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Specific caspase interactions and amplification are involved in selective neuronal vulnerability in Huntington's disease

E Hermel[1,2], J Gafni[1], SS Propp[1], BR Leavitt[3], CL Wellington[3], JE Young[1], AS Hackam[3], AV Logvinova[1], AL Peel[1], SF Chen[1], V Hook[1], R Singaraja[3], S Krajewski[4], PC Goldsmith[1], HM Ellerby[1], MR Hayden[3], DE Bredesen[1,4] and LM Ellerby*[,1]

[1] The Buck Institute for Age Research, Novato, CA, USA;
[2] Touro University College of Osteopathic Medicine, Vallejo, CA, USA;
[3] Department of Pathology and Laboratory Medicine or Centre for Molecular Medicine and Therapeutics, Department of Medical Genetics, University of British Columbia, Vancouver, British Columbia, Canada;
[4] Program on Aging and Apoptosis, The Burnham Institute, La Jolla, CA, USA
* Corresponding author: LM Ellerby, The Buck Institute for Age Research, 8001 Redwood Blvd, Novato, CA 94945, USA. Tel: 415 –209 2088; Fax: 415 –209 2230; E-mail: lellerby@buckinstitute.org

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Abstract

Huntington's disease (HD) is an autosomal dominant progressive neurodegenerative disorder resulting in selective neuronal loss and dysfunction in the striatum and cortex. The molecular pathways leading to the selectivity of neuronal cell death in HD are poorly understood. Proteolytic processing of full-length mutant huntingtin (Htt) and subsequent events may play an important role in the selective neuronal cell death found in this disease. Despite the identification of Htt as a substrate for caspases, it is not known which caspase(s) cleaves Htt *in vivo* or whether regional expression of caspases contribute to selective neuronal cells loss. Here, we evaluate whether specific caspases are involved in cell death induced by mutant Htt and if this correlates with our recent finding that Htt is cleaved *in vivo* at the caspase consensus site 552. We find that caspase-2 cleaves Htt selectively at amino acid 552. Further, Htt recruits caspase-2 into an apoptosome-like complex. Binding of caspase-2 to Htt is polyglutamine repeat-length dependent, and therefore may serve as a critical initiation step in HD cell death. This hypothesis is supported by the requirement of caspase-2 for the death of mouse primary striatal cells derived from HD transgenic mice expressing full-length Htt (YAC72). Expression of catalytically inactive (dominant-negative) forms of caspase-2, caspase-7, and to some extent caspase-6, reduced the cell death of YAC72 primary striatal cells, while the catalytically inactive forms of caspase-3, -8, and -9 did not. Histological analysis of post-mortem human brain tissue and YAC72 mice revealed activation of caspases and enhanced caspase-2 immunoreactivity in medium spiny neurons of the striatum and the cortical projection neurons when compared to controls. Further, upregulation of caspase-2 correlates directly with decreased levels of brain-derived neurotrophic factor in the cortex and striatum of 3-month YAC72 transgenic mice and therefore suggests that these changes are early events in HD pathogenesis. These data support the involvement of caspase-2 in the selective neuronal cell death associated with HD in the striatum and cortex.
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Abbreviations: HD, Huntington's disease; Htt, huntingtin; BDNF, brain-derived neurotrophic factor; GFP, green fluorescent protein; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling; PVDF, polyvinylidene fluoride; GFAP, glial fibrillary acidic protein

Introduction

Expansion of CAG trinucleotide repeats that encode polyglutamine tracts in nine otherwise unrelated proteins is the common mutation underlying nine distinct neurodegenerative diseases. [1–11] Expansion of polyglutamine repeats is associated with a toxic gain-of-function that affects specific neuronal populations in each of these diseases.[12] Huntington's disease (HD), one of the most extensively studied of these diseases, is a debilitating inherited neurodegenerative disorder characterized by involuntary movements, personality changes, dementia, and early death.[13] HD is characterized by selective neuronal loss and fibrillary reactive astrocytosis predominantly within the striatum and cortex.[14,15]

The selectivity of neuronal death seen in HD striatum and cortex is remarkable given that huntingtin protein (Htt) is normally expressed throughout the central nervous system as well as in non-neuronal cells.[16–18] Htt is also widely distributed at the subcellular level and has been found within nuclei, perikarya, neurites, and synaptic elements.[16–21] Considering the widespread expression of Htt, it is interesting that the enkephalin and substance P-containing medium spiny neurons are vulnerable in the striatum, while large cholinergic and medium aspiny neurons are spared.[14,22–24] In the cortex, the pyramidal cortical projection neurons in layers III and V are vulnerable. Markers of apoptosis have been detected in the brains of patients with HD in both of these regions.[25]

Despite the numerous studies *in vitro* and *in vivo* supporting a correlation between Htt length and toxicity, little is known about how cleavage events may be initiated or how the proteolytic processing of Htt or other polyglutamine disease proteins is regulated.[26–29] Cell-type-dependent cleavage by

specific caspases or other proteases may explain some of the selective neurodegeneration seen in these diseases. Evidence for region-specific cleavage of Htt *in vivo* comes from studies of YAC transgenic mice expressing mutant full-length huntingtin (YAC72). In this model, N-terminal fragments of Htt are clearly found in the medium spiny neurons of the striatum[30] and cortex,[31] but not in other regions of the brain unaffected in HD. These results suggest that cleavage of Htt by caspases and other proteases such as calpains[32] may produce toxic fragments. However, it does not explain how cleavage is initiated, which caspases cleave Htt *in vivo*, nor how selective regional neuronal cell loss occurs in HD. We hypothesized that three factors might be involved in this process: (1) the cell-specific expression of proteases within the striatum and cortex that cleave Htt *in vivo*, (2) the ability of Htt to interact with cell-death signaling molecules and be cleaved in a region-specific manner, and (3) the regulation of these caspases by brain-derived neurotrophic factor (BDNF). BDNF levels are directed regulated by wild-type Htt and are reduced in HD transgenic mice and HD patient brains.[33]

We find that caspase-2 cleaves Htt selectively at amino acid 552, which is the Htt caspase cleavage product detected *in vivo* both in early-grade HD postmortem tissue and in YAC transgenic mice expression mutant Htt,[31] and further that Htt directly interacts with specific caspases to form a cell-death complex. We found that caspase-2, -6, and -7 recruit Htt in an apoptosome-like complex. Caspase-2 and -7 bound full-length Htt while caspase-6 bound the N-terminal caspase cleavage product. Furthermore, the binding of initiator caspase-2 was repeat-length dependent, which could explain why the length of the polyglutamine tract correlates with the age of disease onset. Caspase-2 was required for the death of primary striatal cells derived from YAC72 transgenic mice. Analysis of YAC72 transgenic mice[30] and post-mortem brain from HD patients confirmed the contribution of caspase-2 to the pathogenesis of HD. Furthermore, caspase-2 mRNA levels are regulated by trophic support from the growth factor, BDNF. The upregulation of caspase-2 correlates directly with decreased levels of BDNF in the cortex and striatum of 3-month YAC72 transgenic mice and therefore suggests that activation of these pathways maybe an early event in the pathogenesis of HD.

Results

HD is specifically cleaved by caspase-2 at caspase consensus site 552 *in vitro* and *in vivo*

The caspase cleavage sites of Htt are clustered around a 76 amino-acid region of Htt (Figure 1a) and cleavage in this region generates N-terminal fragments that have been shown to be toxic in cells. [34–36] We have previously shown that Htt is cleaved *in vitro* by caspase-3 at amino acids 513 and 552, and by caspase-6 at amino-acid position 586.[36] However, *in vivo*, we found that Htt is specifically cleaved at a caspase consensus site at amino acid 552 in pyramidal cortical neurons, while the caspase consensus site at amino acid 513 does not appear to be utilized.[31] Since caspase-3 cleaves at both D513 and D552, we further evaluated the specificity of caspases in cleaving Htt. Here, we show that Htt is also cleaved by caspase-2, -7, and -8 (Figure 1b). Cleavage of Htt

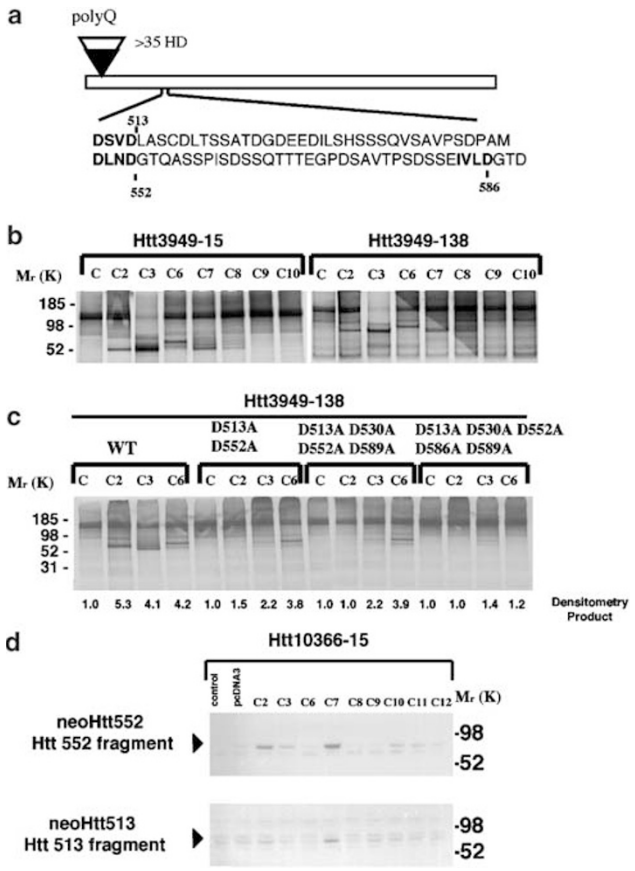

Figure 1 *In vitro* cleavage of Htt with recombinant caspases. (**a**) Htt caspase cleavage sites are clustered around a 76 amino-acid region of Htt. (**b**) Radiolabeled Htt with 15 (pRC-CMV3949-15; construct yielding the first 1212 amino acids of Htt) and 138 glutamine repeats (pRC-CMV3949-138) were incubated with the indicated recombinant caspases (~10–100 nM) or in cleavage buffer (uncleaved). It should be noted that that Htt is cleaved by caspase-8 *in vitro*, although less efficiently. The recombinant caspase-3 and -7 was purified via a different method with higher specific activity and therefore accounts for the difference reported in our previous work.[29] (**c**) Radiolabeled caspase-resistant forms of Htt with 138 repeats (pRC-CMV3949-138) were incubated with the indicated recombinant caspases (~100 nM) or in cleavage buffer (uncleaved) in order to determine the site of caspase-2 cleavage. Densitometry of the caspase cleavage products of Htt indicate that caspase-2, -3, -6 cleaved Htt with similar efficacy and the caspase-resistant forms are resistant to cleavage relative to control. (**d**) Western blot analysis of lysates from 293T cells transfected with the indicated Htt and caspase constructs (48 h after transfection) probed with neoepitope antibodies to Htt cleavage products

by caspase-3 yields a 70 kDa (80 kDa; expanded Htt) fragment and 75 kDa (85 kDa; expanded Htt) fragment (sites at D513, D552), while caspase-6 cleavage yields an 80 kDa (90 kDa; expanded Htt) fragment (cleavage site at D586).[36] As shown in Figure 1b,c, caspase-2 is also capable of cleaving Htt to generate a cleavage product at approximately 75 kDa (85 kDa; expanded Htt), which corresponds to cleavage at D552. Comparison of Htt cleavage by caspase-2, -3, and caspase-6 with various caspase-resistant forms of Htt (Figure 1c) demonstrates that caspase-2 cleaves at D552 in Htt3949-138 yielding an 85 kDa cleavage product. This cleavage product is slightly smaller than the caspase-6-generated cleavage product and common to one of the cleavage products generated by caspase-3. Caspase-2, -3,

and -6 cleave Htt with similar efficacy as demonstrated by densitometry (Figure 1c). Of note, the cleavage of Htt by caspase-2 occurs at a single site, D552, unlike caspase-3, which cleaves at both D513 and D552 suggesting different substrate specificity for these two caspases. The rate of caspase-2 cleavage of Htt is repeat-length independent (data not shown).

To further evaluate the cleavage of Htt by caspases in tissue culture, we coexpressed Htt with cDNA constructs of caspase-2, -3, -6, -7, -8, -9, -10, -11, or -12 in 293T cells. Our results demonstrate that Htt is cleaved by caspase-2 only at the 552 site when probed with antibodies that detect caspase-cleaved Htt fragments (neospecific antibodies to cleavage sites at 513 and 552) (Figure 1d).[31] These results indicate that, *in vitro*, caspase-2 cleaves Htt at amino acid 552 and correlates with our recent results demonstrating that Htt is also cleaved *in vivo* at the D552 caspase consensus site in pyramidal cortical neurons of YAC72 mice and HD brains.[31] However, these results do not necessarily explain how this cleavage event is initiated *in vivo*. We hypothesized that this cleavage event may require the formation of cell-death complexes and therefore Htt might be involved in interactions directly or indirectly with caspases. To explore the hypothesis that Htt is specifically recruited into a protein complex, we carried out the following immunoprecipitation experiments.

Immunoprecipitation of Htt/caspase cell-death complexes

We have previously shown that expression of disease proteins with expanded polyglutamine repeats are able to induce cell death in established cell lines, including COS-7 and 293T cells.[26,27,34,35] We carried out our initial coimmunoprecipitations with caspases and Htt in 293T cells. We cotransfected 293T cells with constructs encoding either 15 or 138 polyglutamine repeats of full-length Htt or with an N-terminal truncated form of Htt (1955-15 and 1955-128; amino acids 1–548 of Htt) and a corresponding epitope-tagged caspase. At 48 h after transfection, the cells were harvested and immunoprecipitation was carried out using epitope-coupled (HA or FLAG) or caspase antiserum-coupled agarose beads. The expression of each Htt construct in the total cell lysates (TL) prior to immunoprecipitation (IP) is shown by Western analysis in Figure 2a–d (left panel). The expression of each caspase prior to immunoprecipitation was also confirmed by Western analysis. Transfection of the truncated form of Htt1955-15 and Htt1955-128 into 293T cells resulted in a band of relative molecular mass of 75 or 85 kDa for proteins containing 15 and 128 glutamines, respectively (Figure 2a, left panel). Transfection of full-length Htt15 and Htt138 resulted in the expression of a protein with relative molecular mass of 348 kDa (Figure 2a, left panel). When extracts from cells transfected with caspase-2 and various Htt constructs were immunoprecipitated with caspase-2 specific antiserum coupled to agarose beads, both the full-length expanded Htt protein and the 85 kDa Htt fragment were pulled down (Figure 2a, right panel). Cell extracts expressing Htt138 showed greater interaction with caspase-2 than Htt15 (Figure 2a, right panel), indicating that Htt interaction with caspase-2 is polyglutamine repeat-length dependent. Htt constructs with 15 (Htt15), 44 (Htt44), and 80 (Htt80) polyglutamine repeats in 293T cells confirmed that the caspase-2/Htt interaction is repeat dependent (Figure 2e). The amount of Htt protein immunoprecipitating with caspase-2 gradually increased with increasing polyglutamine length. Equal amounts of caspase-2 were pulled down and inputs were equal (data not shown). Densitometry revealed an 11.7-fold increase for Htt44, and a 19-fold increase for Htt80 relative to Htt15 in binding caspase-2. Control experiments with beads or preimmune serum were negative for the interaction.

Similar experiments were carried out for caspase-6, -7, -8, -9 and -3, as shown in Figure 2b, c, d. Immunoprecipitation of caspase-7 pulled down both full-length Htt (Figure 2b, right panel) and the 85 kDa Htt fragment (Figure 2b, right panel). Immunoprecipitation of caspase-6 extracted the 85 kDa fragment of Htt (Figure 2b, right panel), but not the full-length protein (Figure 2b, right panel). Cell extracts expressing Htt with 138 repeats yielded the same amount of immunoprecipitated protein as Htt with 15 repeats demonstrating that Htt binding to caspase-7 and -6 is repeat independent (data not shown). Htt did not coimmunoprecipitate with caspase-9 (Figure 2b, right panel), caspase-8 (Figure 2c), or caspase-3 (Figure 2d). These results indicate that only specific caspases are capable of forming complexes with Htt.

To confirm the interaction between caspase-2 and Htt was physiological and not due to overexpression of the proteins in cell culture, we carried out immunoprecipation experiments from YAC72 transgenic and control mouse cortex. Immunoprecipitation of Htt pulled down caspase-2 (Figure 2f). It should be noted that the immunoprecipitation of Htt brings down both the endogenous mouse Htt and the human Htt72. Htt72 is expressed at less than 50% the endogenous levels of mouse Htt. Our results suggest Htt exists in a complex with caspase-2 under physiological conditions. Interestingly, we have shown that purified recombinant Htt (amino acids 1–520) and active caspase-2 interact directly (unpublished data), suggesting that the prodomain of caspase-2 or the caspase cleavage site of Htt are not required for the interaction.

Inhibition of Htt-induced polyglutamine repeat-dependent cell death by catalytically inactive caspases

Given that specific caspases interact with Htt, we tested whether the catalytically inactive forms of these enzymes could act as dominant negatives inhibitors of HD-mediated striatal cell death. We utilized dominant-negative caspases rather than caspase knockout mice since it has been demonstrated that compensatory caspase pathways are utilized in these models.[37] We developed a transient transfection system whereby primary rat striatal neurons expressed green fluorescent protein (GFP) and full-length Htt with normal repeat length (Htt15) or an expanded polyglutamine stretch (Htt138).[35] Expression of Htt138, but not Htt15, induced cell death in the cultured striatal neurons (Figure 3a). Cell body shrinkage, nuclear condensation, and neurite retraction were prominent features of these dying neurons

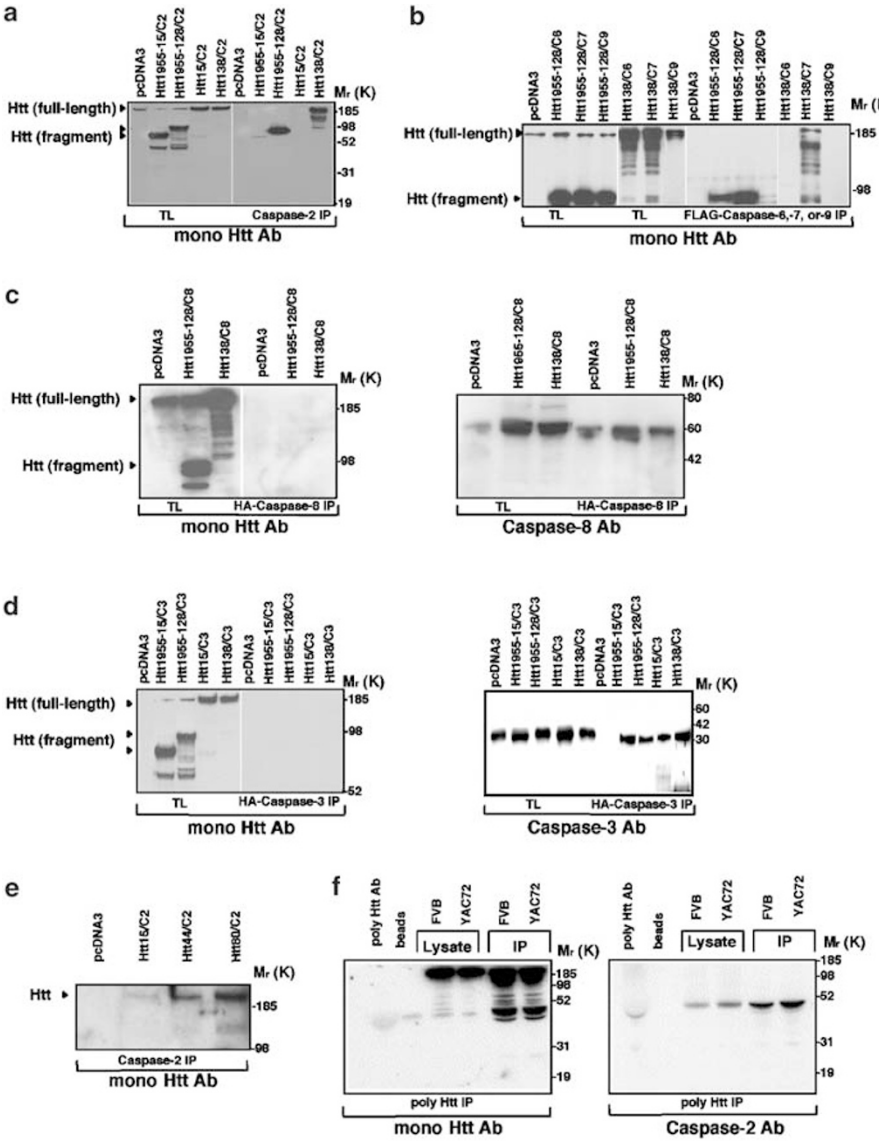

Figure 2 Full-length Htt15 and Htt138 coimmunoprecipitates with caspases-2 and -7. (**a**) 293T cells cotransfected full-length Htt (Htt15), full-length Htt with glutamine expansion (Htt138), N-terminal fragment (Htt1955-15), N-terminal fragment with glutamine expansion (Htt1955-128), and pcDNA3-caspase-2. The total lysate prior to immunoprecipitation (left panel) and immunoprecipitation of caspase-2 from these extracts (right panel) were probed with Htt monoclonal antibody 2166 (Chemicon). Indicated are TL and IP. (**b**) 293T cells cotransfected with Htt138 or Htt1955-128, and FLAG-tagged caspase-6, -7, and caspase-9. The left panel shows the total cell lysate of the cotransfection of N-terminal fragment of Htt1955-128 and full-length Htt (Htt138) with caspase-6, -7, and -9. The right panels show the results of immunoprecipitation of the respective caspases in the presence of the various Htt constructs. Both are probed with Htt monoclonal antibody 2166 (Chemicon). (**c**) Htt probed before (TL) and after immunoprecipitation of caspase-8 (IP) (left panel) with Htt monoclonal antibody 2166 (Chemicon). Caspase-8 was immunoprecipitated and probed with caspase-8 antibody (right panel). (**d**) Htt probed before (TL) and after immunoprecipitation of caspase-3 (IP). Caspase-3 was immunoprecipitated and probed with caspase-3 antibody (right panel). (**e**) 293T cells cotransfected with caspase-2 and pcDNA3, wild-type full-length huntingtin (Htt15), or mutant full-length Htt with 44 (Htt 44) or 80 (Htt80) polyglutamine repeats. Western blot analysis of caspase-2 IP probed with Htt monoclonal antibody 2166. (**f**) Western blot analysis of control and YAC72 cortical lysates (1-year old) before (lysate) and after IP of Htt with an N-terminal polyclonal Htt antibody (left panel). Reprobing blot with caspase-2 antibody (MAB3507) demonstrates coimmunoprecipitation of caspase-2 (right panel)

(Figure 3b). Our results differ from an earlier report using primary culture because we use the full-length Htt construct while they used a Htt-derived fragment mostly composed of polyglutamine repeats.[38] Using a primary culture model where full-length Htt is expressed is more relevant for evaluating the early events in HD disease pathogenesis and proteolysis.

Next, we examined whether coexpression of catalytically inactive forms of caspase-2 (C303A), caspase-3 (C163A), caspase-6 (C163A), caspase-7 (C186A), caspase-8 (C360A), or caspase-9 (C287A) inhibit cell death induced by Htt138 in striatal neurons. These are denoted as dominant-negative caspase (DNC) in the figures. The percentage of cell death was determined at 96 h post-transfection by counting the number of dead (those rounded or shrunken without processes; Hoechst staining of condensed nuclei) and live (those with extensive neurites) GFP-positive neurons. Expression of inactive caspase-2 (C303A) or caspase-7 (C186A) led to a inhibition of neuronal cell death induced by

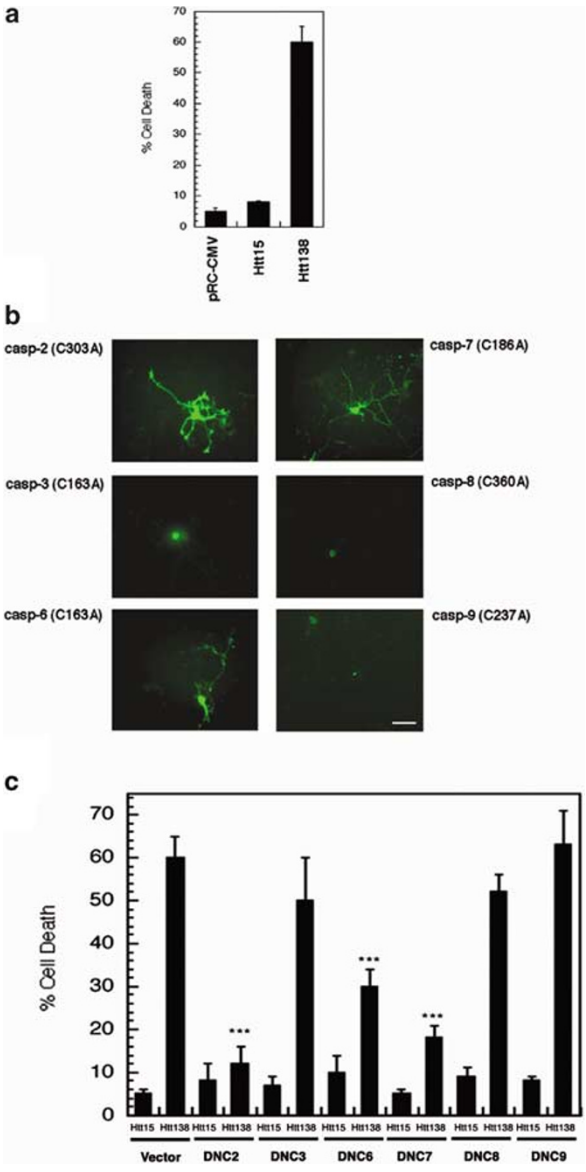

Figure 3 Inhibition of Htt polyglutamine repeat-induced cell death by catalytically inactive caspases. Rat (E17-E18) primary striatal neurons were transfected with vectors either full-length huntingtin (Htt15 or Htt138) or pRC-CMV control with GFP vector included as a marker. Fluorescent micrographs at 96 h after transfection of both the Htt138 and GFP vector transfected striatal neurons showed cell body shrinkage, neuritic contraction, and significant cell loss, while cells transfected with Htt15 did not. (**a**) Rat primary neurons were transfected with vectors encoding GFP and either full-length Htt (Htt15 or Htt138) and the indicated control vector, and 96 h after transfection the percentage of cell death was determined. S.D. was determined from three to six experiments; $P<0.001$ (***) compared to Htt138-transfected cells. (**b**) Caspase-2 (C303A), caspase-7 (C186A), and caspase-6 (C163A) prevented Htt138-induced cell – death, while caspase-3 (C163A), caspase-8 (C360A), and caspase-9 (C237A) did not as shown by the monitoring the GFP-expressing neurons. Fluorescent micrographs at 96 h after transfection of Htt138 and the respective catalytically inactive caspase. (**c**) Rat primary neurons were transfected with vectors encoding GFP, full-length Htt (Htt15 or Htt138) and the indicated catalytically inactive caspase, and 96 h after transfection the percentage of cell death was determined. S.D. was determined from three to six experiments compared to Htt138-transfected cells. Scale bar = 20 μm. Two-factor ANOVA analysis was carried out using GraphPad Prism

Htt138 (Figure 3b,c, $P<0.001$). Dominant-negative caspase-6 caused a moderate inhibition of apoptosis induction by Htt138 (Figure 3b,c, $P<0.001$). Expression of inactive caspase-3 (C163A), caspase-8 (C360A), or caspase-9 (C287A), shown to be functional dominant-negatives in caspase-3, Fas, or bax-induced cell death, respectively (data not shown), had no effect on cell death triggered by Htt138 (Figure 3b,c). These results suggest that specific caspases are involved in the Htt138-induced cell death in primary striatal neurons.

Localization of caspase-2 and -7 in primary striatal cultures correlates with HD-mediated cytotoxicity and caspase recruitment

Given catalytically inactive forms of caspase-2 and -7 blocked Htt138-induced cell death, we investigated whether Htt localized with these caspases in primary striatal cultures derived from control and YAC72 transgenics and confirmed, under physiological conditions, that dominant-negative caspase-2, -6, and -7 blocked cell death in this cell culture model. Further, we examined whether these caspases had altered distribution during cell death given the evidence of caspase relocalization. For example, during ischemic reperfusion, caspase-9 translocates from the mitochondria to the nucleus in some neurons.[39] Caspase-2 has been localized to the Golgi complex and nucleus.[40] Subcellular caspase localization can vary according to tissue and cell type.[39]

Expression of inactive caspase-2 (C303A) or caspase-7 (C186A) led to an inhibition of neuronal cell death in the YAC72 striatal cells (Figure 4a). Dominant-negative caspase-6 caused a moderate inhibition of apoptosis in YAC72 striatal cells (Figure 4a). As shown in Figure 4b (panels a–f), caspase-2 colocalizes with Htt (panels c,f) in a punctate pattern in the cytoplasm of striatal neurons and their distal processes (30.6% $\pm$ 6.6 of the Htt red channel colocalized with the caspase-2 green channel). Caspase-7 colocalizes with Htt in the cytoplasm and the perinuclear zone in striatal cultures (data not shown), and during apoptotic activation, the active form of caspase-7 coexists with Htt in the endoplasmic reticulum (ER) in YAC72 striatal cells (Figure 4b, panels i,l) and not in control primary striatal cells (no immunoreactivity to activated caspase-7, not shown). Interestingly, ER stress response protein GRP78 is induced and colocalizes with Htt in YAC72 striatal cells as shown in Figure 4b (panels g–i). Correspondingly, Htt can be found colocalized with activated caspase-7 in YAC72 striatal cells (See Figure 4b, panels j–l; 96% $\pm$ 3 of the Htt red channel colocalized with the caspase-7 green channel). Caspase-7 has been previously reported to localize in the ER and nucleus during apoptotic stimulation.[41,42] Colocalization of caspase-2 and -7 with Htt is consistent with both our immunoprecipitation results and our proposed role of these caspases in the initiation of Htt-mediated cell death.

Expression of caspases in the cortex and striatum

Initially, we immunostained adult mouse brains (Figure 5, 7) and post-mortem human tissue (Figures 6, 7) with antibodies

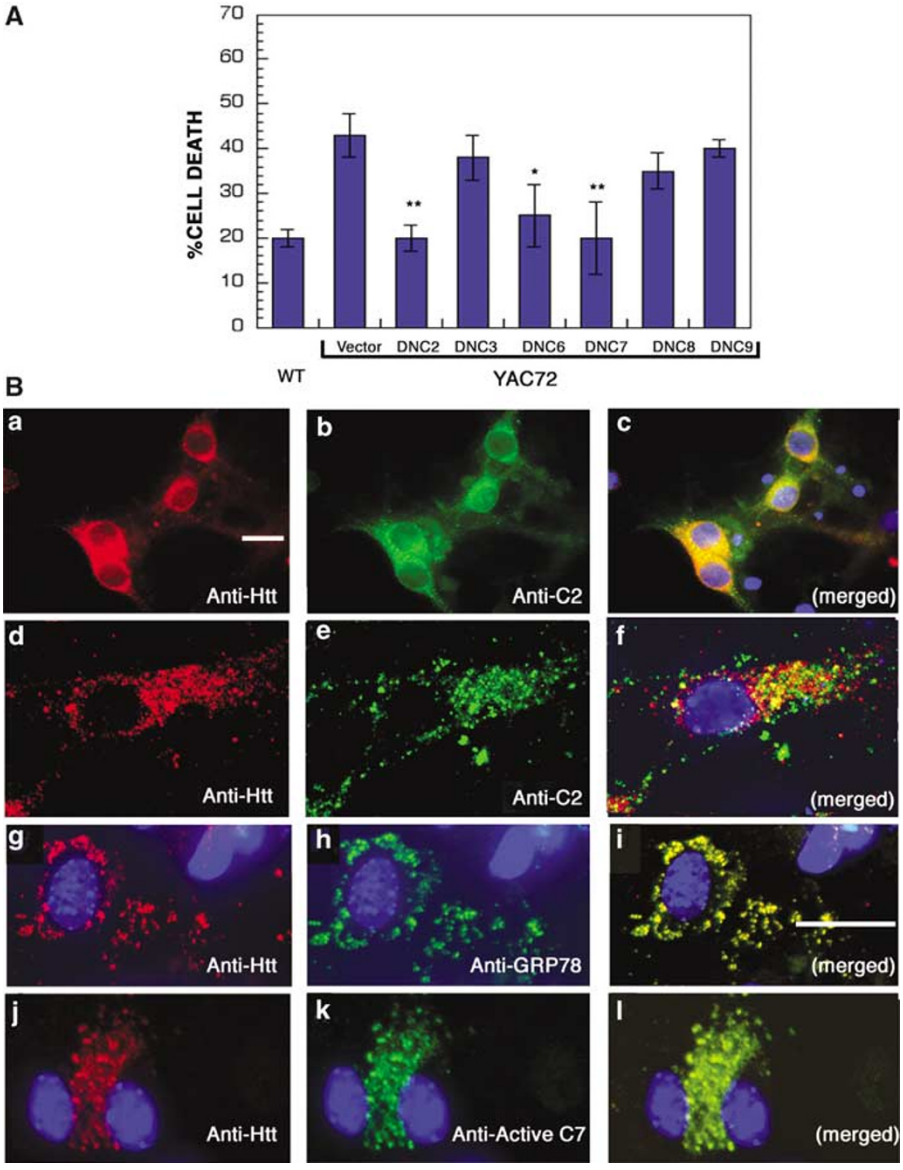

Figure 4 Location of endogenous Htt and caspases in primary striatal cultures. (**a**) Mouse primary neurons derived from YAC72 transgenic mouse (D1) were transfected with vectors encoding GFP and the indicated catalytically inactive caspase, and 96 h after transfection the percentage of cell death was determined. Standard deviation was determined from three to six experiments; $P<0.05$ (*); $P<0.01$ (**); $P<0.001$ (***) compared to pcDNA transfected cells. (**b**) Primary striatal YAC72 neurons were immunostained with either an antibody to Htt (panels a, d, g, j; Texas Red) or an antibody to caspase-2 (panels b, e; FITC), an antibody to GRP78 (panel h; FITC) or an antibody to activated caspase-7 (panel k; FITC). Panels c, f, i and l are the overlay of the first two images. The antibody used for Htt was monclonal 2170 (Chemicon). Scale bar $= 20\ \mu$m

specific for caspase-2, -3, -6, -7, -8, and -9 to determine whether the regional distribution of any of these caspases correlated with the selective neuronal cell death found in HD. We focused on immunostaining in the mouse striatum and cortex (Figure 5). Interestingly, each of these caspases has a unique cell-specific pattern of expression and subcellular location. Particularly noteworthy is the comparison of the expression pattern of caspase-7 to caspase-9 in the murine striatum (Figure 5a,b,f,g). Caspase-7 is expressed in the vulnerable medium-sized neurons (Figure 5f,g; also see Figure 7c,d), while caspase-9 is enriched in the large cholinergic neurons (Figure 5a,b), which are spared in HD. Moreover, the murine striatal level of caspase-7 expression is elevated compared to other regions such as cortex (Figure 5f). We found the same unique pattern of cell-specific staining for caspase-7 and -9 in the human striatum (Figure 7c,d and data not shown). These findings are also consistent with our immunoprecipitation results and colocalization data, supporting our hypothesis that cell-specific expression of caspases may contribute to HD pathogenesis.

Immunostaining also shows that subsets of medium-sized striatal neurons are positive for caspase-6 (Figure 5d,e), whereas caspase-9 immunoreactivity occurs in pyramidal cells cortical layer V (Figure 5h) and caspase-8 is ubiquitously expressed (Figure 5i,j). Since the pyramidal cells in cortical layer V produce trophic factor (BDNF) that undergoes

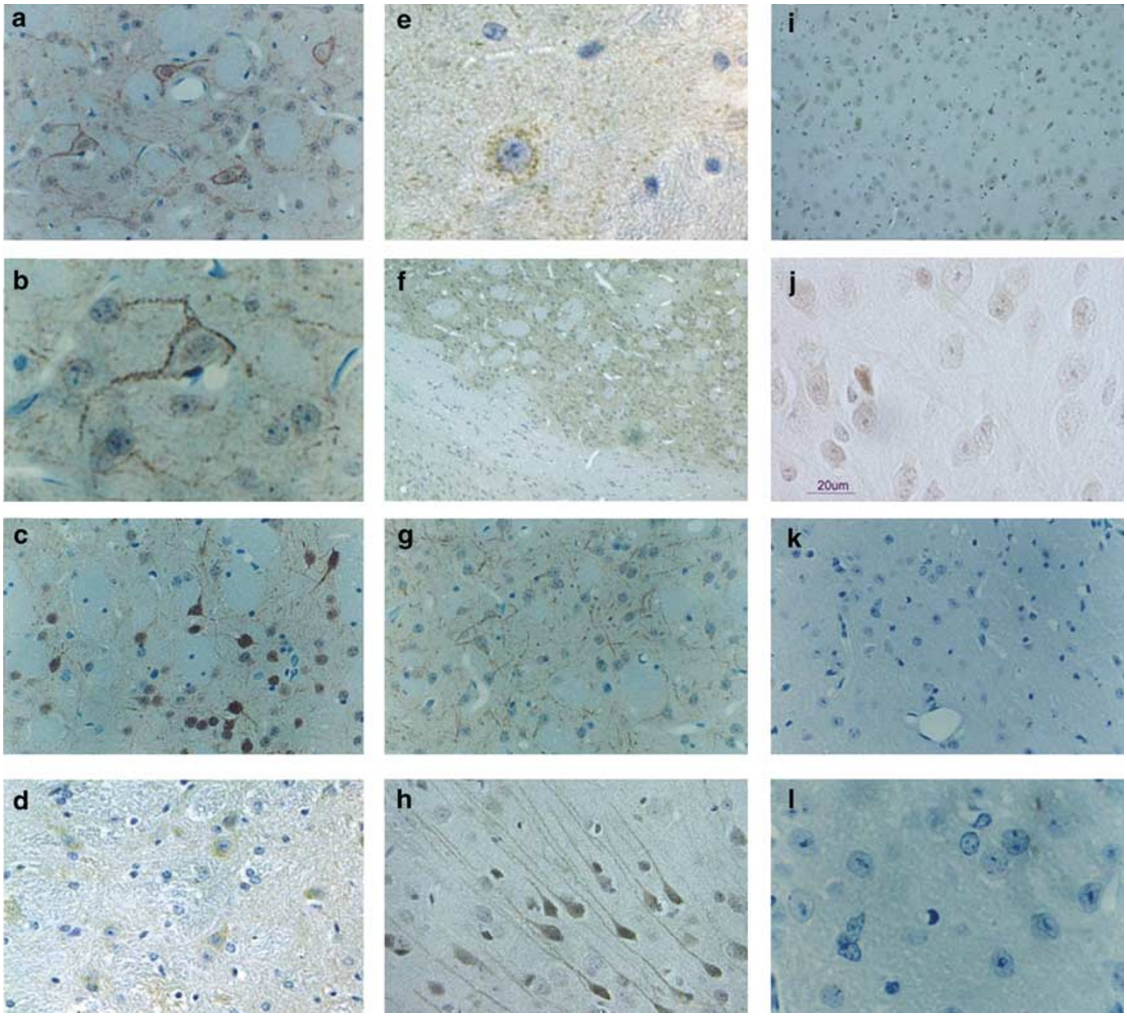

Figure 5 Immunohistochemical analysis of caspase-6, -7, -8, and caspase-9 in mouse striatum and cortex. (**a, b**) Capase-9 immunostaining of the large cholinergic cells of the striatum exhibit an organellar pattern of staining (× 400, × 1000). (**c**) Calbindin immunostaining denotes the medium-sized cells of the striatum (× 400). (**d, e**) Caspase-6 staining shows that a subset of medium-sized striatal neurons is positive for this caspase (× 400, × 1000). (**f, g**) Caspase-7 immunostaining demonstrates enrichment of this caspase in the striatum relative to the cortex and other regions of the brain (× 100) and specific staining in the medium spiny neurons (× 400). (**h**) Caspase-9 stains pyramidal cells in the cortex (× 400). (**I, j**) Caspase-8 stains ubiquitously throughout the cortex and striatum in many cell types (× 100, × 600). Results were confirmed by immunostaining with two separate anticaspase antisera. (**k, l**) Controls for nonspecific immunostaining included substitution of the primary antibody with preimmune serum or antigen-preadsorbed serum and were negative in all tissues. Only two examples are presented here (× 400, × 1000)

anterograde transport to the striatum and these cortical neurons degenerate in HD, the high expression of caspase-9 may be important in the cortical neuropathology. These immunostaining results support the concept that cell-specific expression of caspases may contribute to HD neuropathology and disease progression. Additional *in vivo* evidence from human post-mortem HD tissue supporting the participation of specific caspases in HD is described below.

Detection of activated caspase-3 and -6 in distinct cell populations in human HD striatum

To determine if caspases are activated in HD, we evaluated whether the downstream caspase-3 and -6 are present at the sites of neuronal degeneration in human post-mortem striatal tissue. These antibodies react preferentially with the activated form of the caspases. Using an antibody to activated caspase-3, we found that cells in the caudate of post-mortem HD were immunopositive (Figure 6a), while aged-matched controls were devoid of immunoreactivity (Figure 6k). The caspase-3 immunoreactivity localized to the nucleus in these cells. Double labeling with neuronal marker MAP2 (red) and activated caspase-3 (brown) showed no colocalization (Figure 6b). Double labeling with astrocytic marker glial fibrillary acidic protein (GFAP) (red) demonstrated that the cells immunopositive for activated caspase-3 (brown) in the caudate were all GFAP-immunoreactive astrocytes (Figure 6c). For comparison, single labeling of GFAP astrocytic marker (Figure 6d) and neuron marker MAP2 (Figure 6h) are shown. One of the hallmarks of HD pathology is the extensive gliosis found within the striatum. It is interesting that many of the glial cells contain high levels of activated caspase-3 and these cells are terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) positive.[25] Conversely, using an antibody that

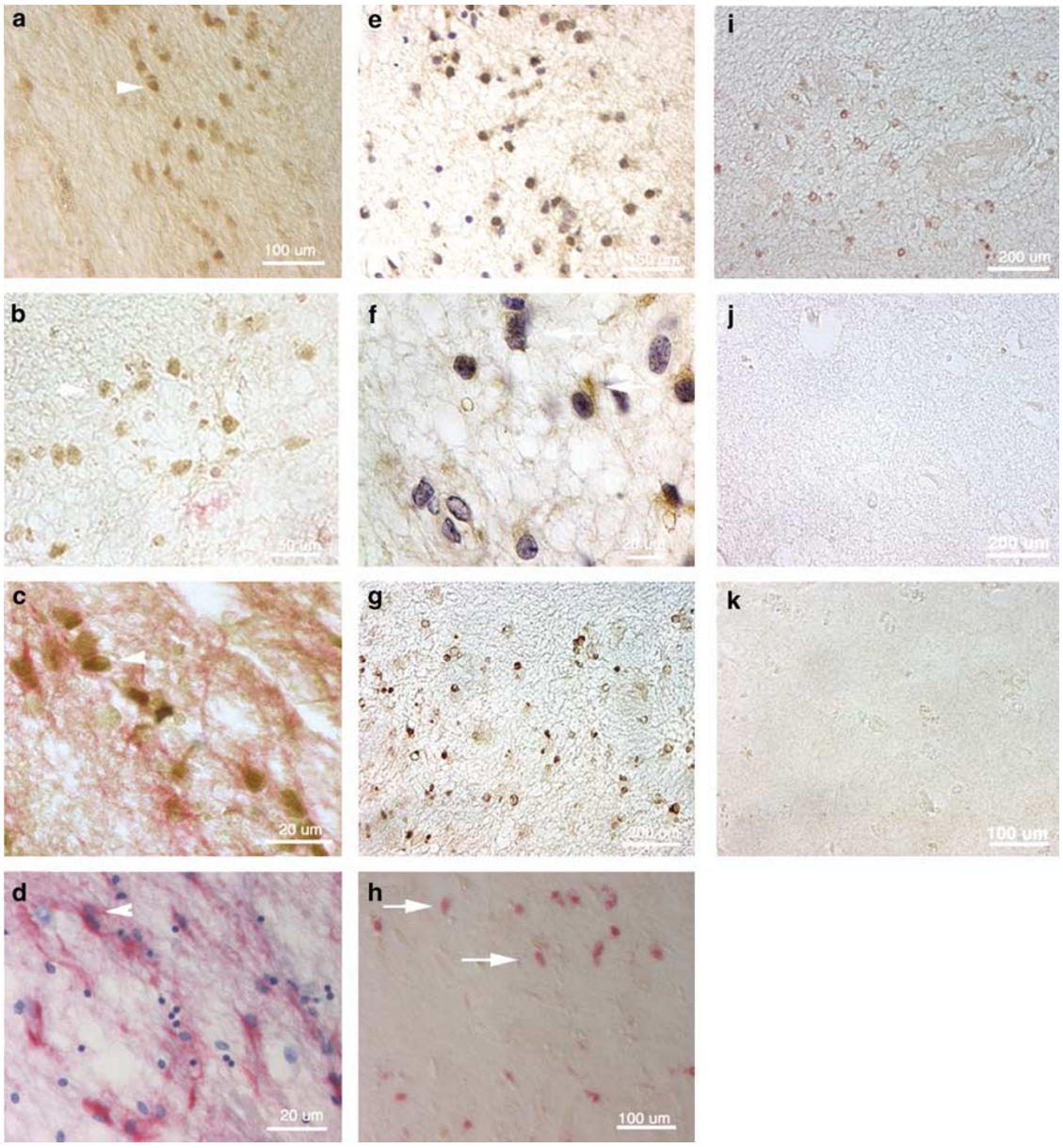

Figure 6 Immunoreactivity of activated caspase-3 in glia and activated caspase-6 in dying neurons and glia in caudate nucleus of HD brain. Frozen sections from HD ($n = 3$) and aged-matched control patients were probed with polyclonal antibody to activated caspase-3. (**a**) Degenerating cells in the caudate immunoreactive to activated caspase-3 are found in HD samples, but not in (**k**) age-matched controls. (**b**) Double-label immunostaining with activated caspase-3 (brown) and MAP2 (red) show only a few neurons are double labeled. (**c**) Double-label immunostaining of activated caspase-3 (brown) with GFAP (red) show colocalization. (**d**) GFAP (red) single-label immunostaining. (**e**, **f**) HD sections were probed with activated caspase-6 antibody in the caudate. (**j**) A representative age-matched control stained with activated caspase-6 staining is shown in the caudate. (**g**) Double-label immunostaining of activated caspase-6 (brown) with calbindin (red) show colocalization. Comparison of serial sections stained with (**h**) MAP2 or (**i**) calbindin antibody show localization of caspase-6 immunoreactivity with either neuronal marker. Controls for nonspecific immunostaining also were performed for all tissues, including use of preimmune serum and antigen-preadsorbed serum. VECTOR Red substrate kit was used for double-labeling experiments according to the manufacturer's instructions. Glial cells are denoted with an arrowhead and neurons with an arrow

preferentially recognizes activated caspase-6, we found that medium-sized neurons and glia in the caudate were immunoreactive to this antibody in post-mortem HD tissue (Figure 6e,f), while aged-matched controls were devoid of immunoreactivity (Figure 6j). These results support the relevance of caspase-mediated cleavage *in vivo*, particularly in the vulnerable medium-sized neurons of the caudate. Double labeling with antibody to activated caspase-6 (brown) and the neuronal marker calbindin (red) indicated that most of the cells immunoreactive to the caspase-6 antibody were neurons (Figure 6g). For comparison, single labeling of neuronal marker MAP2 (Figure 6h) and neuronal marker calbindin (Figure 6i) are shown. These results show distinct populations of cells in the brain activate different caspases and suggests that the majority of the dying neurons in the HD caudate contain activated caspase-6, while glial cells contain high levels of activated caspase-3. The potential caspases involved in initiating HD neurodegeneration are described below.

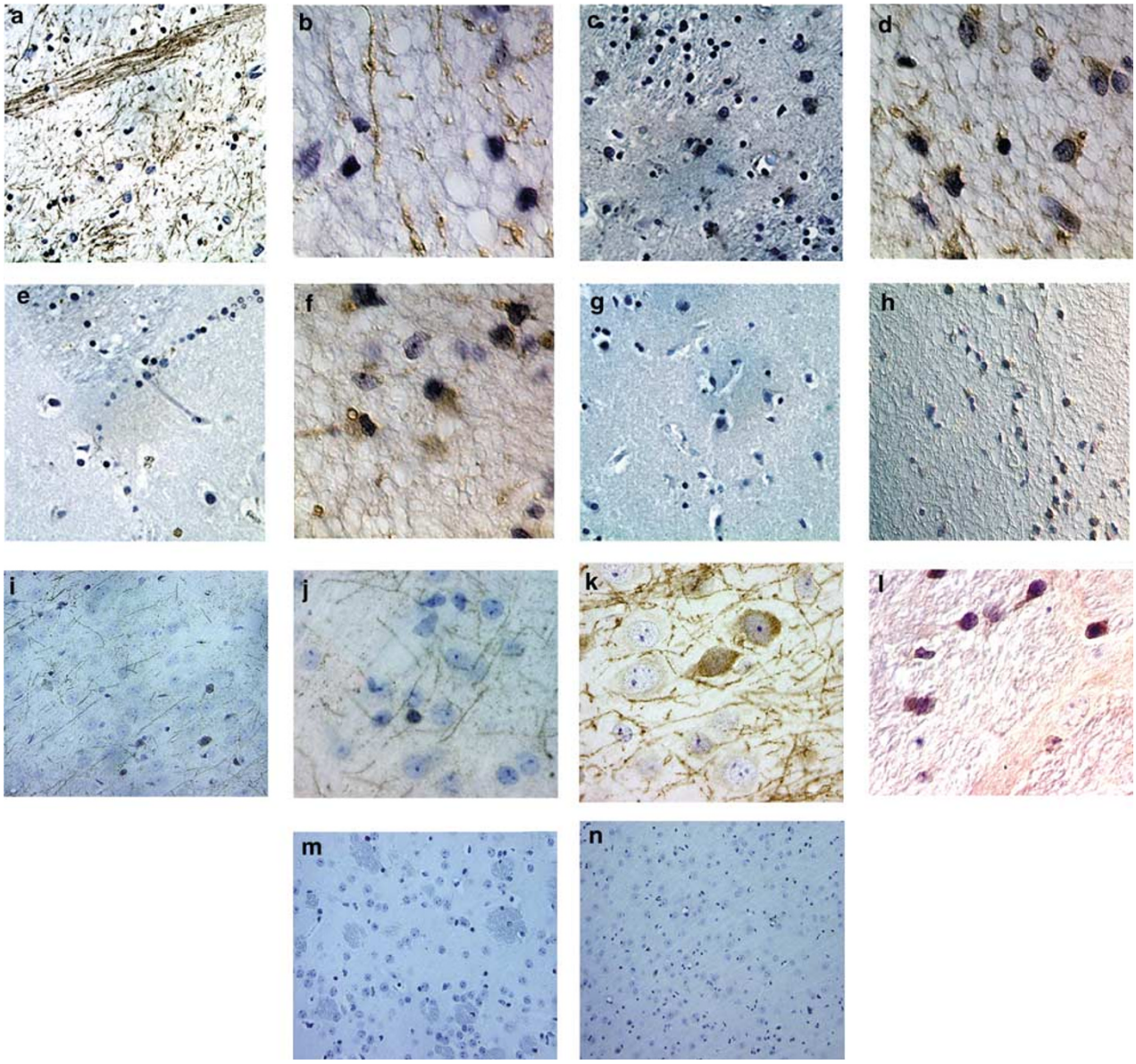

Figure 7 Caspase-2, -6, and caspase-7 immunoreactivity in post-mortem HD tissue and the YAC72 transgenic mouse brain. Fiber bundles and processes of degenerating caudate neurons in human HD brain show intense (**a, b**) caspase-2 immunostaining compared to (**e**) aged-matched control. Medium-sized neurons in the caudate nucleus display enhanced (**c, d**) caspase-7 immunostaining ($\times$ 400 and $\times$ 1000) in post-mortem HD tissue compared to controls (not shown). (**f**) Medium-sized caudate neurons in the post-mortem HD caudate nucleus are immunoreactive to caspase-6 ($\times$ 1000) compared to controls (not shown). (**h**) Medium-sized neurons in the caudate are immunoreactive to antibody raised against cleaved caspase-7 ($\times$ 1000) for post-mortem HD tissue compared to (**g**) controls. All results were confirmed by immunostaining with at least two independent anticaspase antisera and by verifying specificity via Western analysis to recombinant caspases and to endogenous caspases in cell lysates (data not shown). (**i–k**) Intense caspase-2 immunostaining also occurs in neuritic profiles and neurons of the cortex and striatum of YAC72 transgenic mouse YAC72 ($\times$ 200, $\times$ 600, $\times$ 1000, respectively) compared to (**m, n**) age-matched control striatum and cortex. (**l**) Caspase-7 immunoreactivity in the striatum of the YAC72 transgenic mouse ($\times$ 1000). Controls for nonspecific immunostaining also were performed for all tissues, including the use of preimmune serum and antigen-preadsorbed serum and each control showed no immunoreactivity

Enhanced immunoreactivity of caspase-2, -6, and -7 in post-mortem HD tissue and a transgenic HD mouse model

Since we found that full-length Htt interacts with caspase-2 and -7, we evaluated whether brains from cases of HD (grade 3; $n=3$) had altered expression of these two caspases when compared to healthy aged-matched controls ($n=3$). Caspase-2 is expressed in the neuritic profiles (possibly axons) of the HD striatum (Figure 7a,b), but not in control tissue (Figure 7e). The neuronal processes are swollen in appearance, rich in caspase-2 immunoreactivity. As shown in Figure 7c,d, caspase-7 immunoreactivity in post-mortem tissue from HD patients is dramatically enhanced in the medium spiny neurons of the caudate nucleus and neurons in the putamen (data not shown) when compared to age-matched controls (Figure 7g). The increased caspase-7 immunoreactivity seen in the perikarya of the medium spiny neurons (Figure 7d) is at

the site of caspase-6 activation (Figure 6e,f) in the caudate of HD patients. As shown in Figure 7f, caspase-6 immunoreactivity is enriched in the site of neurodegeneration in the caudate of HD patients. Since the enhanced immunoreactivity of caspase-7 and -2 does not prove that these caspases have been activated during HD cell death, we utilized an antibody that recognizes only the cleaved form of caspase-7. As shown in Figure 7h, a number of medium-sized neurons stain positively for activated caspase-7 in HD tissue and not in controls (data not shown), indicating the direct activation of caspase-7 in HD brain.

Since human HD tissue has varying post-mortem intervals, we evaluated our hypothesis in a transgenic mouse model of HD overexpressing wild-type or mutant full-length Htt (YAC18 and YAC72) under the control of the human Htt promoter. This transgenic mouse model shows selective loss of medium spiny neurons in the striatum, translocation of N-terminal fragments to the nucleus, gliosis in the striatum, and loss of neurons in the cerebral cortex by 1 year of age.[30] As shown in Figure 7, immunostaining for caspase-2 (panels i–k) and caspase-7 (panel l) in mouse YAC72 transgenics (1 year of age) was enriched in the striatum and cortex when compared to YAC18 age-matched control (panels m,n). Similar to that found in HD post-mortem tissue, caspase-2 was found in the neuritic profiles of the striatum and cortex of the HD transgenic mouse model (Figure 7i–k). We also immunostained for caspase-8 and -9 where we found no change in immunoreactivity of the striatum between the mouse YAC72 transgenic and the age-matched YAC18 controls (data not shown). Using an antibody to activated caspase-9, we found a number of cortical neurons immunoreactive only in the YAC72 (data not shown). These data further substantiate the involvement of specific caspases in the progression of HD disease in distinct neuronal populations, and support a critical role for caspase-2.

Caspase-2 expression is modulated by BDNF levels

Zuccato *et al*.[33] reported that striatal BDNF levels are regulated by wild-type Htt protein in the cortical projection neurons. BDNF mRNA is not present in the striatum and therefore the supply of BDNF is controlled by corticostriatal projection neurons. Since mutant Htt lowers BDNF levels in YAC72 mice, we tested whether the enhanced immunoreactivity of caspase-2 in the cortical and striatal neurons in this model could be due to altered BDNF levels and whether these alterations preceded the onset of disease progression. It has been shown in mouse sympathetic neurons that caspase-2 is required for trophic factor deprivation (NGF)-induced death.[37] We first analyzed whether exogenous BDNF altered caspase gene transcription. The relative expression of caspases was determined by quantitative polymerase chain reaction (PCR) in the presence and absence of BDNF in primary striatal neurons (Figure 8a). These studies revealed that the caspase-2 transcript is dramatically increased in the absence of BDNF, while no significant changes were observed for transcripts encoding caspase-1, -3, -7, -8, or -9. Caspase-6 and -12 are downregulated.

We therefore evaluated whether the transcription of caspase-2 was altered in presymptomatic YAC72 mice. Using RT-PCR, we found that caspase-2 mRNA was elevated in the cortex and striatum of YAC72 mice, compared with age-matched wild-type littermates (3 months, Figure 8b). Samples run in parallel probing for β-actin mRNA show no difference in expression (Figure 8b). These results are consistent with the enhanced immunoreactivity of caspase-2 found both in YAC72 mice and HD tissue along with a 1.5-fold increase in protein expression as detected by Western analysis (data not shown). Levels of BDNF gene transcription (exon II–IV mRNAs) were also measured in presymptomatic YAC72 mice and compared with age- and sex-matched wild-type littermates (3 months, Figure 8c). As described previously in 9-month-old YAC72 mice,[33] exon II, exon III, and IV mRNA are decreased in YAC72 (3 month) when compared to age-matched wild-type littermates (3 month) in the cortex.

Discussion

We have previously shown that the proteolytic processing of Htt by caspases and cleavage of Htt *in vivo* occurs at amino acid 552. [26–29,43] In the present work, we demonstrate that specific caspases bind to Htt. The caspases that interact with Htt are enriched and activated in the vulnerable cell types in the striatum and cortex and therefore our work offers some potential insight into the selective neuronal cell death found in HD. Our observations support a model whereby the interaction with caspases and the subsequent proteolytic processing of Htt contributes to neuronal cell death in several distinct steps. First, BDNF levels are reduced in the cortex and striatum and this correspondingly increases expression of caspase-2. Second, full-length Htt with a polyglutamine expansion is inappropriately recruited and bound to the initiator caspase-2. Since caspase-2 and Htt colocalize abundantly in the neuronal processes and axonal terminals, this binding would act as a seed to induce neuritic degeneration as Htt is cleaved to yield cytotoxic products. The N-terminal product generated from caspase cleavage has been shown to associate with synaptic vesicles and inhibit glutamate uptake.[44] Since the binding of full-length Htt to caspase-2 is repeat dependent, an apoptotic initiation event need not occur to start a caspase-dependent amplification process. However, several factors such as lower BDNF levels in HD patients and transgenic mouse models may contribute to a feedback loop of caspase initiation and amplification. Lower BDNF levels will increase caspase-2 expression and caspase-2 is known to play a critical role in cell death stimulated by trophic factor deprivation in neurons.[37] Secondly, and perhaps simultaneously, caspase-7, which is specifically expressed in the medium-sized neurons and enriched in the striatum, would bind full-length Htt. With the association of caspase-2 and -7 with Htt, the activity of additional caspases (particularly caspase-6) would accelerate the production of Htt fragments and result in the eventual induction of apoptosis both in the neuronal processes and somata. Active caspase-7 colocalizes with Htt in the ER recruiting Htt during apoptotic cell death and further amplifies the cell-death process.

Support for our proposed model is as follows. We have found that a specific subset of caspases interact with Htt.

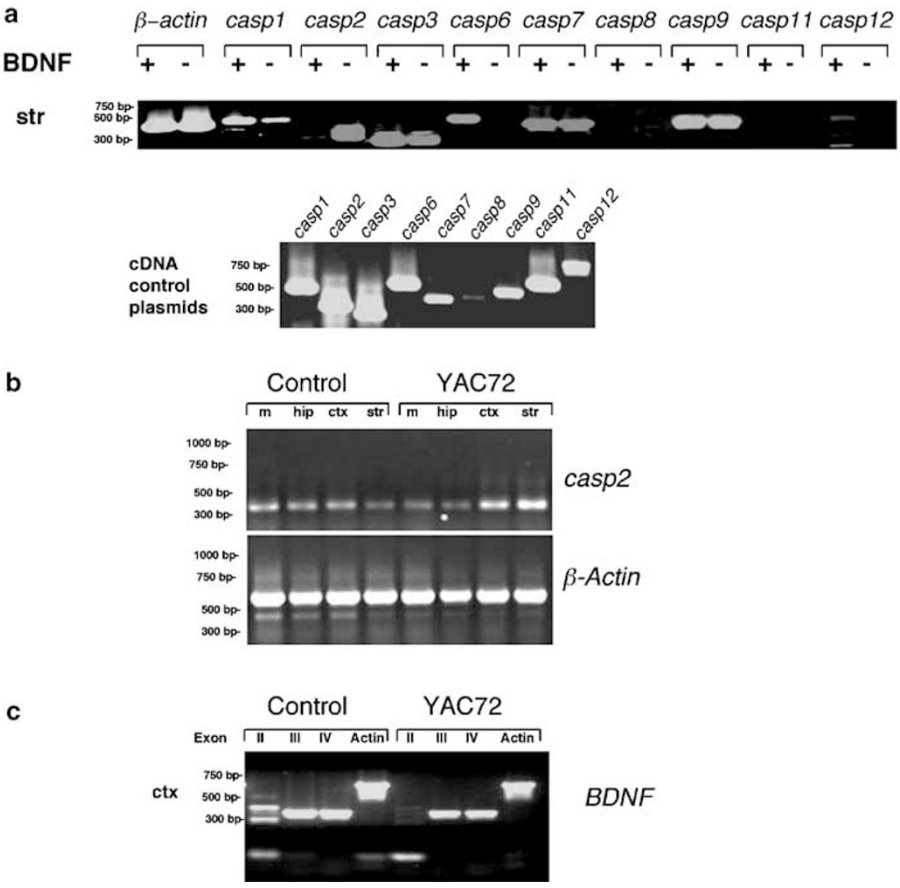

Figure 8 Caspase-2 mRNA is upregulated in presymptomatic YAC72 transgenic mice, and BNDF levels modulate caspase-2 expression. (**a**) Ethidium bromide-stained gels of RT-PCR analysis of caspase-1, -2, -3, -6, -7, -8, -9, -11, and -12 of primary striatal cells treated with BDNF or anti-BDNF (upper panel) run in parallel with β-actin mRNA (upper panel). cDNA controls for each caspase and their respective primers are shown as a positive control (lower panel). (**b**) The upregulation of caspase-2 in the cortex and striatum of YAC72 mice, compared with age- and sex-matched wild-type littermates (3 months, upper panel). Samples run in parallel with β-actin mRNA show no difference in mRNA levels (lower panel). The tissue regions dissected are the following: cortex (ctx), striatum (str), hippocampus (hip), and midbrain (m). (**c**) Levels of BDNF gene transcription were measured in presymptomatic YAC72 mice and compared with age-matched wild-type littermates (3 months). Levels of BDNF exon II–IV mRNAs were measured in the cortex as described in Zuccato *et al*.[33] As described previously in 9-month-old YAC72, exon II mRNA is not detected, and exons III and IV are decreased in YAC72 (3 month) when compared to age-matched wild-type littermates (3 month). The results were repeated in triplicate. It is important to note that caspase-6 is downregulated by the absence of BDNF. However, we found no change in mRNA for caspase-6 when comparing YAC72 to control (data not shown)

Those particular caspases demonstrate increased immunoreactivity in the brains of HD patients, cell-specific expression in the medium-spiny neurons of the striatum and cortex, and increased immunoreactivity in a full-length transgenic mouse model of HD. These same caspases are required for cell death induced specifically by expanded polyglutamine Htt in striatal neurons in culture. Our utilization of a dominant-negative caspase expression system may be preferable to caspase knockout primary cultures since compensatory activation of other caspases in response to the removal of the gene have been reported.[37]

Our results also indicate an important role for caspase-2-dependent cell death in the etiology of HD. Caspase-2 interacts with Htt in a polyglutamine-dependent fashion as shown by greater binding for Htt138 than for Htt15, and then cleaves Htt to liberate a cytotoxic fragment. Caspase-2 is also critical for expanded polyglutamine Htt-induced striatal neuronal cell death, since overexpression of a catalytic caspase-2 mutant blocks Htt-induced cell death. Furthermore, caspase-2 immunoreactivity is markedly increased in the neurites of striatal and cortical neurons in both HD patients and the YAC72 transgenic mouse model of HD. Importantly, the lower levels of BDNF increase the expression of caspase-2.

Although caspase-7 also interacts with Htt, its interaction does not appear to be polyglutamine length dependent. Caspase-7 is capable of cleaving Htt and a catalytic mutant of caspase-7 blocks Htt-induced death of striatal neurons in primary culture. In contrast to caspase-2 and -7, caspase-6 may have a downstream role, since it does not interact with full-length Htt, but does interact with the cleaved Htt fragment. Thus, caspase-6 may enhance the proapoptotic effect of the fragments generated by caspase-2 and -7, since a catalytic mutant of caspase-6 has an inhibitory effect on Htt-induced striatal neuronal cell death. Since caspases interact with Htt, cleave Htt to produce cytotoxic fragments, and are in turn activated by the proapoptotic effects of Htt fragments, mutant Htt has an amplifying effect on caspase activation initiating a feedback loop and activating the caspase cascade, thus potentially causing neuronal death observed in HD patient brains.

The finding that caspase-6 coimmunoprecipitates with an Htt fragment but not with full-length Htt supports the notion that caspase cleavage may lead to a conformational change in Htt, and underscores the need to evaluate potential protein–protein interactions both with full-length Htt and with physiologically relevant proteolytic fragments of Htt. This finding may also explain the apparent differences between the report that a polyglutamine tract demonstrates interaction with caspase-8[38] and our failure to identify caspase-8 interaction with full-length Htt (or to identify caspase-8 requirement in Htt-induced striatal neuronal cell death).

It is important to note that, although there is evidence for caspase cleavage of Htt *in vivo*,[31] definitive proof for the requirement of caspase-2, -3, -6, and -7 *in vivo* will require further studies. Further, other proteases, such as calpains may also be involved.[32] Nevertheless, the results presented here suggest that the interaction between caspase-2 and expanded Htt may be an appropriate target for therapeutic intervention. Further, our recent finding that caspase cleavage of both normal and expanded Htt occurs *in vivo*, with the identified 552 site as a preferred site of caspase cleavage in human HD and YAC72 transgenic mice, correlates well with our proposed role for caspase-2 in HD.[31] Together, these findings have important implications for the treatment of HD. Rather than simply blocking cell death with a general caspase inhibitor, one could design molecules that specifically block the interaction of Htt with the caspase-2 apoptosome complex or alternately downregulate the expression of capase-2. Our findings also raise the possibility that each of the polyglutamine-repeat disease proteins interacts with caspase-specific cell-death complexes and that specificity of neuronal cell-death observed in these diseases can be correlated in part with regional caspase expression.

Our studies do not address how sequential activation of caspases early in HD pathogenesis may affect the functional and neuroanatomic effects taking place over months in neurodegenerative diseases and at this point are correlative in nature. However, it is possible that caspase activation may contribute to early cellular dysfunction and is not just a late stage event. Slowly evolving neurodegenerative diseases may be the result of cotemporal activation of cell death proteases in neuronal cell populations that occurs over weeks and months and is an upstream event. Further, the cotemporal activation of cell death proteases may occur in distinct substructures of the neuron – both soma and the axons of striatal projection neurons where local activation of distinct caspases may contribute to neuronal dysfunction and eventual death. Testing this hypothesis will require further experimentation. Interestingly, careful stereological counting of the loss of striatal and cortical cells in YAC HD mice demonstrates a direct correlation with motor dysfunction.[45] Further studies are required to address the relative contribution of caspases to early and late events in HD pathogenesis.

Materials and Methods

Plasmid construction

Wild-type full-length human Htt (Htt15) and mutant Htt with 44 (Htt44), 80 (Htt80), and 138 (Htt138) repeats, the N-terminal fragment encoding a 548 amino-acid fragment of wild-type Htt (Htt1955-15) and mutant Htt with 128 repeats(Htt1955-128) and the N-terminal fragment encoding a 1212 amino-acid fragment of wild-type Htt (Htt3949-15) and mutant Htt with 138 repeats (Htt3949-138) were described previously.[28,34,36] The following catalytic mutant caspases, which disable the catalytic cysteine residue, were generated using the QuikChange Site-Directed Mutagenesis Kit (Stratagene): caspase-2 (C303A), caspase-3 (C163A), caspase-6 (C163A), caspase-7 (C186A), caspase-8 (C360A), and caspase-9 (C287A).

Cell culture and antibodies

Human embryonic kidney 293T cells were grown as described previously.[26,27] Transient transfections of plasmids in 293T cells were performed with Superfect reagent (Qiagen) following the manufacturer's instructions (10 μg DNA, 300 μl serum-free medium, and 30 μl Superfect added to a 10 cm-dish containing 3–4 ml of media). Control transfections with pcDNA3-GFP were carried out and showed greater than 80% transfection efficiency.

Western analysis of 293T cells transfected with human caspase-1 through caspase-12 confirmed the specificity of each antibody and the lack of crossreactivity between caspase family members. Antibody Bur49 was raised against human caspase-9, and Bur1890 against human caspase-8 following the same methods.[39] The specificity and affinity of these antibodies for their respective caspases were confirmed as previously described.[39] Rabbit and goat polyclonal anticaspase-2 antibody (Santa Cruz, sc-625, sc-625) and rat monoclonal anticaspase-2 (Chemicon, MAB3501 and MAB3507) crossreact with mouse and human caspase-2 and were used for both IP and histology. Rabbit polyclonal antiactive caspase-3 (67341A, Pharmingen), polyclonal active caspase-3 (Cell Signaling, #9661S), and mouse monoclonal caspase-7 antibody (Pharmingen) react with human and mouse. Anticaspase-3 antibody (Transduction Laboratories, C31720) reacts with human only. Rabbit polyclonal caspase-6 antibody was raised against human caspase-6 and also purchased from Upstate Biotechnology (#06–691) reacting with human and mouse. Rabbit polyclonal active-caspase-6 antibody (Cell Signaling, #9761) reacts against mouse and human. Rabbit polyclonal antiactive caspase-7 (Cell Signaling, #9491) reacts with mouse and human. Anticleaved caspase-9 antibody (BioLabs #9501S) reacts with mouse and human. Antibodies were used according to the directions of the manufacturer. Htt neoepitope antibodies were described previously.[31]

In vitro protein synthesis and caspase cleavage

In vitro transcription and translation were performed using the Promega Coupled kit. The constructs (pRC-CMV3949-15; construct yielding the first 1212 amino acids of Htt) and 138 glutamine repeats (pRC-CMV3949-138) were translated, and the protein products were used to assess caspase cleavage. Cleavage with caspases -2, -3, -6, -7, -8, -9, and -10 were performed and assessed as previously described.[26,27]

Caspase interaction assay in cultured cells and mouse cortical tissue

Cells were cotransfected with caspase-2, -3, -6, -7, -8, or -9 and Htt or pRC-CMV vector control only. Cell lysates of cotransfected 293T cells were prepared by incubating cells in NP40 lysis buffer (0.1% NP40, 50 mM Hepes, pH 7.4, 250 mM NaCl, 5 mM EDTA) for 30 min on ice with occasional vortexing. IP was carried out by incubation with anti-FLAG M2

agarose affinity gel (Sigma, St. Louis, MO, USA), anti-HA antibody, or anticaspase-2 agarose beads for 12 h to bind FLAG- or HA-tagged caspases or caspase-2, respectively. The beads were washed five times and resuspended in NP40 lysis buffer and then Laemmli sample buffer. The immunoprecipitated proteins were resolved by 10% SDS-PAGE and were transferred to polyvinylidene fluoride (PVDF) membranes for Western blotting. FLAG M2 antibody, caspase antibodies, or monoclonal Htt antibody (Chemicon 2166) was used for Western analysis. The immunoblots were developed with peroxidase-conjugated secondary antibody and enhanced chemiluminescence.

Cortical tissue from FVB and YAC72 mice were lysed in RIPA buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% SDS, 1% SDOC, 1% NP-40, protease inhibitor (Complete Mini, Roche Molecular Biochemicals), sheered with a 26-gauge needle and clarified by centrifugation at 16 000 *g* for 20 min (4°C). The N-terminal rabbit polyclonal Htt antibody described previously[46] was used for immunoprecipitation. Immunoprecipitation and Western analysis was performed as described above.

Striatal cultures

Primary cultures of striatum were prepared as described previously.[47] Striata of 17- to 18-day-old Sprague–Dawley rat embryos were dissected, minced, and digested with 0.25% trypsin for 5 min. After the addition of 10% horse serum, the tissue was triturated 15–20 times and centrifuged for 5 min (800 *g*). The pellet was resuspended in MEM-PAK (UCSF Cell Culture Facility) supplemented with 30 mM glucose, 2 mM GlutaMAX-1 (Life Technologies), and penicillin/streptomycin (100 U/ml). The suspension was filtered through a 70 μm cell strainer, and 5% horse serum (Life Technologies) was added. Cells at 2–3 $\times$ 10^5/cm^2 were seeded on either 24-well plates or 12-mm coverslips precoated with 50 μg/ml of D-polylysine. After a 30-min incubation, unattached cells were removed and the medium was replaced with glucose-enriched MEM-PAK. Cultures were fed weekly and used between 1 and 3 weeks. Striata were dissected from YAC72 and FVB controls at postnatal day 0–1 (P0–1) mice and primary cultures prepared as described above. Striatal neurons were transfected by the standard calcium phosphate method.[47] The striatal neurons were exposed to calcium phosphate and DNA precipitate for 20 min, washed twice with medium, and then cultured in fresh medium. Cells were transfected at 2–5% transfection efficiency. This initial exposure to calcium stimulated cell death in the Htt138-transfected cells and the YAC72 striatal cells. The percentage of cell death induced by full-length Htt with polyglutamine expansion (Htt138) was determined by transfecting cells with 2 μg of the pRC-CMV-Htt138 expression construct and 0.4 μg of pEGFP-N1 (Clontech) was used as a marker. In cotransfection experiments with catalytically inactive caspase constructs encoding human caspase-2 C303A, caspase-3 C163A, caspase-6 C163A, caspase-7 C186A, caspase-8 C360A, and caspase-9 C286A, a ratio of 1 : 1 with the pRC-CMV-Htt138 expression construct was used. Apoptotic cells were identified by visual inspection of GFP-positive cells with a Nikon inverted fluorescence microscope. Hoechst 33342 was used to examine nuclear morphology and count cells. Each experiment was performed at least in triplicate, with $>$300 cells counted for each determination. BDNF (Sigma B3795) was applied to primary mouse striatal cultures for 24 h at 50 ng/ml. Monclonal anti-human BDNF from Sigma (B5050) was applied to striatal cultures to neutralize the biological activity of endogenous BDNF. Monoclonal Htt antibody (Chemicon 2166, 2168) and rabbit GRP78 (StressGen SPA-826) was used for immunocytochemistry. ANOVA analysis with *post hoc* tests (via GraphPad Prism) for the data presented in Figure 4 and two-factor ANOVA analysis for Figure 3 were preformed with $P^* < 0.05$, $P^{**} < 0.01$, and $P^{***} < 0.001$.

Immunocytochemistry

Formalin-fixed human tissue (from the Harvard Brain Tissue Resource Center), or mouse brains perfused with 4% paraformaldehyde, was paraffin embedded. YAC transgenic animals expressing human Htt with 72 CAG repeats were used as a source of murine tissues. Line 2511 contains 1–2 copies of the YAC72 and has been described previously.[30] Mouse brains were sectioned horizontally (8 μm) on the automated rotary microtome (Leica) and deparaffinized in xylene. Antigen retrieval was carried out by microwaving sections in 10 mM citrate buffer, pH 6.0, for 5 min at 40% power in an 1100 W microwave oven. Blocking was carried out in 10% normal horse serum in TBS –for 1 h at room temperature. Primary antibody was diluted 1 : 100–1 : 1000 in 1% BSA in TBS and incubated for 48 h at 4°C. Rabbit IgG2 (2 μg/ml) or preimmune antiserum was used as a negative control. Biotinylated secondary antibody (1 : 250, Vector) was used for incubation times of 1 h at 37°C in a humidified chamber and followed by TBS washes (3 $\times$ 10 min) at RT. DAB at a final concentration of 0.25 mg/ml with 0.05% H_2O_2 in TBS was used for developing typically for 3.5 min at RT. VECTOR Red substrate kit was used for double-labeling experiments according to the instructions of the manufacturer. Sections were counter stained with Mayers hematoxylin (American MasterTech).

cDNA generation

Total RNA from wild-type or transgenic cortex, hippocampus, mid-cortex, and striatum was extracted twice using Trizol (Gibco/BRL, Gaithersburg, MD,USA). cDNA was generated from 1.0 μg of RNA using SuperScript II reverse transcriptase (Life Technologies, Gaithersburg, MD, USA) according to the instructions of the manufacturer.

Semiquantitative PCR

PCR parameters were 2.5 μl of four-fold-diluted cDNA reaction products, 25 pM primers, 0.5 mM dNTPs, and 2 U of *Taq* polymerase (Roche Molecular Biochemicals, Indianapolis, IN, USA) with 2.5 μl of 10 $\times$ buffer. Total reaction volume was 25 μl. Cycling conditions and PCR primers for BDNF exons 2, 3, 4, and 5 were as described.[33] As a positive control for PCR, mouse β-actin primers (β-actin-5′: CATCGTGGGCCGCTCTAGGCACCAA and β-actin-3′: CAGGGAGGAA-GAGGATGCGGCA) were used. The caspase oligos react with both mouse and human sequences and are as follows: Casp1-5′: acacgtcttgccctcattatctgcaa, Casp1-3′: Atggtt-gttcaaatgaaaatcgaacct; Casp2-5′: tgagggag-ctcatcca-ggcca; Casp2-3′: tccagtgaagtgcacattgctca; Casp3-5′: aaacctcag-tggattcaaaatc-cattaa; Casp3-3′: acacacacaaag-ctgctcc-ttttgct; Casp6-5′: acaagatggac-cacaagaggagagga; Casp6-3′: tgcagctttttggtcagcattgaggc; Casp7-5′: aatgcatcatca-taaacaa-caagaactt; Casp7-3′: gcagagggcctgcacaaaccagga; Casp8-5′: ggatgtt-ggaggaaggcaatctgtc; Casp8-3′: ttgatgatca-gacag-tatccccga; Casp9-5′: tcatcatcaacaatgtgaacttctgcc; Casp9-3′: ggagggactg-caggtcttcag; mCASP11-5′: ctggaaatggag-gaaccagaaga; mCASP11-3′cgatcaatggtgggcatctgggaa; mCASP12-F2: ggaaaaatatttgctggccacatt; mCASP12-3′: tagtgggcatctgggt-cagttca.

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