C8 (C8*) fragment (10). Finally, these complexes are supershifted in the presence of specific 4F11 anti-EN antibody (Fig. 2B). Altogether these experiments show that in vitro, the 4A8 DNA fragment binding within *d htt* binds EN specifically.

We further tested the ability of intron 15 (containing the 4A8 EN binding fragment) to bind EN in vivo. For this purpose, we cloned *d htt* intron 15 as well as another *d htt* intron (intron 21), used as a negative control. Duplicates of blots containing these two *d htt* intronic regions were hybridized with probes corresponding to embryonic chromatin, crosslinked by formaldehyde and immunoprecipitated with either anti-EN antibody (IP-EN), or in the absence of antibody (Mock) (Fig. 2C). In this experiment, the d htt-115 was six times better retained in the presence of anti-EN antibody than d htt-121 that was otherwise not specifically retained by anti-EN, compared to Mock experiment, confirming that intron 15 that contains the 4A8 genomic fragment was preferentially bound by EN. Hence, d htt-115 is a direct binding site of EN in vivo.

In order to test what is the transcriptional effect of EN through this genomic DNA fragment, we constructed transgenic lines where the d htt-115 was placed upstream of a GFP reporter gene (Fig. 2D). These d htt-115-GFP transgenic lines do not express any detectable GFP in larval salivary glands that normally do not express EN (data not shown). By expressing EN in the entire larvae using a hs-Gal4 driver (Fig. 2D1), we identified a clear enhancement of the GFP expression in salivary glands (Fig. 2D2). This result has been confirmed by quantitative RT–PCR on entire d htt-115-GFP transgenic larvae: the level of GFP transcripts is enhanced 10-fold in the presence of an excess of EN (Fig. 2D3). This experiment shows that EN is acting as a transcriptional activator through d htt-115.

We further asked what could be EN overexpression effect on polyQ-hHtt-induced phenotypes. For this purpose we analysed the consequences of mutant polyQ-hHtt expression (human hHtt$^{171\text{aa}-138\text{Q}}$, labelled with anti-HA) in the absence or in the presence of EN expression (Fig. 3A). We used MS1096-Gal4 to express EN in larval salivary glands. We used several forms of EN, either the normal form that can act as a repressor or an activator (Fig. 3A2), or a VP16-EN chimeric form that is only acting as a transcriptional activator (10) (Fig. 3A3).

In this experiment, controls correspond to larvae with one copy of mutant polyQ-hHtt (HA-hHtt$^{171\text{aa}-138\text{Q}}$) transgene in the presence of another neutral UAS-transgene (UAS-LacZ). In these conditions, the mutant protein was detected either in aggregates or diffusely present in both the nucleus and the cytoplasm (Fig. 3A1), illustrating that the aggregation phenotype was milder than when two polyQ-hHtt transgenes are present (Fig. 1A4). Thus, aggregation of mutant polyQ-hHtt is directly linked to its level of expression. When the neutral UAS-LacZ transgene is replaced by a UAS-EN transgene, enabling the concomitant expression of EN in salivary glands, polyQ-hHtt aggregation is diminished (Fig. 3A2 compared with A1). More importantly, VP16-EN chimeric protein that is only acting as a transcriptional activator (10) is able to completely prevent aggregation of mutant human polyQ-hHtt (Fig. 3A3). This suggests that EN is acting the same way as VP16-EN (i.e. activation of transcription), but might not act as strong an activator as VP16-EN. We, therefore, tested whether the addition of EN earlier in salivary glands might be more effective than addition of EN at the same time as polyQ-hHtt (which was the situation with the Gal4-driven expression). For this purpose, we used a tramtrack mutation (itrk$^{eua}$), which results, when heterozygous, in an increase of EN expression at early stages in salivary glands (39). In these conditions, polyQ-hHtt aggregates are completely absent (Fig. 3A4). This was confirmed by quantifying aggregation with a filter retardation assay (40) (Fig. 3B). Altogether these data indicate that EN is acting as a transcriptional activator, like VP16-EN to rescue the aggregation phenotype. Such a protective role of EN has also been found on polyQ-hHtt-induced eye toxicity (data not shown).

Thus, the EN ectopic expression is able to rescue both consequences of polyQ-hHtt expression, protein aggregation and cellular toxicity. Based on our ChIP results and the activator effect of EN through the d htt-115 fragment, a straightforward explanation of this effect is that EN is directly activating endogenous *Drosophila huntingtin* (*d htt*), which in turn would be able to prevent the effects of human polyQ-hHtt.

We, therefore, tested whether EN regulates endogenous d htt, by performing real-time PCR experiment (Fig. 3C). As a control, we first tested whether our quantitative RT–PCR conditions can be used to detect a 2-fold differential amount of d htt transcription level. For this purpose, we compared d htt mRNA levels of expression in salivary glands of animals heterozygous for a chromosome deletion that is uncovering d htt (*Df*(3R)16210/+), and that must express half dose of d htt, to animals heterozygous for a complementary deficiency (*Df*(3R)3450/+), that is deleting most of the genes covered by the *Df*(3R)16210 deletion chromosome, but not d htt, and so the expression of d htt should be at normal level. As expected, the d htt mRNA level of expression in (*Df*(3R)16210+/+) is ~60% lower than that in (*Df*(3R)3450+/+) (Fig. 3C upper graph), confirming that even slight variations in the amount of d htt mRNAs are detectable
Figure 2. Engrailed binding fragments within Drosophila huntingtin. (A) Genomic map of Drosophila huntingtin gene, covering 40 kb (11). 4A8 108 bp DNA fragment has been isolated in vivo as a potential EN binding fragment, and is localized within intron 15 (I15). 4A8 DNA sequence contains several consensus EN binding sequences (10), marked in blue. (B) Gel shift assays are carried out with end-labelled 4A8 DNA, migrating as F when free, and that has been incubated with increasing amounts of EN protein extract, from 0.3 to 1 μl, as indicated. * shows the position of the complexes. Competitions are performed with 1 μl of EN protein, in the presence of cold DNA, corresponding to either DNA fragments that bind EN specifically (D2 or C8), or to DNA that does not bind EN (N and C8*). Supershift experiments are performed in the presence of 4F11 anti-EN antibody, where the supershifted complex is noticed (*). (C) Southern blots where either intron 15 dHtt-I15 that contains 4A8 genomic fragment, or another intronic region corresponding to intron 21 (control), have been transfered onto a membrane and hybridized with probes corresponding to embryonic chromatin immunoprecipitated either by anti-EN antibody (IP-EN), or in its absence (Mock). Quantifications show that intron 15 is 6-fold better retained than intron 21, compared to the Mock experiments. (D) Schematic representation of the d httI15-GFP transgene where the intron 15 of d htt has been cloned upstream to a minimal promoter and a GFP reporter gene. GFP expression of d httI15-GFP transgenic line is analysed in larval salivary glands. In the absence of EN, d httI15-GFP salivary glands do not express detectable GFP (data not shown). In the presence of EN, induced after a 1 h heat-shock (1), GFP expression is activated in salivary glands (2). (3) Quantitative RT–PCR of GFP expression on entire larvae that either do not express EN (d httI15-GFP/+; UAS-EN/+; UAS-EN/hs-Gal4), after a 1 h heat shock. Three independent experiments were performed and compilation of results is depicted here.
Figure 3. Influence of Engrailed on polyQ-hHtt aggregation. (A) Expression in salivary glands of mutant UAS-HA-hHtt\(^{171aa-138Q}\), induced by MS1096-Gal4 driver, followed by anti-HA immunostaining, at 29\(^\circ\)C, in the presence of one copy of (1) UAS-LacZ, (2) UAS-EN, (3) UAS-EN-VP16 (Note that this transgene is also HA tagged and is expressed in the nuclei) and (4) in tramtrack (\(ttk^{804/+}\)) heterozygous mutant background that highly express EN in salivary glands (39). (B) Filtration retardation assay of two independent experiments performed on salivary glands from hHtt171aa-138Q, in the presence of UAS-LacZ neutral transgene (as in A1, control) compared to hHtt171aa-138Q in (\(ttk^{804/+}\)) (as in A4), using anti-HA antibody. Quantification of aggregates has been reported in brackets, as percentages compared to the control evaluated at 100%. (C) The quantitative RT–PCR analysis of \(dhtt\) target gene expression in different genetic backgrounds. Relative mRNA levels of \(dhtt\) were determined in comparison with \(Pgb\), \(Rpl13\) or \(Tpb\) expressions. Three independent experiments were performed, compilation of results are depicted here. On the upper graph, the \(dhtt\) expression level from salivary glands heterozygous for (\(Df\ (3R)\ 16210/+\)) that is covering \(dhtt\) has been compared to a complementary deficiency (\(Df\ (3R)\ 3450/+\)) that is not deleting \(dhtt\). As expected around half dose of \(dhtt\) transcripts are detected using the deficiency that is uncovering \(dhtt\). On the lower graph, the \(dhtt\) expression level from (\(MS1096-Gal4;\ UAS-LacZ/+\)) salivary glands is used as a control, referred as 1 to compare to \(dhtt\) expression levels in the two independent (\(MS1096-Gal4;\ UAS-EN/+\)) RNA preparations. Overexpression of EN in salivary glands leads to an increase of \(dhtt\) expression of about three times.
in our experimental conditions. Using the same conditions of quantitative RT–PCR, we then tested the influence of EN on d htt level of expression. Using the MS1096-Gal4 driver, we ectopically expressed EN in salivary glands and analysed the level of expression of endogenous d htt, compared to salivary glands that do not express EN. When expressing EN, we could detect a 3-fold increase of d htt transcripts, compared to salivary glands that express the neutral UAS-LacZ transgene (Fig. 3C lower graph). This confirms that EN is able to activate d htt transcription.

**Influence of Drosophila Htt on polyQ-hHtt aggregation**

The protective role of EN on polyQ-hHtt-induced phenotypes may be due to the activation of endogenous d htt by EN, as previously shown. Therefore, we tested the protective role of Drosophila dHtt by modifying its level of expression and analysed whether this modifies mutant polyQ-hHtt aggregation in salivary glands. Transgenic flies expressing 620 aa of the endogenous Drosophila dHtt cDNA placed under UAS sequences have been constructed (d htt 620aa). The 620 aa N-terminal part of dHtt shares conserved motifs with its human homolog (Supplementary Material, Fig. S2). We tested whether the expression of this transgene in salivary glands could rescue the strong aggregation of polyQ-hHtt (HA-hHtt 171aa-138Q) induced at 29°C (Fig. 4A). We observed that the polyQ-hHtt aggregation was decreased in the presence of dHtt 620aa (Fig. 4A2), compared with salivary glands expressing LacZ as a control (Fig. 4A1). This effect was confirmed with quantification by filter retardation assay (Fig. 4B).

Since increasing the amount of an N-terminal fragment of wild-type dHtt prevents human polyQ-hHtt-induced aggregation, we further asked whether decreasing wild-type dHtt was also able to influence aggregation. Because null d htt mutations were not available yet, we decreased endogenous Drosophila d htt by RNA interference. The use of UAS-dHtt-RNAi lines had been shown to decrease d htt expression to 10–30% of endogenous levels (31). We also verified that eye phenotypes induced by dHtt-RNAi were enhanced on a chromosome deletion line that uncovers the d htt gene (data not shown). In conditions of mild polyQ-hHtt aggregation at 25°C, mutant polyQ-hHtt formed only few aggregates, in the presence of a control UAS-LacZ transgene (Fig. 4C1). In the presence of a UAS-dHtt-RNAi transgene, 20% of the larvae showed a marked increase in aggregates (Fig. 4C2), which never appeared in control conditions. Quantifications on these particular 20% larvae that show aggregates confirmed that the number, but also to a lesser extent, the size of the aggregates were enhanced (Supplementary Material, Fig. S3). Using filter retardation assay (Fig. 4D), we could confirm that human polyQ-hHtt aggregation was globally enhanced when endogenous dHtt amount was decreased. This enhancement in aggregation could also be visualized on Western blots (Supplementary Material, Fig. S4). These data confirm again that endogenous dHtt level of expression interferes with polyQ-hHtt-induced protein aggregation.

Hence, finally tested whether the protective effect of EN actually required endogenous dHtt, as hypothesized previously. Indeed, we noticed that EN was not able to rescue polyQ-hHtt aggregation when endogenous Drosophila d htt is decreased by RNA interference (Supplementary Material, Fig. S5), confirming that the level of d htt expression is important in the process of rescue by EN.

Altogether, these data clearly indicate that dHtt plays a protective role against protein aggregation induced by polyQ-hHtt expression. We then wondered whether the wild-type human Htt N-terminus could also play such a protective role.

**Conservation of functions between Drosophila and human Huntingtin**

In conditions where strong aggregation (29°C) of polyQ-hHtt (HA-hHtt 171aa-138Q) (Fig. 5A1) was observed, we replaced the control UAS-LacZ by a wild-type UAS-hHtt transgene (UAS-hHtt 548aa-0Q) containing a larger fragment of the hHtt N-terminus (548 amino-acids). We could observe a suppression of aggregation (Fig. 5A2), which was confirmed by Western blot (Supplementary Material, Fig. S4), and by filter retardation assay (Fig. 5B). Notably, we could also notice a clear rescue of the subcellular localization of the mutant polyQ-hHtt protein. This protein colocalized strongly with the microtubule network (Fig. 5A3), such as the wild-type HA-hHtt 171aa-18Q (Fig. 1A) protein. This shows that increasing the amount of unexpanded hHtt N-terminus (first 548 amino acids) can suppress polyQ-hHtt-induced aggregation, and rescue the localization of the mutant protein. Similar effects were also observed with shorter hHtt N-terminal fragments (hHtt 171aa-18Q, hHtt 77aa-20Q), even though they were not so effective (data not shown).

Altogether, these data indicate that the ratio between wild-type and mutant human Htt N-terminal fragments is critical in the formation of aggregates.

**Influence of wild-type hHtt on polyQ-hHtt-induced eye degeneration**

We also tested the rescuing effect of wild-type hHtt N-terminus onto polyQ-hHtt-induced eye degeneration (Fig. 6). Similar eye toxicity phenotypes were obtained using a gmr-Gal4 driver (Fig. 6A) and different polyQ-hHtt Drosophila strains, covering the first 67 aa, 171 aa or 548 aa (Fig. 1 and Supplementary Material, Fig. S6, and data not shown). These phenotypes are age-dependent eye depigmentation, as well as disorganization of the ommatidial array (Supplementary Material, Fig. S6). Because of the limitation of chromosomal localization of the polyQ-hHtt inserts, the following eye toxicity experiments have been carried out with mutant hHtt 77aa-93Q, referred to as mutant polyQ-hHtt.

When normal hHtt 548aa-0Q was coexpressed, the eye depigmentation defect induced by hHtt 67aa-93Q was rescued (Fig. 6A2 compared to A1), as well as the organization of the ommatidia (Supplementary Material, Fig. S6B3 compared to S6A3). Such a rescue was also clearly visible with the hHtt 71aa-18Q (Supplementary Material, Fig. S6C), and to a lesser extent, with the hHtt 67aa-20Q (Supplementary Material, Fig. S6D). Therefore, as shown for the aggregation phenotype, the relative expression level of mutant and wild-type hHtt forms is crucial for eye degeneration since increasing the amount of wild-type hHtt N-terminus can completely rescue the toxicity induced by the expression of mutant polyQ-hHtt.
Next, we analysed how the level of dHtt expression could affect eye phenotypes induced by mutant polyQ-hHtt. Increasing the level of expression of dHtt N-terminus is also able to rescue eye toxicity induced by polyQ-hHtt (Fig. 6A3). Conversely, knock down of dhtt expression induced by RNA interference was shown to enhance eye degeneration induced by mutant polyQ-hHtt (data not shown).

Altogether, our results show that either human or Drosophila wild-type Htt is able to rescue eye degeneration induced by mutant polyQ-hHtt. Moreover, the ability of wild-type hHtt N-terminal fragments to rescue polyQ-hHtt phenotypes led us wonder whether these wild-type hHtt forms were able to rescue eye degeneration induced by RNAi knockdown of endogenous dhtt expression. As already shown, reduction of dhtt expression caused a progressive eye phenotype, characteristic of neurodegeneration (31). Indeed, using a gmr-Gal4 driver, newly born flies that carry UAS-dHtt-RNAi transgene in the presence of one control UAS-GFP transgene presented normal eyes (Fig. 6B1). When grown at 29°C, these flies progressively showed a loss of pigmentation (see 20 days old flies in Fig. 6B2), associated with a rough eye phenotype characterized by abnormal ommatidial arrays (data not shown). In the same conditions, when the control UAS-GFP transgene was replaced by wild-type UAS-hHtt N-terminus, adults presented normal eyes even after 20 days at 29°C (Fig. 6B3). Normal hHtt covering the N-terminal 548 aa gave, however, a better rescue than shorter constructs.

Human and Drosophila Htt share biological properties

Our previous results strongly suggested that the human and Drosophila Htt can fulfil same biological properties in terms of suppressing polyQ-hHtt protein aggregation and toxicity.
This shows that the hHtt N-terminal 548 aa fragment is able to rescue a dhtt knockdown phenotype, confirming that the human and Drosophila Htt homologs share biological properties. In addition, this indicates that the first 548 amino acids of the human Htt protein contain critical protein domains required for Htt function in long-term cell viability.

Wild-type hHtt acts specifically on HD

The next question was to test whether the protective effect of wild-type hHtt with respect to polyQ-hHtt-induced phenotypes could be generalized to phenotypes induced by other polyQ proteins known to be the cause of other neurodegenerative diseases (41). To answer in a general way, we tested whether wild-type hHtt N-terminal 548 aa fragment was able to rescue the eye degeneration phenotype induced by polyQ expansion alone, using a UAS-GFP-75Q transgene expressed in the eye (Fig. 6C). We observed that the expression of hHtt548aa-0Q had no effect on polyQ-induced loss of pigmentation (Fig. 6C2, compared to 6C1 in the presence of control UAS-LacZ). We also verified by scanning electron microscopy that eye roughening was identical in these different genetic backgrounds (data not shown). Hence, expression of wild-type hHtt N-terminal 548 aa fragment is not able to prevent the molecular pathways leading to neurodegeneration that are shared by all polyQ diseases, showing that this fragment is only acting on a specific neurodegenerative pathway induced by polyQ-Htt. This result was confirmed with transgenic lines that express polyQ proteins responsible for other polyglutamine diseases, including SCA1-82Q (42) or SCA3-78Q (43) (data not shown). Therefore, neurodegeneration in the case of HD results from a combination of polyQ toxicity (a mechanism shared by different polyQ diseases) and a specific action of mutant polyQ-Htt. We show here that the specific action of polyQ-Htt, but not the polyQ toxicity, is sensitive to the amount of wild-type Htt.
Ratio between wild-type and mutant polyQ-hHtt is crucial for aggregation in mammalian cells

In order to confirm the protective role of wild-type hHtt in human cells, we further analysed polyQ-hHtt aggregation in HeLa cultured cells. HeLa cells were transfected with a GFP-hHtt171aa-138Q expressing construct. Twenty four hours after transfection, we observed that polyQ-hHtt aggregates were formed in the cytoplasm (Fig. 7A1), as found in vivo in Drosophila cells (Fig. 1A). When a myc-hHtt548aa-0Q was coexpressed with GFP-hHtt171aa-138Q, we could not detect any aggregate in the majority of the cells (Fig. 7B). In addition, the mutant polyQ-hHtt fragment (GFP-hHtt171aa-138Q) became normally diffusely localized in both the nucleus and cytoplasm (Fig. 7B1), like the wild-type hHtt548aa-0Q fragment (Fig. 7B2). These data confirm that the ratio between wild-type...
and mutant polyQ-hHtt is crucial in the formation of aggregates in mammalian cells. Interestingly, GFP-hHtt$^{548aa-138Q}$ aggregate formation potential was also highly reduced when cells were co-expressing a Drosophila myc-dHtt$^{620aa}$ protein (Fig. 7C1 and C2). This further proves the functional conservation between human and Drosophila Htt N-terminus. Quantifications using filter retardation assays also confirm these observations (Fig. 7D). These results show that Drosophila model is able to reconstitute events detected in human cells.

**DISCUSSION**

Using a Drosophila model of HD, we first show that an N-terminal fragment of hHtt, in the presence of expanded
polyQ domain (hHtt^{171aa-138Q}), is sufficient to reconstitute different landmarks of HD, since it forms cytoplasmic aggregates in different cell types (salivary glands and neuronal cells for instance, in Supplementary Material S1) and drives eye toxicity, associated to a depigmentation of the eye that was otherwise correlated with photoreceptor neurodegeneration. These phenotypes corroborate those identified with longer proteins (26,36).

The identification of *Drosophila huntingtin* (dhtt) as a potential direct target of EN regulation, as well as the protective role of EN on polyQ-hHtt-induced phenotypes, led us to analyse what would be the effects of changes in dhtt level of expression on these phenotypes.

We first identified a genomic DNA fragment lying within intron 15 of *d htt* gene that was specifically binding EN both *in vitro* (by gel shift assays) and *in vivo* (by ChIP experiments, or by transcriptional assay). Since EN expression was found to activate d htt (by real time PCR) and since EN was able to prevent polyQ-hHtt aggregation, it suggested that the activation of d htt by EN might be responsible for the protective effect of EN on the formation of aggregates. Accordingly, we have been able to show that wild-type dHtt covering N-terminal 620 aa was able to prevent the accumulation of polyQ-hHtt. Conversely, we also found that knockdown of d htt expression, induced by RNA interference (31), enhanced the aggregation of polyQ-hHtt. These observations strongly suggested that the ratio between wild-type and mutant Htt is important in the onset of polyQ-hHtt cellular toxicity, and that *Drosophila* and human Huntingtin might share biological properties.

**The ratio between wild-type and mutant Htt is crucial in the onset of polyQ-hHtt-induced phenotypes**

In this report, by testing directly the effect of addition of normal hHtt on different polyQ-hHtt-induced phenotypes (protein aggregation and eye degeneration), we could verify that the ratio between wild-type and mutant hHtt N-terminal fragments is crucial in the onset of these different phenotypes, as it has been proposed by several studies (12–15,17,44).

Indeed, we first showed that the level of expression of the mutant polyQ-hHtt is correlated to the level of deleterious phenotypes, since aggregation or eye degeneration varies according to conditions of expression (temperature, number of UAS transgenes). Second, increasing the level of wild-type hHtt is able to prevent polyQ-hHtt aggregation and eye toxicity associated to the accumulation of polyQ-hHtt. We also show here that wild-type hHtt is not only able to prevent the formation of aggregates, but is also able to restore a normal subcellular localization of the mutant polyQ-hHtt along the microtubule network. This strongly suggests that, when not aggregated, polyQ-hHtt might be able to exercise a normal function in an intracellular transport for instance, which also confirms that the presence of long CAG tract does not completely abolish the normal functions of the protein (15,31,45).

That the ratio between wild-type and mutant hHtt N-terminal fragments is crucial for aggregation has been also tested and confirmed in mammalian HeLa cells.

Rescue experiments have been performed with different wild-type hHtt, covering different lengths of the N-terminus of hHtt, that are covering the exon1 (corresponding to hHtt^{67aa-20Q}) (35), or to longer N-terminal regions covering either 171 aa (hHtt^{171aa-18Q}) or 548 aa (hHtt^{38aa-0Q}) (36).

In the experiments presented here, we can notice that influence on eye toxicity is particularly sensitive, since the short human (exon1) protein is sufficient to rescue eye dysmorphology, whereas reduction of aggregation was found to be more effective with longer proteins. This suggests that hHtt exon1 is involved at least in part in the rescue. We could also notice that none of these constructs has been otherwise able to significantly rescue rhabdomere neurodegeneration of the eye (data not shown). In studies in mice, the influence of wild-type Htt on HD has also been tested (16,21), and was found to only partially ameliorate striatal phenotypes induced by polyQ-hHtt. However, the observation that striatal phenotypes were not much worsen in YAC128 mice in htt knock-out background, compared to a wild-type background (21), may be reinterpreted according to our results. Indeed, the persistence of a short 20 kDa protein covering N-terminal 224 aa of endogenous Htt protein in the knock-out background (46) may have a protective effect against neurodegeneration. Still, the converse experiment, i.e. the addition of full-length hHtt in YAC128 mice resulted in a clear, but mild improvement in striatal neuropathology (16).

Moreover, those results suggest that restoring wild-type Htt might not be sufficient to completely cure HD, but it may help to prevent some of the early symptoms of HD. In particular our data confirm, as already noticed by different groups (12,47), that therapeutics that only trigger the gain of function defects of HD, might reduce the expression of both alleles, which could result in a degenerative phenotype.

Interestingly, we also identified that the rescue is specific to polyQ-hHtt since, eye toxicity induced by a polyQ fragment only (Fig. 6C) or other non-HD polyQ disease proteins (data not shown), cannot be rescued by wild-type hHtt. This suggests that the ability of wild-type Htt to protect against HD might set molecular mechanisms different from those involved by other polyQ diseases. Direct interactions between wild-type and polyQ-hHtt N-terminus have been identified using the two-hybrid approach in yeast (unpublished results), which suggests that the rescue of phenotypes by normal N-terminal Htt fragments may involve interactions with the polyQ-hHtt fragments.

Therefore, one strategy to consider in preventing HD will be to design synthetic polypeptides that cover human exon1 or other parts of hHtt 548 aa and test whether these peptides can reduce polyQ-hHtt-induced phenotypes *in vivo*. Precedence for this approach comes from the expression of peptides that inhibit aggregation and cell death (48–50). Domains isolated from Htt exon1 have already been tested for their abilities to interfere on polyQ-hHtt aggregation, among which the first 17 amino acids were found to enhance the propensity of Htt to aggregate, whereas the presence of the proline-rich region appeared to reduce the aggregation potential of Htt (51).

One scenario could be that, once cleaved, mutant proteins will aggregate at a rate that not only depends on the size of the polyQ repeat (52), but also on the amount of wild-type Htt in the cell. Therefore, over time, on one hand the high stability of the mutant protein in aggregates (53), and the sequestration of the wild-type Htt protein in these aggregates (29,54), will be particularly deleterious for the cell survival.
(12,15,31,55). Altogether, these data confirm that dissecting the impact of different Htt domains upon HD phenotypes might help in the development of future therapeutic intervention.

Human and Drosophila Huntingtin homologs share biological properties

An important issue was also to test how far the level of expression of d htt was able to interfere on phenotypes induced by polyQ-hHtt. Indeed, since EN was found to directly activate d htt and to prevent polyQ-hHtt aggregation, one hypothesis was that the process of rescue by EN requires dHtt. We provide here several arguments for such a mechanism. If mutant polyQ-hHtt acts as a dominant negative, resulting in an impairment of wild-type dHtt function, and if dHtt and its human homolog are acting in the same biological process, we would expect polyQ-hHtt aggregation to vary according to d htt expression level. We indeed could show that the expression of dHtt N-terminal fragment was able to prevent polyQ-hHtt aggregation, whereas the aggregation potential was intensified in a context of reduction of the d htt expression mediated by RNA interference. Additionally, we observed that polyQ-hHtt-induced-eye toxicity also varies according to dHtt level of expression.

RNA interference against d htt expression in the eye was shown to drive photoreceptor neurodegeneration mimicking the expression of mutant polyQ-hHtt-induced eye phenotype (31). Interestingly, we observed that eye morphodermorphism resulting from d htt knockout mediated by RNA interference was compensated in the presence of wild-type human hHtt. As well, we found in different tests on eye toxicity or in HeLa cultured cells, that Drosophila or human Htt was able to prevent human polyQ-hHtt accumulation. Whereas dHtt contains only few regions of high similarity with the human Htt ((11) and Supplementary Material, Fig. S2) and in particular does not contain a polyQ domain, we show here that dHtt shares important biological properties with its human homolog, since hHtt can compensate dHtt function when d htt expression was knocked down.

The functional homologies between Drosophila and human Htt proteins, that we have identified in this report, makes Drosophila a privileged organism to study HD pathologic mechanisms in vivo, making the different genetic screen for modifiers of induced polyQ-hHtt neurodegeneration particularly informative and particularly useful to open new avenues to future therapeutic strategies (26,56).

Finally, the implication of wild-type level of expression in the strength of the polyQ-hHtt-induced phenotypes, as shown in this report, shows that studies on transcriptional mechanisms to future therapeutical strategies (26,56).

MATERIALS AND METHODS

Drosophila stocks and transgenes

MS1096-Gal4 (on X) (58); gmr-Gal4 (on II); hs-Gal4 (on III); UAS-GFP (on II); UAS-LacZ (on III) were obtained from the Bloomington Drosophila stock centre. UAS-dHttRNAi was obtained from Goldstein et al. (31). UAS-Htt67aa-20Q and UAS-Htt67aa-93Q were obtained from Leslie Thompson (University of California, Irvine) (35). UAS-Htt548aa-0Q and UAS-Htt548aa-128Q were obtained from J. Troy Littleton (36).

UAS-EN-hHtt171aa-18Q and UAS-EN-hHtt171aa-138Q were made as follows: cdNA encoding an N-terminal fragment (171 amino acids) of human Huntingtin (with 18 glutamine repeats, normal or with 138 glutamine repeats, mutant) obtained from Michael Hayden (24) has been subcloned into pUAST vector. Both forms have been tagged with HA epitope at their 5’-end.

UAS-GFP-75Q-FLAG: CAG repeats were PCR amplified from UAS-MJDtr-78Q flies (43) using the following primers: MJDtrQFLAGF 5’-CGG AAT TCT ACT TTG AAA AAC AGC AG-3’; MJDtrQFLAGR 5’-TGC GCC CGC TCA CTT ATC GTC ATC GTC CTT GTA ATC TCC TGA TAG TGC CCG-3’. The resulting 75Q-FLAG PCR product was sequenced and a contraction of three CAG repeats was detected. The 75Q-FLAG insert was then subcloned as an EcoR1/NolI fragment into the pUAST-GFP vector (a kind gift of Dr Cahir O’Kane; University of Cambridge, UK) to make pUAST-GFP-75Q-FLAG.

Drosophila cDNA Htt, covering the 620 aa in N-terminal part, has been provided by Drosophila Genomics resource Center (DGRC) and has been used as a matrix to be amplified by PCR to get cloned in a gateway pDONR™ vector (invitrogen). It was then recombined with either pcDNA-myc for cell culture expression, or with pUAS-myc tagged in 5’-end, to construct transgenic Drosophila strains. pUAS transgenic lines covering exons 1 to 3 (corresponding to 81 aa) have also been constructed and used in rescue experiments on eye toxicity as shown in Figure 6A3.

DNA from hHtt171aa-138Q and hHtt548aa-0Q (36) transgenic Drosophila lines have been isolated by PCR on genomic fly DNA and cloned in a pDEST53 vector (Invitrogen) to be expressed in Hela cells with a GFP localized in N-terminus.

The d htt-115-GFP construct was obtained by inserting the 1.95 kb intron 15 of d htt into the WH-GFP vector, corresponding to a mini white-based P element vector with multiple cloning sites upstream to a minimal promoter (10). Several transgenic lines have been obtained by injection of the vector, using P element.

Immunostainings

Salivary glands of third instar larvae were dissected and fixed 20 min in fixation buffer [30 mM Pipes (pH 7.4); 160 mM KCl; 40 mM NaCl; 4 mM EGTA; 1 mM spermidine; 0.4 mM spermine; 0.2% β- mercaptoethanol; 0.1% Triton X-100 and 3.7% paraformaldehyde], then washed four times in PBT: PBS±0.1% Triton X-100 and incubated for immunostainings with antibodies in PBT in the presence of 1% BSA. Primary antibodies were polyclonal rabbit anti-HA (SC805, 1:400,
Santa Cruz), secondary detected by Cy3-conjugated antibody; monoclonal mouse anti-β-tubulin (E7, 1:7, Developmental Studies Hybridoma bank), secondary detected by Cy2-conjugated antibody. Nuclei were counterstained with DAPI (4’, 6 diamino-2-phenylindole). Images were acquired with a Biorad 1024 or a Zeiss LSM 510 Meta confocal microscope.

Filter retardation assays

On salivary glands. Assays have been performed according to Ehrnhoefer et al. (40). Salivary glands from 10 larvae have been dissected and mixed with 30 μl of 2% SDS and 50 mM DTT, and denatured at 98°C for 7 min. Aliquots of 5 μg of protein samples in 200 μl of SDS 0.1% were filtered in triplicates.

On HeLa cells. Protein extracts from HeLa cells were performed according to Sittler et al. (59), with the following modifications: after transfection, HeLa cells pellets treated by DNase are resuspended into 150 μl of 1% SDS and 50 mM DTT in PBS. The samples were boiled for 5 min, and 3 × 45 μl of each sample was filtered. Protein samples from salivary glands or HeLa cells are filtered through a cellulose acetate membrane (0.2 μm pore size, Schleicher & Schuell), using a Bio-Rad dot-blot filtration unit. Membranes were then immunodetected with primary antibodies [rabbit polyclonal anti-HA antibody (1:600)], or with anti-hHtt antibody [mouse monoclonal VMA5492 from Abcys (1:5000)], as indicated. Immunoreactive spots were detected with chemiluminescent ECL substrate (Pierce), and quantifications have been performed using chemismart.

Transfections of cultured HeLa cells

Cells were grown in six-well plates, transfected with 1.5 μg total DNA with JetPei reagent (Qbiogene). Equal amounts of expression vector were added for each transfection. When necessary, an empty expression vector (pcDNA-myc) was added to equalize the total amount of plasmid DNA.

Quantitative RT–PCR

cDNAs were generated from 500 ng total RNAs treated with DNase I (Applied Biosystems) by using random hexamers and Superscript II reverse transcriptase (Invitrogen). Real-time PCR was done on an Applied Biosystems 7500 Real-Time PCR System (Courtaboeuf, France) with SYBR Green PCR mix according to the manufacturer’s instructions. PCR was done as follows: 10 min at 95°C followed by 40 cycles: 15 s at 95°C, 60 s at 60°C. Housekeeping genes used to normalize EN protein extract corresponds to a nuclear extract prepared from HSEN Schneider 2 cell line, in conditions described in Serrano et al. (38) and is estimated at (10–8 M). Competitions were performed in the presence of 750 ng unlabelled DNA, corresponding to D2 specific EN binding fragment, to N DNA fragment that do not bind EN (38), to C8 corresponding to a consensus EN binding sequence or to C8* corresponding to mutated consensus (10). Supershift experiments are performed in the presence of 1 μl of 4F11 anti-EN antibody.

Chromatin ImmunoPrecipitation (ChiP) and Southern blot analysis

Probes corresponding to immunoprecipitated chromatin have been prepared according to Chanas et al. (39). Seventy-five nanograms of PCR-amplified DNA purified after ChiP either in the presence of anti EN antibody (IP-EN) or in the absence of antibody (Mock) were radio labelled by the random priming Rediprime kit, and probed on Southern blots containing two intronic regions of htt gene that have been obtained by specific PCR performed on genomic DNA of wild-type adult flies. Equal amounts of amplified 1950 bp intron 15 (that contains the 4A8 fragment), and 1823 bp intron 21 genomic regions have been loaded on 0.8% agarose gel and transferred onto nylon membrane (PerkinElmer life sciences). Duplicates have been hybridized with either a probe corresponding to IP-EN or a probe corresponding to the Mock.

Gel shift assays

Gel shift assays were performed according to Solano et al. (10), where in each lane, 0.5 ng of end-labelled 4A8 DNA has been incubated in the presence of varying quantities of a EN protein extract, lying from 0.3 to 1 μl, as indicated. The EN protein extract corresponds to a nuclear extract prepared from HSEN Schneider 2 cell line, in conditions described in Serrano et al. (38) and is estimated at (10–8 M). Competitions were performed in the presence of 750 ng unlabelled DNA, corresponding to D2 specific EN binding fragment, to N DNA fragment that do not bind EN (38), to C8 corresponding to a consensus EN binding sequence or to C8* corresponding to mutated consensus (10). Supershift experiments are performed in the presence of 1 μl of 4F11 anti-EN antibody.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

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