

Dysregulation of Receptor Interacting Protein-2 and Caspase Recruitment Domain Only Protein Mediates Aberrant Caspase-1 Activation in Huntington's Disease

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Caspase-1 plays a role in the pathogenesis of a variety of neurological diseases. Caspase-1 activation is an early event in models of Huntington's disease (HD). However, mechanisms regulating the activation of this apical caspase in cell death are not known. Receptor interacting protein-2 (Rip2) and caspase recruitment domain (CARD) only protein (Cop) are two CARD proteins with significant homology to the caspase-1 CARD and modulate caspase-1 activation in inflammation. Rip2 is a caspase-1 activator, and Cop is a caspase-1 inhibitor. We demonstrate in models of HD that caspase-1 activation results from dysregulation of caspase-1 activation pathways. Associated with disease progression, we detect elevation of the caspase-1 activator Rip2 and reduction of the caspase-1 inhibitor Cop. Knocking down endogenous Rip2/Cop respectively results in reduced/increased sensitivity to neurotoxic stimuli. Our data provide evidence that caspase-1-mediated cell death is regulated, at least in part, by the balance of Rip2 and Cop, and alterations of this balance may contribute to aberrant caspase-1-mediated pathogenesis in Huntington's disease.

Key words: Cop; Rip2; caspase-1; Huntington's disease; RNA interference; cell death

Introduction

Caspases exist within cells as inactive precursors (Cohen, 1997; Ashkenazi and Dixit, 1998; Chen and Wang, 2002). Aberrant caspase activation can result in inappropriate cell death, contributing to a myriad of diseases (Friedlander, 2003). Several endogenous activators and inhibitors have been described that modulate the activity of different members of the caspase family (Goyal, 2001; Shi, 2002). Caspase-1 plays an important role in pathological cell death, particularly in neurological diseases including Huntington's disease (HD), amyotrophic lateral sclerosis (ALS), and cerebral ischemia (Friedlander et al., 1997a; Schielke et al., 1998; Ona et al., 1999; Zhu et al., 1999; Chen et al., 2000; Li et al., 2000b; Rabuffetti et al., 2000; Ilzecka et al., 2001; Friedlander, 2003; W.H. Zhang et al., 2003). Caspase-1 is activated

early in the disease process. In mouse models of HD, caspase-1 is the earliest caspase that has been demonstrated to be activated (Y. Zhang et al., 2003). Initial low-level caspase-1 activation is followed by a progressive amplification that is associated with disease progression (Chen et al., 2000; Y. Zhang et al., 2003). However, mechanisms regulating initial caspase-1 activation and the observed downstream amplification in cell death and in neurological diseases have not been described. Given the key role that caspases play in neurological diseases, understanding the mechanisms regulating caspase activation in neurological diseases, and in cell death in general, is of primary importance.

Specific adaptor molecules regulate the activation of caspases. Three adaptors have been reported to regulate the activation of caspase-1. They are receptor interacting protein-2 [Rip2; also known as Cardiak and RICK (RIP-like interacting CLARP kinase)] (Druilhe et al., 2001; Lee et al., 2001), apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC), a PYRIN-caspase recruitment domain (CARD) protein (Srinivasula et al., 2002; Stehlik et al., 2003; Mariathasan et al., 2004), and Ice-protease activating factor (Ipafl)/CLAN (caspase-associated recruitment domain, leucine-rich repeat, and NACHT-containing protein)/CARD12, a human *Caenorhabditis elegans* death gene 4/apoptotic protease activating factor-1 family member (Jason et al., 1988; Poyet et al., 2001; Mariathasan et al., 2004). Rip2, ASC, and Ipafl all are CARD-containing proteins; their CARD domains bind to the CARD domain of caspase-1 prodomain via CARD–CARD interactions, induce caspase-1 oligomerization, and promote caspase-1 activation. Caspase-1 acti-

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vation then induces apoptosis and proinflammatory cytokine stimulation (Druilhe et al., 2001; Lee et al., 2001; Poyet et al., 2001; Srinivasula et al., 2002; Stehlik et al., 2003; Mariathasan et al., 2004). Among these caspase-1 activators, Rip2 stands out as a potential candidate for having a role in the CNS. Unlike ASC (which is weakly expressed in the brain) and Ipaf (which is not expressed in the brain), Rip2 mRNA and protein have been demonstrated clearly in human and mouse brain tissues (Jason et al., 1988; Masumoto et al., 1999; Engidawork et al., 2001; W. H. Zhang et al., 2003). Furthermore, Rip2 has been demonstrated to mediate cell death in some cell lines (Inohara et al., 1998; McCarthy et al., 1998). Additionally, we recently reported the role of the Rip2/caspase-1 pathway in hypoxia and ischemia-induced neuronal cell death (W. H. Zhang et al., 2003). Rip2 upregulation has been reported in the brains of patients with Alzheimer's disease (Engidawork et al., 2001). However, a specific role for Rip2 in neurodegeneration has not been described.

Another CARD-containing protein, CARD-only protein (Cop), shares a high degree of homology with the CARD of procaspase-1 (Druilhe et al., 2001; Lee et al., 2001). Binding of Cop to the CARD prodomain of procaspase-1 results in inhibition of caspase-1 activation. Cop binds to both procaspase-1 and Rip2, resulting in inhibition of Rip2-mediated activation of caspase-1 in inflammatory conditions. Given that caspase-1 activation is an early event in a number of described neuronal cell death pathways (Li et al., 2000b; Wang et al., 2003; W. H. Zhang et al., 2003; Y. Zhang et al., 2003), understanding the regulation of this event will provide important insight into disease pathogenesis. Here, we show that Rip2 and Cop dysregulation results in aberrant caspase-1 activation in both of cellular and mouse models of HD.

Materials and Methods

Reagents and antibodies. The following reagents and antibodies were purchased from the indicated suppliers: caspase-1 antibody from Dr. Junying Yuan (Harvard Medical School) as a gift (for cultured cells) or from Santa Cruz Biotechnology (Santa Cruz, CA) (for mouse tissue) or from Biosource (Camarillo, CA); caspase-3 antibodies recognized for procaspase-3 and active caspase-3 or only active caspase-3 from Cell Signaling Technology (Beverly, MA); Rip2 antibody from Alexis Biochemicals (San Diego, CA); Cop antibody from Abcam (Cambridge, UK); β -actin antibody, normal IgG, and protein G-Sepharose beads from Sigma (St. Louis, MO); histone H2A antibody from MBL International (Woburn, MA); horseradish peroxidase-conjugated secondary antibodies and ECL reagents from Amersham Biosciences (Arlington Heights, IL); FITC-conjugated or Texas Red-conjugated secondary antibodies from Vector Laboratories (Burlingame, CA); Lipofectamine and Lipofectamine 2000 from Invitrogen (San Diego, CA).

Cell lines, constructs, and transfection. ST14A cells are striatal neurons conditionally immortalized by transfection with a temperature-sensitive form of the simian virus 40 large T-antigen (nucleotides 1955–128). Stable huntingtin (htt) ST14A cells (mutant-htt ST14A cells and parental ST14A cells) were cultured as described previously (Rigamonti et al., 2000). ST14A cells were kindly provided by Dr. Elena Cattaneo (University of Milan, Milan, Italy). Parental ST14A cells, mutant-htt ST14A cells, and HeLa cells were cultured in DMEM supplemented with 10% fetal bovine serum, 0.1 mM nonessential amino acids, 2 mM L-glutamine, and penicillin/streptomycin in 5% CO₂ at 33°C (ST14A cells) or 37°C (HeLa cells). Primary cerebrocortical neurons (PCNs) [embryonic day 14 (E14) to E16] were performed as described previously (W. H. Zhang et al., 2003). Briefly, cerebral cortex of mouse embryos at E15 were freed from meninges and separated from olfactory bulb and hippocampus. Trypsinized cells were suspended in medium (neurobasal medium with 2% B27 supplement, 2 mM glutamine, 100 U/ml penicillin, and strepto-

mycin) and seeded at a density of 2×10^4 per square centimeter on poly-D-lysine-coated dishes or plates. Cells were used for experiments on day 7 of culture.

Cop and Rip2 plasmids have been described previously (Lee et al., 2001). pU6-siRNA Cop S1, pU6-siRNA Cop S2, pU6-siRNA Rip2 S1, pU6-siRNA Rip2 S2, pU6-siRNA Rip2 S3, and pU6-siRNA Rip2 S4 were constructed by Biomyx Technology (San Diego, CA). Transient transfection was performed by Lipofectamine (Invitrogen) in ST14A and HeLa cells and Lipofectamine 2000 (Invitrogen) in PCNs.

Mouse brain specimens of HD. For mouse brain, 7- and 11-week-old R6/2 mice (The Jackson Laboratory, Bar Harbor, ME) and age-matched wild-type (WT) mice were bred in our facility. The proteins of the mouse brains were extracted and analyzed by Western blotting. Frozen brain sections were obtained for immunohistochemistry assay.

Caspase-1-deficient mice. The mice were created and characterized by BASF (Worcester, MA) and generously provided by Dr. W. Wong (Li et al., 1995). These mice contain a null mutation in the *caspase-1* gene that was generated by homologous recombination in embryonic stem cells.

Apoptosis induction. For RNA interference (RNAi) assay, mutant-htt ST14A cells were transfected by pU6-siRNA, pU6-siRNA Rip2 S1, pU6-siRNA Rip2 S2, pU6-siRNA Rip2 S3, pU6-siRNA Rip2 S4, pU6-siRNA Cop S1, or pU6-siRNA Cop S2 and shifted to a nonpermissive temperature (37°C) for 13–14 h. Cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTS) assay.

Lactate dehydrogenase assay. The assay was performed according to the manufacturer's instructions (Roche Products, Indianapolis, IN).

MTS assay. The assay was performed as described previously (Wang et al., 2003) and according to the manufacturer's instructions (Promega, Madison, WI). The measurements were made on parental ST14A and mutant-htt ST14A cells using the MTS assay, a measure of mitochondrial function and thus cell survival. This method registers a complementary pattern when compared with the lactate dehydrogenase (LDH) assay used with primary cortical cells, because the latter technique measures enzyme release after cell death.

Microscopic determination of cell death. Parental ST14A striatal neurons, mutant-htt ST14A neurons, and PCNs were cotransfected with indicated plasmids and green fluorescent protein (GFP) as the transfection marker. After 48 h transfection, transfected green cells were scored by chromatin condensation and nuclear fragmentation under fluorescence microscopy. For RNAi experiments, see below (RNA interference assay).

Immunoprecipitation and Western blot analysis. Striatal cells and HeLa cells were collected in lysis buffer [20 mM Tris, pH 8.0, 137 mM NaCl, 10% glycerol, 1% Nonidet P-40, 2 mM EDTA with 5 mM Na₂VO₄, protease inhibitor mixture (Roche Products), and 0.2 mM phenylmethylsulfonyl fluoride] on ice, centrifuged at $10,000 \times g$ for 10 min, and lysed by immunoprecipitation (IP) or directly analyzed by Western blot (WB). Fresh mouse brains from wild-type or R6/2 transgenic mice were dissected and lysed in the radioimmunoprecipitation assay buffer with protease inhibitors (Wang et al., 2003) and lysed by IP or directly analyzed by WB. For IP, the soluble lysates were incubated with a Flag or Cop antibody, and immune complexes were harvested with protein G-Sepharose beads, followed by extensive washes, and then boiled in sample buffer. Eluted proteins were separated by SDS-PAGE, transferred to polyvinylidene fluoride membranes, and incubated with primary hemagglutinin (HA), Myc, or Cop antibody. For preparation of nuclear extract, striatal cell lysates were prepared as described previously (Wang et al., 2003). For WB, the protein content of the extracts was measured (Bio-Rad, Hercules, CA), and the total lysate sample (50 μ g per lane) was resolved by SDS-PAGE and immunoblotted with primary antibodies. Blots were developed ECL reagents.

Immunocytochemistry. Mutant-htt ST14A cells, parental ST14A cells, and HeLa cells were untreated or treated, or transfected with Flag-Cop plasmids, as indicated, on chamber slides. The cells were fixed in 4% paraformaldehyde for 15 min, 0.1 M glycine for 15 min, and 1% Triton X-100 for 30 min. Blocking was done in 5% BSA in PBS for 30 min. Cells then were incubated with antibodies Rip2 or Cop and incubated with FITC- or Texas Red-conjugated secondary antibodies. Hoechst 33342

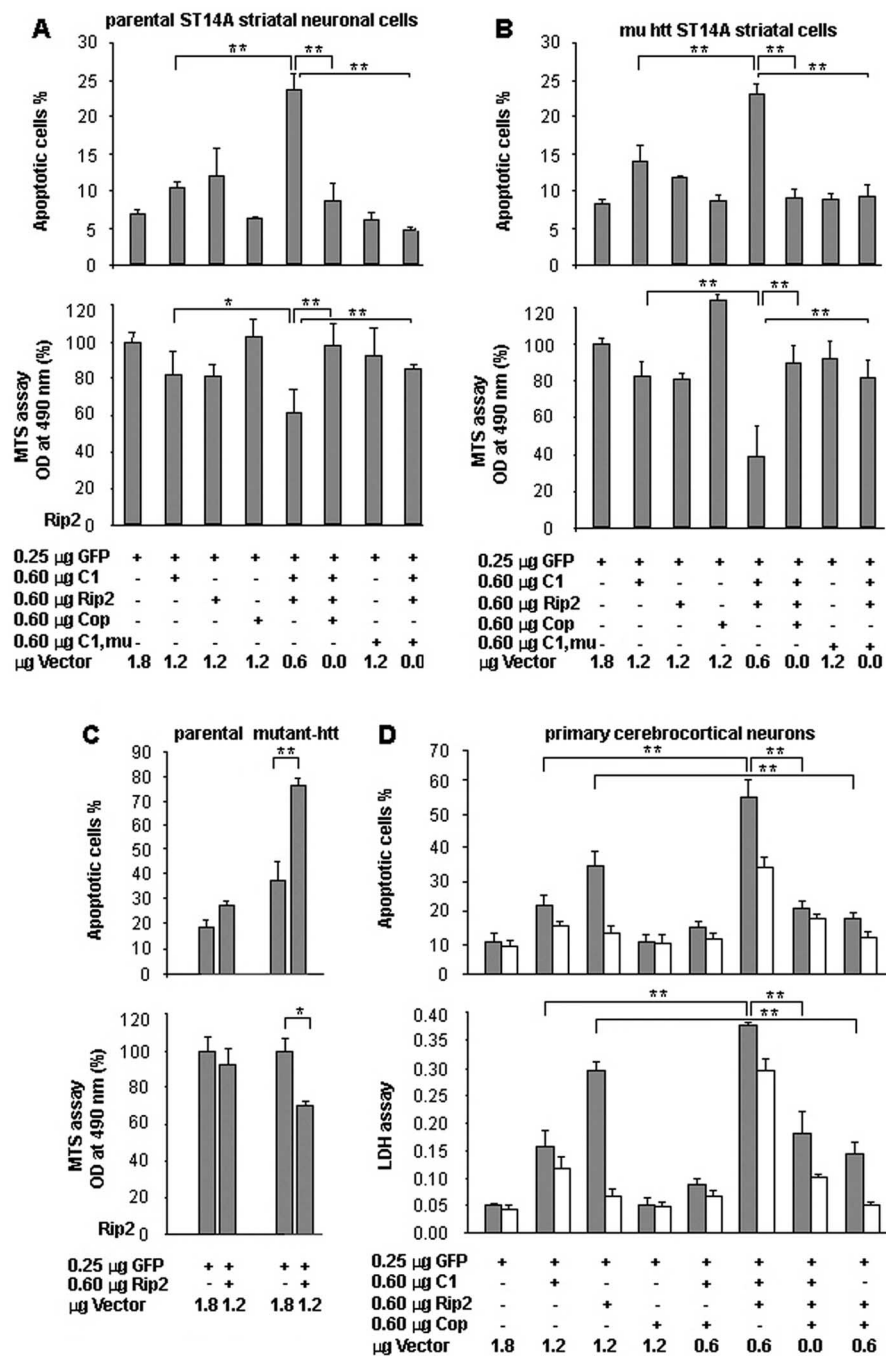


Figure 1. Transient overexpression of Cop makes cells more resistant to cell death. Parental striatal ST14A neuronal cells (**A, C**), mutant-htt striatal ST14A cells (**B, C**), and PCNs from wild-type or caspase-1-deficient mice (**D**) were cotransfected with plasmids encoding Cop in combination with the indicated expression plasmids encoding GFP, Rip2, procaspase-1 (C1), mutant caspase-1 (C1, mu), or plasmids encoding Rip2 or GFP (**C**). The amount of DNA was normalized to 2.05 μg with empty vector pcDNA3. At 48 h after transfection (**A, B, D**) or nonpermissive temperature shift 18 h after 30 h transfection (**C**), apoptotic cells were counted under fluorescence microscopy. Cell viability was measured in parallel by the MTS assay (**A–C**) and LDH-release assay (**D**). The gray bars represent the parental ST14A cells (**A, C**) and mutant-htt ST14A cells (**B, C**) and PCNs from wild-type mice (**D**); the white bars represent the PCNs from caspase-1-deficient mice (**D**). The results represent mean ± SD of three independent experiments. **p* < 0.05; ***p* < 0.001.

staining (Invitrogen) (1:10,000 for 2–5 min) was performed. Deconvoluted images were taken with a Nikon (Tokyo, Japan) Eclipse TE 200 fluorescence microscope and processed by using IP Lab software (Spectra Services, Webster, NY).

Immunohistochemistry. Brains of middle and later stage of R6/2 and matched WT mice were obtained and frozen in cold isopentane after cryoprotection in 30% sucrose. Frozen sagittal sections (10 μm thick)

were washed with 0.05% Tween 20 in PBS and permeabilized with 70% methanol and 0.05% Triton X-100. The sections were then blocked and incubated with Rip2 antibody and neuron-specific antibody (NeuN), washed by 0.05% Tween 20 in PBS, and reacted with FITC- or Texas Red-conjugated secondary antibodies and washed by 0.05% Tween 20 in PBS. Hoechst 33342 was used for counterstaining. The fluorescently stained sections were evaluated using deconvolution protocols with a Nikon Eclipse TE 200 fluorescence microscope.

RNA interference assay. pU6-Rip2 siRNA and pU6-Cop siRNA plasmids were constructed by Biomyx Technology. Synthetic oligonucleotides with the following sequences were introduced into pU6 vector: Rip2 S1, 5'-TGTCATTCAGGCTCATTGCAAATTCCTTTTGTCTTCATCCTATCTAGACAT-3' and 5'-GGAAGTTCAGGCTCATTGCAAATTCCAAACAAGGCTTTTCTCCAAGG-3'; Rip2 S2, 5'-TGCAAACTGGTTCAGATTCTATAATT-TTGTCTTCATCCTATCTAGACAT-3' and 5'-GGAAGAACTGGTTCAGATTCTATAATAACAAGGCTTTTCTCCAAGG-3'; Rip2 S3, 5'-TGCAAGAGGATCCACATGATTCCTCTTTTGTCTTCATCCTATCTAGACAT-3' and 5'-GGAAGAGAGATCCACATGATTCCTCAAACAAGGCTTTTCTCCAAGG-3'; Rip2 S4, 5'-TGTCAGGCAAATTCCTCTCTTGGATGCTTTTGTCTTCATCCTATCTAGACAT-3' and 5'-GGAAGGGCAAATTCCTCTCTTGGATGTCAAACAAGGCTTTTCTCCAAGG-3'; Cop S1, 5'-TGCAAGGTATCGGACCTGCTGAGAGTCCTTTTGTCTTCATCCTATCTAGACAT-3' and 5'-GGAAGGTATCGGACCTGCTGAGAGTCCTTTTCTCCAAGG-3'; Cop S2, 5'-TGTCAGTATTCTGAACATGGCACCTCTGCTTTTGTCTTCATCCTATCTAGACAT-3' and 5'-GGAAGGTATTCTGAACATGGCACCTCTGCAAACAAGGCTTTTCTCCAAGG-3'. All siRNA sequences were verified by DNA sequencing. Transient transfection was performed by Lipofectamine in mutant-htt ST14A cells. After transfection, cells were presented for reverse transcription-PCR (RT-PCR) and WB analysis. For apoptotic induction, mutant-htt ST14A cells were shifted to a nonpermissive temperature (37°C) for 13–14 h. For microscopic determination of cell death, cotransfection with indicated plasmids, and GFP as the transfection marker, transfected green cells were scored by chromatin condensation and nuclear fragmentation under fluorescence microscopy. Cell viability was assessed by MTS assay.

RT-PCR. Total RNA was isolated from mutant-htt ST14A cells using Trizol reagent (Invitrogen). After reverse transcription, cDNA was amplified using PCR (Invitrogen). For RT-PCR, the following primers were used: Rip2, 5'-CCATCCCGTACCACAAGCTC-3' and 5'-GCAGGATGCGGAATCTCAAT-3' (Chin et al., 2002); rat Cop, 5'-ATGGCCGACAAGGTCTCTG-3' and 5'-TCTCCATCTCTCTCTGGTTC-3'; glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5'-ATGGTGAAGTCTGGTGTCAACGGA-3' and 5'-TTACTCCTTGGAGGCCATGTAGGC-3' (Clontech, Mountain View, CA).

DNA sequencing. PCR products of rat Cop and mouse Cop were puri-

fied and ligated into the TA cloning vector pCR2.1 (Invitrogen) according to the manufacturer's protocol. After ligation, the vector was transfected into the INV α F⁺ strain of *Escherichia coli*. Individual bacterial colonies were picked, and plasmid DNA was isolated (Qiagen, Valencia, CA). The sequence was performed by the Brigham and Women's Hospital DNA Sequencing Facility.

Real-time RT-PCR. Mutant-htt ST14A cells were kept at 33°C with regular medium or shifted to 37°C in serum-deprived medium (SDM) for 18 h. Total RNA was extracted by a RNeasy kit from Qiagen. The following primers were used: caspase-3, 5'-ggacctgtggacctgaaaaa-3' and 5'-gcatgccatcatcatctcag-3'; GAPDH, 5'-ctcatgaccacagtcctgc-3' and 5'-ttcagctctgggatgacctt-3' (Invitrogen). Real-time quantitative RT-PCR (qRT-PCR) was performed in a MX3000P instrument (Stratagene, La Jolla, CA); 0.5 μ g of total RNA was added to a mixture of 50 μ l final volume consisting of 25 μ l of 2 \times SYBR qRT-PCR master mix (Stratagene), 200 nM of each primer, 0.75 μ l of 1:500 diluted reference dye, and 0.125 μ l of StrataScript RT/RNase Block enzyme mixture. The RT-PCR was performed by 50°C for 30 min, 95°C for 10 min, followed by 45 cycles of 95°C for 30 s, 55°C for 1 min, and 72°C for 30 s. A dissociation curve was generated to distinguish specific amplicons from nonspecific amplifications. Ct values were determined by an automatic method for amplification-based threshold fluorescence level by MX3000P. A comparative Ct method was used to take average of four replicas of the Ct values of each sample for caspase-3 and GAPDH. The Δ Ct value was obtained by subtracting the average GAPDH Ct value from the average of caspase-3. Average Δ Ct of three control group samples was taken as the calibrator. $\Delta\Delta$ Ct was calculated as substrate calibrator from individual Δ Ct. The mRNA fold change = $2^{-\Delta\Delta Ct}$.

Statistical analysis. Densitometric quantification used the Quantity One program (Bio-Rad). Statistical significance was evaluated by ANOVA followed by a *post hoc* Tukey's test (see Fig. 1A, B, D) or Stat-View (SAS Institute, Cary, NC) software using *t* test. *p* values < 0.05 were considered significant and are indicated by *. *p* values < 0.001 are indicated by **.

Results

Cop inhibits Rip2/caspase-1-mediated neuronal cell death

Striatal and cortical neurons are the targets of selective degeneration in HD (Li, 1999). To evaluate whether Cop may play a role in HD-related neuronal cell death, we overexpressed Cop and evaluated its effect on neuronal cell death pathways. Parental ST14A and stable mutant huntingtin-expressing ST14A (mutant-htt ST14A) striatal cells (Rigamonti et al., 2000) were transiently transfected with plasmids encoding Cop, procaspase-1, Rip2, or mutant procaspase-1 (a caspase-1 dominant-negative inhibitor). When cultured at the permissive temperature (33°C) (Fig. 1A, B), none of these plasmids individually resulted in significant induction of cell death compared with controls. However, cotransfection of procaspase-1 and Rip2 induced a significant increase in cell death, suggesting that, in these cell types, the combination of caspase-1 and a caspase-1 activator Rip2 was required for effective induction of cell death. Caspase-1/Rip2-mediated cell death was almost completely inhibited by the addition of Cop to the transfection (Fig. 1A, B). Shifting temperature-sensitive mutant-htt ST14A cells from 33°C (permissive temperature) to 37°C (nonpermissive temperature) in SDM causes mutant-htt ST14A cells to die in a time- and polyglutamine-dependent manner (Rigamonti et al., 2000; Wang et al., 2003). At the nonpermissive temperature, parental ST14A cells also die but to a significantly lesser degree than the mutant cell line (Rigamonti et al., 2000). Unlike at the permissive temperature, in the absence of exogenous caspase-1, transient transfection of Rip2 at the nonpermissive temperature results in sig-

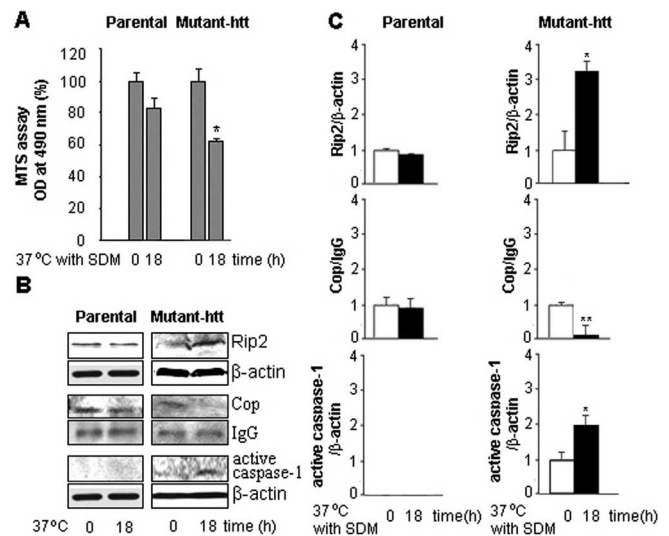


Figure 2. Nonpermissive stress sensitizes mutant-htt ST14A cells to Rip2-mediated cell death. Both parental and mutant-htt ST14A striatal cells were placed at 33°C with regular media (permissive) or placed at 37°C in SDM (nonpermissive) conditions for 18 h. Cell viability was measured by the MTS assay (A). Immunoblotting demonstrates changes of Rip2/Cop/caspase-1 proteins in the indicated cell lines and conditions (B). Densitometry was performed to quantify each lane. The white bars represent the cells no treatment, and the black bars represent the cells under 18 h apoptotic treatment (C). The data represent the mean \pm SD. $n \geq 3$; * p < 0.05; ** p < 0.001.

nificant mutant-htt ST14A cell death (Fig. 1C). However, at the nonpermissive temperature, Rip2 does not induce cell death of the parental cell line in the absence of exogenous caspase-1 (Fig. 1C). These data suggests that in these particular cell lines, an additional stressor needs to be present in order for Rip2 to be able to induce cell death.

Transgenic mice expressing a dominant-negative mutant of caspase-1 in neurons have been shown *in vivo* to have remarkable protective properties in a broad number of experimental models of both acute (stroke, traumatic brain, and spinal cord injury) (Friedlander et al., 1997a; Fink et al., 1999; Li et al., 2000a) and chronic (HD, ALS, human immunodeficiency virus, and Parkinson's disease) (Friedlander et al., 1997b; Klevenyi et al., 1999; Ona et al., 1999; Garden et al., 2002) neurodegenerative diseases. Consistent with these findings, a mutant caspase-1 expression plasmid inhibited caspase-1/Rip2-mediated killing of ST14A cells (Fig. 1A, B).

Given the role of caspase-1 in neuronal cell death, we evaluated the potential role of the Rip2/Caspase-1/Cop cell death axis in PCNs. Interestingly, Rip2 transfection induced cell death in the absence of exogenous caspase-1 in these cells, and Rip2-mediated PCN cell death is dependent on endogenous caspase-1, because Rip2 is unable to induce cell death in caspase-1-deficient neurons (Fig. 1D) (W. H. Zhang et al., 2003). As in ST14A cells, Rip2/caspase-1 cotransfection resulted in greater cell death than either vector alone. Cop inhibited both Rip2 and Rip2/caspase-1-mediated cell death (Fig. 1D). Together, the above-described experiments demonstrate a protective role of Cop in inhibiting Rip2/caspase-1-mediated neuronal death.

Immunocytochemical analysis of cultured neurons confirms the correlation of Rip2 accumulation, Cop depletion, caspase-1 and caspase-3 activation, and cell death

We then investigated whether endogenous Rip2 and/or Cop might play a role in a cell death model in which caspase-1 is

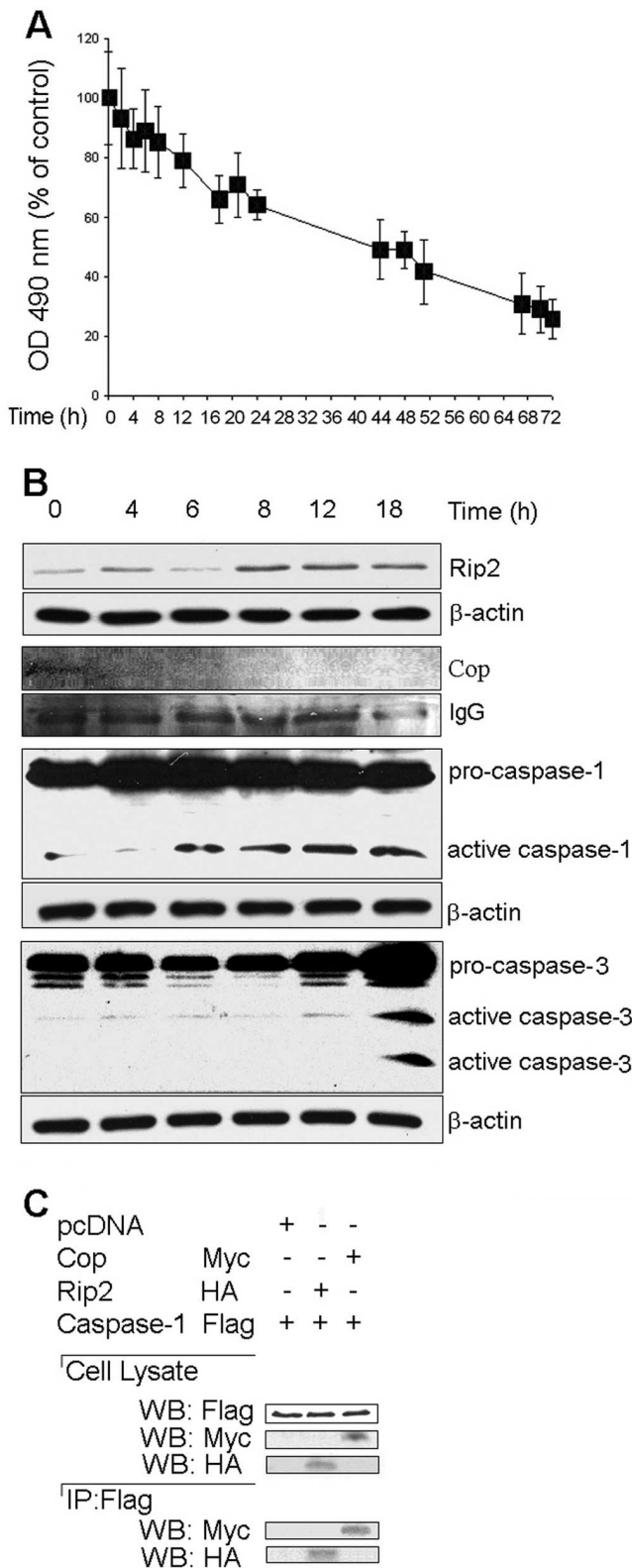


Figure 3. Activation of caspase-1 and caspase-3 associated with the Rip2 upregulation and Cop downregulation. *A*, Mutant-htt ST14A cells were shifted to 37°C in SDM. Cell viability was measured by the MTS assay as a function of time from temperature shift. The data represent the mean \pm SD ($n \geq 3$). *B*, Change in protein levels of endogenous Rip2, Cop, caspase-1, and caspase-3 in mutant-htt ST14A cells after shift to 37°C in SDM. Rip2 (1:1000), caspase-1 (1:500), and caspase-3 (1:1000) are direct immunoblots [β -actin (1:5000) is a loading control]. Cop was detected after immunoprecipitation (1:100) and immunoblotting using the Cop antibody (1:100) (IgG is a loading control). *C*, To demonstrate the interaction of Cop and of Rip2 with procaspase-1, mutant-htt ST14A cells were cotransfected with indicated plasmids Flag-

activated in a context involving a polyglutamine huntingtin mutant. Shifting temperature-sensitive mutant-htt ST14A cells from 33°C (permissive temperature) to 37°C (nonpermissive temperature) in SDM causes mutant-htt ST14A cells to die in a time- and polyglutamine-dependent manner and results in the activation of multiple caspases, including caspase-1 (Rigamonti et al., 2000; Wang et al., 2003). After shifting mutant-htt ST14A cells to 37°C, as described previously, we detect progressive loss of viability in mutant-htt ST14A cells (Figs. 2*A*, 3*A*) and significantly lesser degree cell death in parental ST14A cells (Fig. 2*A*). Associated with cell death, we detect early caspase-1 activation followed by caspase-3 activation in mutant-htt ST14A cells (Figs. 2*B,C*, 3*B*) but not in parent ST14A cells (Fig. 2*B,C*). To further evaluate the potential role of Rip2 and Cop in this cell death model of HD, we evaluated for alterations in levels of these proteins. Before caspase-1 activation, we detected significant depletion of endogenous Cop in mutant-htt ST14A cells (Figs. 2*B,C*, 3*B*) and only slight decrease of endogenous Cop in parental ST14A cells (Fig. 2*B,C*). Furthermore, associated with progressive caspase-1 activation, we detected a progressive increase of endogenous Rip2 protein in mutant-htt ST14A cells (Figs. 2*B,C*, 3*B*) but not in parental ST14A cells (Fig. 2*B,C*). As has been demonstrated previously in human embryonic kidney 293 cells (Druilhe et al., 2001; Lee et al., 2001), we confirm that both Cop and Rip2 bind to procaspase-1 in mutant-htt ST14A cells (Fig. 3*C*).

In these experiments, we noted in mutant-htt ST14A cell lysates, that associated with cell death, in addition of increased active caspase-3, we also detect increased procaspase-3 protein (Fig. 3*B*). This finding is similar to what we found in R6/2 mice, in which associated with disease progression, we detected increased procaspase-3 and active caspase-3; moreover, in those mice, we detected progressive increase caspase-3 mRNA (Chen et al., 2000). We therefore evaluated whether increased caspase-3 mRNA is also detected in these cells as they progress through the cell death cascade. Using real-time RT-PCR, we demonstrate a 62% significant increase of caspase-3 mRNA in mutant-htt ST14A cells as they undergo cell death compared with controls (data represent the mean \pm SD; $n = 3$; $*p < 0.05$).

We detected increased Rip2 signal by immunofluorescence staining of mutant-htt ST14A cells at the nonpermissive temperature, correlating with increased amounts of Rip2 detected by immunoblotting. Interestingly, associated with the progression of the cell death process, we detected progressive translocation of Rip2 from the cytoplasm to the nucleus (Fig. 4*A*). At 18 h after the temperature shift, we detect by immunohistochemistry and immunoblot a clear increase of cytoplasmic Rip2 (Fig. 4*A*, lane 2, *B*, cytosolic fraction) and early evidence for nuclear translocation (Fig. 4*A*, lane 3, *B*, nuclear fraction). By 48 h, in whole-cell lysate, there is progressive accumulation of Rip2 (Fig. 3*B*), with most of the additional Rip2 being found in the nucleus (Fig. 4*B*). The two images in Figure 3*B*, lanes 4 and 5, are representative of the 48 h time point, one demonstrating a greater proportion of cells with cytoplasmic Rip2 and the other with more nuclear Rip2. Evaluating the cytoplasmic and nuclear fractions, it becomes apparent

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caspase-1, HA-Rip2, and Myc-Cop. The same amount of DNA was normalized with empty vector pcDNA3. At 48 h after transfection, cells were collected and lysed by IP or directly analyzed by WB with anti-Flag, anti-HA, or anti-Myc antibody. For the IP, the lysates were incubated with a Flag antibody bound to protein A/G-agarose beads. Eluted proteins were analyzed with primary antibody (anti-HA or anti-Myc) and secondary antibody.

that most of the increase of Rip2 identified in the whole lysate becomes translocated to the nucleus (Fig. 4B). Nuclear fractionation, confirmed a time-dependent, cell death-associated enrichment of Rip2 in the nuclear fraction, whereas a more modest increase of Rip2 is identified in the cytoplasm (Fig. 4B). Rip2 nuclear translocation has not been described previously, and its precise nuclear function is a subject of future investigation. Furthermore, correlating with the depletion of Cop detected by immunoblotting, immunofluorescence staining demonstrates significant reductions of Cop protein (Figs. 3B, 4C). To confirm the specificity of the Cop antibody, HeLa cells were transfected with a FLAG-tagged Cop construct. Immunoprecipitation (anti-Flag) and immunoblotting (anti-Cop) verified the ability of the Cop antibody to recognize Cop protein (supplemental Fig. S1A, available at www.jneurosci.org as supplemental material). Caspase-1 has a nuclear localization signal and has been reported previously to translocate to the nucleus (Mao et al., 1998; Fankhauser et al., 2000). We therefore evaluated caspase-1 subcellular localization in mutant-htt ST14A cells during nonpermissive temperature shift using immunofluorescence. In this condition, we find that in parallel with Rip2 translocation, caspase-1 also progressively translocates from the cytoplasm to the nucleus (Fig. 4D).

Caspase-1 activation in R6/2 mice is associated with Rip2 upregulation and Cop downregulation; histological analysis reveals the subcellular localization of Rip2 protein

Critical insight into the potential pathophysiological role of Rip2 and Cop requires *in vivo* confirmation of dysregulation in neurological disease models associated with caspase-1 activation. We have demonstrated previously caspase-1 activation in brains of a transgenic mouse model (R6/2) and in humans with HD (Ona et al., 1999; Chen et al., 2000; Y. Zhang et al., 2003). Recently, we reported that Rip2 is an upstream modulator of apical caspase-1 activation in ischemia-induced neuronal cell death *in vivo* (W. H. Zhang et al., 2003). Consistent with our previous reported data (Ona et al., 1999; Chen et al., 2000; Y. Zhang et al., 2003), immunoblotting demonstrated caspase-1 activation in brains of symptomatic R6/2 mice (Fig. 5A). In parallel, we evaluated protein levels of Rip2 and Cop in brains of early (7 weeks) and later (11 weeks) symptomatic R6/2 mice and in age-matched wild-type littermates. We detected progressive increases of Rip2 protein in brains of R6/2 mice compared with controls. In these same brain samples, we found progressive depletion of Cop protein (Fig. 5A). To determine whether Rip2 accumulation in brains of R6/2 mice occurred within neurons, we performed double-immunofluorescence staining using a NeuN and Rip2. In brains of wild-type mice, we detected stable/low levels of Rip2 staining,

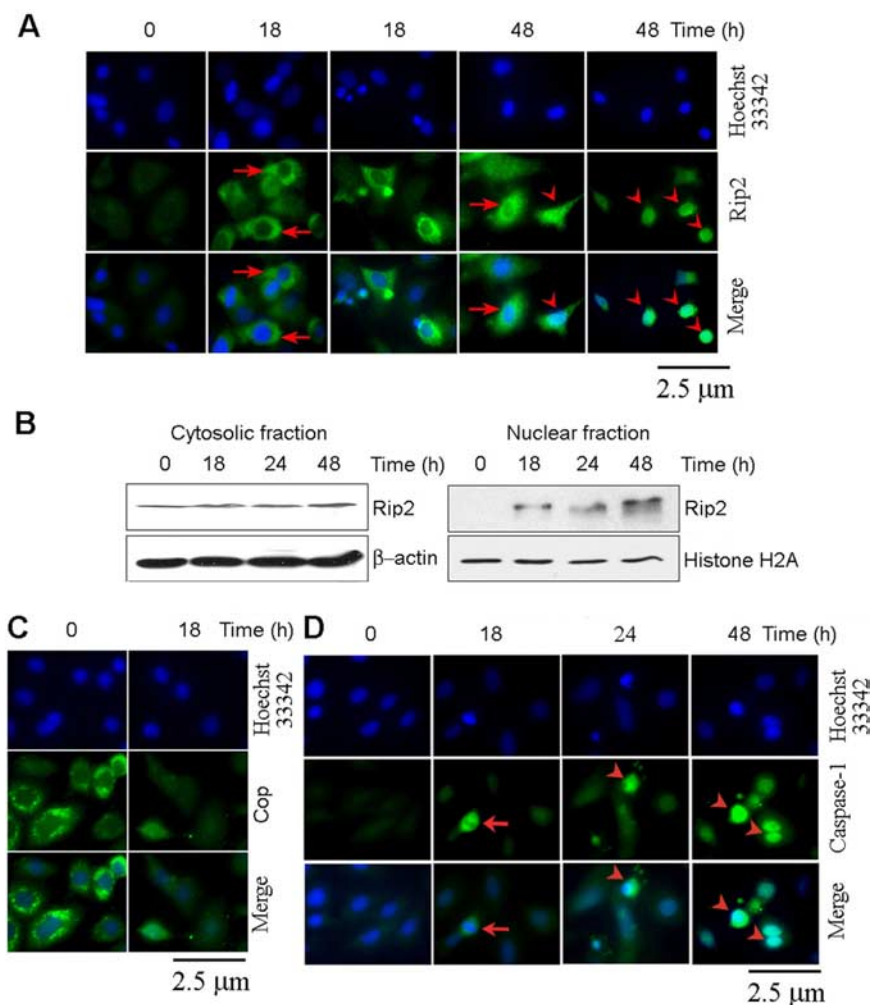


Figure 4. Translocation of Rip2 and active caspase-1 from the cytoplasm to the nucleus associated with cell death. Mutant-htt ST14A cells at 33°C with regular medium were shifted to 37°C in SDM for the indicated time points. Cells were immunostained with anti-Rip2 antibody (1:500) (A), anti-Cop antibody (1:100) (C), or anti-caspase-1 antibody (1:10) (D) and FITC-conjugated secondary antibody (for Rip2, 1:300; for Cop and caspase-1, 1:200) and then stained with Hoechst 33342 or extracted to obtain cytosolic and nuclear fraction for Western blotting analysis with anti-Rip2 antibody (B). The arrows and arrowheads indicate cytoplasmic and nuclear staining of Rip2 (A) and caspase-1 (D), respectively. β -Actin (1:5000) is a loading control for cytosolic fraction, and histone H2A (1:250) was used as a nuclear fraction loading control (B).

In contrast, in brains of R6/2 mice, we observed progressive/increased Rip2 staining, most prominently noted in cortex (Fig. 5B), but also present, to a lesser degree, in the striatum and hippocampus (data not shown). Rip2 is clearly detected in NeuN-positive cells in the brain tissue of these mice, confirming its neuronal localization. Furthermore, similar to the results *in vitro*, we detected a greater accumulation of Rip2 *in vivo* within the cytoplasm of cells in mid-disease-stage mice and within the nucleus of cells in late-disease-stage mice (Fig. 5B). Together, these observations provide evidence of dysregulation of caspase-1 regulatory pathways in a mouse model of HD. It is noteworthy that caspase-1 is the first caspase to be activated in this mouse model and that it plays an important functional role in disease progression (Ona et al., 1999; Chen et al., 2000; Y. Zhang et al., 2003).

RNAi-mediated Rip2/Cop knock-down effectively reduces/increases the vulnerability to neuronal cell death

We next evaluated whether endogenous Rip2 and Cop play a functional role in modulating cell death. We used RNAi as a tool

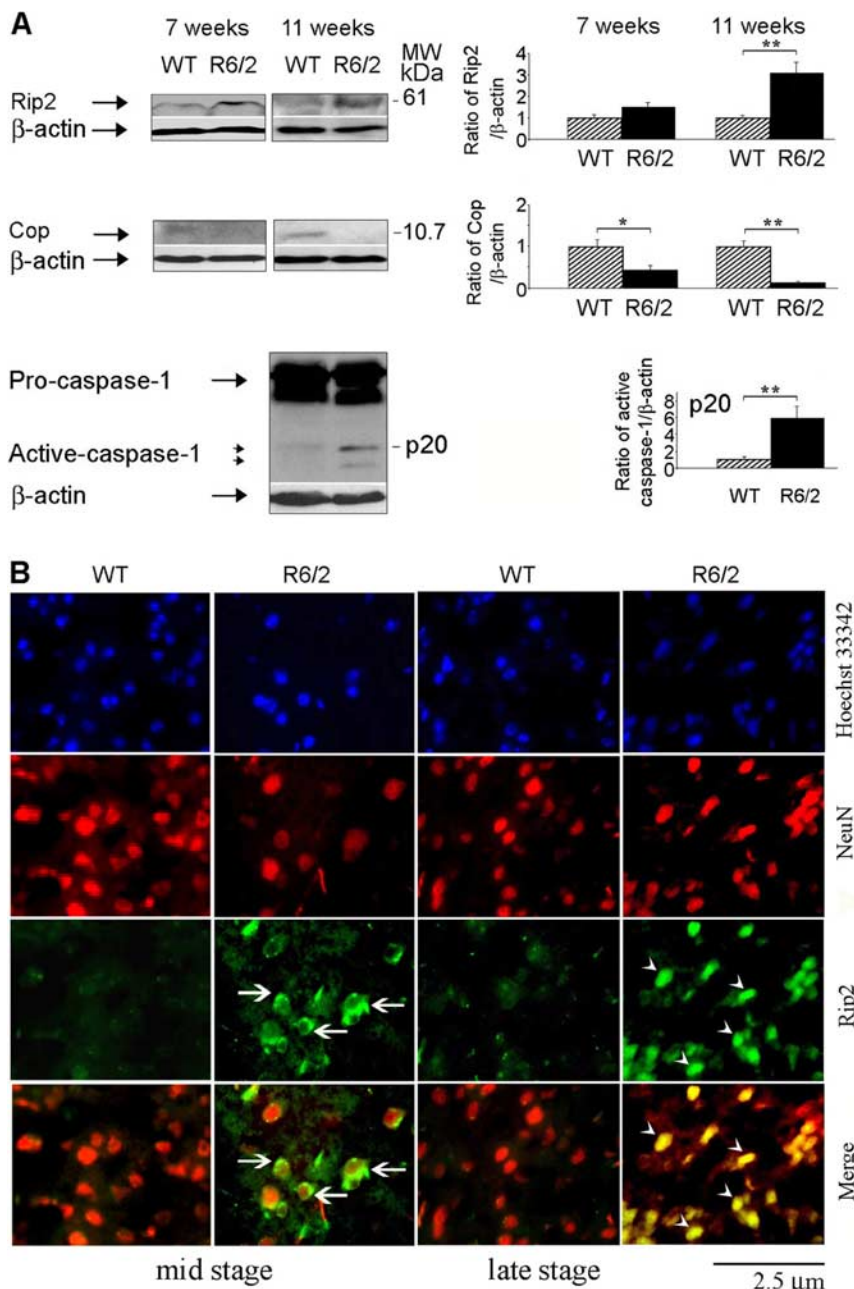


Figure 5. Cop is downregulated and Rip2 is upregulated in brain of R6/2 mice. **A**, Brains from 7-week-old (early disease stage) and 11-week-old (advanced disease stage) R6/2 and age-matched WT littermate mice were analyzed by immunoblotting with Rip2, Cop, or caspase-1 antibodies, respectively. The same blot was reprobed with a β -actin antibody. **B**, Brains of mid- and advanced-disease-stage R6/2 and wild-type control mice were stained with Hoechst 33342, anti-Rip2 antibody (1:300) (secondary FITC-conjugated antibody; green), and anti-mouse monoclonal NeuN antibody (1:1000) (secondary Texas Red-conjugated antibody; 1:500). The staining is a representative of three R6/2 mice and three wild-type mice at each stage. Images were obtained using deconvolution protocols. The arrows indicate cytoplasmic localization of upregulated Rip2. The arrowheads indicate nuclear localization of Rip2. The same blot was reprobed with a β -actin antibody. Densitometry was performed to quantify each lane. $n = 6$ per condition; * $p < 0.05$; ** $p < 0.001$.

to modulate endogenous levels of these proteins. Initially, we confirmed that Rip2 and Cop mRNA were expressed in mutant-htt ST14A cells. As demonstrated in Figure 6A, we detected Rip2 and Cop mRNA in this cell line [rat Cop and mouse Cop (data not shown) was confirmed by DNA sequencing with the product from the band demonstrating a high degree of sequence homology with human Cop; see Materials and Methods]. Four expression vectors were then designed for Rip2 knock-down. One vec-

tor was significantly effective at diminishing Rip2 mRNA and protein levels (pU6-Rip2-S4) (Fig. 6A) and was chosen for evaluation. The pU6-Rip2-S1, which was ineffective in reducing Rip2 mRNA, served as a control (Fig. 6A). Two vectors were designed for Cop knock-down. One vector was effective at reducing Cop mRNA and protein levels (pU6-Cop-S2), whereas a second vector that was ineffective (pU6-Cop-S1) served as a control (Fig. 6A) (transfection efficiency, 40–60%). Transfected cells were shifted to the nonpermissive temperature of 37°C with SDM. When cells were cotransfected with GFP and the control pU6-siRNA vector, transfected (green) cells were observed to undergo cell death, as evidenced by cell shrinkage and chromatin condensation after shift to 37°C compared with the cells at 33°C (Fig. 6B, top, arrows, C, top). Quantification of cell death at 33 and 37°C was confirmed in parallel using the MTS assay (Fig. 6C, bottom). Cells were cotransfected with GFP and with the ineffective pU6-Rip2 S1 siRNA or pU6-Cop-S1 siRNA vectors. No alterations in cell death were detected compared with controls (Fig. 6C). When cells were cotransfected with the GFP and pU6-Rip2 S4 siRNA vectors, Rip2 knock-down rendered cells significantly more resistant to cell death (Fig. 6B, middle, C). In contrast, cells cotransfected with the GFP and pU6-Cop S2 siRNA vectors resulted in Cop knock-down and demonstrated significantly increased vulnerability to cell death (Fig. 6B, bottom, arrows, C). Moreover, the death pathway repressed by elimination of Rip2 appeared to be mediated, at least in part, by inhibition of caspase-1 activation. Activated caspase-1 was detected in cells transfected with pU6-si RNA and pU6-Rip2 S1 siRNA, or pU6-Cop S1 siRNA control vectors, but was significantly inhibited in cells transfected with the pU6-Rip2 S4 siRNA vector (Fig. 6D). Conversely, elevated vulnerability to cell death by Cop knock-down was associated with enhanced caspase-1 activation (Fig. 6D). Similar enhanced activation and inhibition of caspase-3 was detected by Cop and Rip2 knock-down, respectively, suggesting that the Rip2/Cop/Caspase-1 axis plays a role in caspase-3 activation (Fig. 6D). These

data provide evidence of the importance of the balance between an activator (Rip2) and an inhibitor (Cop) of caspase-1 in this model of cell death.

Discussion

Over the past decade, an increasing level of understanding into the mechanisms of cell death pathways has resulted in the real-

ization that the process is active and is highly regulated. Significant evidence has been generated demonstrating the role for caspases as key active executioners of cell death pathways. Caspase-1 appears to be an important, early regulator of certain cell death pathways (Friedlander et al., 1997a; Schielke et al., 1998; Ona et al., 1999; Zhu et al., 1999; Chen et al., 2000; Li et al., 2000b; Rabuffetti et al., 2000; Benchoua et al., 2001; Ilzecka et al., 2001; Friedlander, 2003; Wang et al., 2003; W. H. Zhang et al., 2003; Y. Zhang et al., 2003). However, the regulation of activation of caspase-1 in neurological diseases has not been described previously. We provide evidence from a variety of complementary approaches that dysregulation of molecules controlling the activation of caspase-1 occurs in a number of neurological diseases featuring caspase-1 activation.

Rip2 and Cop are two proteins that bind to procaspase-1 via CARD–CARD interaction. The interactions of Rip2 and Cop to procaspase-1 have antagonistic effects: Rip2 causes activation of enzymatic function and serves as a scaffold about which procaspase-1 molecules assemble, whereas Cop competes with Rip2 for binding to procaspase-1 and therefore prevents such activation by sequestering procaspase-1 (Druihe et al., 2001; Lee et al., 2001). In the current study, we demonstrate that elevation of endogenous Rip2 levels and concomitant reduction of endogenous Cop levels are important modulators of pathologic caspase-1 activation. There is a balance between Cop and Rip2 in the regulation of caspase-1 activation in mutant-htt ST14A cells and in mouse models of HD. Cop and Rip2 compete with each other for binding to the CARD domain of procaspase-1. Once cells are under apoptotic stress, or mice in a disease situation, we observe reduction or depletion of Cop. Given that at the baseline, Rip2 is present, reduction of Cop levels provides the unhindered ability for Rip2 to activate caspase-1. These interactions underlie the antagonistic action of Cop and Rip2 in HD, as illustrated in supplemental Figure S 2 (available at www.jneurosci.org as supplemental material). However, an important point to note in this study is the fact that Cop has not been identified in the rat or mouse genome. The facts that the Cop antibody recognizes a protein from rat cells and mouse brain of the correct molecular weight and that we were able to PCR a band with a high degree of sequence homology with human Cop (data not shown) strongly suggest that indeed a Cop homolog exists in rats and mice. This is a subject of current investigation.

In health conditions, either in cultured cells or in wild-type mice, there is abundant expression of Cop and lower expression of Rip2 (Figs. 2–5), resulting in an inhibitory balance regarding caspase-1 activation (supplemental Fig. S 2, left, available at www.jneurosci.org as supplemental material). In HD, mutant-htt expression results in the downstream detrimental alterations in

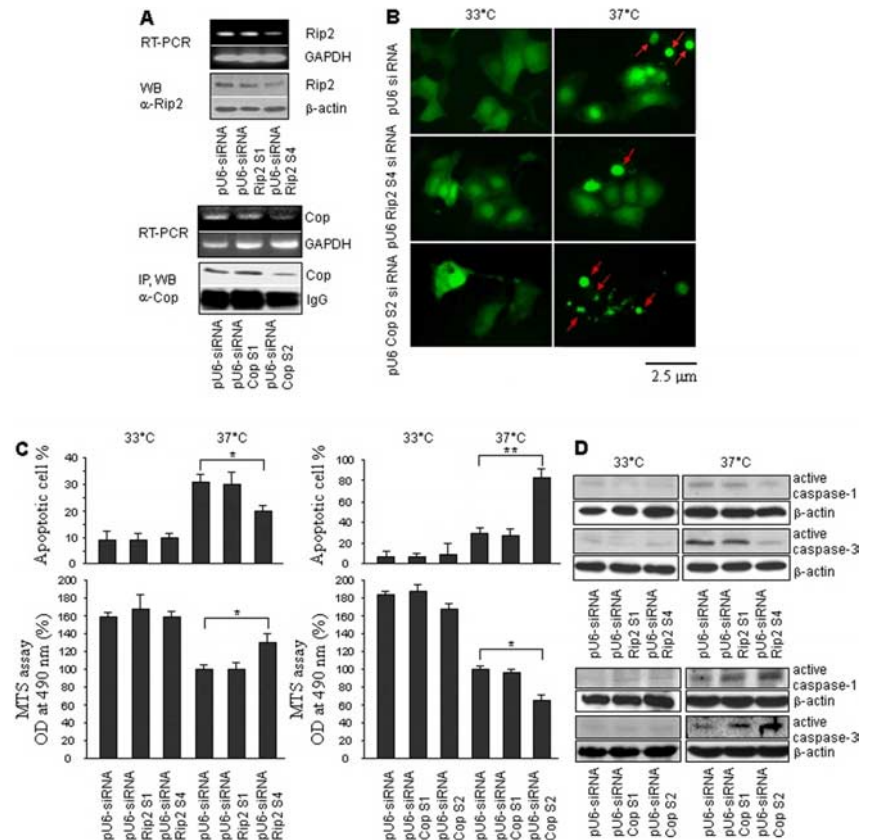


Figure 6. Cop/Rip2 knock-down results in increased/reduced vulnerability to cell death. Mutant-htt ST14A cells were transfected with pU6-siRNA, pU6-siRNA Rip2 S1, pU6-siRNA Rip2 S4, pU6-siRNA Cop S1, or pU6-siRNA Cop S2 and cotransfected with GFP by Lipofectamine. The pU6-siRNA Rip2 S4 and pU6-siRNA Cop S2 constructs are effective at knocking down their respective messages and protein, whereas pU6-siRNA Rip2 S1 and pU6-siRNA Cop S1 are not effective and served as controls. **A**, At 38–40 h (mutant-htt ST14A cells) after transfection, cells were either extracted for RT-PCR or lysed and either directly analyzed by WB with anti-Rip2 (1:1000) antibodies or immunoprecipitated and analyzed by immunoblot with anti-Cop antibodies (1:100). GAPDH is the internal control for the RT-PCR, β -actin (1:5000) for the Rip2 immunoblot, and the IgG band for the Cop immunoprecipitation immunoblot. **B**, For apoptotic induction, cells were shifted to 37°C for 14 h. Transfected green cells were scored as either alive (flat) or dead (round) (arrows) under fluorescence microscopy, and deconvoluted images were recorded. **C**, Cell viability was assessed by the MTS assay, and percentage cell death was determined by visual inspection. Results are representative from three independent experiments. Quantification of cell death was data represent the mean \pm SD. $n \geq 3$; * $p < 0.05$; ** $p < 0.001$. **D**, Transfected cells were extracted and analyzed for the activation of caspase-1 (1:500) and caspase-3 (1:750) by immunoblot.

the balance of Cop and Rip2, resulting in aberrant caspase-1 activating. The precise mechanism by which mutant-htt mediates Cop and Rip2 dysregulation is not yet understood. Li et al. (2000) demonstrated that an N-terminal fragment of mutant-htt mediates transcriptional upregulation of caspase-1. This correlates with our previously published data demonstrating increased caspase-1 transcription in R6/2 mice, resulting in increased caspase-1 protein (Chen et al., 2000). Furthermore, in brains of R6/2 mice, caspase-1 is the earliest caspase activity to be clearly detected (Y. Zhang et al., 2003). In this study, we demonstrate that expression of mutant huntingtin results in elevation of Rip2 and reduction of Cop levels (Figs. 2–4). Therefore, mutant huntingtin expression results in the initiation (elevation of caspase-1 expression) and propagation (reduced Cop and increased Rip2) of the caspase-1-mediated cell death pathway. Upregulated Rip2 therefore catalyzes oligomerization of procaspase-1 molecules mediating caspase-1 activation. Concomitantly, reduced Cop levels results in increased cellular vulnerability caused by unopposed Rip2-mediated caspase-1 activation. Active caspase-1 triggers downstream events including the activation of apoptotic executor caspase-3, therefore resulting in neuronal dysfunction and

death (supplemental Fig. S 2, right, available at www.jneurosci.org as supplemental material). Furthermore, the elevated vulnerability to cell death resulting from the active reduction of endogenous Cop levels, and the elevated resistance to cell death demonstrated by the active reduction of Rip2 levels, provide functional data as to the physiological roles of these proteins (Fig. 6). Hereafter, we suggest that the modulation of caspase-1 activity is regulated by Rip2 (activator) and by Cop (inhibitor) in HD *in vitro* and *in vivo*.

This is the first report in a neurological disease demonstrating the synchronous dysregulation of a caspase inhibitor and activator resulting in caspase activation. Understanding the triggering events resulting in neuronal cell death in neurological diseases is key to developing targeted therapies for the treatment of these devastating diseases. Furthermore, we propose that enhancement of levels of activators of cell death, such as Rip2, or reduction of levels of inhibitors of cell death, such as Cop, may alter the sensitivity threshold to cell death and therefore to neurodegeneration. Returning homeostasis to caspase-1 regulation pathways may be an important approach in the treatment of these diseases.

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