

Wild-type huntingtin protects neurons from excitotoxicity

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Abstract

Huntingtin is a caspase substrate, and loss of normal huntingtin function resulting from caspase-mediated proteolysis may play a role in the pathogenesis of Huntington disease. Here we tested the hypothesis that increasing huntingtin levels protect striatal neurons from NMDA receptor-mediated excitotoxicity. Cultured striatal neurons from yeast artificial chromosome (YAC)18 transgenic mice over-expressing full-length wild-type huntingtin were dramatically protected from apoptosis and caspase-3 activation compared with cultured striatal neurons from non-transgenic FVB/N littermates and YAC72 mice expressing mutant human huntingtin. NMDA receptor activation induced by intrastriatal injection of quinolinic acid initiated a form of apoptotic neurodegeneration within the striatum of mice that was associated with caspase-3 cleavage of huntingtin in neurons and astrocytes, decreased levels of full-length hunt-

ingtin, and the generation of a specific N-terminal caspase cleavage product of huntingtin. *In vivo*, over-expression of wild-type huntingtin in YAC18 transgenic mice conferred significant protection against NMDA receptor-mediated apoptotic neurodegeneration. These data provide *in vitro* and *in vivo* evidence that huntingtin may regulate the balance between neuronal survival and death following acute excitotoxic stress, and that the levels of huntingtin may modulate neuronal sensitivity to excitotoxic neurodegeneration. We suggest that further study of huntingtin's anti-apoptotic function will contribute to our understanding of the pathogenesis of Huntington's disease and provide insights into the selective vulnerability of striatal neurons to excitotoxic cell death.

Keywords: Excitotoxicity, Huntington's disease, Huntingtin, neurodegeneration, neuroprotection.
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Huntingtin (htt) is a widely expressed 350-kDa protein with little overall functional homology to other known proteins (Sharp *et al.* 1995). Huntington's disease (HD) is a progressive neurodegenerative disorder caused by an expansion of polyglutamine residues within the N-terminal region of htt (Huntington's Disease Collaborative Research Group 1993), and this polyglutamine expansion has been proposed to result in a novel toxic gain of function of the mutant protein (MacDonald and Gusella 1996). The normal function of htt is essential for proceeding through gastrulation during embryonic development (Duyao *et al.* 1995; Nasir *et al.* 1995; Zeitlin *et al.* 1995), and an anti-apoptotic function of wild-type htt has been demonstrated recently both *in vitro* (Rigamonti *et al.* 2000; Ho *et al.* 2001) and *in vivo* in peripheral cells (Leavitt *et al.* 2001). Loss of normal htt function was first suggested to be involved in HD based on the finding of neurodegeneration in adult mice heterozygous for targeted deletion of the *Hdh* gene (Zeitlin *et al.* 1995).

More recently, mice were generated that develop postnatal conditional targeting of the *Hdh* gene (Dragatsis *et al.* 2000). These mice also exhibit adult-onset neurodegeneration in the basal ganglia, supporting the hypothesis that decreasing levels of full-length wild-type htt sensitizes neurons to cell

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Abbreviations used: BDNF, brain-derived neurotrophic factor; DARPP, dopamine- and camp-regulated phosphoprotein, 32kDa; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HD, Huntington's disease; htt, huntingtin; MSN, medium-sized spiny striatal neuron; NeuN, neuronal nuclei; P, postnatal day; PBS, phosphate-buffered saline; QA, quinolinic acid; TUNEL, TdT-mediated duTP-biotin nick end labeling; YAC, yeast artificial chromosome.

death and that loss of htt function may play a role in the selective neurodegeneration of HD (Nasir *et al.* 1995).

Increased sensitivity of striatal neurons to glutamatergic activation of NMDA receptors leading to excitotoxic neurodegeneration has been postulated to be a critical factor in the pathogenesis of HD (Beal 1992). Mutant htt causes increased neuronal response to glutamatergic stimulation in cultured cells (Chen *et al.* 1999; Zeron *et al.* 2001) and in medium-sized spiny striatal neurons (MSNs) of several transgenic mouse models of HD (Levine *et al.* 1999; Cepeda *et al.* 2001; Zeron *et al.* 2002). An increased sensitivity to excitotoxic neuronal death in transgenic HD mice provided the first direct *in vivo* link between the CAG expansion in the HD gene and raised levels of excitotoxicity (Zeron *et al.* 2002). Glutamate over-activity in neurons leads to Ca²⁺-induced cellular dysfunction and activation of a variety of proteolytic enzymes, including caspase-3 and calpain (Goffredo *et al.* 2002; Wellington *et al.* 2002).

In this report, we provide the first evidence that increased levels of full-length wild-type htt protect against NMDA-mediated excitotoxicity. Direct caspase-3-mediated cleavage of htt and apoptotic neurodegeneration occur *in vivo* in response to stimulation of NMDA receptors in the striatum, a brain region selectively affected by HD. Caspase-mediated proteolytic cleavage decreases the endogenous levels of wild-type htt and may predispose striatal neurons to degeneration. Conversely, increasing wild-type htt levels within neurons is neuroprotective, and may represent a novel therapeutic approach for neurodegenerative diseases.

Materials and methods

We previously generated yeast artificial chromosome (YAC) transgenic mice that appropriately express full-length human htt with 18 (YAC18) or 72 (YAC72) polyglutamines (Hodgson *et al.* 1996, 1999). In this report we used three lines of transgenic mice maintained on a pure FVB/N strain background and their non-transgenic wild-type littermates. Two independent lines of YAC18 mice were utilized to assess the gene-dose effect of increasing htt levels *in vivo*. All mice were treated according to our institutional animal care guidelines under approved protocols, and were provided with food and water *ad libitum* for all experiments. Mice were killed by overdose of intraperitoneally administered avertin anesthesia.

Cultures of primary striatal neurons were generated and treated with glutamate or NMDA as described previously (Zeron *et al.* 2002). Briefly, striata from postnatal day (P)0/P1 wild-type, YAC72 or YAC18 mice were dissected and treated with papain followed by trypsin inhibitor. At this age the transgenic mice have no overt phenotypic differences compared with control mice. Dissociated neurons were plated on poly-D-lysine-coated glass coverslips and allowed to mature for 9 days *in vitro*, then cultures were treated with balanced salt solution (BSS, containing 50 μ M glycine) alone (control) or BSS containing NMDA (100 and 500 μ M), caffeine (1 mM) or glutamate (100 μ M) for 10 min. Twenty-four hours later, neurons were fixed with 4% paraformaldehyde in phosphate-

buffered saline (PBS) and stained with an antibody specific for activated caspase-3 or with propidium iodide and TUNEL, according to the manufacturer's instructions (Roche, Indianapolis, IN, USA). We defined stringent and rigorous criteria for counting apoptotic cells, requiring both morphological (nuclear blebbing with propidium iodide) and biochemical (TUNEL or caspase-3 staining) evidence of apoptosis. Staurosporine (10 μ M) was added directly to the media for 24 h followed immediately by fixation and staining. Over 1000 neurons were counted for all conditions and counts were done in triplicate for each batch of cultured neurons and repeated on 4 different days.

NMDA receptor-dependent excitotoxic degeneration of striatal neurons occurs in response to intrastriatal injection of the selective NMDA receptor agonist quinolinic acid (QA), and is a model of the selective neurodegeneration in HD (Beal *et al.* 1986). In this study 53 YAC18 mice and 43 wild-type FVB/N mice 4–11 months of age each received bilateral stereotaxic injections of 12 nmol QA or an equal volume of vehicle (PBS) while under inhalation (isoflurane) anesthesia as described previously (Zeron *et al.* 2002). Intrastriatal injections of QA caused brief episodes of stereotypy and/or rotational behavior in all injected mice. Each mouse was observed continuously for at least 2 h following recovery from QA microinjections. The occurrence, severity and duration of stereotypic movements was graded and recorded.

Some 18 h, 24 h, 72 h or 1 week after QA microinjection, brains were removed from some of the mice and either frozen immediately for immunocytochemistry, or specific brain regions were microdissected and immediately frozen in isopentane on dry ice for western blotting. A second group of microinjected mice were injected with heparin before terminal anesthetization by intraperitoneal injection of 2.5% avertin and intracardiac perfusion with 3% paraformaldehyde in PBS. Brains were left in the skull for 24 h in 3% paraformaldehyde then removed, weighed and stored in PBS at 4°C. All brains were cryopreserved in 25% sucrose before freezing in Tissue-TEK OCT (Sakura, Torrance, CA, USA). Serial 25-mm coronal cryostat sections were cut through the entire striatum. After every 200 mm, two sections were removed for quantitative analysis, TUNEL and immunohistochemical staining. The volume of the striatal lesion containing degenerating neurons was identified in striatal sections by FluoroJade B histochemistry (Histo-Chem, Jefferson, Arkansas, USA). FluoroJade B is a fluorescent stain that labels degenerating neurons in fixed brain sections. The total cross-sectional area of degenerated striatal neurons labeled with FluoroJade was recorded for each section selected for quantitative analysis. Alternate sections were stained with cresyl violet or used for immunocytochemical analysis. Primary antibodies included the Htt52 antibody, the neuronal marker NeuN antibody (raised against neuronal nuclei) (1 : 100; Chemicon, Temecula, CA, USA), dopamine- and camp-regulated phosphoprotein, 32kDa (DARPP-32), a marker for MSNs (1 : 200; Chemicon) or an activated caspase-3 antibody (1 : 500; Cell Signaling technology, Beverly, MA, USA). Sections were then incubated with either fluorescently labeled anti-mouse secondary antibody or biotinylated anti-mouse antibody (1 : 200; Vector Laboratories, Burlingame, CA, USA) before signal amplification with an ABC Elite kit (Vector Laboratories) and detection with diaminobenzidine (Pierce, Rockford, IL, USA).

Striatal neuronal profile counts and striatal lesion volumes were determined by a blinded investigator from a matched series of stained and mounted sections using unbiased techniques and StereoInvestigator software (MicroBrightfield, Williston, VT, USA). Briefly, striatal neuronal profile counts were estimated by counting neurons within $30 \times 30 \mu\text{m}$ counting frames spaced evenly throughout matched sections containing the striatum (striatal grid size was $300 \times 300 \mu\text{m}$) using a $100 \times$ objective. Slide-mounted sections were viewed with an Axioskop 2 fluorescence microscope (Zeiss, Toronto, Canada) fluorescence microscope and digital photomicrographs were captured with a cooled charged couple device (CCD) camera (Princeton, Piscataway, NJ, USA).

To assess htt protein levels and evaluate the role of caspase-mediated htt cleavage following intrastriatal QA injection, striata were microdissected from serial frozen sections, pooled and homogenized in buffer containing 0.25 M sucrose, 20 mM Tris HCl, pH 7.2, 1 mM MgCl_2 , 0.5 mM EDTA, complete protease inhibitor (Roche) and $5 \mu\text{M}$ zVAD-fmk. Lysates were then homogenized with a pestle in 1.5-mL eppendorf tubes and cleared of debris by centrifugation for 15 min at $817 g$ at 4°C . Striatal homogenates were electrophoresed through sodium dodecyl sulfate-polyacrylamide gels (7.5%) and transferred to a polyvinylidene difluoride membrane. Blots were probed with Htt552 antibody, 2166 antibody (Chemicon), anti- β -actin antibody (Sigma, St Louis, MO, USA) or anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (Chemicon) for 1 h in 5% skimmed milk in PBS. After several washes in PBS with 0.5% Tween 20 (PBST), horseradish peroxidase-conjugated secondary antibodies were added for 30 min at 22°C . The blots were washed in PBST, developed using enhanced chemiluminescence (Amersham Biosciences, Monmouth Junction, NJ, USA), scanned in using a Hewlett Packard ScanJet 6300C scanner (Hewlett Packard, Palo Alto, CA, USA), and the image file inverted. The density of bands was measured using Bio-Rad Quantity One software (Bio-Rad, Hercules, CA, USA).

Quantitative data are expressed as mean \pm SEM, with significance determined using a two-tailed Student's *t*-test.

Results

Mutant htt increases glutamate-induced neuronal cell death

We confirmed our previous finding that MSNs from YAC72 transgenic mice expressing mutant htt have an increased sensitivity to excitotoxic stressors compared with neurons from wild-type mice (Zeron *et al.* 2002). In these previous studies NMDA was used to induce excitotoxicity in cultured MSNs. Here we validated these findings using the endogenous receptor agonist glutamate, and showed that caspase-3 is activated during this process. Administration of $100 \mu\text{M}$ glutamate to primary cultures of striatal neurons caused activation of caspase-3 (Fig. 1a). Caspase-3 activation in response to glutamate exposure could be blocked using the specific caspase inhibitor DEVD-CHO (data not shown). Striatal neurons from YAC72 transgenic mice had signifi-

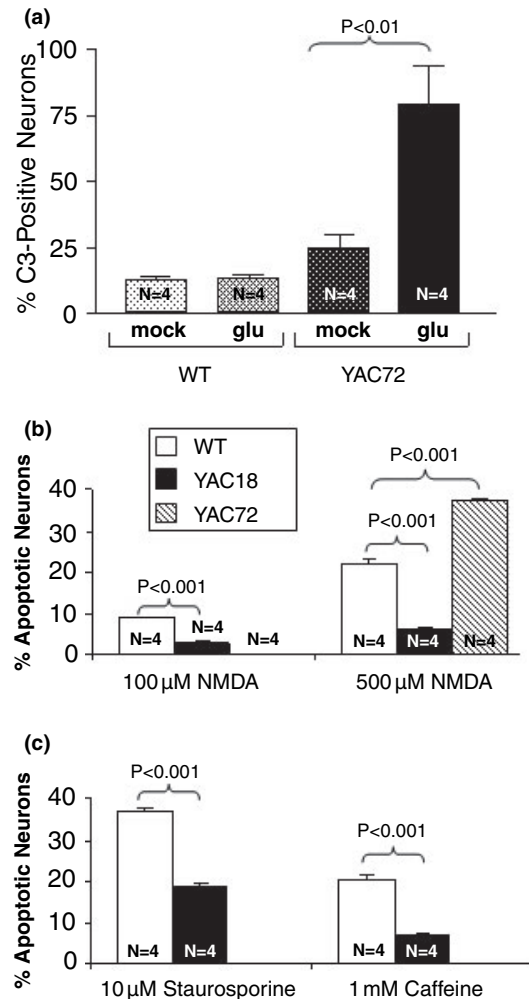


Fig. 1 Caspase-3 activation and apoptotic cell death in response to glutamatergic stimulation of MSNs in culture. (a) MSNs from YAC72 mice showed a dramatic and significant increase in activated caspase-3 (C3) expression ($n = 4$, $p < 0.001$) following glutamate stimulation compared with MSNs from wild-type (WT) littermates. Glutamate exposure did not significantly increase the levels of activated caspase-3 in wild-type or YAC18 MSNs at $100 \mu\text{M}$, nor did mock treatment, in which the medium was replaced without addition of glutamate. (b, c) Quantification of apoptotic cell death as measured by TUNEL staining and nuclear condensation and blebbing by propidium iodide staining in MSNs from wild-type (WT), YAC18 and YAC72 mice following exposure to toxic stressors (b) NMDA ($n = 4$), and (c) staurosporine ($n = 4$) and caffeine ($n = 4$). MSNs cultured from YAC18 transgenic mice had decreased apoptosis in response to NMDA, staurosporine and caffeine exposure compared with MSNs cultured from wild-type littermates, and MSNs from YAC72 mice had a dramatic increase in apoptotic cell death following stimulation with $500 \mu\text{M}$ NMDA. Values are mean \pm SEM percentage of caspase-3-positive or apoptotic neurons, with significance determined using a two-tailed Student's *t*-test.

cantly increased caspase-3 activation compared with cultures from wild-type FVB/N mice exposed to glutamate ($p < 0.001$) (Fig. 1a).

Exposure to glutamate did not significantly increase activated caspase-3 expression in wild-type MSNs (Fig. 1a). Importantly, similar to wild-type MSNs, no significant caspase-3 activation was observed in MSNs from YAC18 mice at this normally sublethal glutamate dose (data not shown).

Increased htt protects neurons from NMDA receptor-mediated death

To specifically examine the pro-survival effects of increased htt expression, we examined apoptosis induced by NMDA stimulation at a dose that induced significant levels of death in MSNs from wild-type cultures. Quantification of apoptotic cell death in MSNs from wild-type, YAC18 and YAC72 mice following NMDA, staurosporine and caffeine exposure is summarized in Fig. 1. Twenty-four hours after transient exposure to 100 μM NMDA, $8.9 \pm 0.2\%$ of wild-type MSNs were undergoing apoptosis compared with only $2.8 \pm 0.7\%$ of YAC18 MSNs ($p < 0.001$) (Fig. 1b). At 500 μM NMDA $22.1 \pm 1.3\%$ of wild-type MSNs underwent apoptosis compared with only $6.2 \pm 0.3\%$ of YAC18 MSNs ($p < 0.001$), representing a greater than 70% protection against NMDA-induced apoptosis as a result of increasing full-length wild-type htt levels. In contrast, YAC72 MSNs expressing full-length mutant htt had significantly increased apoptosis ($37.2 \pm 0.9\%$, $p < 0.001$) compared with wild-type MSNs at the 500 μM dose of NMDA, similar to our previous data (Zeron *et al.* 2002) and were included in these experiments as a positive control. This demonstrated that polyglutamine expansion converts the anti-apoptotic activity of htt to a death-promoting activity with several classical morphological hallmarks of apoptosis.

Striatal neurons cultured from YAC18 mice also had decreased levels of apoptosis induced by two other forms of neuronal cell death not directly mediated by NMDA receptor activation. Twenty-four hours after transient exposure to 10 μM staurosporine YAC18 MSNs showed significant protection from apoptosis compared with that in striatal neurons cultured from wild-type littermates (18.6 ± 1.0 vs. $36.9 \pm 0.9\%$ respectively; $p < 0.001$) (Fig. 1c). YAC18 MSNs were also significantly protected from apoptosis induced by treatment with 1 mM caffeine compared with MSNs cultured from wild-type littermates (6.8 ± 0.6 vs. $21.4 \pm 0.8\%$ respectively 24 h after treatment; $p < 0.001$).

NMDA receptor stimulation *in vivo* causes htt cleavage by caspase-3

Because apoptotic stimuli caused caspase-mediated cleavage of htt in culture, we evaluated whether full-length htt was cleaved by caspase-3 in striatal neurons in response to apoptotic stress *in vivo*. NMDA receptor-dependent excitotoxic degeneration of striatal neurons occurs in response to intrastriatal injections of the selective NMDA receptor

agonist QA. Mice were given intrastriatal injections of QA as described previously (Zeron *et al.* 2002), and subsequently analyzed for neurodegeneration (FluoroJade B), neuronal apoptosis (TUNEL) and caspase-3-specific htt cleavage (htt552 staining). Htt552 antibody is specific for the novel epitope generated at the C-terminal end of the N-terminal fragment of htt upon cleavage at amino acid 552. Western blots previously revealed that this antibody specifically detects the 552-amino acid caspase-3 htt cleavage product (Wellington *et al.* 2002).

In wild-type FVB/N mice, intrastriatal injection of QA caused apoptotic neurodegeneration in the striatum as identified by FluoroJade (Fig. 2a) and TUNEL staining (Fig. 2d). No significant FluoroJade or TUNEL staining was seen in the contralateral hemisphere following microinjection of PBS (Figs 2b and e). Numerous FluoroJade- and TUNEL-labeled neurons were identified in wild-type brains following QA treatment, which displayed a morphology typical of apoptotic neurons (Figs 2c and f). This finding supports the hypothesis that striatal neurons stained with FluoroJade are dying through apoptotic pathways following NMDA stimulation.

In parallel with the degenerating apoptotic striatal neurons, we detected caspase-3-specific htt cleavage within both neuronal cell bodies and processes (Fig. 2g) and in activated astrocytes (Fig. 2h) using the htt552 antibody. Neurons stained for caspase-cleaved htt fragments using the htt552 antibody were detected predominantly at the periphery of the NMDA-induced striatal lesion as delineated by FluoroJade (Fig. 2a) and TUNEL (Fig. 2d) staining, whereas astrocytes positive for htt552 immunostaining were generally located at the core of the lesion.

NMDA receptor stimulation *in vivo* decreases full-length htt levels and increases levels of N-terminal htt fragments

Intrastriatal QA injection resulted in significantly decreased levels of full-length htt ($p = 0.04$) (Figs 2i and j). Western blots performed on striata following QA and PBS injections, using the htt552 antibody and β -actin as a control for protein loading (Fig. 2k) showed that QA caused a dramatic increase in levels of the htt552 caspase-cleavage fragment of htt (Fig. 2l). Taken together, these results provide direct *in vivo* evidence of htt cleavage by caspases in neurons and astrocytes following NMDA-mediated excitotoxic stress. This cleavage of htt has two important consequences relevant to HD: the generation of toxic N-terminal fragments of mutant htt and reduced levels of full-length anti-apoptotic wild-type htt within neurons.

Over-expression of htt is neuroprotective *in vivo*

Increased vulnerability to glutamate excitotoxicity has long been suggested to play a major role in the selective loss of striatal neurons in HD (McGeer and McGeer 1976; Coyle *et al.* 1983; Hodgson *et al.* 1996), but the mechanism linking

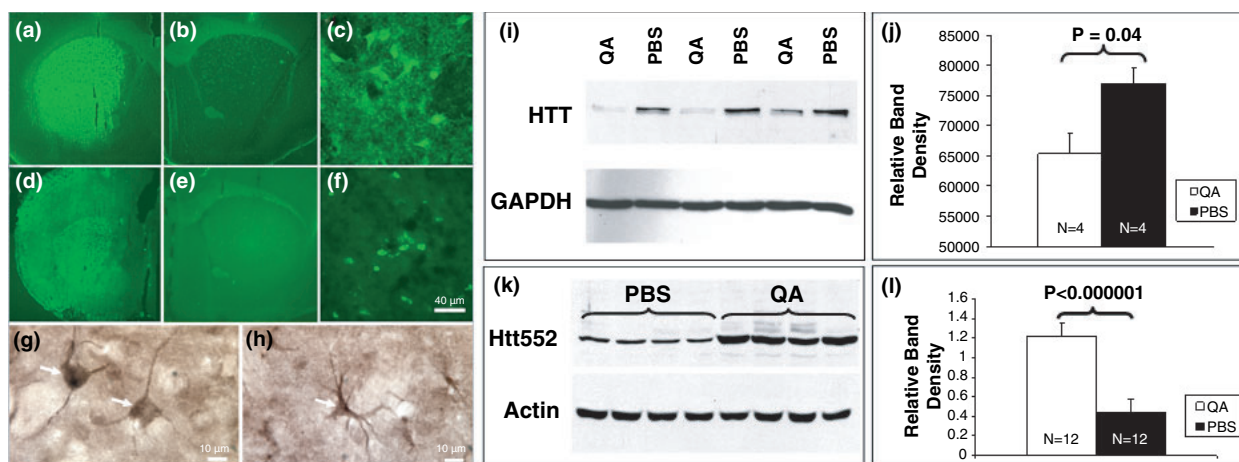


Fig. 2 Apoptotic neurodegeneration and caspase-mediated cleavage of wild-type htt in striatal neurons following intrastriatal QA injections. Degenerating striatal neurons were identified within the striatum of QA-treated mice using FluoroJade labeling (a and c), but none were observed in the contralateral striatum of the same mice injected with PBS (b). Degenerating neurons were found to be TUNEL positive (d and f) on the QA-injected side, but no TUNEL staining was found in the striatum treated with PBS (e). Caspase-cleaved htt was identified *in vivo* within neurons (g) and astrocytes (h) undergoing excitotoxic neurodegeneration using the htt-552 antibody, but not in the PBS-

treated striatum (not shown). Western blots of striatal tissue from mice injected with QA were probed with the htt-specific antibody 2166 and for GAPDH (i) and showed a significant decrease in full-length htt levels compared with striatal tissue from mice injected with PBS (j). Western blots of striatal tissue from mice injected with QA probed with the htt552 antibody and β -actin (k) showed a significant increase in caspase-3 cleavage fragments of htt compared with tissue from mice injected with PBS (l). Values in bar charts are mean \pm SEM, with significance determined using a two-tailed Student's *t*-test.

the polyglutamine expansion in htt with increased susceptibility to excitotoxicity is unknown (McGeer and McGeer 1976; O'Kusky *et al.* 1999). We now demonstrate that over-expression of full-length wild-type htt in YAC18 transgenic mice confers protection against neuronal apoptosis following intrastriatal QA injections. There were no differences in duration or severity of stereotypy induced by QA injections between YAC18 transgenic mice and non-transgenic FVB/N mice. No neurodegeneration occurred in mice of any genotype following intrastriatal injection of PBS (Fig. 2b).

In vivo, over-expression of wild-type htt significantly protected MSNs from NMDA-mediated excitotoxic degeneration within the striatum as demonstrated by DARPP-32 staining in YAC18 versus non-transgenic littermates (Fig. 3a) and by NeuN staining (Fig. 3b). YAC18 mice had 38% smaller striatal lesions than control animals, based on FluoroJade staining (Fig. 3c). Over-expression of htt in YAC18 mice had a significant protective effect on total QA-induced lesion volume in the striatum compared with wild-type mice (2.0×10^9 vs. 3.2×10^9 mm³ respectively; $p = 0.007$) (Fig. 3d). Increased total numbers of surviving striatal neurons (NeuN positive) were seen in YAC18 mice compared with non-transgenic littermate mice injected with QA ($p = 0.027$) (Fig. 3e).

Two independent lines of YAC18 mice (212 and B60) were used to assess a possible gene-dose effect of increasing htt levels *in vivo* on the apoptotic neurodegeneration induced

by intrastriatal QA injections (Fig. 3f). There was a significant decrease in striatal lesion size in YAC18 mice with increasing levels of transgenic protein expression. YAC18 line B60+/- mice, which have 50% of the transgenic human htt levels of B60+/+ mice, had significantly larger striatal lesions ($p < 0.01$) (Fig. 3f). YAC18 line 212 mice had the highest htt levels and the smallest striatal lesions following QA injection. The relative levels of transgenic human htt in the three YAC18 lines are shown in the western blot in Fig. 3(f).

Discussion

These data provide compelling evidence that wild-type htt plays a neuroprotective role *in vivo*, and that htt levels modulate the sensitivity of neurons to apoptotic death elicited by NMDA receptor-mediated excitotoxicity. YAC18 transgenic mice over-expressing wild-type human htt demonstrate significant protection against apoptotic neurodegeneration induced by the NMDA receptor agonist QA, and the level of htt expression has a dose-dependent protective effect on NMDA excitotoxicity *in vivo*. NMDA receptor-mediated excitotoxic neurodegeneration has been postulated to be a critical factor in the pathogenesis of HD (Beal 1992), and intrastriatal injections of QA are an acute insult used to model this form of cell death *in vivo*. The synchronous and rapid death of large numbers of striatal neurons induced in

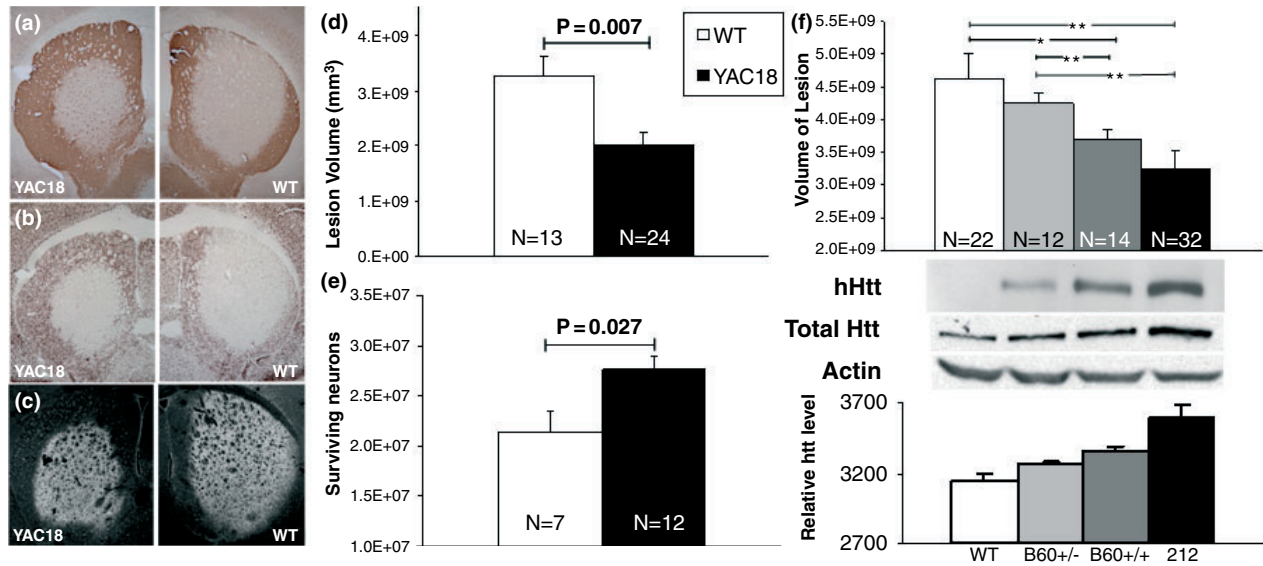


Fig. 3 Over-expression of wild-type htt in YAC18 transgenic mice causes decreased neurodegeneration following intra-atrial injection of QA. Representative sections from YAC18 (line 212) and wild-type (WT) littermates following intra-atrial QA injections stained for the MSN-specific marker DARRP32, the neuron-specific marker NeuN (b) and FluoroJade, a marker of degenerating neurons (c). Striatal lesion volumes following QA injections in YAC18 transgenic mice over-expressing wild-type htt (YAC18, $n = 24$) and littermate controls (FVB/N, $n = 13$) were reduced by 38% in YAC18 mice compared with control littermates (WT) (d). Significantly higher numbers of striatal neurons (identified by NeuN) survived in YAC18 mice compared with

wild-type (WT) mice following QA microinjection (e). (f) There was a significant decrease in striatal lesion size in YAC18 mice with increasing levels of transgenic protein expression. B60+/- mice, which have 50% of the transgenic human htt levels of B60+/+ mice, had significantly larger striatal lesions and mice with the highest overall levels of htt (YAC18 line 212) had the smallest striatal lesions following QA injection ($*p < 0.05$, $**p < 0.01$). Data were normalized to YAC18 (212) and expressed as mean \pm SEM lesion volume, with significance determined using a two-tailed Student's *t*-test. Striatal levels of transgenic human htt (hHtt) and total htt for each of the YAC18 lines are shown in a western blot.

this model is not seen in HD, but the model is useful for investigation of the role of htt in excitotoxicity (Zeron *et al.* 2002). These *in vivo* findings were recapitulated using primary striatal neurons prepared from P0 or P1 mice. Glutamate or NMDA exposure of wild-type neurons results in activation of caspase-3 and subsequent apoptotic neuronal death, and neurons from YAC72 mutant mice were even more sensitive (Zeron *et al.* 2002). Primary striatal neurons over-expressing wild-type htt are protected from NMDA-induced excitotoxicity. Our results thus confirm and extend results from studies using immortalized cell lines (Rigamonti *et al.* 2000), and provide further data that wild-type htt has anti-apoptotic neuroprotective functions.

Evidence for proteolytic cleavage of htt by caspase-3 in apoptotic cells was initially produced in 1996 (Goldberg *et al.* 1996). Cleaved N-terminal fragments of mutant htt are found in the brains of patients with HD (DiFiglia *et al.* 1997; Mende-Mueller *et al.* 2001; Wellington *et al.* 2002) and mice transgenic for full-length mutant htt (Hodgson *et al.* 1999). Direct evidence that caspase-mediated cleavage of mutant htt occurs in the HD brain as an early step in the pathogenesis of HD was established recently (Wellington *et al.* 2002). These findings, in combination with the discovery that N-terminal fragments of mutant htt are more toxic than full-length

mutant htt in many systems (Wellington and Hayden 1997; Martindale *et al.* 1998; Wellington *et al.* 2000), led to the development of the 'toxic fragment hypothesis' model of HD pathogenesis, linking htt cleavage with striatal neurodegeneration (Wellington and Hayden 1997).

Further support for this hypothesis has recently been obtained by site-directed mutagenesis experiments in which the caspase cleavage sites in htt were altered to generate caspase-resistant mutant htt, which was found to have significantly reduced toxicity in neuronal cells (Wellington *et al.* 2000). This model suggests that, in addition to the well established gain-of-function toxicity caused by fragments of mutant htt, depletion of endogenous wild-type htt by a variety of cellular processes, including caspase-mediated cleavage, should result in a loss of the anti-apoptotic function of htt, rendering neurons more vulnerable to cellular stress and neurodegeneration. Caspase activation has also been suggested to play a critical role in a variety of neurodegenerative disorders including polyglutamine disorders (Wellington *et al.* 1998; Ellerby *et al.* 1999; Ona *et al.* 1999; Li *et al.* 2000), Alzheimer's disease (Gervais *et al.* 1999) and amyotrophic lateral sclerosis (Friedlander *et al.* 1997).

Huntingtin promotes neuronal cell survival. The anti-apoptotic function of htt appears to act before caspase-3

activation during neuronal cell death (Rigamonti *et al.* 2001). The present studies further explore the possibility that the selective neuropathology in HD may reflect both loss-of-function and gain-of-function pathways (Cattaneo *et al.* 2001). For example, proteolytic cleavage of wild-type htt by a variety of processes could be expected to inactivate the pro-survival functions of htt. In contrast, generation of htt cleavage products containing an expanded polyglutamine tract would initiate a number of toxic pathways, including formation of aggregates and recruitment of caspase-8 (Sanchez *et al.* 1999), transcriptional dysregulation (Cha 2000; Luthi-Carter *et al.* 2000; Chan *et al.* 2002; Sugars and Rubinsztein 2003) and proteasome inhibition (Wytenbach *et al.* 2000; Jana *et al.* 2001). Because htt proteolysis may be a key event in tipping the balance from pro-survival to pro-death roles of htt, it is of interest to elucidate the pathways that lead to htt cleavage in HD and other neurodegenerative diseases.

Here we provide direct *in vivo* evidence for specific caspase-3-mediated cleavage of htt within neurons, depletion of wild-type htt levels in the striatum during excitotoxic stress, generation of N-terminal htt fragments, and an anti-apoptotic function for htt in the CNS. How could this anti-apoptotic function of htt relate to the pathogenesis of HD? The pathogenic polyglutamine expansion in htt is associated with the generation of N-terminal htt fragments that are both directly toxic to neurons (Hodgson *et al.* 1999) and capable of forming insoluble cellular aggregates (DiFiglia *et al.* 1997). Interestingly, both the mutant and wild-type htt proteins undergo cleavage (Wellington *et al.* 2000), and are recruited and sequestered into htt aggregates (Martindale *et al.* 1998), leaving less full-length wild-type htt available to counteract pro-apoptotic stimuli. We demonstrate that, during excitotoxic stress, htt is cleaved by caspases resulting in decreased levels of full-length htt.

No evidence for a neuroprotective effect of mutant htt in either our cell culture assays or *in vivo* models of NMDA-mediated excitotoxicity has been demonstrated, and over-expression of mutant htt has previously been shown to sensitize striatal neurons to this form of cell death. All patients heterozygous for the HD mutation (one mutant and one wild-type allele) express 50% wild-type htt and 50% mutant htt, and have 50% of the wild-type htt levels of a normal individual (two wild-type alleles). Because mutant htt does not appear to have the neuroprotective function of wild-type htt, this probably represents a 50% loss of wild-type htt neuroprotective function and, together with htt cleavage and sequestration of wild-type htt in aggregates, may combine to further decrease functional htt levels in HD.

Mice heterozygous for targeted disruption of the *Hdh* gene express half the normal levels of wild-type htt, and these mice develop neuronal degeneration in the basal ganglia in adulthood (Nasir *et al.* 1995). Recently, postnatal deletion of

the *Hdh* gene in mouse was found to result in neurodegeneration and a motor phenotype similar to that described in transgenic mouse models of HD (Dragatsis *et al.* 2000). Targeted disruption of the *Hdh* gene leads to reduced body size, movement abnormalities and increased ventricular volume in mice (Auerbach *et al.* 2001).

A possible mechanism by which wild-type htt may exert its anti-apoptotic effect is via regulation of brain-derived neurotrophic factor (BDNF) levels. Evidence for this is that cell lines and transgenic mice expressing increased full-length wild-type htt levels have raised BDNF levels, mediated by positive regulation of the transcriptional activity of the *BDNF* gene by wild-type htt (Cattaneo *et al.* 2002). BDNF is co-localized with htt in cortical neurons that project to the striatum, and striatal BDNF levels correlate with decreased levels of htt following QA administration (Fusco *et al.* 2003).

One of the normal functions of htt may be to provide protection against apoptotic stress in the brain by modulating striatal levels of survival factors such as BDNF and/or by decreasing the activation of caspases. We provide data to support a hypothesis first raised in 1995 (Nasir *et al.* 1995), suggesting that both a toxic gain of function and a partial loss of the normal function of htt may be involved in the selective vulnerability of striatal neurons to degeneration in HD (Cattaneo *et al.* 2001). The gene products of many of the polyglutamine disorders [except spinocerebellar ataxia (SCA1)] are substrates for cleavage by various caspases (Wellington *et al.* 1998) raising the possibility that loss of function of these proteins may be involved in the clinical phenotype and progression of each of these disorders. A polyglutamine-mediated gain of toxicity may be the common basis for the many clinical and neuropathological similarities in this group of neurodegenerative disorders, but loss of the specific function of the wild-type proteins may also contribute significantly to the differences.

Our results demonstrate a relationship between normal htt function and neuronal susceptibility to excitotoxicity. Increasing levels of wild-type htt expression or preventing cleavage of full-length htt would be predicted to have a therapeutic benefit in HD and other neurodegenerative disorders that involve excitotoxic or apoptotic death programs. When attempting to develop treatments for HD based on decreasing the expression of mutant htt in the brain, the possible adverse effects of concurrent decreases in wild-type htt levels and induction of neuronal apoptosis owing to loss of the pro-survival function of htt must be carefully considered.

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References

- Auerbach W., Hurlbert M. S., Hilditch-Maguire P., Wadghiri Y. Z., Wheeler V. C., Cohen S. I., Joyner A. L., MacDonald M. E. and Turnbull D. H. (2001) The HD mutation causes progressive lethal neurological disease in mice expressing reduced levels of huntingtin. *Hum. Mol. Genet.* **10**, 2515–2523.
- Beal M. F. (1992) Does impairment of energy metabolism result in excitotoxic neuronal death in neurodegenerative illnesses? *Ann. Neurol.* **31**, 119–130.
- Beal M. F., Kowall N. W., Ellison D. W., Mazurek M. F., Swartz K. J. and Martin J. B. (1986) Replication of the neurochemical characteristics of Huntington's disease by quinolinic acid. *Nature* **321**, 168–171.
- Cattaneo E., Rigamonti D., Goffredo D., Zuccato C., Squitieri F. and Sipione S. (2001) Loss of normal huntingtin function: new developments in Huntington's disease research. *Trends Neurosci.* **24**, 182–188.
- Cattaneo E., Rigamonti D. and Zuccato C. (2002) The enigma of Huntington's disease. *Sci. Am.* **287**, 92–97.
- Cepeda C., Ariano M. A., Calvert C. R., Flores-Hernandez J., Chandler S. H., Leavitt B. R., Hayden M. R. and Levine M. S. (2001) NMDA receptor function in mouse models of Huntington disease. *J. Neurosci. Res.* **66**, 525–539.
- Cha J. H. (2000) Transcriptional dysregulation in Huntington's disease. *Trends Neurosci.* **23**, 387–392.
- Chan E. Y., Luthi-Carter R., Strand A. *et al.* (2002) Increased huntingtin protein length reduces the number of polyglutamine-induced gene expression changes in mouse models of Huntington's disease. *Hum. Mol. Genet.* **11**, 1939–1951.
- Chen N., Luo T., Wellington C., Metzler M., McCutcheon K., Hayden M. R. and Raymond L. A. (1999) Subtype-specific enhancement of NMDA receptor currents by mutant huntingtin. *J. Neurochem.* **72**, 1890–1898.
- Coyle J. T., Ferkany J. W. and Zaczek R. (1983) Kainic acid: insights from a neurotoxin into the pathophysiology of Huntington's disease. *Neurobehav. Toxicol. Teratol.* **5**, 617–624.
- DiFiglia M., Sapp E., Chase K. O., Davies S. W., Bates G. P., Vonsattel J. P. and Aronin N. (1997) Aggregation of huntingtin in neuronal intranuclear inclusions and dystrophic neurites in brain. *Science* **277**, 1990–1993.
- Dragatsis I., Levine M. S. and Zeitlin S. (2000) Inactivation of Hdh in the brain and testis results in progressive neurodegeneration and sterility in mice. *Nat. Genet.* **26**, 300–306.
- Duyao M. P., Auerbach A. B., Ryan A., Persichetti F., Barnes G. T., McNeil S. M., Ge P., Vonsattel J. P., Gusella J. F. and Joyner A. L. (1995) Inactivation of the mouse Huntington's disease gene homolog Hdh. *Science* **269**, 407–410.
- Ellerby L. M., Andrusiak R. L., Wellington C. L. *et al.* (1999) Cleavage of atrophin-1 at caspase site aspartic acid 109 modulates cytotoxicity. *J. Biol. Chem.* **274**, 8730–8736.
- Friedlander R. M., Brown R. H., Gagliardini V., Wang J. and Yuan J. (1997) Inhibition of ICE slows ALS in mice. *Nature* **388**, 31.
- Fusco F. R., Zuccato C., Tartari M., Martorana A., De Marchi Z., Giampa C., Cattaneo E. and Bernardi G. (2003) Co-localization of brain-derived neurotrophic factor (BDNF) and wild-type huntingtin in normal and quinolinic acid-lesioned rat brain. *Eur. J. Neurosci.* **18**, 1093–1102.
- Gervais F. G., Xu D., Robertson G. S. *et al.* (1999) Involvement of caspases in proteolytic cleavage of Alzheimer's amyloid-beta precursor protein and amyloidogenic A beta peptide formation. *Cell* **97**, 395–406.
- Goffredo D., Rigamonti D., Tartari M., De Micheli A., Verderio C., Matteoli M., Zuccato C. and Cattaneo E. (2002) Calcium-dependent cleavage of endogenous wild-type huntingtin in primary cortical neurons. *J. Biol. Chem.* **277**, 39 594–39 598.
- Goldberg Y. P., Nicholson D. W., Rasper D. M. *et al.* (1996) Cleavage of huntingtin by apopain, a proapoptotic cysteine protease, is modulated by the polyglutamine tract [see comments]. *Nat. Genet.* **13**, 442–449.
- Hansson O. A. P. N., Leist M., Nicotera P., Castilho R. F. and Brundin P. (1999) Transgenic mice expressing a Huntington's disease mutation are resistant to quinolinic acid-induced striatal excitotoxicity. *Proc. Natl Acad. Sci. USA* **96**, 8727–8732.
- Ho L. W., Brown R., Maxwell M., Wytenbach A. and Rubinsztein D. C. (2001) Wild type Huntingtin reduces the cellular toxicity of mutant Huntingtin in mammalian cell models of Huntington's disease. *J. Med. Genet.* **38**, 450–452.
- Hodgson J. G., Smith D. J., McCutcheon K. *et al.* (1996) Human huntingtin derived from YAC transgenes compensates for loss of murine huntingtin by rescue of the embryonic lethal phenotype. *Hum. Mol. Genet.* **5**, 1875–1885.
- Hodgson J. G., Agopyan N., Gutekunst C. A. *et al.* (1999) A YAC mouse model for Huntington's disease with full-length mutant huntingtin, cytoplasmic toxicity, and selective striatal neurodegeneration. *Neuron* **23**, 181–192.
- Huntington's Disease Collaborative Research Group (1993) A novel gene containing a trinucleotide repeat that is expanded and unstable on Huntington's disease chromosomes. The Huntington's Disease Collaborative Research Group [see comments]. *Cell* **72**, 971–983.
- Jana N. R., Zemskov E. A., Wang G. and Nukina N. (2001) Altered proteasomal function due to the expression of polyglutamine-expanded truncated N-terminal huntingtin induces apoptosis by caspase activation through mitochondrial cytochrome *c* release. *Hum. Mol. Genet.* **10**, 1049–1059.
- Leavitt B. R., Guttman J. A., Hodgson J. G., Kimel G. H., Singaraja R., Vogl A. W. and Hayden M. R. (2001) Wild-type huntingtin reduces the cellular toxicity of mutant huntingtin *in vivo*. *Am. J. Hum. Genet.* **68**, 313–324.
- Levine M. S., Klapstein G. J., Koppel A. *et al.* (1999) Enhanced sensitivity to *N*-methyl-D-aspartate receptor activation in transgenic and knockin mouse models of Huntington's disease. *J. Neurosci. Res.* **58**, 515–532.
- Li S. H., Lam S., Cheng A. L. and Li X. J. (2000) Intranuclear huntingtin increases the expression of caspase-1 and induces apoptosis. *Hum. Mol. Genet.* **9**, 2859–2867.
- Luthi-Carter R., Strand A., Peters N. L. *et al.* (2000) Decreased expression of striatal signaling genes in a mouse model of Huntington's disease. *Human Mol. Genet.* **9**, 1259–1271.
- MacDonald M. E. and Gusella J. F. (1996) Huntington's disease: translating a CAG repeat into a pathogenic mechanism. *Curr. Opin. Neurobiol.* **6**, 638–643.
- Martindale D., Hackam A., Wiczorek A. *et al.* (1998) Length of huntingtin and its polyglutamine tract influences localization and frequency of intracellular aggregates. *Nat. Genet.* **18**, 150–154.
- McGeer E. G. and McGeer P. L. (1976) Duplication of biochemical changes of Huntington's chorea by intrastriatal injections of glutamic and kainic acids. *Nature* **263**, 517–519.

- Mende-Mueller L. M., Toneff T., Hwang S.-R., Chesselet M.-F. and Hook V. Y. H. (2001) Tissue-specific proteolysis of Huntingtin (htt) in human brain: evidence of enhanced levels of N- and C-terminal htt fragments in Huntington's disease striatum. *J. Neurosci.* **21**, 1830–1837.
- Nasir J., Floresco S. B., O'Kusky J. R., Diewert V. M., Richman J. M., Zeisler J., Borowski A., Marth J. D., Phillips A. G. and Hayden M. R. (1995) Targeted disruption of the Huntington's disease gene results in embryonic lethality and behavioral and morphological changes in heterozygotes. *Cell* **81**, 811–823.
- O'Kusky J. R., Nasir J., Cicchetti F., Parent A. and Hayden M. R. (1999) Neuronal degeneration in the basal ganglia and loss of pallido-subthalamic synapses in mice with targeted disruption of the Huntington's disease gene. *Brain Res.* **818**, 468–479.
- Ona V. O., Li M., Vonsattel J. P. *et al.* (1999) Inhibition of caspase-1 slows disease progression in a mouse model of Huntington's disease [see comments]. *Nature* **399**, 263–267.
- Rigamonti D., Bauer J. H., De Fraja C. *et al.* (2000) Wild-type huntingtin protects from apoptosis upstream of caspase-3. *J. Neurosci.* **20**, 3705–3713.
- Rigamonti D., Sipione S., Goffredo D., Zuccato C., Fossale E. and Cattaneo E. (2001) Huntingtin's neuroprotective activity occurs via inhibition of procaspase-9 processing. *J. Biol. Chem.* **276**, 14 545–14 548.
- Sanchez I., Xu C. J., Juo P., Kakizaka A., Blenis J. and Yuan J. (1999) Caspase-8 is required for cell death induced by expanded polyglutamine repeats [see comments]. *Neuron* **22**, 623–633.
- Sharp A. H., Loev S. J., Schilling G., Li S. H., Li X. J., Bao J., Wagster M. V., Kotzok J. A., Steiner J. P. and Lo A. (1995) Widespread expression of Huntington's disease gene (IT15) protein product. *Neuron* **14**, 1065–1074.
- Sugars K. L. and Rubinsztein D. C. (2003) Transcriptional abnormalities in Huntington disease. *Trends Genet.* **19**, 233–238.
- Wellington C. L. and Hayden M. R. (1997) Of molecular interactions, mice and mechanisms: new insights into Huntington's disease. *Curr. Opin. Neurol.* **10**, 291–298.
- Wellington C. L., Ellerby L. M., Hackam A. S. *et al.* (1998) Caspase cleavage of gene products associated with triplet expansion disorders generates truncated fragments containing the polyglutamine tract. *J. Biol. Chem.* **273**, 9158–9167.
- Wellington C. L., Singaraja R., Ellerby L. *et al.* (2000) Inhibiting caspase cleavage of huntingtin reduces toxicity and aggregate formation in neuronal and nonneuronal cells. *J. Biol. Chem.* **275**, 19 831–19 838.
- Wellington C. L., Ellerby L. M., Gutekunst C. A. *et al.* (2002) Caspase cleavage of mutant huntingtin precedes neurodegeneration in Huntington's disease. *J. Neurosci.* **22**, 7862–7872.
- Wytenbach A., Carmichael J., Swartz J., Furlong R. A., Narain Y., Rankin J. and Rubinsztein D. C. (2000) Effects of heat shock, heat shock protein 40 (HDJ-2), and proteasome inhibition on protein aggregation in cellular models of Huntington's disease. *Proc. Natl Acad. Sci. USA* **97**, 2898–2903.
- Zeitlin S., Liu J. P., Chapman D. L., Papaioannou V. E. and Efstratiadis A. (1995) Increased apoptosis and early embryonic lethality in mice nullizygous for the Huntington's disease gene homologue. *Nat. Genet.* **11**, 155–163.
- Zeron M. M., Chen N., Moshaver A., Lee A. T., Wellington C. L., Hayden M. R. and Raymond L. A. (2001) Mutant huntingtin enhances excitotoxic cell death. *Mol. Cell. Neurosci.* **17**, 41–53.
- Zeron M. M., Hansson O., Chen N., Wellington C. L., Leavitt B. R., Brundin P., Hayden M. R. and Raymond L. A. (2002) Increased sensitivity to *N*-methyl-D-aspartate receptor-mediated excitotoxicity in a mouse model of Huntington's disease. *Neuron* **14**, 849–860.