Neuron, Vol. 22, 623–633, March, 1999, Copyright ©1999 by Cell Press

Caspase-8 Is Required for Cell Death Induced by Expanded Polyglutamine Repeats

Ivelisse Sánchez,* Chi-Jie Xu,* Peter Juo,* Akira Kakizaka,† John Blenis,* and Junying Yuan*‡
*Department of Cell Biology
Harvard Medical School
Boston, Massachusetts 02115
†The Fourth Department
Osaka Biosciences Institute
Osaka 565–0874
Japan

Summary

We show here that caspase-8 is required for the death of primary rat neurons induced by an expanded polyglutamine repeat (Q79). Expression of Q79 recruited and activated caspase-8. Inhibition of caspase-8 blocked polyglutamine-induced cell death. Coexpression of Q79 with the caspase inhibitor CrmA, a dominant-negative mutant of FADD (FADD DN), Bcl-2, or Bcl-x_L, but not an N-terminally tagged Bcl-x_L, prevented the recruitment of caspase-8 and inhibited polyglutamine-induced cell death. Furthermore, Western blot analysis revealed the presence of activated caspase-8 in the insoluble fraction of affected brain regions from Huntington's disease (HD) patients but not in those from neurologically unremarkable controls, suggesting the relocation and activation of caspase-8 during the pathogenesis of HD. These results suggest an essential role of caspase-8 in HD-related neural degenerative diseases.

Introduction

Expansion of CAG trinucleotide repeats that encode polyglutamine tracts in otherwise unrelated proteins is now known to be the underlying cause of eight neurodegenerative diseases, including Huntington's disease (HD) and spinocerebellar ataxia 3 (Huntington's Disease Collaborative Research Group, 1993; Kawaguchi et al., 1994). Such expansion of polyglutamine repeats appears to constitute a toxic gain-of-function mutation that is selectively deleterious to the neurons affected in these diseases (Paulson and Fischbeck, 1996). Expression of cDNAs that encode truncated polypeptides constituting mostly the expanded polyglutamine repeats, but not of those that encode the corresponding full-length proteins, has been shown to induce cell death by apoptosis (Ikeda et al., 1996; Davies et al., 1997). These observations have led to the hypothesis that a protein fragment derived from the full-length protein associated with each of these diseases may adopt a conformation that is toxic to neurons (Trottier et al., 1995) and thereby result in neuronal degeneration (DiFiglia et al., 1997). Such truncated proteins have been shown to form aggregates or inclusions, as has been observed in cultured cells overexpressing a truncated ataxin-3 (the product of the *SCA3* gene) with an expanded polyglutamine tract (Ikeda et al., 1996), in transgenic mice expressing a truncated huntingtin (the product of the *HD* gene) (Bates and Davies, 1997), in *Drosophila* expressing a truncated ataxin-3 (Warrick et al., 1998), and in postmortem brains of individuals with SCA3 or HD (DiFiglia et al., 1997; Paulson et al., 1997). Although it has been proposed that such abnormal inclusions participate in inappropriate protein–protein interactions that lead to cell death, the nature of such interactions and the mechanism by which cell death is induced remain unclear.

Mammalian apoptosis is regulated by evolutionarily conserved pathways whose critical components are homologs of those that mediate programmed cell death in the nematode *Caenorhabditis elegans* (Cryns and Yuan, 1998). The members of the mammalian Bcl-2 family of proteins are homologs of CED-9, which suppresses cell death in *C. elegans*. Expression of Bcl-2 or Bcl-x_L, two major anti-apoptotic members of the family, inhibits apoptosis induced by many different stimuli. Mammalian caspases are homologs of the product of the *C. elegans* cell death gene *ced-3* and play important roles in regulating apoptosis (Cryns and Yuan, 1998). A cytokine response–modifier gene (*crmA*) of cowpox virus encodes a serpin that is a specific inhibitor of two mammalian caspases, caspase-1 and caspase-8 (Zhou et al., 1997). Two polyglutamine-containing proteins, huntingtin and the DRPLA, are indeed cleaved by caspases during apoptosis, observations that have led to the hypothesis that caspases may be responsible for cleavage of the mutant proteins containing expanded polyglutamine tracts and the generation of toxic protein fragments in vivo (Goldberg et al., 1996; Miyashita et al., 1997; Wellington et al., 1998). However, a critical role for caspases in the generation of such toxic protein fragments, or a possible role for these enzymes after the initial cleavage event, remains to be demonstrated.

The Fas pathway of apoptosis plays an important role in the immune system, contributing to cytotoxic T lymphocyte–mediated cytotoxicity and downregulation of immune responses. Activation of the Fas pathway by Fas ligand or agonistic antibodies induces oligomerization of the Fas receptor, which results in exposure of its intracellular protein–protein interaction domain, known as the death domain, and in the formation of death-inducing signaling complex (DISC), which transduces the Fas death signal (Kischkel et al., 1995). The death domain of the Fas receptor thus interacts with and recruits Fas/APO-1–associated death domain protein (FADD), an adapter protein that contains a death domain in its C-terminal half and another protein–protein interaction domain, termed the death effector domain, in its N-terminal half (Chinnaiyan et al., 1995). FADD, in turn, recruits caspase-8, which contains two death effector domains in its N-terminal region and a caspase domain in its C-terminal region. Expression of a truncated FADD (FADD DN) containing only the C-terminal death domain

‡To whom correspondence should be addressed (e-mail: jyuan@hms.harvard.edu).

has been shown to inhibit Fas-induced cell death (Chinnaiyan et al., 1996).

Given the importance of caspases in mammalian apoptosis, we have investigated the role of these enzymes in polyglutamine-induced cell death. We now show that caspase-8 is recruited and activated by expanded polyglutamine repeats in cultured cells and primary neurons and that inhibition of caspase-8 recruitment and activation blocks the cell death induced by these polyglutamine repeats. Furthermore, we provide preliminary evidence for the contribution of caspase-8 to the pathogenesis of HD in vivo.

Results

Inhibition of Polyglutamine Repeat–Induced Cell Death by Bcl-2, CrmA, and FADD DN

To investigate the mechanism by which polyglutamine inclusions induce cell death, we developed a transient transfection system whereby primary neurons were engineered to express green fluorescent protein (GFP) and one of four different versions of ataxin-3. We first established cultures of primary cortical, striatal, and cerebellar neurons from embryonic day 17 (E17) rat embryos in serum-free medium as described by Brewer (1995). We then transfected the cultured neurons with a GFP vector and expression constructs encoding truncated ataxin-3 that contained either 35 (Q35) or 79 (Q79) glutamine residues (Igarashi et al., 1998). Expression of Q79, but not of Q35, induced cell death in the cultured striatal and cerebellar neurons and, to a lesser extent, in the cortical neurons (Figures 1A and 1C, bottom panel). Neurons expressing Q79 show cell body shrinkage and chromosomal DNA condensation (Figure 1B). Given that both Q79 and Q35 expression constructs encode only 21 amino acid residues (nine of which are the hemagglutinin [HA] tag), in addition to the polyglutamine tract, its product is essentially a polyglutamine peptide, and our results should be relevant to HD and other polyglutamine expansion diseases, including SCA3.

Because anti-apoptotic members of the Bcl-2 family are potent inhibitors of apoptosis in a variety of systems, we examined whether expression of Bcl-2 or Bcl-x_L inhibits cell death induced by Q79. Cerebellar neurons were transfected with the GFP vector as well as with expression constructs encoding Q79, Q79, and Bcl-2; Q79 and Bcl-x_L; or Q79 and Bcl-x_L tagged at its N terminus with the Flag-epitope (N-Bcl-x_L). Forty-eight hours after transfection, the percentage of cell death was determined by counting the numbers of dead (those rounded or shrunken without processes) or live (those with extensive processes) GFP-positive neurons. In some experiments, uptakes of propidium iodide and trypan blue were used to confirm the counting of dead cells, and staining with Hoechst dye was used to monitor apoptotic nuclear morphology. Expression of Bcl-2 or Bcl-x_L inhibited neuronal cell death induced by Q79 (Figures 1B and 1C). In contrast, N-Bcl-x_L, which markedly inhibits induction of apoptosis by the proapoptotic Bcl-2 family member Bid (Li et al., 1998), had no effect on cell death triggered by Q79 (Figures 1C and 1E). These results suggest that a free N terminus is required for Bcl-x_L to inhibit polyglutamine repeat–induced cell death.

To determine whether caspases play a role in polyglutamine-induced cell death, we investigated the effect of cotransfecting primary cultured neurons with expression constructs encoding Q79 and CrmA, a cowpox virus serpin that is a specific inhibitor of caspases. Expression of CrmA inhibited Q79-induced cell death in all three types of neurons (Figures 1C and 1D), suggesting that caspases contribute to the death program activated by polyglutamine repeats. The role of caspase activity in Q79-induced neuronal death was also examined by transfecting neurons with the Q79 vector in the absence or presence of 100 μM zVAD.fmk, a synthetic peptide inhibitor of caspases. The survival of the transfected neurons was markedly increased by the presence of zVAD.fmk (Figure 1C, top panel), again suggesting that caspase activity is required for polyglutamine-induced cell death.

Fas-induced apoptosis is one of the best understood pathways of programmed cell death. We therefore investigated whether the Fas apoptotic pathway plays a role in neuronal cell death induced by polyglutamine repeats. We examined the effect of FADD DN, a dominant-negative inhibitor of Fas-induced apoptosis, on polyglutamine repeat–induced neuronal death by cotransfecting cultures of striatal, cerebellar, or cortical neurons with vectors encoding Q79 and FADD DN. FADD DN markedly inhibited Q79-induced cell death, as assessed 48 hr after transfection, suggesting that mediators of the Fas pathway may contribute to this process (Figures 1C and 1D).

Thus, three different types of apoptosis inhibitors, Bcl-2, CrmA, and FADD DN, all inhibited polyglutamine repeat–induced neuronal cell death. Coexpression of Bcl-2, CrmA, or FADD DN not only prevented the cell death induced by Q79 but also preserved the extensive neurites of the cultured neurons (Figures 1B and 1D). In contrast, although Bcl-2 and CrmA each inhibit neuronal cell death induced by trophic factor deprivation, they do not prevent the loss of neurites associated with trophic factor removal (Allsopp et al., 1993; Gagliardini et al., 1994; see also Figure 1B). Our data thus indicate that Bcl-2, CrmA, and FADD DN may preserve neuronal function in addition to preventing cell death in neurons expressing polyglutamine repeats.

Although polyglutamine repeat–induced cell death in primary neuronal cultures most closely resembles the neuronal degeneration apparent in individuals with polyglutamine expansion diseases, it is difficult to obtain large numbers of transfected neurons for biochemical analysis. Given that expression of polyglutamine repeats has been shown to induce cell death in established cell lines, including COS-7 and 293T cells (Paulson et al., 1997; Igarashi et al., 1998), we analyzed a similar transfection system with cell lines. We cotransfected HeLa cells with constructs encoding either 35 or 79 polyglutamine repeats of ataxin-3 and a GFP marker. Forty-eight hours after transfection, the percentage of cell death was determined by counting the numbers of round (dead) versus flat (live) GFP-positive cells. Similar to the results obtained with the primary neurons, Q79, but not Q35, induced apoptosis in HeLa cells (Figure 1E, left panel). Furthermore, coexpression of CrmA, FADD DN, Bcl-2, or Bcl-x_L, but not N-Bcl-x_L, inhibited Q79-induced HeLa cell death (Figure 1E, middle panel). Although as

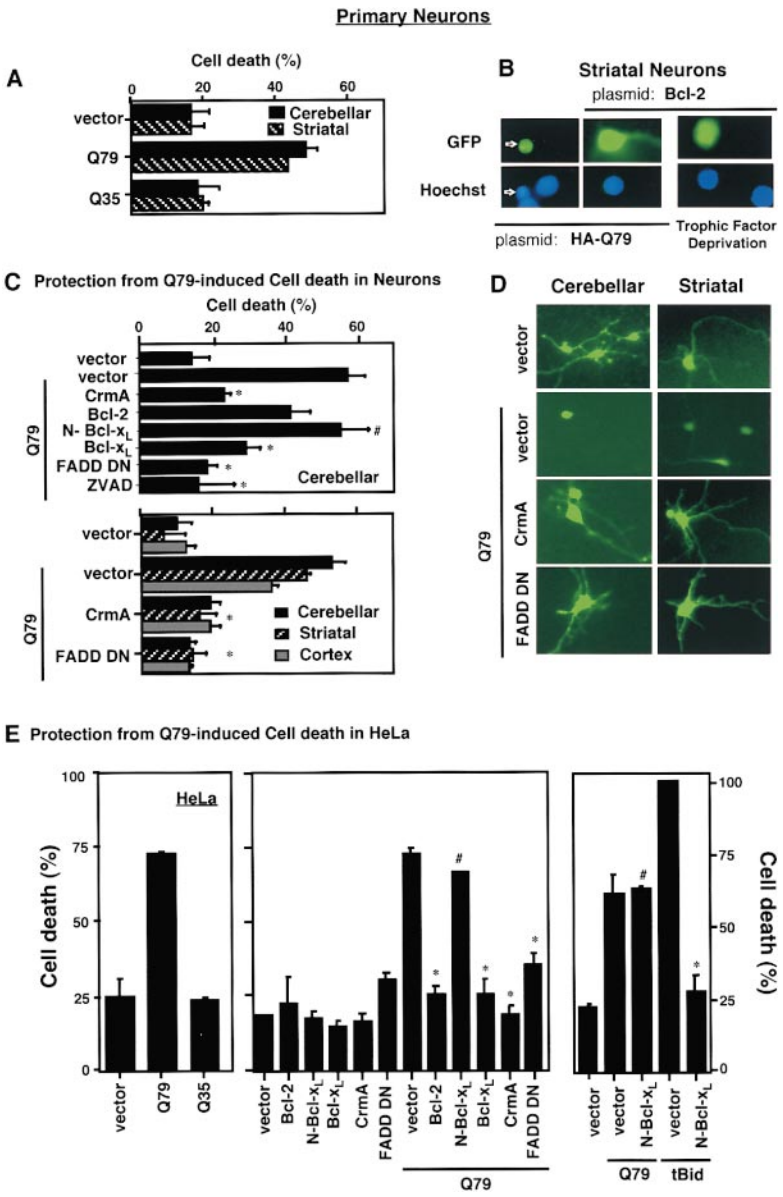

Figure 1. Induction of Cell Death by Q79 and Its Inhibition by CrmA, FADD DN, Bcl-2, and Bcl-x_L in Primary Neurons and HeLa Cells

(A) Induction of cell death in primary neurons by expression of Q79 but not by that of Q35. Cultures of cerebellar and striatal neurons from E17 rat embryos were transfected with both a vector encoding a GFP marker and expression constructs encoding the indicated ataxin-3 proteins. The percentage of cell death was determined 48 hr after transfection (left panel); data are means from a representative experiment done in duplicates.

(B) Nuclear morphology of striatal neurons expressing HA–Q79 and comparison of Bcl-2 protection against Q79 and serum withdrawal–induced cell death. Striatal neurons were transfected with HA–Q79/GFP or HA–Q79/Bcl-2/GFP in the presence of serum or Bcl-2/GFP in the absence of serum and stained with Hoechst dye. The neuron expressing HA–Q79 showed cell body shrinkage and nuclear condensation (arrow) The neuron expressing both HA–Q79 and Bcl-2 showed normal cell body and nuclear size, as well as neurites. The neuron expressing Bcl-2 in the absence of serum showed normal cell body and nuclear size but without neurites.

(C) Prevention of Q79-induced cell death in primary neurons by coexpression of CrmA, FADD DN, Bcl-2, or Bcl-x_L but not N-Bcl-x_L. Primary cerebellar neurons (upper panel), or cerebellar, striatal, or cortical neurons (lower panel), were transfected with vectors encoding GFP and Q79 as well as with expression constructs encoding either CrmA, FADD DN, Bcl-2, Bcl-x_L, or N-Bcl-x_L in the presence or absence of zVAD.fmk. Cell death was quantitated 48 hr after transfection. Data are means from a representative experiment done in duplicate. The experiments were repeated at least twice with similar results.

(D) Fluorescence photomicrographs of primary cerebellar (left panels) or striatal (right panels) neurons 48 hr after transfection with both the GFP vector and the indicated combinations of expression constructs encoding Q79, CrmA, and FADD DN.

(E) Induction of apoptosis in HeLa cells by Q79 and its inhibition by CrmA, FADD DN, Bcl-2, and Bcl-x_L. HeLa cells were transfected with the GFP vector and either with expression constructs encoding Q79 or Q35 (left panel) or with the Q79 vector and constructs encoding CrmA, FADD DN, Bcl-2, Bcl-x_L, or N-Bcl-x_L as indicated (middle panel). The right panel is a comparison of the effect of N-terminal-tagged Bcl-x_L in cell death induced by Q79 and tBid. The percentage of cell death was determined 48 hr after transfection. Data are mean ± SD from one representative experiment out of four similar experiments.

previously shown (Li et al., 1998), N-terminal-tagged Bcl-x_L inhibited cell death induced by truncated Bid very efficiently, it did not prevent Q79-induced cell death (Figure 1E, right panel). These data suggest that the biochemistry of polyglutamine repeat–induced cell death in HeLa cells is similar to that in primary cultured neurons, thus validating the use of HeLa cells as a model system for further biochemical characterization of this process.

Requirement of Caspase-8 Activity for Polyglutamine Repeat–Induced Cell Death

The inhibition of polyglutamine repeat–induced cell death by CrmA and zVAD suggested a role for caspases in this process. Because in vitro kinetic analyses revealed that CrmA inhibits caspase-1 and caspase-8 most potently (Zhou et al., 1997), with its effects on caspases-3, −6, and −7 requiring concentrations several orders of magnitude greater than those effective for caspases-1 and −8 (rendering caspase-3, −6, and −7 physiologically irrelevant targets for CrmA), our data implicated caspase-1 or caspase-8 in polyglutamine repeat–induced cell death.

To investigate further the role of caspase-1 in cell death induced by polyglutamine inclusions, we transfected mouse embryonic fibroblasts that lack caspase-1 (*caspase-1*$^{-/-}$) with the Q79 vector. The extent of Q79-induced cell death in *caspase-1*$^{-/-}$ cells did not differ

significantly from that observed in the corresponding wild-type (*caspase-1*$^{+/+}$) cells (Figure 2A, left panel), indicating that caspase-1 is not required for polyglutamine-induced cell death. Similarly, the sensitivity of embryonic fibroblasts that lack caspase-2 or caspase-11 to Q79-induced cell death was similar to that of wild-type cells (Figure 2A, left panel), suggesting that these two caspases also do not play a critical role in this process. Furthermore, MCF7 cells, which do not express caspase-3 (Janicke et al., 1998), are equally susceptible to Q79-induced cell death (Figure 2A, middle panel), suggesting that caspase-3 is also not essential for polyglutamine repeat–induced death.

A mutant Jurkat cell line specifically lacking *caspase-8* was generated by ethylmethane sulfonate–induced mutagenesis (Juo et al., 1998). We transfected the parental and *caspase-8* mutant Jurkat cells with the Q79 construct and determined the percentage of cell death on the basis of propidium iodide uptake. Whereas the wild-type Jurkat cells were sensitive to Q79-induced cell death, the *caspase-8* mutant cells were resistant (Figure 2A, right panel), suggesting caspase-8 is critical for polyglutamine repeat–induced cell death. The role of caspase-8 in polyglutamine-induced cell death was further investigated in Jurkat cells transfected with a construct encoding a dominant-negative mutant of caspase-8 (C360S), in which the cysteine residue from the active site is replaced by serine, which inhibits apoptosis induced by Fas or tumor necrosis factor (Vincenz and Dixit, 1997). Expression of caspase-8 C360S inhibited Q79-induced cell death in the wild-type Jurkat cells but did not further reduce the cell death in *caspase-8* mutant Jurkat cells (Figure 2A, right panel). These results indicate that inhibition of caspase-8 activity, either by a loss-of-function mutation or by expression of a dominant-negative mutant, results in inhibition of polyglutamine repeat–induced cell death. In addition, our data suggest that the dominant-negative caspase-8 mutant specifically inhibits caspase-8, since caspase-8 C360S had no effect in *caspase-8* mutant cells.

To determine whether polyglutamine-induced death of primary neurons also requires caspase-8 activity, we cotransfected cerebellar, striatal, and cortical neurons with the vectors encoding Q79 and the dominant-negative caspase-8 C360S mutant. Expression of caspase-8 C360S inhibited Q79-induced death in all three types of neurons (Figure 2B). Together, these results indicate that caspase-8 is required for polyglutamine repeat–induced neuronal cell death.

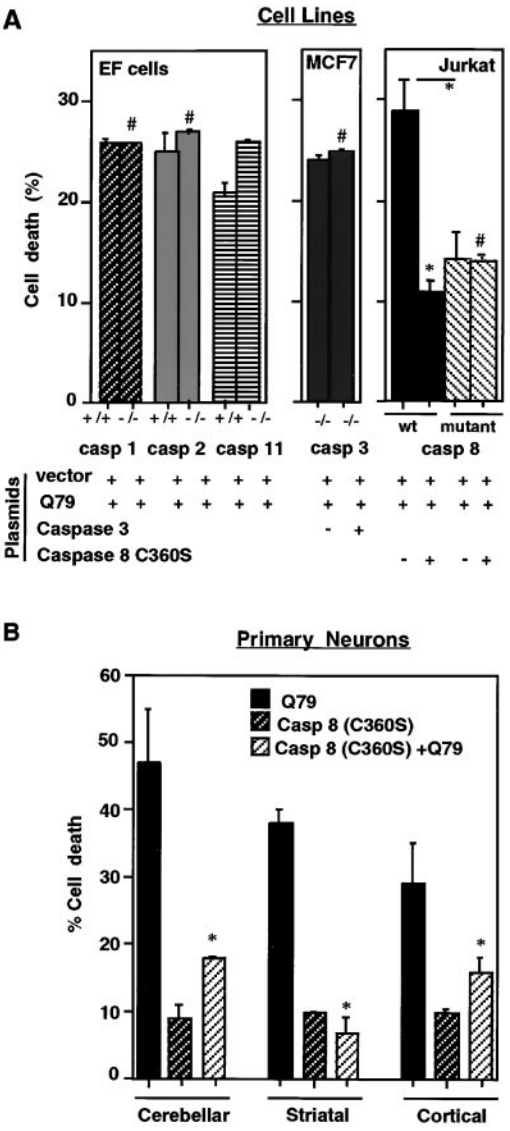

Figure 2. Requirement of Caspase-8 for Polyglutamine Repeat–Induced Cell Death

(A) Embryonic fibroblasts (EF cells) that lack caspases-1, −2, or −11 or wild-type control cells (left panel), MCF7 cells (which lack caspase-3) (middle panel), and Jurkat cells (right panel) were transfected with the vectors encoding GFP and Q79 (in the absence or presence of a vector encoding the caspase-8 mutant C360S in the case of Jurkat cells). The percentage of cell death was determined 48 hr after transfection. Data are means from a representative experiment; $p > 0.05$ (pound sign), $p < 0.05$ (asterisk) versus corresponding control cells (Student's t test). Cells lacking procaspase-8 or expressing caspase-8 C360S but not cells mutant for caspase-1, -2, -3, or -11 were resistant to expression of Q79.

(B) Rat (E17) primary neurons were transfected with vectors encoding GFP and either Q79 or caspase-8 C360S (or both) as indicated, and 48 hr after transfection, the percentage of cell death was determined. Data are mean ± SD from three experiments; $p < 0.05$ (asterisk) versus Q79 alone (Student's t test). The dominant-negative mutant of caspase-8 C360S was able to block Q79-induced cell death in different populations of neurons.

Caspase-8 Recruitment and Activation by Polyglutamine Repeats

We next investigated the mechanism of caspase-8 activation by polyglutamine repeats. Given that oligomerization of caspase-8 is sufficient to induce its activation (Yang et al., 1998), it was possible that polyglutamine repeats provide a surface for protein–protein interaction and recruit caspase-8 directly or indirectly. To determine whether Q79 recruits caspase-8, we examined the effect of Q79 expression on the intracellular localization of a GFP-tagged version of caspase-8 C360S in 293T cells. In cells transfected only with a low concentration of the GFP–caspase-8 C360S vector (0.3 μg per well in a 6-well dish), the encoded protein was distributed evenly throughout the cytoplasm (Figure 3A). In cells also transfected

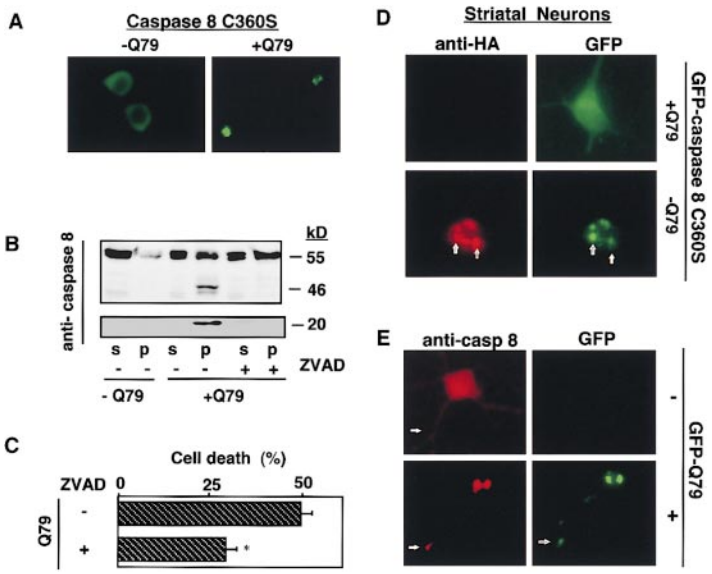

Figure 3. Recruitment and Activation of Caspase-8 by Polyglutamine Repeats

(A) 293T cells were transfected with a vector encoding GFP-tagged caspase-8 C360S in the absence (right panel) or presence (left panel) of a vector encoding HA-tagged Q79. The subcellular distribution of the caspase-8 mutant was subsequently examined by GFP immunofluorescence analysis.

(B) HeLa cells were transfected (or not) with a Q79 vector in the absence or presence of 100 μM zVAD. After continued incubation of cells in the absence or presence of zVAD for 48 hr, the distribution and activation of caspase-8 were examined by immunoblot analysis of soluble (s) and insoluble (p) subcellular fractions with a mAb to caspase-8 (α-casp-8). The positions of 55, 46, and 20 kDa immunoreactive bands are indicated.

(C) HeLa cells were transfected with the Q79 expression vector and incubated in the absence or presence of zVAD as in (B), after which the percentage of cell death was determined. Data are mean ± SD of two experiments.

(D) Striatal neurons were transfected with a vector encoding GFP-tagged caspase-8 C360S in the absence or presence of HA-tagged Q79 in the presence of 100 μM zVAD. The subcellular localization of polyglutamine inclusions and the caspase-8 mutant were subsequently detected by immunostaining with a mAb to HA and GFP fluorescence, respectively. Caspase-8 C360S colocalized with polyglutamine inclusions.

(E) Striatal neurons were transfected with a control vector or a vector encoding a C-terminal GFP-tagged Q79 in the presence of 100 μM ZVAD, and the subcellular distributions of endogenous caspase-8 and Q79 were examined by immunostaining with a mAb to caspase-8 and GFP fluorescence, respectively. Endogenous caspase-8 was recruited to the Q79 inclusions present in the cell body and neurite (arrow).

with a construct encoding Q79 tagged with the hemagglutinin-epitope, GFP-caspase-8 C360S formed aggregates that closely resembled Q79 inclusions (Figure 3A), suggesting that caspase-8 is recruited by polyglutamine repeats.

To determine whether endogenous caspase-8 is recruited by polyglutamine repeats, we examined the distribution of the endogenous protein in HeLa cells expressing Q79. Since overexpression of Q79 results in formation of insoluble inclusions, we reasoned that if caspase-8 is recruited by such inclusions, it would likely exhibit a shift in subcellular distribution from the soluble fraction to the insoluble fraction. We thus subjected the soluble and insoluble fractions of control cells and of cells expressing Q79 to Western blot analysis with a monoclonal antibody to caspase-8. In control cells, caspase-8 was present mostly in the soluble fraction, whereas in cells expressing Q79, it was recruited to the insoluble fraction (Figure 3B). Caspase-8 was also activated in cells expressing Q79, but not in control cells, as indicated by the appearance of 46 and 20 kDa anti-caspase-8 immunoreactive bands that are the reported molecular weights of activated caspase-8 subunits (Martin et al., 1998), in addition to the 55 kDa proenzyme. Activation of caspase-8, but not its recruitment into the insoluble fraction in the Q79-expressing cells, was prevented by performing transfection and subsequent incubation in the presence of zVAD (Figure 3B). The caspase inhibitor also suppressed Q79-induced death in HeLa cells (Figure 3C). Thus, caspase-8 is activated in cells expressing Q79, and inhibition of caspase-8 activation prevented polyglutamine repeat–induced cell death.

To investigate whether caspase-8 colocalizes with polyglutamine inclusions in neurons, we transfected cultured striatal neurons with vectors encoding GFP-tagged caspase-8 C360S and HA-tagged Q79. We chose to use caspase-8 C360S here, because it only differs from wild-type caspase-8 by one amino acid change at the active site that renders it inactive and unable to induce cell death, unlike the wild-type caspase-8. GFP fluorescence revealed that the tagged caspase-8 mutant colocalized with the polyglutamine inclusions (Figure 3D), indicating that GFP–caspase-8 C360S was recruited by these inclusions.

The possible recruitment of endogenous caspase-8 by polyglutamine inclusions in neurons was examined by immunofluorescence analysis of primary striatal neurons with the mAb to caspase-8. In control striatal neurons, caspase-8 immunoreactivity was evenly distributed throughout the cytoplasm; however, in neurons expressing GFP-tagged Q79, caspase-8 appeared highly aggregated at the sites of polyglutamine inclusions (Figure 3E). The polyglutamine inclusions were detected around or within nuclei as well as in neuronal processes (Figure 3E), similar to the pattern observed in affected neurons in brain tissue from individuals with HD (DiFiglia et al., 1997). These observations indicate that endogenous caspase-8 is recruited by polyglutamine inclusions in neurons and that such recruitment may result in the activation of caspase-8.

Inhibition of Polyglutamine-Mediated Caspase-8 Recruitment by Bcl-2, CrmA, or FADD DN

The mechanisms by which Bcl-2, CrmA, and FADD DN inhibit polyglutamine repeat–induced cell death were investigated by first examining the possibility that these

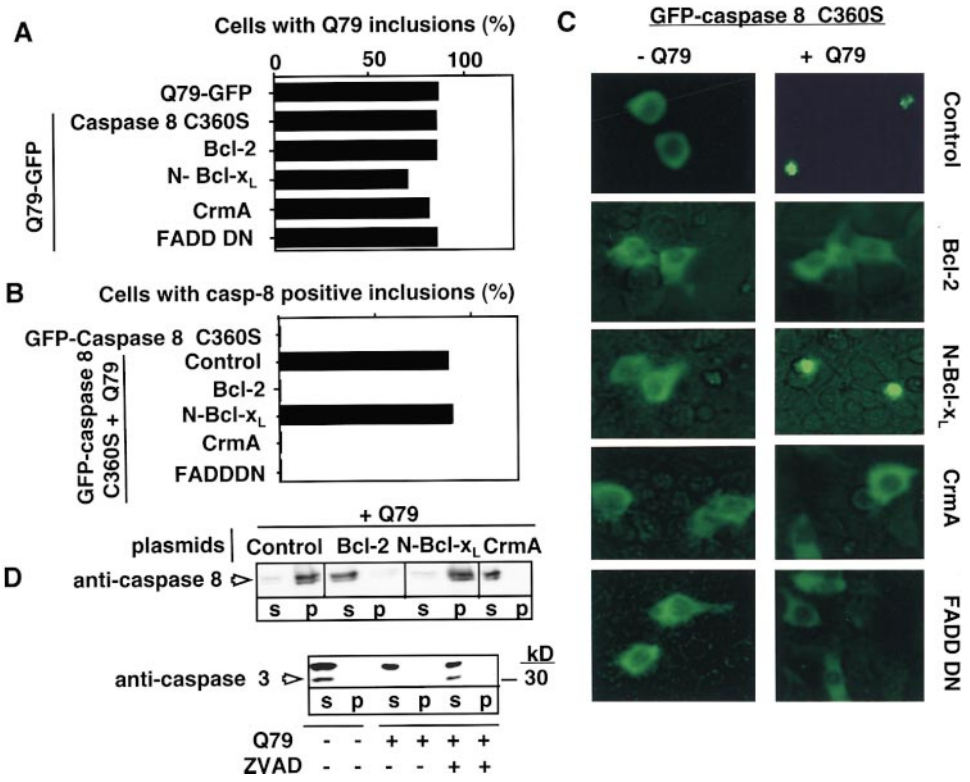

Figure 4. Inhibition of Polyglutamine Repeat–Mediated Recruitment of Caspase-8 by Bcl-2, CrmA, and FADD DN

(A) 293T cells were transfected with a vector encoding GFP-tagged Q79 in the absence or presence of vectors encoding caspase-8 C360S, Bcl-2, N-Bcl-x_L, CrmA, or FADD DN. Forty-eight hours after transfection, the percentage of cells containing polyglutamine inclusions was determined by GFP fluorescence. Data are means from a representative experiment. The formation of polyglutamine inclusions was unaffected by cotransfection with Bcl-2, CrmA, or FADD DN.

(B and C) 293T cells were transfected with a vector encoding GFP-tagged caspase-8 C360S in the absence or presence of vectors encoding HA-tagged Q79, Bcl-2, N-Bcl-x_L, CrmA, or FADD DN, as indicated. The number of cells containing GFP-positive inclusions was examined by fluorescence microscopy (C) and quantitated (B). Caspase-8 C360S was recruited to Q79 inclusions, while it remained cytoplasmic when coexpressed with Bcl-2, CrmA, or FADD DN but not N-tagged Bcl-x_L (N-Bcl-x_L).

(D) HeLa cells were transfected with a Q79 vector in the absence or presence of vectors encoding Bcl-2, N-Bcl-x_L, or CrmA. The soluble (s) and insoluble (p) fractions of cell lysates were subsequently subjected to immunoblot analysis with a mAb to caspase-8 (upper panel, arrow) or with antibodies to caspase-3 (lower panel, arrow). In control HeLa cells as well as HeLa cells coexpressing Q79 and Bcl-2 or CrmA but not N-Bcl-x_L, endogenous caspase-8 is present in the soluble fraction, whereas in HeLa cells expressing Q79 alone, caspase-8 is present in the insoluble fraction. Expression of Q79 also induced caspase-3 activation, as shown by the disappearance of the proform (arrow); however, caspase-3 was not recruited into insoluble fractions when its activation was inhibited by zVAD. The top band in the caspase-3 Western blot is an unrelated cross-immunoreactive protein shown here as an internal loading control.

anti-apoptotic proteins prevent the formation of polyglutamine inclusions. We thus transfected 293T cells with a vector encoding GFP-tagged Q79 in the absence or presence of vectors encoding the test proteins and subsequently determined the percentage of cells containing inclusions. We chose 293T cells for these experiments, because they express the SV40 large T antigen and contain only a low concentration of caspase-8, and the resulting delay in Q79-induced cell death allows the frequency of inclusion formation to be assessed before cell death occurs. The percentage of cells containing inclusions was not affected by Bcl-2, CrmA, or FADD DN; similarly, caspase-8 C360S and N-Bcl-x_L had no effect on this parameter (Figure 4A). Thus, CrmA, Bcl-2, and FADD DN must inhibit a step in polyglutamine-induced cell death other than inclusion formation.

To examine whether Bcl-2, CrmA, or FADD DN inhibit the recruitment of caspase-8 by polyglutamine inclusions, we cotransfected 293T cells with vectors encoding GFP-tagged caspase-8 C360S, HA-tagged Q79, and each of the test proteins. Subsequent quantitation of inclusions containing the GFP-tagged caspase-8 mutant revealed that Bcl-2, CrmA, and FADD DN, but not N-Bcl-x_L, prevented the formation of caspase-8 aggregates in the presence of Q79 (Figures 4B and 4C). These results suggest that the recruitment of caspase-8 is critical for the induction of cell death by polyglutamine repeats.

To examine whether prevention of recruitment of endogenous caspase-8 underlies the ability of Bcl-2 and CrmA to inhibit polyglutamine repeat–induced cell death, we determined the distribution of endogenous caspase-8 in HeLa cells expressing Q79 in the absence or presence of an inhibitor. Western blot analysis with the mAb to caspase-8 revealed that caspase-8 was present mostly in the soluble fractions of control cells and of cells coexpressing Q79 with Bcl-2 or CrmA, whereas it was localized predominantly in the insoluble fractions of cells expressing Q79 alone and of those coexpressing Q79 and N-Bcl-x_L (Figure 4D). In contrast,

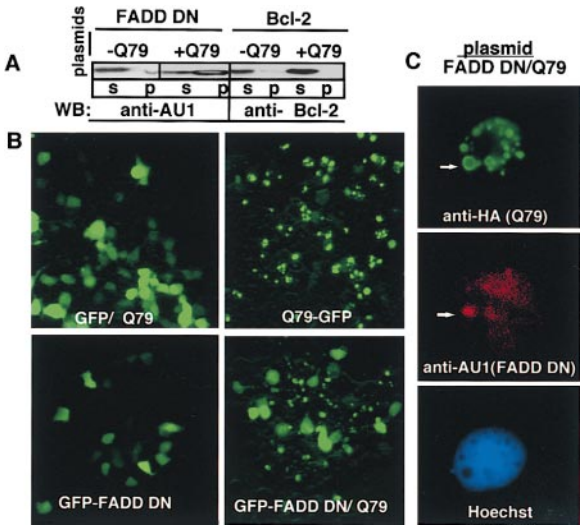

Figure 5. Recruitment of FADD DN, but Not of Bcl-2, by Polyglutamine Repeats

(A) HeLa cells were transfected with vectors encoding either AU1-tagged FADD DN or Bcl-2 in the absence or presence of a Q79 vector, as indicated. Soluble (s) and insoluble (p) fractions of cell lysates were subsequently subjected to immunoblot analysis with antibodies to AU1 or Bcl-2, as indicated.

(B) HeLa cells expressing GFP and HA-Q79 (GFP/Q79), GFP-tagged Q79 (Q79–GFP), GFP-tagged FADD DN (GFP–FADD DN), or GFP–FADD DN and Q79 (GFP–FADD DN/Q79) were visualized by fluorescence microscopy 24 hr after the transfection.

(C) HeLa cells expressing HA-tagged Q79 and AU1-tagged FADD DN (24 hr after the transfection) were immunostained with antibodies to HA and AU1 and examined by confocal microscopy; nuclear morphology was also revealed by staining with Hoechst dye.

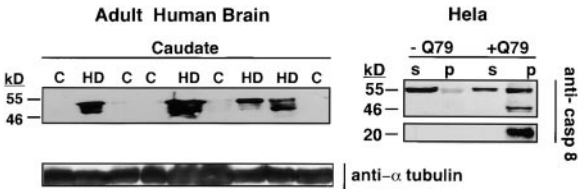

Figure 6. Recruitment and Activation of Caspase-8 in Caudate Tissue from Individuals with HD

The insoluble fractions of caudate tissue from four different HD patients and five age-matched neurologically unremarkable individuals (control) were subjected to immunoblot analysis with a mAb to caspase-8 (upper left panel). Anti-caspase-8 immunoreactive bands of ~46 kDa were detected in HD samples that were similar in molecular weight to the activated caspase-8 in the insoluble fraction of HeLa cells expressing Q79 (right panel). Antibodies to tubulin were used to confirm that similar amounts of protein were loaded in each lane (lower left panel).

caspase-3 was largely restricted to the soluble fractions of both control and Q79-expressing cells (Figure 4D). These results indicate that expression of Q79 recruits caspase-8 to the insoluble fraction and that inhibition of Q79-induced cell death by Bcl-2, CrmA, or FADD DN may be related to the prevention of such caspase-8 recruitment.

Recruitment of FADD DN by Polyglutamine Repeats

Inhibition of polyglutamine repeat–induced cell death by FADD DN (Figure 1) suggests that the Fas pathway contributes to this process. To examine the possible role of extracellular signaling via the Fas pathway, we investigated the effect of a neutralizing monoclonal antibody to Fas ligand (NOK-1, Pharmingen) on Q79-induced cell death. This antibody had no effect on Q79-induced HeLa cell death at a concentration of 1 μg/ml (data not shown), which is twice the concentration previously shown to be sufficient to inhibit Fas ligand–induced apoptosis (Kayagaki et al., 1995; Oyaizu et al., 1997). Thus, extracellular signaling via the Fas pathway is not involved in polyglutamine inclusion–induced cell death.

The mechanism by which FADD DN inhibits Q79-induced cell death was further investigated by examining the subcellular localization of FADD DN in HeLa and 293T cells in the absence or presence of Q79 expression. We transfected HeLa cells with a vector encoding AU1-tagged FADD DN in the absence or presence of a Q79 vector and subsequently subjected soluble and insoluble fractions of cell lysates to Western blot analysis with a mAb to AU1 (Figure 5A). Whereas FADD DN was localized predominantly to the soluble fraction when expressed by itself, a substantial portion of the protein was present in the insoluble fraction of cells also expressing Q79, suggesting that Q79 is able to recruit FADD DN. A similar experiment revealed that Bcl-2 was not recruited by Q79 (Figure 5A).

The recruitment of FADD DN by Q79 was also apparent in 293T cells expressing GFP-tagged FADD DN and Q79. Consistent with previous observations (Perez and White, 1998), we showed that GFP-tagged FADD DN is diffusely distributed within the cytoplasm and nucleus of cells not expressing Q79 (Figure 5B). However, coexpression of FADD DN with Q79 resulted in the formation of FADD DN aggregates (Figure 5B). To determine whether FADD DN colocalizes with Q79 inclusions, we examined the localizations of HA-tagged Q79 and AU1-tagged FADD DN in transfected HeLa cells by immunocytochemistry and confocal microscopy. A substantial portion of AU1-tagged FADD DN colocalized with the polyglutamine inclusions. These results suggest that polyglutamine inclusions are able to recruit FADD DN by interacting with its death domain and that such interaction may inhibit the recruitment of cell death–inducing proteins.

Detection of Caspase-8-Like Immunoreactivity in the Insoluble Fraction of Affected Brain Regions from HD Patients

Finally, we investigated whether recruitment of caspase-8 by polyglutamine inclusions could be detected in postmortem brain tissue from individuals with HD. We thus determined the distribution of caspase-8 between soluble and insoluble fractions of caudate nuclei. In four out of four caudate samples from HD patients, but in none of the five samples from neurologically unremarkable control individuals, we detected one or two bands of ~46 kDa in the insoluble fraction that reacted with the mAb to caspase-8; these proteins were similar in size to activated caspase-8 in the insoluble fraction of HeLa cells expressing Q79 (Figure 6). In four out of four HD cerebellum samples, which are unaffected in HD, we did not detect similar caspase-8-like immunoreactivity

in the insoluble fraction (data not shown). In addition, the amount of caspase-8-like immunoreactivity in HD caudate samples was markedly greater than that in control caudate tissue, although no substantial difference was apparent between the soluble fractions of HD and control caudate samples (I. S. and J. Y., unpublished data). These preliminary results suggest that caspase-8 or a related caspase-8-like protein is specifically recruited to insoluble components of human caudate cells and subsequently becomes activated.

Discussion

The mechanism of caspase activation has been the subject of intensive study. Activation of caspase-8 can be achieved through FK506 binding protein– (FKBP-) mediated dimerization of an FKBP–caspase-8 fusion protein (Muzio et al., 1998; Yang et al., 1998), These observations suggest that oligomerization of caspase-8 is sufficient to induce its activation. In contrast, caspase-3, which contains a much shorter prodomain, cannot be activated by a similar mechanism (Yang et al., 1998). To date, Fas-induced apoptosis has been the only physiological process in which the recruitment and activation of caspase-8 have been demonstrated. Polyglutamine repeats may promote a pathological mechanism of protein–protein interaction that results in the recruitment and activation of caspase-8. We have shown here that caspase-8 is likely to be recruited and activated specifically in the caudate nuclei of HD patients, suggesting that the recruitment and activation of caspase-8 may be responsible for neuronal degeneration in HD. The mutant polyglutamine repeat–containing proteins have been shown to recruit a number of proteins, including proteins involved in ubiquitination (Davies et al., 1997; Gourfinkel-An et al., 1998) and molecular chaperone HDJ-2 (HSDJ) (Cummings et al., 1998). Of all the known proteins recruited by polyglutamine repeats, none is potentially more dangerous to the cell than caspase-8, which can trigger the activation of downstream components of the apoptotic pathway and neuronal cell death. Although our data appear to rule out the recruitment of caspases, such as caspase-3, that contain a short prodomain, it remains to be examined whether other caspases with a long prodomain are also recruited by polyglutamine inclusions.

Apoptosis induced by oligomerization and activation of caspase-8 requires only hours (Muzio et al., 1998; Yang et al., 1998), whereas neurodegenerative diseases such as HD develop over decades. Various rate-limiting factors may contribute to this apparent discrepancy. The first and most important rate-limiting factor is the generation of a truncated protein consisting predominantly of polyglutamine repeats. Expression of full-length huntingtin or related proteins such as ataxin-3, regardless of the length of the polyglutamine repeat, is not toxic to cells, whereas truncated versions of these proteins containing a sufficient number of polyglutamine repeats are highly cytotoxic (Goldberg et al., 1996; Miyashita et al., 1997; Wellington et al., 1998; the present study). The decades required for these diseases to develop may thus reflect the time required for sufficient accumulation of the corresponding truncated protein. The mechanism responsible for the generation of the truncated protein in each disease also may underlie the tissue and cell-type specificity of neuronal degeneration; that is, the specific conditions required for cleavage of the disease-associated protein may be present only in the neuronal populations affected in each disease. Although polyglutamine inclusions are detected only in specific populations of vulnerable neurons in patients with disease, other cell types are also sensitive to polyglutamine repeat–induced cell death when forced to express a truncated protein containing such repeats. A second possible rate-limiting factor may be the availability of appropriate caspases. We propose that the presence of polyglutamine repeats in the nucleus may play a role in the amplification of the apoptotic response, including the induction of caspase-8 or reduction of caspase-8 degradation during decades of disease incubation period.

Two inhibitors of caspase-8 recruitment, CrmA and FADD DN, appear to act by distinct mechanisms. Because CrmA is a pseudosubstrate type of inhibitor of caspases, forming a tight complex with these enzymes (Komiyama et al., 1994), CrmA likely interacts directly with caspase-8 to block its recruitment. The size of CrmA may be critical here, since zVAD.fmk, which is also a pseudosubstrate inhibitor, did not inhibit the recruitment of caspase-8. In fact, full-length caspase-8 has been shown to interact with CrmA (Muzio et al., 1998). In contrast, FADD DN may interact directly with polyglutamine repeats and thereby prevent the recruitment of caspase-8. FADD DN consists predominantly of a death domain that interacts homophilically with the death domain of the Fas receptor. Although the mechanism by which caspase-8 is recruited to polyglutamine repeats remains unclear, we hypothesize that an adapter protein similar to FADD, which contains a death domain and a death effector domain, may act as a bridge between the polyglutamine repeats and caspase-8. Our data thus suggest that an alternative intracellular Fas pathway plays a critical role in mediating neuronal degeneration induced by polyglutamine inclusions and that inhibition of the recruitment of caspase-8 may be able to prevent or retard the progression of neuronal loss in polyglutamine expansion diseases.

We have shown that polyglutamine repeat–induced cell death is inhibited by expression of Bcl-2 or Bcl-x_L but not N-Bcl-x_L. Expression of Bcl-2 or Bcl-x_L has previously been shown to inhibit apoptosis induced by a wide variety of stimuli. Two major mechanisms have been proposed to explain the actions of Bcl-2 and Bcl-x_L, based on the observations that these proteins form ion channels (Minn et al., 1997; Schendel et al., 1997) or interact directly with other mediators of apoptosis, such as CED-4 and Apaf-1 (Chinnaiyan et al., 1997; Pan et al., 1998). Although it is not clear whether these two modes of action coexist, a free N terminus of Bcl-x_L is required for the protein to protect cells from apoptosis only in certain instances. We have recently shown that Bid, a proapoptotic member of the Bcl-2 family, becomes a potent inducer of apoptosis after cleavage by caspase-8 (Li et al., 1998). Whereas an N-terminally tagged form of Bcl-x_L was highly efficient in inhibiting

cell death induced by truncated Bid, it could not inhibit polyglutamine repeat–induced cell death. A free N terminus of Bcl-x_L is required for its biochemical and functional interaction with CED-4 and the CED-4-like domain in Apaf-1 (Chinnaiyan et al., 1997; Pan et al., 1998); our results suggest that a similar interaction may be required for Bcl-x_L to inhibit polyglutamine inclusion–induced cell death. Since caspase-8 can interact with both Apaf-1 (Hu et al., 1998) and Bcl-x_L (Chinnaiyan et al., 1997), the competition between caspase-8 binding to Apaf-1 and Bcl-x_L has been hypothesized to play a role in mediating caspase-8 activation. We suggest that Bcl-x_L may inhibit polyglutamine repeat–induced cell death by competing for binding of caspase-8.

Studies with transgenic mice expressing exon 1 of the *huntingtin* gene and mutant *SCA1* as well as with brain tissue from patients with SCA3 or HD have shown that expanded polyglutamine repeats can form aggregates within nuclei (Davies et al., 1997; DiFiglia et al., 1997; Paulson et al., 1997). These observations have led to the hypothesis that the formation of such intranuclear inclusions is a critical step in neuronal degeneration. Recently, however, two groups have provided evidence against a critical role of intranuclear inclusions in inducing neurodegeneration (Klement et al., 1998; Saudou et al., 1998). Our study does not directly address the role of inclusions, since induction of caspase-8 oligomerization by polyglutamine repeats may not require the formation of microscopically visible inclusions. The system used by Saudou et al. (1998) is similar to ours, except that they used a long version of huntingtin (amino acids 1–480 of huntingtin) that may need to be further cleaved to activate cell death, explaining a delay of up to 9 days before inducing cell death; in contrast, we used a construct expressing a polyglutamine repeat only, and cell death can be induced directly by this peptide without further cleavage.

Although our data do not distinguish between the roles of nuclear versus cytoplasmic repeats, we propose that caspase-8 recruitment by such repeats occurs predominantly within the cytoplasm for the following reasons: first, caspase-8 is normally localized in the cytoplasm rather than in nuclei, and second, most anti-apoptotic proteins, including Bcl-2 and Bcl-x_L, normally reside on the outer mitochondrial membrane (Merry and Korsmeyer, 1997). Thus, we suggest that recruitment and activation of caspase-8, as well as inhibition of caspase-8 by Bcl-2 and Bcl-x_L, occur primarily in the cytoplasm. In addition, although most caspase-8 molecules appeared to be associated with the Q79 inclusions, we cannot rule out the possibility that the interaction of caspase-8 with the polyglutamine repeats occurs before aggregate formation; aggregation of the polyglutamine repeats may then simply serve to bring together multiple caspase-8 molecules. In the case of the Fas receptor, the interaction of two caspase-8 molecules is sufficient to induce their activation (Muzio et al., 1998; Yang et al., 1998).

If the cytoplasmic polyglutamine repeats play a primary role in caspase recruitment and activation, what then is the role of the intranuclear repeats? We propose that the presence of expanded polyglutamine repeats in the nucleus may be sufficient to induce a stress response, which may initially result in the induction of stress-related proteins, such as members of the Bcl-2 and c-Jun N-terminal kinase (JNK) families, but not enough to kill, since caspases are not activated; eventually, however, the stress response system may be overwhelmed, and an apoptotic response, which could include upregulation of caspases or reduction of caspase turnover, may ensue. Such upregulation of pro- and anti-apoptotic proteins, such as bad, bax, and bcl-2, have been demonstrated in human tissues affected with other neurodegenerative diseases (Kitamura et al., 1998). This scenario may be consistent with the apparent induction of caspase-8-like immunoreactivity in caudate tissue from HD patients. The levels of caspase-8 in control caudate tissue are low compared with that in HeLa, Jurkat, and MCF7 cells (I. S. and J. Y., unpublished data), whereas the amount of caspase-8 in the insoluble fraction of HD caudate tissue is markedly increased. Such upregulation of caspase-8 may be required for recruitment and activation of caspase-8 by cytoplasmic polyglutamine repeats. After such cytoplasmic activation, caspase-8 may enter the nucleus, together with other downstream caspases, to complete the apoptotic process.

Concluding Remarks

We have shown here that the recruitment of caspase-8 by polyglutamine repeats may contribute to the pathogenesis of HD and related diseases. We suggest that such recruitment may not be limited to HD-related neurodegenerative disorders. Formation of intracellular aggregates occurs in several other diseases, as exemplified by the Lewy bodies associated with Parkinson's disease and the recent demonstration of aggregates of an amyotrophic lateral sclerosis–associated mutant form of superoxide dismutase in transgenic mice (Bruijn et al., 1998). Given that oligomerization may be a fundamental mechanism of caspase activation, other intracellular aggregates with properties similar to those of polyglutamine inclusions also might be able to recruit and activate caspases and induce cell death.

Experimental Procedures

Plasmid Construction

A cDNA encoding the GFP-tagged C360S mutant of caspase-8 was constructed by digesting a caspase-8 C360S expression construct (kindly provided by V. Dixit) with KpnI and XhoI and cloning the released fragment into pEGFP-N2 (Clontech). The caspase-8 reading frame (lacking the sequence encoding 45 amino acids of the small subunit at the C terminus) was thus placed in the reverse orientation but within appropriate restriction sites. The resulting plasmid was digested with BamHI and XhoI, and the released fragment was cloned into pEGFP-N2 that had been digested with BglII and SalI. The resulting vector encodes caspase-8 C360S (lacking 45 amino acids) fused at its C terminus with GFP. The vector encoding FADD DN fused at its C terminus with GFP was generated by digesting a FADD expression construct (kindly provided by V. Dixit) with SalI and BamHI and cloning the released fragment into pEGFP-C1 (Clontech) that had been digested with the same two restriction enzymes. The vector encoding Q79 fused at its C terminus with GFP was prepared by digesting Q79-pIND with HindIII and ApaI and cloning the released fragment into pEGFP-N2 that had been digested with the same two enzymes. The Bcl-2 expression construct was RR/1 (Gagliardini et al., 1994). The construct encoding Bcl-x_L tagged at its N terminus with the Flag-epitope (N-Bcl-x_L) was as described by Li et al. (1998).

Cell Lines and Primary Neuronal Cultures

Cells were seeded at a density of 2×10^4 per 35 mm well in Dulbecco's modified Eagles medium supplemented with 10% fetal bovine serum (FBS) (Hyclone) and were transfected by the standard calcium phosphate method. Striatal, cerebellar, and cortical neurons from E17 rat embryos (Taconic) were cultured in neurobasal medium containing B27 supplement (Gibco) as described by Brewer (1995). Neurons were plated at a density of 1×10^5 per well in 6-well plates. After 4 days in culture, neurons were transfected by the standard calcium phosphate method. One hour before transfection, the culture medium was adjusted to 1% FBS, and half of the medium was then removed, to be added back after transfection. The neurons were exposed to the calcium phosphate and DNA precipitate for 60 min, washed twice with neurobasal medium, and then cultured in the conditioned medium for 48 hr in the presence of amphidocolin (3 μg/ml) (Sigma). For the trophic factor deprivation experiments, neurons were transfected as mentioned above and cultured for 12 hr before the media were exchanged for neurobasal culture media devoid of B27 supplement or serum.

The percentage of cell death induced by Q79 was determined by transfecting cells with 1 μg of the CMX–Q79 (HA-tagged) expression construct and 0.2 μg of pEGFP-N1 (Clontech) as a marker. In cotransfection experiments, constructs encoding FADD DN, CrmA, Bcl-2, Bcl-x_L, or N-Bcl-x_L were used at a ratio of 5:1 with the CMX–Q79 expression construct. Caspase-8 C360S was used at a ratio of 2:1 with the CMX–Q79 expression construct. Apoptotic cells were identified by visual inspection of GFP-positive cells with a Nikon inverted fluorescence microscope. In early experiments, uptake of trypan blue and propidium iodide was used to confirm the quantitation of cell death based on cell morphology, and staining with Hoechst 33342 was used to examine nuclear morphology. Each experiment was performed at least in duplicate, with >100 cells counted for each determination.

Antibodies and Immunoblot Analysis

The mAb to caspase-3, -7 and -8 was generated by injecting rats with appropriate recombinant caspases (Li et al., 1998). Anti-human Bcl-2 antibody was purchased from Dako. For immunoblot analysis, lysates of cells or of human tissue samples that had been grounded in liquid N_2 were prepared in a solution containing 150 mM NaCl, 1% NP-40, 0.1% SDS, and 50 mM Tris-HCl (pH 8.0), by vortexing and passing through syringe needles. A soluble fraction was obtained by collecting the supernatant after centrifugation of the lysate in a microfuge at 13,000 rpm for 15 min. The remaining insoluble pellets were dissolved in SDS sample buffer (2% SDS, 100 mM dithiothreitol, 60 mM Tris-HCl [pH 6.8], and 0.01% bromophenol blue) by boiling, vigorous mixing, and then adding urea to achieve a final concentration of 2 M, before SDS–PAGE electrophoresis.

Immunofluorescence and Confocal Microscopy

For immunostaining, HeLa cells and MCF7 cells were grown in Labtek slide culture chambers at a density of 2×10^3 per chamber and transfected by the calcium phosphate method. Twenty-four or forty-eight hours after transfection, the cells were fixed with 2% paraformaldehyde or, when quenching of GFP fluorescence was required, with ice-cold acetone for 10 min. Slides were washed with two changes of phosphate-buffered saline (PBS) and then incubated for 20 min at room temperature in PBS containing 5% FBS and 0.1% Triton X-100. Cells were then incubated for 2 hr with primary antibodies diluted in PBS containing 1% FBS and 0.1% Triton X-100, and subsequently for 30 min with secondary antibodies; they were washed three times with PBS after each antibody incubation. After incubation for 10 min with Hoechst 33342, the cells were mounted in PBS containing 90% glycerol and phenylamine (10 mg/ml). Primary antibodies included mAb 11.1 to the HA-epitope and a mAb to the AU1-epitope (Babco). Slides were viewed with a Zeiss Axiophot inverted confocal microscope.

Acknowledgments

We thank Ethan Signer for sparking our interest in this problem as well as for support and encouragement during the experiments and helpful comments on the manuscript; Vishva Dixit for providing the caspase-8 and FADD expression constructs and MCF7 cell line; Suyue Wang for *caspase-1*$^{-/-}$ and *caspase-11*$^{-/-}$ cells; Honglin Li for the Bcl-x_L construct; Honglin Li and Roberto Sanchez-Olea for helpful suggestions; Louise Bergeron for *caspase-2*$^{-/-}$ cells; Hong Zhu for technical assistance; Ruchika Gupta for critical reading of the manuscript; and Frederic Saudou and Michael E. Greenberg for communicating unpublished results. The human tissue samples were kindly provided by the Harvard Brain Tissue Resource Center, which is supported in part by National Institutes of Health grant NS31862. This work was supported in part by a contract from the Hereditary Disease Foundation and an American Heart Association Established Investigatorship (to J. Y.).

Received November 5, 1998; revised January 25, 1999.

References

Allsopp, T.E., Wyatt, S., Paterson, H.F., and Davies, A.M. (1993). The proto-oncogene bcl-2 can selectively rescue neurotrophic factor-dependent neurons from apoptosis. Cell *73*, 295–307.

Bates, G.P., and Davies, S.W. (1997). Transgenic mouse models of neurodegenerative disease caused by CAG/polyglutamine expansions. Mol. Med. Today *3*, 508–515.

Brewer, G.J. (1995). Serum-free B27/neurobasal medium supports differentiated growth of neurons from the striatum, substantia nigra, septum, cerebral cortex, cerebellum, and dentate gyrus. J. Neurosci. Res. *42*, 674–683.

Bruijn, L.I., Houseweart, M.K., Kato, S., Anderson, K.L., Anderson, S.D., Ohama, E., Reaume, A.G., Scott, R.W., and Cleveland, D.W. (1998). Aggregation and motor neuron toxicity of an ALS-linked SOD1 mutant independent from wild-type SOD1. Science *281*, 1851–1854.

Chinnaiyan, A.M., O'Rourke, K., Tewari, M., and Dixit, V.M. (1995). FADD, a novel death domain–containing protein, interacts with the death domain of Fas and initiates apoptosis. Cell *81*, 505–512.

Chinnaiyan, A.M., Tepper, C.G., Seldin, M.F., O'Rourke, K., Kischkel, F.C., Hellbardt, S., Krammer, P.H., Peter, M.E., and Dixit, V.M. (1996). FADD/MORT1 is a common mediator of CD95 (Fas/APO-1) and tumor necrosis factor receptor–induced apoptosis. J. Biol. Chem. *271*, 4961–4965.

Chinnaiyan, A.M., O'Rourke, K., Lane, B.R., and Dixit, V.M. (1997). Interaction of CED-4 with CED-3 and CED-9: a molecular framework for cell death. Science *275*, 1122–1126.

Cryns, V., and Yuan, J. (1998). Proteases to die for. Genes Dev. *12*, 1551–1570.

Cummings, C.J., Mancini, M.A., Antalffy, B., DeFranco, D.B., Orr, H.T., and Zoghbi, H.Y. (1998). Chaperone suppression of aggregation and altered subcellular proteasome localization imply protein misfolding in SCA1. Nat. Genet. *19*, 148–154.

Davies, S.W., Turmaine, M., Cozens, B.A., DiFiglia, M., Sharp, A.H., Ross, C.A., Scherzinger, E., Wanker, E.E., Mangiarini, L., and Bates, G.P. (1997). Formation of neuronal intranuclear inclusions underlies the neurological dysfunction in mice transgenic for the HD mutation. Cell *90*, 537–548.

DiFiglia, M., Sapp, E., Chase, K.O., Davies, S.W., Bates, G.P., Vonsattel, J.P., and Aronin, N. (1997). Aggregation of huntingtin in neuronal intranuclear inclusions and dystrophic neurites in brain. Science *277*, 1990–1993.

Gagliardini, V., Fernandez, P.A., Lee, R.K., Drexler, H.C., Rotello, R.J., Fishman, M.C., and Yuan, J. (1994). Prevention of vertebrate neuronal death by the *crmA* gene. Science *263*, 826–828.

Goldberg, Y.P., Nicholson, D.W., Rasper, D.M., Kalchman, M.A., Koide, H.B., Graham, R.K., Bromm, M., Kazemi-Esfarjani, P., Thornberry, N.A., Vaillancourt, J.P., and Hayden, M.R. (1996). Cleavage of huntingtin by apopain, a proapoptotic cysteine protease, is modulated by the polyglutamine tract. Nat. Genet. *13*, 442–449.

Gourfinkel-An, I., Cancel, G., Duyckaerts, C., Faucheux, B., Hauw, J.J., Trottier, Y., Brice, A., Agid, Y., and Hirsch, E.C. (1998). Neuronal distribution of intranuclear inclusions in Huntington's disease with adult onset. Neuroreport *9*, 1823–1826.

Hu, Y., Benedict, M.A., Wu, D., Inohara, N., and Nunez, G. (1998). Bcl-XL interacts with Apaf-1 and inhibits Apaf-1-dependent caspase-9 activation. Proc. Natl. Acad. Sci. USA *95*, 4386–4391.

Huntington's Disease Collaborative Research Group (1993). A novel gene containing a trinucleotide repeat that is expanded and unstable on Huntington's disease chromosomes. Cell *72*, 971–983.

Igarashi, S., Koide, R., Shimohata, T., Yamada, M., Hayashi, Y., Takano, H., Date, H., Oyake, M., Sato, T., et al. (1998). Suppression of aggregate formation and apoptosis by transglutaminase inhibitors in cells expressing truncated DRPLA protein with an expanded polyglutamine stretch. Nat. Genet. *18*, 111–117.

Ikeda, H., Yamaguchi, M., Sugai, S., Aze, Y., Narumiya, S., and Kakizuka, A. (1996). Expanded polyglutamine in the Machado-Joseph disease protein induces cell death in vitro and in vivo. Nat. Genet. *13*, 196–202.

Janicke, R.U., Ng, P., Sprengart, M.L., and Porter, A.G. (1998). Caspase-3 is required for alpha-fodrin cleavage but dispensable for cleavage of other death substrates in apoptosis. J. Biol. Chem. *273*, 15540–15545.

Juo, P., Kuo, C.J., Yuan, J., and Blenis, J. (1998). Essential requirement for caspase-8/FLICE in the initiation of the Fas-induced apoptotic cascade. Curr. Biol. *8*, 1001–1008.

Kawaguchi, Y., Okamoto, T., Taniwaki, M., Aizawa, M., Inoue, M., Katayama, S., Kawakami, H., Nakamura, S., Nishimura, M., Akiguchi, I., et al. (1994). CAG expansions in a novel gene for Machado-Joseph disease at chromosome 14q32.1. Nat. Genet. *8*, 221–228.

Kayagaki, N., Kawasaki, A., Ebata, T., Ohmoto, H., Ikeda, S., Inoue, S., Yoshino, K., Okumura, K., and Yagita, H. (1995). Metalloproteinase-mediated release of human Fas ligand. J. Exp. Med. *182*, 1777–1783.

Kischkel, F.C., Hellbardt, S., Behrmann, I., Germer, M., Pawlita, M., Krammer, P.H., and Peter, M.E. (1995). Cytotoxicity-dependent APO-1 (Fas/CD95)-associated proteins form a death-inducing signaling complex (DISC) with the receptor. EMBO J. *14*, 5579–5588.

Kitamura, Y., Shimohama, S., Kamoshima, W., Ota, T., Matsuoka, Y., Nomura, Y., Smith, M.A., Perry, G., Whitehouse, P.J., and Tanigushi, T. (1998). Alteration of proteins regulating apoptosis, Bcl-2, Bcl-x, Bax, Bak, Bad, ICH-1, and CPP32, in Alzheimer's disease. Brain Res. *780*, 260–269.

Klement, I.A., Skinner, P.J., Kaytor, M.D., Yi, H., Hersch, S.M., Clark, H.B., Zoghbi, H.Y., and Orr, H.T. (1998). Ataxin-1 nuclear localization and aggregation: role in polyglutamine-induced disease in SCA1 transgenic mice. Cell *95*, 41–53.

Komiyama, T., Ray, C.A., Pickup, D.J., Howard, A.D., Thornberry, N.A., Peterson, E.P., and Salvesen, G. (1994). Inhibition of interleukin-1 beta converting enzyme by the cowpox virus serpin CrmA. An example of cross-class inhibition. J. Biol. Chem. *269*, 19331–19337.

Li, H., Zhu, H., Xu, C.-J., and Yuan, J. (1998). Cleavage of BID by caspase-8 mediates the mitochondrial damage in the Fas pathway of apoptosis. Cell *94*, 491–501.

Martin, D.A., Siegel, R.M., Zheng, L., and Lenardo, M.J. (1998). Membrane oligomerization and cleavage activates the caspase-8 (FLICE/MACHalpha) death signal. J. Biol. Chem. *273*, 4345–4349.

Merry, D.E., and Korsmeyer, S.J. (1997). Bcl-2 gene family in the nervous system. Annu. Rev. Neurosci. *20*, 245–267.

Minn, A.J., Velez, P., Schendel, S.L., Liang, H., Muchmore, S.W., Fesik, S.W., Fill, M., and Thompson, C.B. (1997). Bcl-x(L) forms an ion channel in synthetic lipid membranes. Nature *385*, 353–357.

Miyashita, T., Okamura-Oho, Y., Mito, Y., Nagafuchi, S., and Yamada, M. (1997). Dentatorubral pallidoluysian atrophy (DRPLA) protein is cleaved by caspase-3 during apoptosis. J. Biol. Chem. *272*, 29238–29242.

Muzio, M., Stockwell, B.R., Stennicke, H.R., Salvesen, G.S., and Dixit, V.M. (1998). An induced proximity model for caspase-8 activation. J. Biol. Chem. *273*, 2926–2930.

Oyaizu, N., Adachi, Y., Hashimoto, F., McCloskey, T.W., Hosaka, N., Kayagaki, N., Yagita, H., and Pahwa, S. (1997). Monocytes express Fas ligand upon CD4 cross-linking and induce $CD4^+$ T cell apoptosis: a possible mechanism of bystander cell death in HIV infection. J. Immunol. *158*, 2456–2463.

Pan, G., O'Rourke, K., and Dixit, V.M. (1998). Caspase-9, Bcl-XL, and Apaf-1 form a ternary complex. J. Biol. Chem. *273*, 5841–5845.

Paulson, H.L., and Fischbeck, K.H. (1996). Trinucleotide repeats in neurogenetic disorders. Annu. Rev. Neurosci. *19*, 79–107.

Paulson, H.L., Perez, M.K., Trottier, Y., Trojanowski, J.Q., Subramony, S.H., Das, S.S., Vig, P., Mandel, J.L., Fischbeck, K.H., and Pittman, R.N. (1997). Intranuclear inclusions of expanded polyglutamine protein in spinocerebellar ataxia type 3. Neuron *19*, 333–344.

Perez, D., and White, E. (1998). E1B 19K inhibits Fas-mediated apoptosis through FADD-dependent sequestration of FLICE. J. Cell Biol. *141*, 1255–1266.

Sandou, F., Finkbeiner, S., Devys, D., and Greenberg, M.E. (1998). Huntingtin acts in the nucleus to induce apoptosis but death does not correlate with the formation of intranuclear inclusions. Cell *95*, 55–66.

Schendel, S.L., Xie, Z., Montal, M.O., Matsuyama, S., Montal, M., and Reed, J.C. (1997). Channel formation by antiapoptotic protein Bcl-2. Proc. Natl. Acad. Sci. USA *94*, 5113–5118.

Trottier, Y., Lutz, Y., Stevanin, G., Imbert, G., Devys, D., Cancel, G., Saudou, F., Weber, C., David, G., Tora, L., et al. (1995). Polyglutamine expansion as a pathological epitope in Huntington's disease and four dominant cerebellar ataxias. Nature *378*, 403–406.

Vincenz, C., and Dixit, V.M. (1997). Fas-associated death domain protein interleukin-1beta-converting enzyme 2 (FLICE2), an ICE/Ced-3 homologue, is proximally involved in CD95- and p55-mediated death signaling. J. Biol. Chem. *272*, 6578–6583.

Warrick, J.M., Paulson, H.L., Gray-Board, G.L., Bui, Q.T., Fischbeck, K.H., Pittman, R.N., and Bonini, N.M. (1998). Expanded polyglutamine protein forms nuclear inclusions and causes neural degeneration in *Drosophila*. Cell *93*, 939–949.

Wellington, C.L., Ellerby, L.M., Hackam, A.S., Margolis, R.L., Trifiro, M.A., Singaraja, R., McCutcheon, K., Salvesen, G.S., Propp, S.S., Bromm, M., et al. (1998). Caspase cleavage of gene products associated with triplet expansion disorders generates truncated fragments containing the polyglutamine tract. J. Biol. Chem. *273*, 9158–9167.

Yang, X., Chang, H.Y., and Baltimore, D. (1998). Autoproteolytic activation of pro-caspases by oligomerization. Mol. Cell *1*, 319–325.

Zhou, Q., Snipas, S., Orth, K., Muzio, M., Dixit, V.M., and Salvesen, G.S. (1997). Target protease specificity of the viral serpin CrmA. Analysis of five caspases. J. Biol. Chem. *272*, 7797–7800.

A Note Added in Proof

After our paper was accepted, Moulder et al. (1999) and Kim et al. (1999) published their work on cell death induced by expanded polyglutamine repeats in primary rat cerebellar granular neurons and in striatal/neuroblastoma fusion cell line N18TG2, respectively. Consistent with our results, both papers showed that the expression of expanded poly-Q-induced activation of caspases and the inhibition of caspases reduced or delayed neuronal toxicity. While the inhibition of caspases clearly has an effect on poly-Q-induced cell death, Moulder et al. stressed that cerebellar granule neurons expressing poly-Q in the presence of Boc-aspartyl-(OMe)-fluoromethylketone (BAF) still die with a delayed time course over 5 days. While it is unclear whether BAF inhibits caspase-8 in the dose used in Moulder's experiment, we cannot exclude the existence of a caspase-independent alternative pathway that is triggered after caspase inhibition. Such a pathway is proposed in cerebellar granular cells in the work done by the same group (Miller and Johnson, 1996). We are currently exploring the molecular basis of a possible caspase-independent pathway and testing whether such a mechanism is induced following the inhibition of the recruitment and activation of caspase-8.

Kim, M., Lee, H.S., LaForet, G., McIntyre, C., Martin, E.J., Chang, P., Kim, T.W., Williams, M., Reddy, P.H., Tagle, D., et al. (1999). Mutant huntingtin expression in clonal striatal cells: dissociation of inclusion formation and neuronal survival by caspase inhibition. J. Neurosci. *19*, 964–973.

Miller, T.M., and Johnson, E.M., Jr. (1996). Metabolic and genetic analyses of apoptosis in potassium/serum-deprived rat cerebellar granule cells. J. Neurosci. *16*, 7487–7495.

Moulder, K.L., Onodera, O., Burke, J.R., Strittmatter, W.J., and Johnson, E.M., Jr. (1999). Generation of neuronal intranuclear inclusions by polyglutamine-GFP: analysis of inclusion clearance and toxicity as a function of polyglutamine length. J. Neurosci. *19*, 705–715.