

## Review

# Caspase inhibition: a potential therapeutic strategy in neurological diseases

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**Summary.** Caspases are intracellular proteases that participate in apoptotic pathways in mammalian cells, including neurons. Here we review evidence that caspase inhibition, through pharmacological or molecular means, may inhibit neuronal cell death in a number of in vitro and in vivo models of neurological disease. It has recently become clear that, at least in most cell culture models, caspase inhibition offers only transient protection, and that a caspase-independent death eventually occurs. This may be due to irreversible caspase-independent alterations at the level of the mitochondria. Despite concerns that targeting caspases alone may prove insufficient to truly reverse the effects of various death stimuli, in vivo studies indicate that caspase inhibition promotes survival and functional outcome in a variety of neurological disease models. In addition, studies of human post-mortem material suggest that caspases are activated in certain human neurological diseases. Caspase inhibition may therefore provide a novel strategy for the treatment of such disorders. Caspases, through the generation of toxic fragments of critical protein substrates, may also be involved in earlier steps of neuronal dysfunction, such as protein aggregation in Huntington's and Alzheimer's disease, and therefore caspase inhibition may be of additional value in the treatment of these particular disorders.

**Key words:** Caspase, Apoptosis, Neuronal death, Neurodegenerative, Therapy, Toxic fragment, Protein aggregation, Mitochondria, Human

### Caspases within the apoptotic pathway

Caspases are intracellular cysteine proteases that participate in apoptotic cell death pathways (Steller, 1995; Fraser and Evan, 1996; Green and Kroemer, 1998; Green, 1998; Earnshaw et al., 1999). The foundations for current concepts of the biochemical and molecular

pathway of apoptosis were laid by the groundbreaking work of Horvitz and colleagues, who identified a number of genes that play a role in programmed cell death in the nematode *C. elegans* (Ellis and Horvitz, 1986; Metzstein et al., 1998). Most notable amongst these were *ced-3* and *ced-4*, which promoted programmed cell death, and *ced-9*, which inhibited it (Avery and Horvitz, 1987; Yuan and Horvitz, 1990; Hengartner et al., 1992; Hengartner and Horvitz, 1994). Shortly thereafter Horvitz and colleagues discovered a homology between *ced-3* and the mammalian gene encoding Interleukin 1- $\beta$  converting enzyme (ICE), a cysteine protease involved in Interleukin 1- $\beta$  (IL1- $\beta$ ) processing (Yuan et al., 1993). Subsequently, a number of investigators cloned a large number of ICE homologues, which comprise the family of caspases (Fraser and Evan, 1996; Earnshaw et al., 1999). To date, 14 members of the caspase family have been identified. They can be classified in various ways, depending for example on their substrate specificity, the presence or absence of a large prodomain, or their perceived position and role within the apoptotic pathway. Based on the latter, they are classified into ICE (caspase 1)-like caspases, which also play a role in inflammatory responses, caspase 3-like caspases, which are generally thought to be the final death effectors, and caspase 8-like caspases, which generally act as initiators or propagators of apoptotic signaling (Earnshaw et al., 1999). Mammalian homologues of *ced-4*, most notably Apaf-1, and of *ced-9*, comprising the *bcl-2* family of proteins, have also been identified (Hengartner and Horvitz, 1994; Zhou et al., 1997; Adams and Cory, 1998).

These genetic data, together with important work

**Abbreviations.** Parkinson's disease: PD; 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine: MPTP; 6-hydroxydopamine: 6OHDA; Alzheimer's Disease: AD; Huntington's Disease: HD; Cu/Zn superoxide dismutase: SOD1; interleukin 1 $\beta$ : IL-1 $\beta$ ; interleukin 1 $\beta$  converting enzyme: ICE; amyloid precursor protein: APP;  $\beta$ -amyloid: A $\beta$ ; amyotrophic lateral sclerosis: ALS; apoptosis initiating factor: AIF; DNA fragmentation factor: DFF; chloromethylketone: CMK; fluoromethylketone: FMK; mutant Superoxide Dismutase 1: mSOD1; dominant negative inhibitor of caspase 1: DN 1; X-chromosome-linked inhibitor of apoptosis: XIAP

regarding the role of mitochondria, on one hand, and death receptors, on the other, in transducing death stimuli, have led to current concepts of the manner in which caspases are activated (Green, 1998; Green and Kroemer, 1998; Earnshaw et al., 1999). There are two main apoptotic pathways, which are not mutually exclusive. In the death receptor pathway, following the binding of the ligand to the receptor, a death complex is formed, through homophilic interactions, that leads to the recruitment and activation of caspase 8, and subsequent activation of other caspases (reviewed by Peter and Kramer, 1998). In the mitochondrial pathway, the death stimulus leads to alterations in members of the bcl-2 family, most notably of the pro-apoptotic protein Bax, that cause, through as yet unknown mechanisms, release of apoptogenic factors, such as cytochrome c, from the mitochondria to the cytosol or to the nucleus. Cytosolic cytochrome c then binds to Apaf-1, the mammalian homologue of ced-4, in the presence of dATP, and this leads to the recruitment and activation of caspase 9, and subsequent activation of downstream caspases, such as caspase 3 (Liu et al., 1996; Li et al., 1997; Zhou et al., 1997; Green, 1998; Green and Kroemer, 1998). Downstream caspases induce death by cleaving critical substrates at aspartate residues, thus causing the nuclear changes typical of apoptosis and the disintegration of the cell (Fraser and Evan, 1996; Earnshaw et al., 1999).

### **Caspases in neuronal cell death**

A wealth of evidence indicates that caspases are essential components of neuronal apoptotic pathways. For example, cell culture studies show that caspases are activated prior to trophic factor deprivation-induced death (Deshmukh et al., 1996; Stefanis et al., 1996, 1998) and that caspase inhibition, through pharmacological or molecular means, protects trophic factor-deprived neurons from death (Gagliardini et al., 1994; Deshmukh et al., 1996; Stefanis et al., 1996; Troy et al., 1996). The pharmacological inhibitors used are generally irreversible pseudosubstrate inhibitors that mimic the preferred cleavage sites of the caspases. Both general caspase inhibitors, such as BAF and zVAD-FMK, and more specific ones, such as YVAD-CMK, more specific for caspase 1, or DEVD-FMK, more specific for caspase 3, have been used in these studies. It should be noted however that the specificity of targeting individual caspases with pharmacological inhibitors is only relative (Garcia-Calvo et al., 1998) and that at least some of these inhibitors may inhibit other proteolytic systems, such as cathepsins (Schotte et al., 1999).

In most cases it appears that neurons depend on the mitochondrial pathway in order to undergo apoptosis (but see also Sanchez et al., 1999; Velier et al., 1999; and Matsushita et al., 2000 for evidence of receptor complex-related caspase activation). In the trophic deprivation paradigm in sympathetic neurons, for example, antibodies to cytochrome c prevent apoptosis

(Neame et al., 1998) and neurons derived from Bax deficient mice are resistant to death (Deckwerth et al., 1996).

Even more direct evidence for the involvement of caspases, and in particular those activated through the mitochondrial pathway, in neuronal cell death is provided by knock-out mice in which particular members of the caspase family have been genetically deleted. For example, mice null for caspase 9 or caspase 3 demonstrate reduced developmental neuronal cell death, leading to profound abnormalities of brain development and a remarkable increase in brain size (Kuida et al., 1996; Hakem et al., 1998). A similar neuronal phenotype is observed in mice null for Apaf-1 (Ceconi et al., 1998; Yoshida et al., 1998), implicating the mitochondrial pathway of caspase activation in developmental neuronal cell death.

### **Caspase involvement in neurological diseases**

There is substantial evidence for caspase involvement in a variety of acute-subacute neurological insults, as well as neurodegenerative diseases. For the purposes of this review, we will address caspase involvement in ischemia and trauma, and in four major chronic neurodegenerative diseases, namely Parkinson's disease (PD), Huntington's disease (HD), Alzheimer's disease (AD), and Amyotrophic Lateral Sclerosis (ALS). For each one, we will review cell culture, animal models, and human neuropathological material data pertaining to caspase involvement.

#### **Parkinson's Disease (PD)**

##### *Cell culture-transplantation studies*

The cellular systems generally employed to model PD *in vitro* use the neurotoxins 6-OHDA or MPP<sup>+</sup>. These agents have been shown to induce neuronal death, however by different mechanisms depending on the dose and cell type studied (see Choi et al., 1999; Lotharius et al., 1999). Several groups have reported activation of downstream effector caspases, such as caspase-3, demonstrated by cleavage of labeled substrates, following treatment of primary cultures of cerebellar granule neurons (CGNs), dopaminergic neurons, or dopaminergic cell lines with 6-OHDA (Dodel et al., 1998, 1999; Ochu et al., 1998). Additionally, pharmacological inhibition of caspases ameliorates the apoptotic death induced by 6-OHDA in CGNs (Dodel et al., 1999), primary dopaminergic neurons (Lotharius et al., 1999), or dopaminergic cell lines (Choi et al., 1999). However, caspase inhibition was unable to prevent the toxicity induced by higher concentrations of 6-OHDA in the dopaminergic PC12 cell line (Ochu et al., 1998).

Similarly, it has also been shown that treatment with low concentrations of MPP<sup>+</sup> leads to the activation of caspase 3-like caspases in CGNs (Du et al., 1997), and can induce the death of primary dopaminergic neurons

via an apoptotic mechanism that is inhibited by treatment with the broad spectrum caspase inhibitor ZVAD-FMK or the caspase 3 like inhibitors DEVD-FMK or DEVD-CHO (Du et al., 1997; Dodel et al., 1998). However, as has been observed with 6-OHDA, as the concentration of MPP<sup>+</sup> increases, the mechanism of death shifts from one that is morphologically apoptotic and inhibitable by caspase inhibition, to one that does not manifest the morphological features of apoptosis and is not responsive to caspase inhibition (Choi et al., 1999).

The transplantation of embryonic dopaminergic tissue has been found to provide some functional benefit in certain patients. The major problem, however, is the significant loss of donor tissue viability, mainly prior to and during the implantation procedure. It has been estimated that less than 10% of the initial graft tissue survives and innervates the host striatum (Kordower et al., 1998), necessitating the use of a considerable amount of donor tissue. It has been shown that a proportion of the neuronal loss in embryonic grafts is via apoptosis (e.g. Mahalik et al., 1994), indicating that neuroprotective anti-apoptotic agents might improve graft survival. Brundin and colleagues have shown that pre-treatment of dissociated embryonic nigral cell suspensions with the ICE-like caspase inhibitor YVAD-CMK significantly improved not only pre-implant survival, but also long term graft survival and innervation as evidenced by enhanced functional improvements in 6-OHDA lesioned animals (Schierle et al., 1999). These findings were extended with the observation that the caspase 3-like inhibitor DEVD-CMK, but surprisingly not the broad spectrum caspase inhibitor ZVAD-FMK, improved the survival of embryonic nigral grafts to over 30% (Hansson et al., 2000). These data suggest that it may be possible to improve the success of fetal tissue transplantation, and reduce the substantial amount of donor tissue required, using caspase inhibition to increase graft tissue viability.

**Table 1.** Summary of caspases found to be activated in animal models of human neurological disease, and the pharmacological and molecular inhibitors of caspase activation found to have neuroprotective effects. Results from mouse transgenic studies are indicated in italicized text (DN-1, overexpression of dominant negative caspase 1; XIAP, overexpression of X-chromosome-linked inhibitor of apoptosis protein).

	CASPASE ACTIVATION	NEUROPROTECTION
MPTP/6-OHDA	2,3	ZVAD, <i>DN-1</i>
Hunt Overexpression	1	ZVAD, <i>DN-1</i>
SOD1 Mutant	1, 3	ZVAD, <i>DN-1</i>
Focal/Global Ischemia	1, 3, 8, 9	YVAD, DEVD, BAF, ZVAD, XIAP, <i>DN-1</i>
Brain/Spinal Cord Trauma	1, 3	ZVAD, YVAD, DEVD, <i>DN-1</i>

### Animal models

The animal models traditionally used to study PD employ injection of the neurotoxins MPTP (the parent compound of MPP<sup>+</sup>) or 6-OHDA, because these agents cause selective death of dopaminergic neurons of the substantia nigra, mimicking PD pathology. Animal models based on genetic defects in PD are gradually emerging. However, data on the mechanisms of cell death in these genetic models are not yet available. In accordance with the cell culture studies, 6-OHDA and MPTP administration may cause death that is primarily necrotic or apoptotic, depending on the exact regimen used and the developmental stage of the animal (for example, see Jackson-Lewis et al., 1995; Tatton and Kish, 1997 for an acute and chronic MPTP regimen causing respectively necrosis and apoptosis).

Activation of caspase 3, using immunohistochemistry with an antibody raised against the active p17 fragment of caspase 3, was shown in apoptotic and pre-apoptotic substantia nigra neurons following 6-OHDA injection in neonatal rats (Jeon et al., 1999). The antibody used for this study, designated CM1, and characterized by Srinivasan and co-workers (1998), does not react with the inactive proform of caspase 3 and thus is a useful tool for the in situ identification and intracellular localization of activated caspase 3 (and perhaps caspase 7) in apoptotic cells. Another study, by Cutillas et al. (1999) showed that caspase inhibition with the general caspase inhibitor zVAD-FMK offered protection against 6-OHDA-induced neurodegeneration in adult rats.

There is also evidence that MPTP-induced death involves caspases: Mice that received striatal injections of MPTP showed processing, indicative of activation, of caspase 2 (Yang et al., 1998), which was prevented by overexpression of the anti-apoptotic protein Bcl-2. In addition, transgenic mice expressing a dominant negative inhibitor of caspase 1 (DN 1) were resistant to chronic systemic MPTP-induced apoptosis of nigral dopaminergic neurons (Klevenyi et al., 1999).

### Human studies

As in the animal models, the examination of caspase involvement in PD is closely linked to the issue of whether apoptotic death occurs in the human disease. This is a controversial issue, with some groups providing

**Table 2.** Summary of caspases found to be activated in acute and chronic human neurological disease.

	CASPASE ACTIVATION
Parkinson's Disease	3?
Alzheimer's Disease	3
Huntington's Disease	1, 8
Amyotrophic Lateral Sclerosis	1, 3-like
Stroke	3
Brain/Spinal Cord Trauma	1, 3

evidence of apoptotic neuronal death (Mochizuki et al., 1996; Anglade et al., 1997; Tompkins et al., 1997; Tatton et al., 1998) and others failing to report it (Banati et al., 1998; Wullner et al., 1999). It seems apparent that methodological differences could account, at least in part, for the disagreement on this issue. This raises the question of the value of solely determining the mechanism of neuronal death, not just in PD but in other neurodegenerative diseases as well, based on the observation of nuclear apoptotic features without examining apoptosis-related biochemical changes within the tissue. Moreover, given that neurodegenerative diseases such as PD, AD, and HD progress over many years, examining post-mortem tissue, which represents the disease state at a single point in time, for morphological evidence of apoptotic neuronal death is not likely to yield a large return. From a technical standpoint, reliance on a single biochemical measure to identify apoptotic neurons, such as end-labeling fragmented DNA (e.g. TUNEL, or ISEL), may not be as reliable an indication as the combination of end-labeling with an examination of nuclear morphology (see Tatton et al., 1998 for discussion).

Hartmann et al. (2000) utilized the CM1 antibody raised against the active conformation of caspase 3 to examine caspase 3 activation in post-mortem tissue from PD patients. Although the absolute numbers of neuromelanin containing neurons that were positive for active-caspase 3 was significantly lowered in PD tissue compared to age-matched controls, when the authors normalized this number to the estimated remaining dopaminergic neurons in the substantia nigra, the proportion was approximately 5-fold higher in PD brain. However, they were unable to detect processed caspase 3 fragments by western blotting of substantia nigra lysates, which was likely due to the small numbers of positively stained neurons observed in tissue sections. In contrast, in a thorough screening of many apoptosis-related proteins in PD and diffuse Lewy body disease post-mortem tissue, Jellinger (2000) failed to detect evidence of activated caspase 3 using the same active conformation-specific antibody (CM1). In this study, there were also no differences observed between PD and control tissue in the levels or distribution of other apoptosis-related proteins such as Bcl-2, Bax, Bcl-XL, or p53. Differences in tissue processing for immunostaining may account for the discrepancy between these studies. At this point, it is unclear whether caspase 3 is activated in PD substantia nigra.

### **Alzheimer's disease (AD)**

#### *Cell culture models*

The extracellular accumulation of  $\beta$ -amyloid peptide (A $\beta$ ) is thought to lead to the neuronal loss associated with AD. The application of A $\beta$  to cultured neuronal cells has accordingly been used as a cellular model of AD. A $\beta$  has been shown to cause apoptotic cell death in

these model systems (e.g. Loo et al., 1993), and to induce caspase activation (Ivins et al., 1999; Uetsuki et al., 1999; Giovanni et al., 2000; Selznick et al., 2000; Troy et al., 2000). In addition, pharmacological or molecular inhibition of particular members of the caspase family has been reported to offer partial or complete protection. In particular, caspases 2, 3, 8, and 12 have been invoked (Ivins et al., 1999; Giovanni et al., 2000; Nakagawa et al., 2000; Troy et al., 2000). However, Selznick et al. (2000) reported that cortical neurons derived from caspase 3 knock-outs were not protected from A $\beta$ -induced neuronal death. Differences in this respect, in particular with the study of Giovanni et al. (2000), who used the same experimental system, may be accounted for by different criteria used to assess death and/or different periods of observation. These data in conjunction suggest that multiple caspases may be involved in the apoptotic death of neurons exposed to A $\beta$ . In most cases however it appears that caspase inhibition offers only transient protection, and non-apoptotic death eventually occurs (Giovanni et al., 2000; Selznick et al., 2000).

A $\beta$  is derived from  $\gamma$ -secretase mediated processing of the amyloid precursor protein (APP). Emerging evidence suggests however that APP may also be cleaved by caspases at sites distinct from classic secretase processing. Caspases 3, 6 and 8 are able to cleave purified APP (Gervais et al., 1999; LeBlanc et al., 1999; Pellegrini et al., 1999). Neuronal cell lines induced to undergo apoptosis display cleavage of APP by caspase 3 or caspase 6, and such cleavage may increase the ultimate conversion of APP to A $\beta$  (Gervais et al., 1999; LeBlanc et al., 1999). These results have raised the possibility of a dual role for caspases in neuronal death in AD. According to this hypothesis, low levels of caspase activity, which are insufficient to induce neuronal death, may process APP, thus favoring the  $\gamma$ -secretase-mediated production of the toxic A $\beta$  fragment. A $\beta$  deposits are subsequently formed, leading to initiation of the apoptotic pathway, culminating in caspase activation. This scenario is rendered more likely by the recent discovery that even caspase proforms can have low levels of enzymatic activity (Yang et al., 1998; Kumar, 1999).

#### *Animal models*

There is no convincing report of caspase activation in animal models of AD. This may in part be due to the paucity of neuronal cell death observed in the most commonly used model, the overexpression of mutated APP. Selznick et al. (1999), using the CM1 antibody, did not detect activated caspase 3 in such animals, which already had evidence of marked amyloid deposition and behavioral deficits.

#### *Human studies*

In studies of AD post-mortem tissue, Stadelmann et

al. (1999) and Selznick et al. (1999) observed CM1-positive immunoreactivity in hippocampal neurons. The CM1 labeling occurred in neurons with apoptotic nuclear features such as condensed and fragmented nuclei and in neurons with evidence of granulovacuolar degeneration. However, no specific CM1 labeling was observed within neurons associated with senile plaques or neurofibrillary tangles. This contrasts with studies in which caspase-cleaved actin, termed fractin, or a caspase-cleaved fragment of APP were localized with specific antibodies in areas with senile plaques in AD hippocampus (Yang et al., 1998; Gervais et al., 1999). The rod-like inclusions found in processes of hippocampal CA1 neurons (Hirano bodies) also stained with the fractin antibody. The fractin positive neurons, however, did not co-localize with activated caspase 3/CM1 staining (Rossiter et al., 2000). Taken together, these results suggest that there is probably limited caspase 3 activation in AD brains, perhaps restricted to the hippocampus. It is possible that the more widespread evidence of caspase-cleaved fragments within areas of amyloid deposition reflects cleavage by other caspases.

### Huntington's disease (HD)

#### *Cell culture studies*

In a similar fashion to AD, a self-amplification cascade of caspase activation has been proposed for neuronal toxicity in polyglutamine disorders such as HD (Wellington and Hayden, 2000). Caspases, most prominently caspase 3, have been shown to cleave the wild type or mutant huntingtin protein, generating truncated fragments. Such truncated fragments containing expanded polyglutamine repeats have shown increased toxicity and propensity to aggregate compared to the full length protein, and may also influence the intracellular localization of aggregates (Goldberg et al., 1996; Lunkes and Mandel, 1998; Martindale et al., 1998; Wellington et al., 1998). Wellington et al. (2000) mutated the predicted caspase 3 cleavage site on the huntingtin protein. This resulted in reduced toxicity and aggregate formation, demonstrating a role for caspase-mediated cleavage of huntingtin in inclusion formation and death, at least within this cellular context.

Another site of potential caspase involvement in the pathogenesis of HD was suggested by Sanchez et al. (1999). These authors showed that caspase 8 was recruited to intracellular aggregates, most likely via its adaptor FADD, and subsequently activated, in neuronal cells expressing an expanded polyglutamine repeat. Caspase inhibition prevented death, but not inclusion formation. Similarly, Saudou et al. (1998), using the caspase 3-like inhibitor DEVD-CHO, prevented death, but not inclusion formation, in cultured striatal neurons expressing truncated forms of huntingtin with expanded polyglutamine repeats. In contrast, Kim et al. (1999), using immortalized striatal cells expressing truncated mutant forms of huntingtin, found that the caspase 3-like

inhibitor DEVD-FMK inhibited inclusion formation, but not death. The general caspase inhibitor zVAD-FMK prevented death, but not inclusions. The requirement of caspase activation for polyglutamine induced neuronal death was questioned by Moulder and co-workers (1999) where it was reported that caspase inhibition only slightly delayed rather than prevented neuronal death induced by a polyglutamine-GFP fusion protein. The discrepancies in these studies may be accounted for, at least in part, by differences in the cellular systems and forms of polyglutamine proteins used.

Taken together, these data suggest a scenario for HD (that is applicable to most other polyglutamine disorders as well, for example see Ellerby et al., 1999) in which mutant huntingtin is cleaved by low levels of caspase activity insufficient to kill the cell. This cleavage generates fragments that form aggregates and enhance susceptibility to apoptotic stimuli. Caspase-cleaved fragments may be further cleaved and thus enter the nucleus, which may be a critical event in the triggering of the death of the cell (Saudou et al., 1998). Aggregates may serve as the nidus for the recruitment and activation of caspases, as suggested by Sanchez et al. (1999). This would lead to an amplification cascade, in which further caspases are activated, leading to further huntingtin cleavage, aggregation and eventually death of the cell. Thus, caspases may be involved at multiple steps in this process. It is possible that different caspases are preferentially activated at each level (Wellington and Hayden, 2000).

#### *Animal models*

Transgenic mice (designated R6/2) that overexpress a fragment of human huntingtin protein with an extended polyglutamine region show reduced survival, neuronal aggregates, and behavioral deficits similar to HD (Mangiarini et al., 1996). In human HD cases there is widespread neuronal loss throughout the caudate, putamen and frontal lobes; however, in this mouse model, there have been conflicting reports concerning the presence of neuronal death and whether this death is apoptotic (Reddy et al., 1998; but see also Turmaine et al., 2000 for evidence of non-apoptotic neuronal loss in R6/2 HD mouse). In another recently described transgenic model, full-length mutant huntingtin is expressed under the control of its natural promoter. In this mouse, intranuclear aggregates of N-terminal huntingtin are seen as well as evidence of neuronal degeneration with some features of apoptotic death, such as condensation and marginization of chromatin, particularly in the lateral regions of the striatum (Hodgson et al., 1999).

Ona and colleagues (1999) recently showed evidence of caspase 1 (ICE) activity in the brains of the R6/2 mice. In addition, in double transgenic mice that also express a dominant negative inhibitor of ICE, disease onset and the formation of neuronal inclusions was significantly delayed, and survival was extended.

Single transgenic mice expressing only the mutant huntingtin protein that were treated with a general caspase inhibitor showed similar improvements (Ona et al., 1999). Other evidence of the involvement of caspases from animal models of HD is provided by the observation that caspases 1 and 3 are upregulated at the level of mRNA in the R6/2 HD transgenic mouse (Chen et al., 2000). The differences in neuropathological outcomes in the different transgenic models currently available make interpretation of the evidence concerning caspase activation difficult, particularly for the placement of caspase activation within the context of disease progression (i.e. cleavage of mutant or wild type huntingtin and/or downstream pro-apoptotic effects).

#### *Human studies*

Extending their studies from the cell culture and animal models of the disease, Ona et al. (1999) and Sanchez et al. (1999) have shown evidence for activation of caspase 1 and caspase 8 in HD brains. Activation of caspase 1 was ascertained through activation of its substrate, IL-1 $\beta$ . It is unclear however whether the activation of caspase 1 occurred within neurons. It is possible that the increase in IL-1 $\beta$  reflects instead an inflammatory reaction from microglial cells. Sanchez et al. (1999) showed that activated caspase 8 was present within insoluble fractions of HD brains, further supporting the idea that caspase 8 is recruited to aggregates and is subsequently activated.

#### **Amyotrophic Lateral Sclerosis (ALS)**

##### *Cell Culture Studies*

Mutant Superoxide Dismutase 1 (mSOD1) is responsible for a proportion of familial ALS cases, possibly through the triggering of oxidative stress. Overexpression of mSOD1 has accordingly been used in both cell culture and animal models of ALS. Ghadge et al. (1997), using neuronal PC12 cells, showed protection from mSOD1 expression-mediated apoptotic death with treatment with the general caspase inhibitor zVAD-FMK or the more specific ICE inhibitor YVAD-CMK. Pasinelli et al. (1998) showed activation of ICE in neuroblastoma cell lines expressing mSOD1. Inhibition of ICE-like caspases was partially effective in protecting these cells when they were exposed to oxidative stress. Using a different strategy, Troy et al. (1996) induced apoptotic death of PC12 cells and sympathetic neurons by inhibiting endogenous SOD1. They found that inhibitors with relative specificity for ICE were protective and that IL-1 $\beta$  was activated. In conjunction, these data suggest involvement of at least caspase 1 (ICE) in cell culture models of ALS.

##### *Animal models*

Several studies now indicate that transgenic mice

overexpressing mSOD1 also show evidence of caspase activation. Pasinelli et al. (1998), Li et al. (2000b), and Vukosavic et al. (2000) have shown processing and activation of caspase 1 in spinal cord lysates from such transgenic ALS mice. Spooren and Hengerer (2000), Li et al. (2000b) and Vukosavic et al. (2000) have also shown activation of caspase 3, by demonstrating positive CM1 staining, caspase 3 processing and caspase 3-like enzymatic activity. In one of these studies, it was shown that caspase 3 was activated subsequent to caspase 1 (Vukosavic et al., 2000). In view of these findings, Li et al. (2000b) suggested that early caspase 1 activation may lead to the processing and release of IL-1 $\beta$  which may act as a diffusible pro-apoptotic signaling molecule (see Troy et al., 1996; Fink et al., 1999). However, at this point it is not known whether caspase 1 is situated directly upstream of caspase 3. Activation of both caspases was significantly delayed in double transgenic mice that also overexpress the anti-apoptotic protein Bcl-2 (Vukosavic et al., 2000), suggesting that the previously reported beneficial effects of Bcl-2 in this model occur upstream of the caspases, likely at the level of the mitochondria (Kostic et al., 1997; Vukosavic et al., 1999). There is also evidence that caspase inhibition protects against mSOD1-induced motor neuron death. Age of onset of the disease was delayed, mortality was improved and behavioral benefits were observed in mice treated with a general caspase inhibitor (Li et al., 2000a,b). Double transgenics overexpressing, in addition to mSOD1, a dominant negative inhibitor of caspase 1 (DN 1) showed similar beneficial effects (Friedlander et al., 1997). Recently, it has also been reported that cytochrome c is released from mitochondria in asymptomatic transgenic mice expressing mutant SOD1, suggesting propagation of an apoptotic death pathway (Guegan et al., 2000). Additionally, double transgenic mice also expressing a dominant negative inhibitor of caspase 1 showed attenuated release of cytochrome c. Taken together these data suggest that a) there exists an early activation of caspases upstream of mitochondria in the spinal cord of transgenic ALS mice, and b) it is possible that an early inflammatory reaction may occur (e.g. the processing of IL-1 $\beta$ ) that initiates an apoptotic pathway and that the beneficial effects of caspase inhibition, particularly of caspase 1, may be due to the disruption of this response rather than of downstream effector caspase activity.

##### *Human studies*

Taken together these data from cell culture and animal studies suggest that caspases may be relevant to motor neuron death in ALS. Importantly, caspase inhibition appears to significantly delay the disease process in these models. A study of pathological material from human ALS cases further supports caspase involvement. Martin (1999) detected cleavage of DNA fragmentation factor-40 (DFF-40), a substrate of caspase 3, in both motor cortex and in spinal cord anterior horn

compared to normal controls, suggestive of caspase 3 activity. Evidence of caspase 3 activity was further provided by cleavage of labeled caspase 3-like pseudosubstrate by lysates from motor cortex, but not anterior horn. The lack of caspase 3 like activity detected in the anterior horn is surprising given the high levels of cleaved DFF-40 in this region; however this may have been related to the sampling technique employed by the author (tissue micropunches rather than dissected anterior horn; Martin, 1999). An increase of IL-1 $\beta$  in spinal cord of ALS patients was also reported by Li et al. (2000b), suggesting caspase 1 activation. As in HD, it is unclear whether this activation occurs within neurons, and whether it is related to apoptosis or to an inflammatory response.

## Ischemia

### *Animal models*

The involvement of caspases in the neuronal damage caused by stroke has been demonstrated in several systems using different models of ischemia. For example, cleavage of fluorogenic caspase 3-like substrates has been detected in brain homogenates following brief middle cerebral artery (MCA) occlusion (Chen et al., 1998; Fink et al., 1998); however the increase was not detected until approximately 9 hr following restoration of normoxia. Additionally processed fragments of the pro-form of caspase 3 were detected using western blotting. Moreover, infarct volume and caspase cleavage were reduced by treatment with a preferential caspase 3 like inhibitor. Molecular inhibition of caspase 3 has also been shown to reduce neuronal death and improve behavioral recovery in transient forebrain ischemia (Xu et al., 1999). This inhibition was induced by overexpression of X-chromosome linked inhibitor of apoptosis protein (XIAP), and confirmed by the reduction in appearance of immunoreactivity to the active p17/12 subunit of caspase 3 in transgenic mice. Conversely, while the caspase 3 like inhibitor DEVD-FMK was able to reduce the delayed neuronal death in the hippocampus and inhibit caspase 3 like activity, it did not prevent the deficits in long term potentiation in the CA1 region of the hippocampus following transient forebrain ischemia (Gillardon et al., 1999).

Evidence of caspase 1 like activation has also been reported in animal models of stroke. Transient MCA occlusion led to increased levels of IL-1 $\beta$  in cerebral tissue indicative of processing by ICE like caspases (Hara et al., 1997) which was inhibited, along with infarct size, by ICV administration of a broad spectrum caspase inhibitor ZVAD-FMK. Similar reductions in infarct size have also been seen in cortex and striatum by ICV administration of ZVAD-DBC 24 hr following transient focal ischemia (Loddick et al., 1996). Moreover, functional recovery was also observed in animals treated with ZVAD-FMK, the caspase 3 like inhibitor DEVD-FMK or the caspase 1 like inhibitor

YVAD-FMK, showing that caspase inhibition can not only ameliorate pathological changes associated with ischemic insults but also behavioral deficits. Additionally, transgenic mice expressing a dominant negative caspase 1 inhibitor, which show impaired processing of IL-1 $\beta$ , are also resistant to neuronal damage induced by focal ischemia (Friedlander et al., 1997) and show reduced neurological deficits compared to control animals.

In a model of transient spinal cord ischemia, the involvement of the Fas-caspase 8 death pathway was implicated (Matsushita et al., 2000). The active p18 subunit of caspase 8 was detected in neurons within the intermediate and ventral horn of ischemic spinal cord as early as 90 min following re-perfusion, followed temporally by activation of caspase 3. Additionally, Fas expression was found to increase within the same population of neurons, and evidence of the formation of Fas-caspase 8 death receptor complexes, using co-immunoprecipitation from spinal cord lysates, was reported. This sequential activation of caspase 8 followed by caspase 3 was also reported by Velier and co-workers (1999) using a panel of novel antibodies recognizing the active conformations of caspase 3 and 8 both *in situ* and in brain lysates. In a canine model of global ischemia, caspase 9, of which a proportion normally is located within the intermembrane space of mitochondria (e.g. Susin et al., 1999), translocates from the mitochondria to cytosolic and nuclear compartments (Krawecki et al., 1999) in neurons that appear pre-apoptotic.

Considerable evidence exists pointing to the involvement of IL-1 $\beta$  maturation in post-ischemic damage (see Touzani et al., 1999 for review). As outlined above, processing of IL-1 $\beta$  occurs relatively early, and it is thought that of the cysteine proteases currently known, only caspase 1 is capable of processing this cytokine (Talanian et al., 1997). Inhibition of caspase 1, either with pharmacological agents or by expressing dominant negative inhibitors, and the subsequent attenuation of IL-1 $\beta$  maturation may be the critical target for abrogation of ischemic damage. In support of this idea, IL-1 $\beta$  receptor antagonists have provided neuroprotection in models of ischemia and oxidative stress (e.g. Troy et al., 1996), thus indicating that activated IL-1 $\beta$  may initiate apoptotic pathways in neuronal cells. Animal studies of caspase activation in stroke point to several pathways involving different caspases. Additionally, the Fas-caspase 8 pathway and the subsequent activation of caspase 3 has been shown to play a role in the delayed neuronal death occurring following ischemic insults. However to our knowledge, the temporal relationship between caspase 1 mediated processing of IL-1 $\beta$  and activation of the caspase 8 pathway has not yet been examined.

### *Human studies*

It is now believed that apoptosis contributes to the loss of neurons during the post-ischemic period in a

wave of delayed neuronal death (see Choi, 1996; Charriaut-Marlangue et al., 1998 for review). Activation of caspase 3 has been observed in human brain following transient global ischemia (cardiac arrest), using immunoreactivity with the CM1 antibody (Love et al., 2000a), and elevated levels of caspase 3 immunoreactivity, using a non-specific antibody that recognizes both the active and pro-forms, has also been reported along with DNA fragmentation indicative of apoptotic neuronal death (Love et al., 2000b).

## Trauma

### *Animal studies*

As in ischemic insults, evidence exists that a significant loss of neurons, via an apoptotic mechanism, occurs during the period following the initial traumatic injury in both brain and spinal cord. Reproducible animal models are available to examine the biochemical pathways that mediate this death.

In the rodent model of spinal cord injury, there is evidence of both caspase 1 (Li et al., 2000a,b) and caspase 3 activation (Springer et al., 1999; Li et al., 2000a). Increased levels of mature IL-1 $\beta$ , indicative of caspase 1 activity, in addition to the appearance of processed active subunits of both caspase 1 and caspase 3 in spinal cord lysates, were seen (Li et al., 2000a). Localized application of the broad spectrum caspase inhibitor ZVAD.fmk or overexpression of a dominant negative caspase 1, significantly reduced not only apoptotic neuronal death and caspase 1 and 3 activity, but also significantly increased behavioral recovery. Of particular note was that caspase inhibition, either pharmacological or molecular, while ongoing since the initial injury, did not prevent early functional deterioration, but started showing a beneficial functional effect 5 days following injury (Li et al., 2000a). This suggests that caspase inhibition specifically targeted neurological deficits due to delayed neuronal (and non-neuronal) apoptotic death rather than the initial insult.

Similar techniques are utilized to model trauma that occurs to the brain as are used in spinal cord injury models. Here, there is also evidence of activation of caspase 1, from increased levels of processed IL-1 $\beta$  (Fink et al., 1999), and of caspase 3, from detection of enzymatic activity and of cleavage of the caspase 3 target proteins DNA-dependent protein kinase and inhibitor caspase-activated deoxyribonuclease (Clark et al., 2000). Additionally, neuroprotection and a reduction in caspase activation was observed in animals that were pretreated or simultaneously treated with the broad spectrum caspase inhibitor ZVAD.fmk or the caspase 1 like inhibitor YVAD.fmk (Fink et al., 1999), and the caspase 3 like inhibitor DEVD.fmk (Clark et al., 2000). Transgenic animals that overexpressed the dominant negative caspase 1 gene were also protected against brain trauma and showed amelioration of functional deficits (Fink et al., 1999). However, while inhibition of

caspase 3 like caspases had a significant effect on neuronal loss and biochemical changes following brain injury, there was no impact on functional recovery seen (Clark et al., 2000). In this model, an additional insult of brief hypoxemia was imposed which may have obscured any functional improvements afforded by caspase inhibition. It is also possible that in brain injury, inhibition of caspase 1, but not 3, may offer functional benefits, if the latter lies too far downstream in the cell death pathway. As in ischemic injury, it is possible that the critical point for intervention may be the caspase 1 mediated processing of IL-1 $\beta$ , which may lie upstream of initiation of a pro-apoptotic signaling cascade. However, a careful examination of the time course of IL-1 $\beta$  maturation with respect to the activation of downstream caspases is still lacking.

### *Human studies*

There is limited data available concerning the activation of caspases following traumatic brain or spinal cord injury in humans. However, evidence of both apoptotic neuronal and non-neuronal death and activation of caspase 3, using the active conformation-specific antibody CM1, has recently been reported in post-mortem tissue examined from human spinal cord injury patients (Emery et al., 1998). Additionally, tissue samples obtained from traumatic brain injury patients during surgical decompression procedures, revealed evidence of both apoptotic neuronal death and activation of caspase 1 and caspase 3, by the detection of the processed active subunits (Clark et al., 1999).

## Pitfalls

Despite compelling evidence that caspases are involved in neuronal cell death pathways that are relevant to neurological disease states, there are a number of pitfalls that need to be taken into account before one enthusiastically subscribes to the notion of administering caspase inhibitors for all neurological diseases in which cell death plays a role. These reservations are based on theoretical concerns, but also on some experimental data, mostly from neuronal cell culture experiments.

Although initially it appeared that caspase inhibition provided a prolonged protection from cell death in a variety of neuronal cell culture systems, it gradually became apparent that this protection was only transient. For example, DNA-damaged embryonic cortical neurons are protected from death for 12 hr through the use of general caspase inhibitors, but undergo a delayed cell death at 24-48 hrs (Stefanis et al., 1999). NGF-deprived sympathetic neurons are protected from death via caspase inhibition for 48-72 hrs, but they also die later on (Deshmukh et al., 2000). Similar observations have been made, as we already mentioned, in the models of A $\beta$ - and polyglutamine-induced death (Moulder et al., 1999; Giovanni et al., 2000). Why does caspase

inhibition in post-mitotic neurons provide only transient respite from death? We and others have proposed that this is due to the fact that caspase involvement lies downstream of the mitochondrial checkpoint (Green, 1998; Stefanis et al., 1999; Deshmukh et al., 2000). Indeed, inhibiting caspases does not prevent cytochrome c release from the mitochondria (Green, 1998; Neame et al., 1998; Stefanis et al., 1999; Deshmukh et al., 2000). Cytochrome c loss from the mitochondria would be expected to lead to dysfunction of the respiratory chain and energy depletion. This notion is supported by the fact that the mitochondria in the caspase inhibitor-treated cultures gradually lose their transmembrane potential, at the same time that they become committed to the delayed death (Stefanis et al., 1999; Deshmukh et al., 2000). Loss of mitochondrial function and transmembrane potential could lead to death through energy depletion or through other mechanisms, such as release of reactive oxygen species or calcium from the dysfunctioning mitochondria. Interestingly, a recent report suggests that the commitment point to death of NGF-deprived sympathetic neurons may depend on their functional state, and in particular on the presence or absence of axons. Axotomized BAF-treated neurons show no sustained survival compared to untreated neurons and, remarkably, eventually show depletion of mitochondria, despite preservation of the other intracellular organelles (Fletcher et al., 2000). These findings further support the idea that mitochondrial dysfunction may be a limiting factor for caspase-targeted therapies, at least in certain settings. Apart from cytochrome c, other apoptogenic factors may be released from the mitochondria, independent of caspase inhibition. One such factor is Apoptosis Inducing Factor (AIF), which translocates to the nucleus and causes cell death even in the presence of caspase inhibition (reviewed by Daugas et al., 2000). Other mechanisms have also been postulated to account for the transient effects of caspase inhibition on neuronal survival. For example, another type of cell death, autophagy, in which the cell creates double membrane structures that engulf and ultimately digest intracellular organelles, has been linked to the death that occurs in the presence of caspase inhibition (Xue et al., 1999).

Another issue, that is linked to the above, is the functional status of the neurons that survive through caspase inhibition. Neurons, for example the BAF-treated NGF-deprived sympathetic neurons, which are transiently protected from death, show decrease of cell size and metabolic rate. Never the less, these neurons, when treated again with NGF, rapidly increase their metabolic rate and enlarge (Deshmukh et al., 1996). These neurons are also able to repopulate their mitochondria with cytochrome c (Martinou et al., 1999; Fletcher et al., 2000). The caspase inhibitor-saved neurons also show neurophysiological alterations, which are reversible with reinstatement of NGF treatment (Werth et al., 2000). These data in conjunction indicate that the caspase inhibitor-saved neurons are functional, but at a lower level compared to healthy neurons.

Caspase inhibition in this setting serves to buy time for the neurons, until a more drastic intervention, in this case readdition of trophic factor, truly reverses the effects of the death stimulus.

There is also evidence that the dependence on caspases declines with neuronal maturation. Pharmacological caspase inhibition delays the death of DNA-damaged embryonic cortical neurons, but not of neurons derived from post-natal cultures (Johnson et al., 1999a,b). In addition, whereas cortical neurons derived from E16 caspase 3 knock-out mice are partially protected from DNA damage-induced death, neurons derived from E19 embryos are not (Keramaris et al., 2000). Caspase inhibition may therefore be more effective in preventing death induced at earlier developmental stages. This raises concerns about the use of caspase inhibitors in neurodegenerative diseases or stroke, which predominantly occur in older adults.

A theoretical concern that arises with the use of caspase inhibitors and other anti-apoptotic strategies is that normal cell turnover that occurs throughout life may be adversely affected by apoptosis inhibition. Another theoretical concern is that inhibiting apoptosis may lead to enhanced tumorigenesis. In addition, caspases may play other roles in the cells apart from mediating cell death. In this case, inhibiting caspases, especially long-term, may lead to disruption of cell homeostasis. However, no such role has been identified up till now, except for a role for caspase 1-like caspases in inflammatory responses. Such concerns, albeit theoretical, may have an impact on the design of caspase inhibitors. It appears that distinct members of the caspase family may be involved in neuronal death, depending on the initiating stimulus and the cell type (Troy et al., 1997; Park et al., 1998). One could envision therefore therapeutic strategies targeted against specific members of the caspase family, depending on the insult and the affected neuronal population. Thus, a more general inhibition of caspases and apoptotic pathways could be avoided.

## **Conclusion**

In conclusion, there is substantial evidence indicating that caspase inhibition may provide neuroprotective effects for a variety of neurological diseases. Recent animal studies in particular suggest that caspase inhibition significantly delays death and neurological dysfunction in a number of models of neurodegenerative disease and acute-subacute neurological insults, such as trauma and ischemia. In most cases however, it is unclear whether these beneficial effects are due to the anti-inflammatory properties that inhibition of caspase 1 affords, or are due to an effect on apoptotic pathways. In cell culture models, caspase inhibition generally provides only transient protection, most likely because of caspase-independent damage to the mitochondria. It remains to be seen whether in the animal models caspase inhibition

targets events upstream of the mitochondria. Emerging, but still controversial, evidence in certain neurodegenerative diseases suggests that such an upstream event targeted by caspase inhibitors could be the formation of toxic aggregates. Caspase inhibition may be more useful in acute or subacute insults, in which the death stimulus is present for only a limited period of time. Caspase inhibitors could also be used in conjunction with other treatments, which would be directed to the initiating events leading to cellular dysfunction and death. In this way, caspase inhibition may serve to "buy time" for neurons that are undergoing cell death, until more definitive treatment is administered.

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## Caspases in neurological disease

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