DJ-1 modulates aggregation and pathogenesis in models of Huntington’s disease

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The oxidation-sensitive chaperone protein DJ-1 has been implicated in several human disorders including cancer and neurodegenerative diseases. During neurodegeneration associated with protein misfolding, such as that observed in Alzheimer’s disease and Huntington’s disease (HD), both oxidative stress and protein chaperones have been shown to modulate disease pathways. Therefore, we set out to investigate whether DJ-1 plays a role in HD. We found that DJ-1 expression and its oxidation state are abnormally increased in the human HD brain, as well as in mouse and cell models of HD. Furthermore, overexpression of DJ-1 conferred protection against neurodegeneration in yeast and Drosophila. Importantly, the DJ-1 protein directly interacted with an expanded fragment of huntingtin Exon 1 (httEx1) in test tube experiments and in cell models and accelerated polyglutamine aggregation and toxicity in an oxidation-sensitive manner. Our findings clearly establish DJ-1 as a potential therapeutic target for HD and provide the basis for further studies into the role of DJ-1 in protein misfolding diseases.

INTRODUCTION

Huntington’s disease (HD) is associated with the occurrence of both oxidative stress and polyglutamine (polyQ) misfolding/aggregation and this has sparked much interest in the therapeutic roles of molecular chaperones (1). Molecular chaperones are able to modulate polyQ aggregation and toxicity in vitro and in vivo and have also been implicated in the regulation of cellular redox homeostasis (1–3). Certain molecular chaperones may themselves also be regulated by changes in the oxidation status of a cell, which is critical to coordinate their response (4,5).

The DJ-1 protein is such an oxidation-sensitive chaperone (6,7) and has been suggested to play a role in several diseases associated with aging, including cancer and neurodegenerative conditions (8,9).

Ageing itself leads to increased oxidative modification of the DJ-1 protein in different animal models and in human brain samples (10,11). Importantly, mutations in the gene encoding for DJ-1 are associated with autosomal recessive forms of Parkinson’s disease (PD) (12). Recently, DJ-1 was found to play a crucial role in neuronal survival in the substantia nigra during aging (13). DJ-1 levels have also been shown to rise in patients with Alzheimer’s disease (AD), PD, amyotrophic lateral sclerosis and dementia with Lewy bodies (14–16). In addition, there is accumulation of monomers, acidic and basic isoform dimers in AD and sporadic PD patients (17). Interestingly, irreversible oxidative damage to DJ-1 is associated with AD and sporadic PD (17).

Since age-related neurodegenerative diseases share common mechanisms of oxidative stress, and DJ-1 is known to exert its protective functions under oxidizing conditions (18–20), we decided to investigate whether DJ-1 levels and its oxidation state are changed during HD and whether increased or decreased levels of DJ-1 in vivo modulated polyQ pathiology. We

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demonstrate that DJ-1 expression and oxidation is abnormally increased in the human HD brain, in the brain of an HD mouse model and in various HD cell models. Importantly, DJ-1 overexpression conferred protection against neurodegeneration in a fly model of HD. Furthermore, we found that DJ-1 directly interacted with an expanded fragment of huntingtin Exon 1 (httEx1) in the test tube and in cell models, providing a basis for the aggregation and toxicity modulating effects we observed. While the precise mode of DJ-1 action during polyQ pathology remains to be established, we provide data that clearly highlight DJ-1 as a therapeutic target for HD. Given that pharmacological compounds are available which interact with and modulate DJ-1 function (21,22), our studies provide important support for future in vivo studies to establish the potential role of DJ-1 during HD, and its efficacy as a therapeutic target for this disorder.

RESULTS

DJ-1 expression and its oxidation is increased in the HD brain and models of HD

We first investigated whether the levels of DJ-1 were changed in the HD brain by performing biochemical analysis using immunoblotting of HD Vonsattel Grade 2 human brain samples in comparison to age-matched control brain samples. We found that DJ-1 protein levels were significantly increased in the frontal cortex of HD brain, but not in the cerebellum (Fig. 1A–C). Coomassie gels were run in parallel to control the frontal cortex of HD brain samples, but not in the cerebellum found that DJ-1 protein levels were significantly increased in comparison to age matched control brain samples. We therefore next investigated whether the increase of DJ-1 levels in the HD brain and models of HD

First, we employed an established yeast model of mutant htt toxicity (28,29) to address the effect of DJ-1 on mutant htt toxicity and aggregation. As previously described, expression of a mutant htt fragment with an expanded polyQ stretch (HttQ72) leads to htt aggregation and cellular toxicity in yeast (Fig. 2). Saccharomyces cerevisiae cells bear four DJ-1 homologs (Hsp31, Hsp32, Hsp33, Hsp34), with Hsp31 being the best characterized. In addition to Hsp31 being required for response to oxidative stress (30), the crystal structure of Hsp31 is similar to human DJ-1 (31), suggesting Hsp31 performs parallel functions to DJ-1 in yeast. We found that Hsp31 overexpression suppresses httQ72 toxicity (Fig. 2A), which correlated with a reduction in aggregated mutant htt protein (Fig. 2B). This suggests that Hsp31 overexpression modulates httQ72 aggregation, thereby altering its toxicity. In contrast, the expression of human DJ-1 led to an increase in HttQ72 aggregation. No consequent changes were observed on toxicity, though enhancement of this phenotype cannot be assessed as httQ72 completely abolishes growth of the yeast.

We next exploited a well-characterized fruit fly model of HD to further explore the modulatory role of DJ-1. In this model, pan-neuronal expression of an exon 1 mutant htt fragment (httEx1Q93) using the elavGAL4 driver leads to robust degeneration of photoreceptor neurons (rhabdomers) in the fly eye, as well as other disease-relevant metrics (32). Fruit flies express two DJ-1 orthologs, DJ-1α and DJ-1β which have been found to play a role in mitochondrial function and protection from oxidative stress (10,33,34). We found that knockout of either Drosophila DJ-1 isomorph does not affect the neurodegeneration of rhabdomers (Supplementary Material, Fig. S3). However, overexpression of DJ-1α in the same tissues as httQ93 ameliorates neurodegeneration, rescuing up to ~45% of neuronal death (Fig. 3A, two-way ANOVA, \( F_{1,48} = 88.52, P < 0.0001 \)). While overexpression of the DJ-1β isoform appeared to confer slight neuroprotection when measured at Day 1, no significant rescue of neurodegeneration was observed in total in the experiment (Fig. 3B, two-way ANOVA, \( F_{1,47} = 1.59, P = 0.2138 \)). Importantly, we performed a GAL4 titration control by co-expressing dsRED with httQ93 and found no protection (Supplementary Material, Fig. S4), indicating that DJ-1α is specifically protective in this HD model.

DJ-1 overexpression ameliorates mutant htt toxicity in yeast and fruit flies

To test whether DJ-1 plays a role in HD pathogenesis, we first interrogated its potential function in yeast and Drosophila models of HD. First, we employed an established yeast model of mutant htt toxicity (28,29) to address the effect of DJ-1 on mutant htt toxicity and aggregation. As previously described, expression of a mutant htt fragment with an expanded polyQ stretch (HttQ72) leads to htt aggregation and cellular toxicity in yeast (Fig. 2). Saccharomyces cerevisiae cells bear four DJ-1 homologs (Hsp31, Hsp32, Hsp33, Hsp34), with Hsp31 being the best characterized. In addition to Hsp31 being required for response to oxidative stress (30), the crystal structure of Hsp31 is similar to human DJ-1 (31), suggesting Hsp31 performs parallel functions to DJ-1 in yeast. We found that Hsp31 overexpression suppresses httQ72 toxicity (Fig. 2A), which correlated with a reduction in aggregated mutant htt protein (Fig. 2B). This suggests that Hsp31 overexpression modulates httQ72 aggregation, thereby altering its toxicity. In contrast, the expression of human DJ-1 led to an increase in HttQ72 aggregation. No consequent changes were observed on toxicity, though enhancement of this phenotype cannot be assessed as httQ72 completely abolishes growth of the yeast.
Having found that DJ-1 activity can modulate both mutant httEx1 aggregation and toxicity in yeast and flies, we next investigated whether this was due to a direct interaction between these proteins by exploiting bimolecular fluorescence complementation (BiFC) assays (35). In order to ascertain whether DJ-1 and mutant httEx1 interact directly, and to quantify the level of any interaction, we transfected constructs encoding either a httEx1 fragment (with either Q25 or Q103) or DJ-1 fused to non-fluorescent halves of a fluorescent reporter into H4 human neuroglioma cells. Using this approach, if htt and DJ-1 interact, the two non-fluorescent halves would be brought together, reconstituting the functional fluorophore. Flow cytometry analyses showed that transfection with all BiFC pairs (Q25/Q25, Q103/Q103, Q25/DJ-1 and Q103/DJ-1) produced fluorescence (Fig. 4A), and this was confirmed by microscopy (Fig. 4B). Thus, DJ-1 appears to interact directly with both httEx1-Q25 and httEx1-Q103 in living human cells. Not surprisingly, the

Figure 1. DJ-1 expression is increased in the frontal cortex of HD brain tissue, R6/2 mouse tissue and in a mutant httEx1-expressing Tet-inducible cell system. (A) Immunoblot analysis showing expression levels of DJ-1 in the frontal cortex and cerebellum. (B) Quantification of DJ-1 expression levels in the frontal cortex of HD samples relative to controls. (C) Quantification of DJ-1 expression levels in the cerebellum of HD samples relative to controls. (D) Immunoreactivity of an antibody recognizing oxidized Cysteine 106 of DJ-1 in the frontal cortex and cerebellum. (E) Quantification of oxidized DJ-1 expression levels in the frontal cortex relative to controls. (F) Quantification of oxidized DJ-1 expression levels in the cerebellum relative to controls. Unpaired t-test was performed for statistical analysis (*P < 0.05, n = 5, frontal cortex and cerebellum, HD = Huntington’s disease patient tissue and C = age matched control tissue). (G) Immunoblot showing DJ-1 expression levels in the frontal cortex and cerebellum of transgenic animals (R6/2) versus wild-type littermate controls. (H) Quantification of DJ-1 expression levels in the frontal cortex of R6/2 relative to controls. (I) Quantification of DJ-1 expression levels in the cerebellum of R6/2 relative to controls. Unpaired t-test was used for statistical analysis (*P < 0.05, n = 5 except R6/2 cortex; n = 4). (J) Time course analysis of httEx1Q23-EGFP (21.20) versus httEx1Q74-EGFP (72.10) inducing cells after 24 h of induction with 1 μM of doxycycline (n = 2–4, one sample t-test was performed for statistical analysis, *P < 0.05). All quantifications were normalized with loading controls (Coomassie gels). Pixel intensity values were quantified by using Odyssey and/or ImageJ software. Error bars represent standard deviation.
Q103/Q103 pair yielded the highest fluorescence levels of pairs tested, suggesting that mutant huntingtin is more prone to dimerize/oligomerize than to interact with DJ-1. Next we explored the effect of DJ-1 on mutant htt aggregation in H4 cells. Our initial fluorescent microscopy studies revealed that DJ-1 and mutant htt co-aggregate in these cells (Fig. 4E). Furthermore, while DJ-1 does not modify the number of aggregates per cell (Fig. 4C) or the percentage of small- and medium-size aggregates (Fig. 4D), it produces a significant increase in the percentage of large aggregates (>3 μm) (Fig. 4D). These results complement our yeast filter trap data (Fig. 2B), and strongly suggest that mutant htt and DJ-1 directly interact and co-aggregate. We have previously observed that in this BiFC system Q103/Q103 pairs induce higher levels of toxicity than Q25/Q25 (Supplementary Material, Fig. S5) (36). In contrast to the work described above in yeast and flies, we found that cellular toxicity was significantly increased upon expression of DJ-1 with either httEx1-Q25 or httEx1-Q103 BiFC constructs. Due to this intriguing finding, we further explored this observation in another cell line (HeLa cells) and in primary cells (astrocytes) in order to test whether DJ-1 also increased the production of large httEx1 aggregates and toxicity in these cell types. We have measured percentage aggregates by counting mRFP positive cells with inclusion bodies (IB). Similarly, cell death was measured by scoring abnormal nuclear morphology of cells co-transfected with DJ-1 or control vector and httEx1Q97-mRFP.

The effects of exogenous overexpression of DJ-1 in HeLa cells and primary astrocytes were similar to the results in H4 cells. DJ-1 significantly increased IB formation of an httEx1Q97-mRFP construct at Days 2 and 3 after transfection in HeLa cells (Fig. 5A and B) and at Day 3 in astrocytes (Fig. 5E). We also observed a trend towards an increase in SDS-insoluble httEx1Q97-mRFP when coexpressed with DJ-1 in HeLa cells as shown by a dot blot analysis, although this did not reach statistical significance (Supplementary Material, Fig. S6). Overexpression of DJ-1 in transient cotransfection experiments was confirmed by immunoblotting and immunocytochemical (ICC) analysis (Supplementary Material, Fig. S7). This increased aggregation in HeLa cells and astrocytes was associated with a moderate, but significant increase in cell toxicity (Fig. 5C, D and F), similar to what we found in the H4 cells (see above). Surprisingly and in contrast to the above results we found that DJ-1 overexpression slightly reduced baseline toxicity induced by httEx1Q25-mRFP (Supplementary Material, Fig. S8). Using ICC, we also checked whether we could detect DJ-1 co-localization with IBs. We found that endogenous DJ-1 occasionally co-localized with IBs in a ring-like structure (Supplementary Material, Fig. S9). When DJ-1 was overexpressed exogenously we also found ring-like co-localization, but more frequently (Supplementary Material, Fig. S9 and data not shown).

Together our cell data suggest that DJ-1 likely associates with httEx1 and accelerates its aggregation when a polyQ expansion is present. It remains unclear why increased expression of DJ-1 was not protective in mammalian cell models (in contrast to our results with DJ-1 orthologues in yeast and Drosophila). We speculated that DJ-1 perhaps exerted different functions in relation to its oxidation status, which is well established to directly affect the chaperone function of DJ-1. Hence, we investigated whether modulation of the cellular redox state influenced the protective function of DJ-1 in mammalian cell models of HD and whether this depended on DJ-1 cysteine oxidation.

**DJ-1 modulates aggregation of httEx1 in an oxidation-sensitive manner**

In order to test the effect of the cellular redox state of DJ-1 on aggregation of httEx1 we transfected HeLa cells with the respective constructs and then exposed the cells to a sub-lethal dose of an exogenous pro-oxidant (H2O2) or a compound known to produce endogenous free radicals (antimycin A). Under these conditions, the increase in mutant httEx1 aggregation and toxicity due to DJ-1 co-expression (as shown in the first two bars of the histogram in Fig. 5A and B) was lost as DJ-1 reduced both polyQ IB formation (Fig. 5A and B) and toxicity (Fig. 5D) of httEx1Q97-mRFP back to baseline. This loss in aggregation acceleration of expanded httEx1 by DJ-1 was also detected using the filter trap assay (Supplementary Material,
Fig. S6). Identical results were found when performing experiments with primary astrocytes (Fig. 5). These results clearly show that the acceleration of aggregation and increase in toxicity due to DJ-1 co-expression with httEx1Q97 is lost when cells are exposed to oxidizing conditions as applied in these experiments. A possible redox-modulation of cysteines within the DJ-1 protein may hence be able to modulate polyQ aggregation (and toxicity).

Figure 3. Overexpression of DJ-1α ameliorates neurodegeneration in HD model flies. Quantification of mean rhabdomeres (± SEM) per ommatidium in flies pan-neuronally expressing both httEx1Q93 and either DJ-1α or DJ-1β using the elavGAL4 driver using the pseudopupil assay. Overexpression of DJ-1α results in significant rescue of rhabdomere death at Days 1, 4, and 7 posteclosion (A) (F1,48 = 88.52, P < 0.0001), while overexpression of DJ-1β has no significant effect on neurodegeneration (B) (F1,47 = 1.59, P = 0.2138). Wild type and control flies (UAS alone, GAL4 alone, htt20Q) exhibit seven rhabdomeres per ommatidium (50). Statistical comparisons by ANOVA with Bonferroni post hoc tests. n = 5–13 samples per genotype (**** P < 0.0001).

Figure 4. DJ-1 interacts and colocalizes with mutant htt. H4 cells were transfected with httEx1-Q103-Venus1 in combination with either httEx-1Q103-Venus2 or DJ1-YFP2 BiFC constructs (A). After 24 h, cells were analyzed by flow cytometry (B) and widefield fluorescent microscopy (C–E). (B) Flow cytometry showed an increase in the fluorescence of cells transfected with both httEx1Q25/DJ1 and httEx1Q103/DJ1 pairs of biFC constructs, indicating that DJ-1 interacts with htt exon 1. Q25/Q25 and Q103/Q103 pairs were used as a positive controls. (C–E) These results were confirmed by microscopy. Cells transfected with the Q103/Q103 and Q103/DJ1 pairs showed similar patterns of fluorescence and aggregation. Aggregates were counted and classified according to their longer diameter in three classes: <1 μm, 1 μm but <3 μm, and 3 μm. DJ-1 did not modify the number of aggregates per cell, though the average size of Q103/DJ1 aggregates was significantly larger than aggregates generated by the Q103/Q103 pair. Scale bar, 20 μm. Statistical comparisons by Student’s t-test. Results depicted are the mean ± SEM of three independent experiments (* P < 0.05 versus Q103 pair).
To test this hypothesis, we co-expressed httEx1Q97-mRFP with a DJ-1 plasmid in which the cysteine 106 (Cys106) was replaced by a Serine (C106S-DJ-1). Thus, the C106S-DJ-1 could not be redox-modified by changing the cellular redox-state and should abolish its potential chaperone activity in a pro-oxidant cellular environment (such as treatment with H2O2 or antimycin A). Several studies have shown that Cys106 is fundamental to DJ-1 function and that it is the most oxidative stress susceptible cysteine residue within DJ-1 (6,18,37,38). Surprisingly, expression of C106S-DJ-1 further increased IB formation in both HeLa cells (Fig. 6A) and astrocytes (Fig. 6C) compared with cells co-transfected with wild-type DJ-1 (data not shown). The increase in polyQ aggregation was mirrored by a parallel increase in toxicity (Fig. 6B and D). As expected and in contrast to wild-type DJ-1, treatment with H2O2 or antimycin A neither modulated IB levels nor httEx1-Q97-mRFP toxicity when C106S-DJ-1 was co-expressed (Fig. 6B and D). Therefore, the influence of wild-type DJ-1 in a pro-oxidant cellular state upon IB formation and toxicity were lost with the C106S-DJ-1 mutant protein.

**DJ-1 protein increases httEx1 oligomerization and fibrillization in vitro in an oxidation-sensitive manner**

Having shown that DJ-1 was able to interact with httEx1 within cells - and that it co-localized to IBs and modulated polyQ aggregation in an oxidation-dependent manner – we speculated that...
DJ-1 may transiently associate with httEx1 to modulate early steps during the polyQ aggregation process. In order to test whether DJ-1 modulated polyQ oligomerization and fibril formation we performed in vitro analyzes using recombinant DJ-1 and httEx1 under normal and oxidizing conditions in combination with atomic force microscopy (AFM) as described previously (39). We co-incubated recombinant httEx1Q53 with recombinant DJ-1 at 1:1 stoichiometry and found that DJ-1 significantly enhanced mean httEx1Q53 oligomer height and accelerated mean fibril length and the number of fibrils per 10 μm² (Fig. 7). However, after pretreatment of DJ-1 with H₂O₂ (2 mM, see Materials and Methods) the DJ-1-dependent polyQ aggregation enhancing effect was lost or modestly inhibited (Fig. 7). The identity of recombinant DJ-1 and its ability to oxidize was confirmed by electrospray ionization mass spectrometry (ESI-MS; data not shown).

**DISCUSSION**

In this study, we show that DJ-1 protein expression and its oxidation is significantly increased in the human HD brain. In the R6/2 mouse model and several cell models of HD, we confirmed that DJ-1 expression is also upregulated due to the expression of expanded polyQ stretches. Hence, these data support the use of these models to study the functional consequences of enhanced expression of DJ-1 in HD. When exogenously overexpressed in an HD fly model we found protection against neurodegeneration, but in cell models DJ-1 overexpression increased aggregation and toxicity in a manner dependent upon the oxidative environment. These results suggest a complex mechanism by which DJ-1 modulates polyQ aggregation and its toxicity likely dependent on DJ-1 chaperone function and it is currently unclear whether its upregulation in vivo in patients contributes to HD pathology or not. Nonetheless, our in vivo results in an animal model presented here suggest that this upregulation is a protective response to the toxic insult of mutant htt.

In the fly model only DJ-1α was protective against polyQ toxicity, but not overexpression of DJ-1β. One possible explanation for this result is that DJ-1β is ubiquitously expressed in the fly (34), and therefore its activity may already be saturated. In contrast, DJ-1α appears to be primarily restricted to the male germ-line, and consequently its overexpression in neuronal tissue may result in an overall increase in neuronal resistance to the toxic insult of mutant httEx1 expression. Given that the absence of DJ-1 did not modulate polyQ neurodegeneration in the fly amorphs, we favor the idea that increased expression of DJ-1 is beneficial. This model is also supported by the yeast data...
where overexpression of the DJ-1 homolog Hsp31 rescued polyQ toxicity.

In both primary astrocytes and immortalized cells mild pro-oxidant conditions (acting via DJ-1 cysteine 106) likely activate the chaperone function of DJ-1 and therefore making its overexpression non-detrimental, and perhaps even beneficial (see Fig. 6)—supporting the findings in the fly and the yeast model. DJ-1 has been shown to reside in various oxidation states (18,19,40) and this fact may also contribute to some of the differences that we observed in cell models versus the fly model. Assuming that DJ-1 elevation is beneficial as shown in the fly and the yeast model where the DJ-1 homolog protected against httEx1 toxicity, its increased expression in the HD brain may indicate a protective stress response to protein misfolding and oxidative stress occurring during HD.

Irrespectively of whether DJ-1 elevation is protective or detrimental, DJ-1 is likely to exert its actions via a direct interaction with httEx1. This idea is supported by our interaction studies in cells and co-incubation experiments in the test tube. It seems that DJ-1 is able to interfere early during the polyQ aggregation pathways as we observe a difference in oligomerization when DJ-1 is exposed to a pro-oxidant (Fig. 7). Within primary astrocytes and immortalized cell lines, the aggregation-accelerating actions of DJ-1 appear not to eliminate toxic misfolded protein species by shifting the balance to less toxic fibrillar protein structures or IB, as we observed an increase in toxicity. However, to precisely define the interplay between DJ-1-induced modulation of polyQ aggregation and toxicity, further studies are necessary.

Our findings also suggest that DJ-1 is oxidatively modified in the HD brain, reminiscent of studies in AD and PD (17,41). As we have shown that mhttEx1 directly interacts with DJ-1—and early aggregation events of httEx1 with polyQ expansions lead to increased free radical production (22)—it may be possible that mhttEx1 aggregation leads to posttranscriptional modifications of DJ-1 such as a partial oxidation at cysteine 106 that is required for its protective chaperone function. However, when a further and complete oxidation of cysteine 106 (Cys106-So3H) occurs it may lead to a loss of DJ-1 function leading to a detrimental cellular outcome. It is important to consider that DJ-1 is mainly expressed in astrocytes under physiological conditions in the human brain (42) and these cells play

**Figure 7.** Recombinant DJ-1 accelerates httEx1Q53 oligomerization and fibril formation in an oxidation-dependent manner. DJ-1 redox activated chaperone activity was tested in *in vitro* using recombinant DJ-1 protein (both non-oxidized and oxidized form) in co-incubation experiments with httEx1Q53 at 1:1 stoichiometry. (A) DJ-1 increases mhttEx1Q53 oligomeric height whereas after pretreatment of DJ-1 with 2 mM H2O2 the oligomer height enhancing effect is lost. (B and C) DJ-1 accelerated the number of mhttEx1 fibrils per 10 \( \mu \)m² (B) and fibril length (C), whereas after pretreatment of DJ-1 with 2 mM H2O2, the aggregation enhancing effect is lost or polyQ aggregation is even modestly inhibited. (D) Examples of AFM images showing mhttEx1Q53 oligomers when incubated without DJ-1 or with DJ-1 or with oxidized DJ-1 (E) Examples of AFM images showing mhttEx1Q53 fibrils when incubation without DJ-1 or with DJ-1 or with oxidized DJ-1. Error bars represent standard deviation. Unpaired *t*-test was performed for statistical analysis (*P < 0.05, **P < 0.005, n = 2).
crucial roles in providing protection against oxidative insults (43,44). Despite this, we believe that it is possible that the observed oxidation of DJ-1 in the human HD brain may indicate a form of oxidative damage that perhaps contributes to toxicity in HD.

It is now important to understand the role of DJ-1 oxidation in HD pathology. This can be further studied by using DJ-1 modulating compounds (UCP0045037 and UCP0054278) that bind to the cysteine 106 region of the reduced form and partially oxidized form of DJ-1 (21). These compounds prevent the complete oxidation of DJ-1 by stabilizing the reduced form or by mimicking the partially oxidized form required for chaperone functions of DJ-1 (21,22), and have been shown to also inhibit ROS production, as well as oxidative stress induced toxicity, in SH-SY5Y cells and primary neuronal cell culture (21). Such compounds may thus have great therapeutic relevance in HD by mitigating free radical production and modulating polyQ-dependent pathology. It is now critical to explore the efficacy of these and related compounds in animal models of HD and to further elucidate the mechanistic underpinnings of how DJ-1 modulates pathogenesis in HD. Ultimately, such studies may inform novel therapeutic strategies for this devastating disorder.

MATERIAL AND METHODS

Plasmids, cell culture and transfection experiments

The chemicals used in this study were purchased from Sigma unless otherwise stated. Plasmids (pCNA3.1) containing the sequence for htt exon 1 (hEx1) with either 25 or 97 glutamine repeats fused to monomeric Red Fluorescent Protein (mRFP) at the C-terminus were used for transfections or adenoaviral infections (MOI 10–20) as described previously (45). HeLa cells were cultured in DMEM with 2 mM L-glutamine, 10% fetal bovine serum (FBS) and 100 U/ml penicillin with 100 µg/ml streptomycin at 37 °C, 10% CO2. The PC12 hEx1Q25/103-mRFP tebufenozide-inducible cell line (kindly provided by E. Schweitzer (46)) was cultured in DMEM with 5 mM HEPES, 5% FBS, 5% Horse serum, 2 mM L-glucose, 100 U/ml penicillin with 100 µg/ml streptomycin and G418 (0.5 mg/ml) at 37 °C, 5% CO2. PC12 Tet-inducible cells (21,20 and 72.10) were cultured in RPMI 1640 (GIBCO, Paisley, UK) 5% FBS, 10% Horse serum (GIBCO) 4.5 g/l D-glucose, 2 mM L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate, 100 U/ml penicillin and 100 µg/ml streptomycin, 75 µg/ml hygromycin (Invitrogen, Paisley, UK) and 100 µg/ml G418 (Invitrogen). One micromolar tebufenozide or 1 mM doxycycline was added to induce expression of HttEx1 in tebufenozide and Tet-inducible cell system, respectively. All PC12 cells were cultured on pre-coated poly-L-lysine surfaces. After 24 h of seeding, HeLa cells were exposed to the appropriate DNA construct/s and lipofectamine (Invitrogen) for 5 h in serum-free medium (OPTI-MEM, Invitrogen) for both transfection and co-transfection procedures as described previously (26,39).

Mouse primary astrocytes were cultured by dissecting P0 mouse cortices and meninges were removed. Cortices were cut into small cubes (<1 mm³), suspended in 20 ml of DMEM (maximum of five brains) and subsequently vortexed at maximum speed for 90 s in a 50-ml centrifuge tube (Greiner Bio One) and then the cell suspension was sieved through a 40-µm cell strainer (BD Falcon). Cell filtrate was mixed with DMEM supplemented by 10% Hybond FBS (characterized, Hyclone) and 100 U/ml penicillin with 100 µg/ml streptomycin (Sigma, Dorset, UK) and seeded in a 1 × T75cm2 laminin (20 µg/ml Sigma)-coated flask. Twenty-four hours after seeding, the cells were washed twice with 1 × PBS (GIBCO) and the media was replaced. Cell media was changed every 3 days and cells reached confluency after 9–12 days. Confluent flasks lids were sealed with parafilm, and shaken for 15 h at 180 rpm in a 37 °C heated incubator. After 15 h the media was removed and cells were washed with PBS. Cells were then trypsinized with 0.025% trypsin/EDTA (GIBCO) and seeded in a 6-well poly-L-lysine-coated plate with/without coverslips (200,000 cells per well for 6-well plate). These secondary cultures were then used after 3–4 days of seeding for transfection- and other studies (final age of astrocytes were normally between 15 and 20 days). Cells in astrocyte cultures were >80% GFAP positive and >99% cells were connexin 43 positive hence suggesting a high astrocyte purity. No oligodendrocytes or microglia cells could be detected by immunocytochemistry (data not shown).

Protein extraction, immunoblot and dot blot analysis

Cell transiently transfected, inducible cell lines (Tet-on and Tebufenozide-inducible cell system) and primary astrocytes were collected at different time points. Samples were lysed in SDS-containing lysis buffer (65.2 mM Tris–HCl, pH 6.8, 2% (w/v) SDS and 10% sucrose and complete protease inhibitor cocktail) followed by a 20-min incubation on ice and then stored at −20 °C. Seventeen-week-old R6/2 (25) and WT littermates were sacrificed and cortex and cerebellum brain regions were collected. Samples were extracted initially with 10% (w/v) homogenization buffer (20 mM HEPES and 100 mM KCl (pH 7.4) with complete protease inhibitors). Subsequently, total SDS soluble protein extraction was then performed by boiling the samples (both soluble and insoluble material) at 95 °C for 4 min with 2% SDS. Samples were then spun at 6000 rpm for 3 min to remove the SDS-insoluble material. Supernatants were collected and stored at −80 °C until further needed. Prescissed brain regions (cortex and cerebellum) from human HD patients and control groups were extracted with an ice-cold lysis buffer (40 mM β-glycerolphosphate, 1 mM EDTA, 1 mM NaF, 50 mM Tris–HCl (pH 7.5), 1% (v/v) NP-40, 120 mM NaCl, 1 mM benzamidine, antifoam (1 : 1000, Sigma) and complete protease inhibitors (Roche West Sussex, UK)). Human HD patients and control samples were then re-extracted using 2% SDS. Samples were stored until further required. Samples were thawed and the Bio-Rad DC protein assay was performed to quantify total soluble protein. Twenty to 40 µg of total protein was used and immunoblotting was performed as described previously (39,47). Membranes were incubated with primary antibodies, rabbit anti-DJ-1 antibody (1:2500; Neuronics, Minneapolis, MN, USA), rabbit anti-DJ-1 antibody (1:1000; cell signaling), human anti-oxidized DJ-1 (1:50; Serotec, UK), for 1 h at room temperature, unless otherwise stated. Membranes were washed three times 10 min with 1 × TBS-T followed by incubation with secondary antibodies (sheep anti-rabbit 800 (1 : 10 000) Rockland, Gilbertsville, PA, USA; Goat anti-rabbit HRP (1 : 10 000), Vector, UK and Human F(ab′)2 HRP (1 : 5 000), Jackson Immunoresearch,
West Grove, PA, USA). Fluorescent secondary antibody-labeled membranes were scanned with an Li-COR scanner at 800 nm and quantified by using odyssey v1.2 while HRP labeled secondary antibodies were analyzed by using an Enhanced Chemiluminescence (ECL) detection kit (GE Healthcare Life Sciences, Buckinghamshire, UK). Coomassie gels were used for loading control and the signal from the entire lane was used for normalization.

For dot blot analysis, HeLa cells were cotransfected with httEx1Q25/Q97-mRFP and DJ-1/EV (1:3) and treated with 5 μM antimycin A and 100 μM H2O2 as mentioned earlier. Cells lysates were collected after 72 h of transfection and processed as described previously (47). Dot blot lysates were normalized for total protein and filtered through a 2% SDS pre-equilibrated cellulose acetate membrane with a pore size of 0.2 μm. Membranes were washed twice with 0.2% SDS followed by incubation with 3% skimmed milk in 1× TBS. The membranes were then incubated with sheep anti-httEx1 (S830, Gilian Bates, Kings College London, London) antibody at a concentration of 1:5000 for 1 h at room temperature followed by an incubated with anti-sheep HRP (1:10 000, Jackson Immunoresearch) secondary antibody. Finally, membranes were developed by using ECL kit. Dot blot quantification was performed by using ImageJ software (http://rsbweb.nih.gov/ij/).

**Analysis of cellular httEx1 polyQ-aggregation and interaction studies, toxicity and immunocytochemistry**

For the BiFC interaction analysis, Htt-Venus BiFC constructs were used which have been previously described (36). The DJ-1 BiFC construct was generated by PCR-based subcloning with complementary pairs of BiFC constructs using the Xtreme-pcDNA3.1. H4 human neuroglioma cells were transfected of DJ-1 and either the N-terminal or C-terminal fragment of DJ-1 BiFC construct was generated by PCR-based subcloning. For the BiFC interaction analysis, Htt-Venus BiFC constructs were used which have been previously described (36). The DJ-1 protein was purchased from Abcam and quality control was performed by western blotting and ESI-MS. Samples were imaged by AFM in air with an uncoated silicon cantilevers (FM-W, Nanoworld Innovative Technologies, Switzerland, nominal spring constant 2.8 N/m) operating in tapping mode. The total number of fibrils per area was calculated by counting the number of fibrils in each 10 μm² image with at least five random images obtained from several experiments. A fibril was defined as an elongated structure of >200 nm in length. Western blotting was performed as described (39), but for the detection of epitopes we used both ECL (Amersham, Amersham, UK) using Kodak Scientific Imaging film and fluorescent secondary antibody-labeled membranes that were scanned with the Li-COR scanner at 700 or 800 nm and quantified by using Odyssey v1.2 software.

**Protein purification, in vitro aggregation and AFM analysis**

Expression of GST-httEx1-Q20/Q53 plasmids was performed as described (39). The DJ-1 protein was purchased from Abcam and quality control was performed by western blotting and ESI-MS. Samples were imaged by AFM in air with an uncoated silicon cantilevers (FM-W, Nanoworld Innovative Technologies, Switzerland, nominal spring constant 2.8 N/m) operating in tapping mode. The total number of fibrils per area was calculated by counting the number of fibrils in each 10 μm² image with at least five random images obtained from several experiments. A fibril was defined as an elongated structure of >200 nm in length. Western blotting was performed as described (39), but for the detection of epitopes we used both ECL (Amersham, Amersham, UK) using Kodak Scientific Imaging film and fluorescent secondary antibody-labeled membranes that were scanned with the Li-COR scanner at 700 or 800 nm and quantified by using Odyssey v1.2 software.

**Analysis of mutant htt toxicity and aggregation in yeast**

Yeast strains used in this study are in the W303 (MATa can1–100 ade2-1 his3-11, 15 trp1-1 ura3-1 leu23,112) genetic background and carry an integrated human htt fragment construct, encoding either 25 or 72 glutamines (28,29). Yeast were transformed independently with pYX212/DJ-1, pBG1805/HSP31 (Open Biosystems) (49) or an empty vector control. Yeast cultures were grown overnight in complete media lacking uracil (SC-URA), containing yeast nitrogen base and glucose (2%). Yeast growth was synchronized by diluting the cultures to an optical density (OD600) of 0.1. After reaching mid-log phase, cultures were normalized for the same OD600, serially diluted (5-fold), and spotted onto SC-URA solid media-containing glucose (2%) or galactose (2%) as carbon sources. These carbon sources were used to repress or induce htt and Hsp31p expression, respectively. DJ-1 was under control of the TPI constitutive promoter.

For protein extraction, mid-log phase cultures were washed twice to remove glucose from the media and were diluted to an OD600 of 0.1 in SD-URA containing 2% of galactose. Cells were harvested after 8 h of induction and lysed by mechanical
disruption with acid-washed glass beads (425–600 nm, Sigma, St. Louis, MO, USA) in lysis buffer (50 mM Tris, pH 7.5; 1 × protease inhibitor cocktail, Roche), vortexing three times for 30 s each in a mini-bead beater (Biospec), with 1 min incubations on ice in between. Glass beads and cell debris were removed by centrifugation and protein extracts were quantified using the BCA protein assay kit (Pierce Biotechnology, Inc.). For SDS–PAGE, extracts were mixed with 4 × Protein Sample Buffer (200 mM Tris–HCl, 8% SDS, 40% glycerol, 0.4% bromophenol blue, 6% β-mercaptoethanol), boiled 10 min and subjected to electrophoresis using a 12% SDS–PAGE. The proteins were transferred to nitrocellulose membranes and detected by immunoblotting. Htt tagged with CFP was identified by a monoclonal α-GFP antibody (Santa Cruz Biotechnology, Inc.), used at a 1:1000 dilution. Hsp31p, tagged with HA, was detected with a polyclonal α-HA antibody (Santa Cruz) diluted 1:5000. α-CFP detection was done by an α-Mouse Ig horseradish peroxidase conjugated antibody (Amersham Bioscience, Piscataway, NJ, USA) at 1:10 000, whereas α-DJ-1 and α-HA were done by an α-Rabbit Ig horseradish peroxidase-conjugated antibody (Amersham Bioscience) also at 1:10 000. ImmunobLOTS were developed by ECL (Millipore, Billerica, MA, USA) and exposed to X-ray films. For filter trap experiments, cell lysates were obtained as described above and mixed with 1% SDS. A total of 100 µg of protein extract was loaded in a dot blotting apparatus and filtered by vacuum in acetate cellulose membranes (0.22 µm pore; GE Water & Process Technologies, Fairfield, CT, USA). Slots were washed two times with PBS 1% SDS. Detection of htt in the filter was performed by immunoblotting as described above.

Analysis of mutant htt-mediated neurodegeneration in fruit flies

Flies were raised on maize media, in LD12:12 at 25°C. The elav-GAL4<sup>cl55</sup> driver and the DJ-1<sup>α</sup>Δ<sup>72</sup> and DJ-1<sup>β</sup>Δ<sup>93</sup> deficiency lines (described in 34) were obtained from the Bloomington Stock Centre, Bloomington, IN, USA. The w<sup>++;UAS HttQ93</sup> exon 1 flies were a gift from Larry Marsh and Leslie Thompson (University of California, Irvine, CA, USA) (32). The UAS DJ-1α and DJ-1β overexpression lines were a gift from Alex Whitworth (University of Sheffield) (34). Neurodegeneration was assayed using the pseudopupil assay. Briefly, the number of visible rhabdomeres per ommatidium was scored for 50–200 ommatidia per fly, with 5–25 flies examined per genotype at Day 1, 4 or 7 posteclosion. Heads from appropriately aged flies were fixed to glass slides using clear fingernail polish and rhabdomeres were examined at ×500 magnification using an Olympus BH2 microscope.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

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