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# Caspase 6 activity initiates caspase 3 activation in cerebellar granule cell apoptosis

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## Abstract

**Using a well documented *ex vivo* system consisting of rodent cerebellar granule cells (CGCs) the activation of caspases 3 and 6 during apoptosis induced by withdrawal of trophic support was analyzed. At the time of deprivation, the addition of the irreversible, broad-spectrum caspase inhibitor zVADfmk or the cell permeable, caspase 6 inhibitor CP-VEID-cho can transiently suppress the appearance of apoptosis, including the early appearance of DNA fragmentation. Using immunoblotting and fluorogenic peptide assays we observe deprivation-induced activation of caspases 3 and 6, but not caspase 9. Furthermore, active caspase 6 is capable of processing and activating procaspase 3 in cellular extracts prepared from non-apoptotic CGCs, whereas caspase 3 failed to activate caspase 6. In consonant with this, the cell permeable caspase 6 inhibitor prevented deprivation-induced caspase 3 activation whereas a cell permeable caspase 3 inhibitor, CP-DEVD-cho, had no effect on caspase 6 activation. This would indicate that caspase 6 is a significant inducer of the early caspase 3 activity in apoptotic CGCs.** *Cell Death and Differentiation* (2000) **7,** 984–993.

**Keywords:** apoptosis; caspase 3; caspase 6; cerebellar granule cells; zVADfmk; trophic support withdrawal

**Abbreviations:** CGCs, cerebellar granule cells; KSD, high potassium/serum deprivation; KS, high potassium/serum; zVADfmk, benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone; zFAfmk, benzyloxycarbonyl-Phe-Ala-fluoromethylketone; DMSO, dimethyl sulphoxide; Ac-DEVD-amc, acetyl-Asp-Glu-Val-Asp-amino methyl coumarin; Ac-YVAD-amc, acetyl-Tyr-Val-Ala-Asp-amino methyl coumarin; Ac-DEVD-cho, acetyl-Asp-Glu-Val-Asp-1-al; Ac-VEID-amc, acetyl-Val-Iso-Glu-Asp-amino methyl coumarin; Ac-VEID-cho, acetyl-Val-Iso-Glu-Asp-1-al; Ac-IETD-amc, acetyl-Iso-Glu-Thr-Asp-amino methyl coumarin; Ac-IETD-cho, acetyl-Iso-Glu-Thr-Asp-1-al; Ac-LEHD-amc, acetyl-Leu-Glu-His-Asp-amino methyl coumarin; Ac-LEHD-cho, acetyl-Leu-Glu-His-Asp-1-al; CP-VEID-cho, Ac-Ala-Ala-Val-Ala-Leu-Leu-Pro-Ala-Val-Leu-Leu-Ala-Leu-Leu-Ala-Pro-Val-Glu-Ile-Asp-al; CP-DEVD-cho, Ac-Ala-Ala-Val-Ala-Leu-Leu-Pro-Ala-Val-Leu-Leu-Ala-Leu-Leu-Ala-Pro-Asp-Glu-Val-Asp-al

## Introduction

Caspase 3 or a caspase 3-like enzyme is activated in cerebellar granule cells (CGCs) when deprived of serum and/or the extracellular potassium is reduced from 25 to 5 mM (KSD).[1–5] The apoptosis induced by potassium and serum deprivation *in vitro* is proposed to mimic that which occurs naturally during morphogenetic cell death *in vivo* (naturally occurring cell death). Following deprivation there is an early increase in caspase 3 mRNA (~4 h) prior to the peak appearance of caspase 3-like cleavage activity and the appearance of the processed large, caspase 3 subunit. These changes precede the period of peak neuronal death following deprivation, consistent with descriptions of caspase 3 activation by death stimuli in other cell types.[6] In addition caspase 3-like activity has been detected in CGCs dying in culture in response to the addition of gluatamate[7] and the toxin 1-methyl-4-phenylpyridinium ($MPP^+$).[8]

Studies of mice homozygous null (−/−) for caspase 3 indicate that this enzyme is required for apoptosis in the developing brain as deletion results in proliferative zone enlargement with hypercellularity.[9] Caspase 9 −/− mice show a striking brain phenotype similar to caspase 3 −/− mice[10,11] with widespread failure of apoptosis and caspase 3 processing.[10] It is therefore likely that caspase 3 activation in neurons undergoing naturally occurring cell death due to trophic support withdrawal also requires the activation of other caspases.

To date there has been no detailed description of the expression or activation of caspases other than caspase 3-like activity in the CGC model of naturally occurring cell death (but see Schulz *et al*[12] and D'Mello *et al*[13]). The broad spectrum, irreversible caspase inhibitor zVAD-fmk (benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone) and inhibitors of macromolecular synthesis can both suppress cell death, caspase 3 processing and activation in this system. However caspase 3 is expressed in viable CGCs maintained in the presence of serum and depolarising potassium.[3] As activation of caspase 3 is also likely to be post-translational, this suggests that unidentified, upstream caspase(s) might be required for the activation of caspase 3 in CGCs. Many previous studies using extracts from non-neuronal cells have shown that caspases 8, 9 and 6[14,15] are capable of activating caspase 3. In partial characterisation of procaspase 3-activating caspases in deprived CGCs we observe that caspase 6 is processed, activated and necessary for caspase 3 activation.

## Results

Post-natal, rodent cerebellar granule cells (CGCs) in tissue culture remain viable for several weeks in the presence of serum and high potassium (25 mM; KS) medium.[16] When CGCs deprived of serum were switched to fresh medium containing a reduced concentration (5 mM) of potassium (KSD), greater than 70% of the neurons died within 36 h (Figure 1). The irreversible, broad-spectrum caspase inhibitor, zVADfmk, suppressed the death induced by KSD in a concentration dependent manner. Greater than 70% of those neurons that would have died with KSD treatment, retained statistically significant cellular dehydrogenase activity with KSD (Tukey test; $P<0.05$) and in the presence of the maximum effective concentration of zVADfmk (80 $\mu$M). Importantly an equimolar concentration of a control fluoromethyl ketone modified peptide, zFAfmk, a cathepsin B inhibitor which lacks aspartate in the preferred $P_1$ position, was ineffective. zVADfmk was however unable to prevent the death of all CGCs at its maximally effective concentration (Figure 2c; arrows). A fraction of the CGCs exposed to KSD that survive in the presence of zVADfmk developed the refractile, shrunken appearance typical of apoptotic neurons in KSD alone (Figure 2b). Thus treatment of KSD neurons with zVADfmk was able to prevent some, but not all the cellular changes associated with apoptosis, or was able to block all the changes but only in a subpopulation of CGCs.

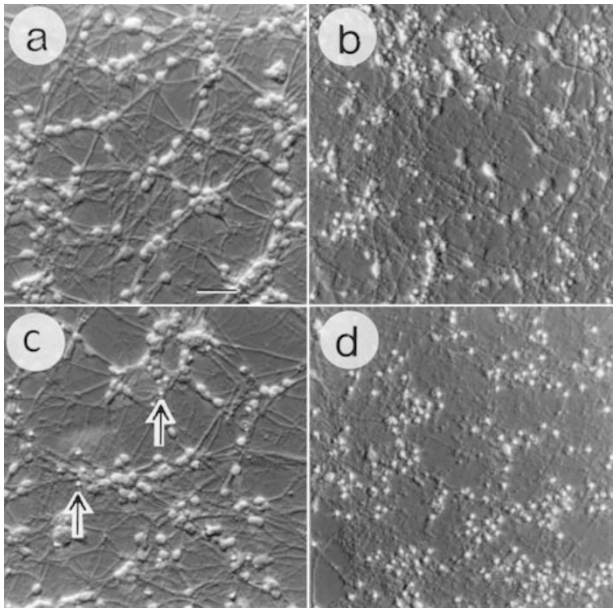


**Figure 2** zVADfmk can suppress apoptosis in cerebellar granule cells. Micrographs of neurons grown either continuously in the presence of KS (**a**), treated with KSD for 24 h (**b**) or treated with KSD but maintained either in the presence of zVADfmk (80 $\mu$M) (**c**) or the cysteine protease inhibitor, zFAfmk (**d**). Although a substantial number of KSD neurons survive in the presence of zVADfmk, some display the condensed morphology of apoptotic neurons (arrows in (**c**)). Bar=50 $\mu$m

### Active caspase profile in apoptotic CGCs

Cleavage activity appropriate for caspase 3-like (Ac-DEVD-amc) and caspase 6-like (Ac-VEID-amc) enzymes were readily detected in the supernatants from fractionated extracts prepared after 24 h with KSD (Figure 3B,C) but barely detectable in extracts prepared from viable, control neurons. The specificity of either caspase 3- or caspase 6-like enzyme activity was further characterised using reversible, aldehyde inhibitors for caspase 3 (Ac-DEVD-cho) or caspase 6 (Ac-VEID-cho). Prior analysis of these inhibitors using preparations of purified, recombinant human caspase 3 or 6 had shown the concentration of Ac-VEID-cho required to inhibit caspase 6 activity by 50% ($IC_{50}$=25 nM) to have no effect on maximum caspase 3 activity (data not shown). At this concentration the inhibitor Ac-VEID-cho totally inhibited the Ac-VEID-amc cleavage activity present in the CGC extracts (Figure 3B). Likewise Ac-DEVD-cho ($IC_{50}$=10 nM) totally inhibited the caspase 3-like activity (Figure 3C) in CGC extracts.

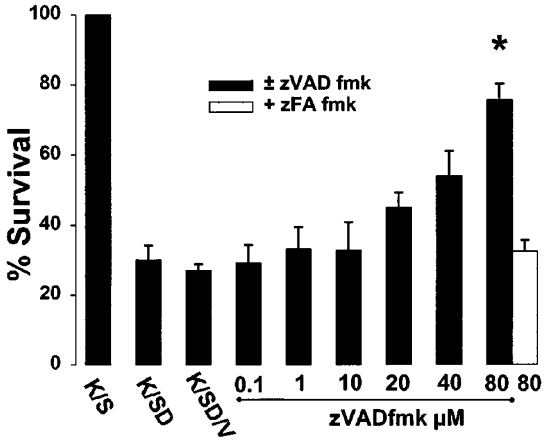


**Figure 1** The caspase inhibitor zVADfmk promotes the survival of high potassium and serum deprived (KSD) cerebellar granule cells. Bar chart illustrating the concentration dependent ability of a caspase inhibitor (zVADfmk; benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone) to promote the short-term survival of neurons exposed to KSD (5 mM potassium with no serum) for 36 h. Alternatively, deprived neurons were exposed to a control peptide, zFAfmk (benzyloxycarbonyl-Phe-Ala-fluoromethylketone). Survival was assessed using the MTS assay. Data are the means and standard error of the means for five separate experiments (*$P<0.05$ one-way ANOVA; Tukey test)

Despite the large amount of caspase 3- compared with caspase 6-like activity, we failed to detect any activity using the substrate Ac-IETD-amc (Figure 3A). The peptide sequence –IETDS- corresponds to the caspase recognition site at the junction between the large and small subunits in the zymogen form of caspase 3 and is thought to represent the first cleavage site that leads to caspase 3 activation.[18] Despite a failure to detect Ac-IETD-amc cleavage activity in CGC extracts, this peptide was readily utilised by caspase 8-like activity in extracts prepared from T cell hybridomas undergoing an activation-induced cell death (data not shown).

Using the peptide substrates Ac-WEHD-amc and Ac-LEHD-amc we failed to detect activity for caspases 1 and 9 respectively in extracts prepared at any time following KSD (data not shown). These substrates were readily utilised by

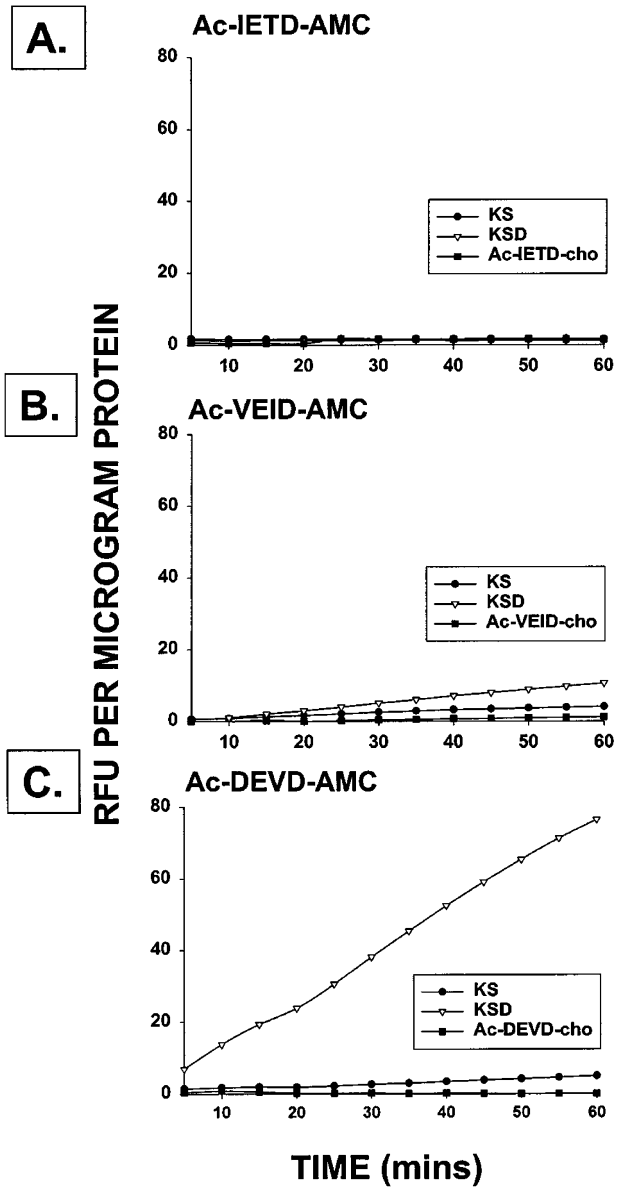


**Figure 3** Induction of caspase 3- and caspase 6-like activity in cerebellar granule cells with KSD. Plots of caspase activity in extracts from either normal neurons (KS) or from KSD neurons (24 h). Data is plotted as the relative units of fluorescence generated per minute from peptide substrates for caspase 8/procaspase 3 (Ac-IETD-AMC) (**A**), caspase 6-like (Ac-VEID-AMC) (**B**) and caspase 3-like (Ac-DEVD-AMC) activity (**C**). Specific activities were inhibited by the appropriate, reversible inhibitors [caspase 3-like, Ac-DEVD-cho (10 nM); caspase 6-like, Ac-VEID-cho (25 nM)]

the appropriate, purified recombinant enzyme in cell free assays. The inability to detect caspase 9 activity was a surprising result as previous studies had shown a release of mitochondrial cytochrome C with high potassium deprivation of CGCs[19,20] and cytochrome C in combination with dATP, apaf-1 and caspase 9 form a potent 'apoptosome' for caspase 3 activation. To independently assess whether caspase 9 was expressed in deprived CGCs, extracts were immunoblotted with an anti-caspase 9 antibody that detects zymogen and processed large subunit (Figure 4). In all CGC samples, bands were detected at 49, 40 kD and a prominent band at approximately 32 kD (double eschelon). The largest band probably corresponds to the zymogen, whereas the 32 kD band represents either a processed intermediate, or the caspase 9 variant that lacks the large subunit and catalytic site.[21,22] No band corresponding to the processed, large subunit of caspase 9 was detected in any of the deprived KSD samples. Reactivity of the antibody to processed caspase 9 was confirmed using an extract prepared from heat shocked Hela cells, which displayed a single band for the subunit (single eschelon).

## Caspases 6 and 3 are processed and concomitantly activated in cerebellar granule cells

As initial experiments had indicated abundant caspase 3 and some caspase 6 activity in CGCs treated with KSD for 24 h, an additional series of experiments were performed to study the relationship between the activities of these enzymes at various times after the onset of KSD. Extracts were prepared from KSD neurons at 1, 8, 24, 48 and 72 h of treatment. Caspase 3 and caspase 6-like activity was analyzed using fluorogenic peptide substrates as described previously. Caspase activity increased in excess of the very low detectable levels present in extracts from control neurons (KS) and caspase 3-like activity reached a maximum at 24 h of deprivation. This coincided with the peak of cell death, as assessed by counting the percentage of propidium iodide-stained dying profiles in similar KSD cultures (Figure 5A). Interestingly approximately 50% of the maximum activity of caspase 3 was attained within the first 8 h of KSD at a time when cell death, as judged by propidium iodide staining, was only about 30% complete. Although the cell counts indicated a high percentage of cell death at later time points, consistent with failure of the dead cells to be cleared, there was a decline in caspase 3 activity. Caspase 6-like activity was elevated within 1 h of KSD (Figure 5A) and remained approximately constant at subsequent analysis times.

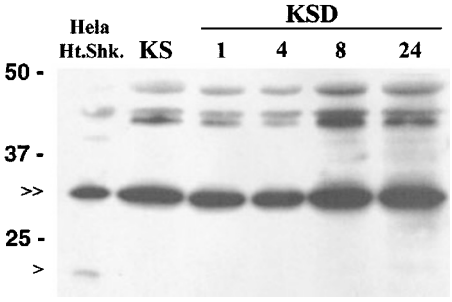


**Figure 4** Immunoblot to show the lack of detectable caspase 9 catalytic subunit in cerebellar granule cells with KSD. Immunoblot depicting the major anti-caspase 9 immunoreactive bands in CGC extracts with KSD (extracts resolved on 12% SDS–PAGE). A band (~49 kD), possibly representing zymogen, is present in all samples including those of neurons deprived for between 1 to 24 h (KSD). A prominent band at the 32–34 kD size is detected (double eschelon). Compared with a sample from heat shocked Hela cells that displays zymogen processing into the large subunit (single eschelon), no large subunit is detectable in any of the CGC samples

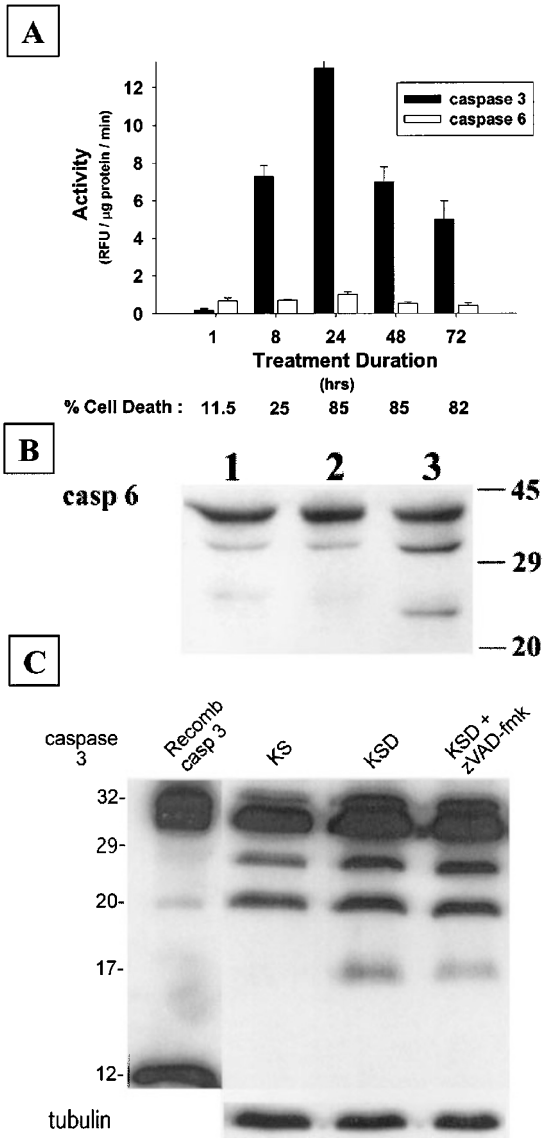


**Figure 5** Caspase 6 is activated and processed early following KSD of cerebellar granule cells. (**A**) Bar chart of levels for caspase 3- (Ac-DEVD-AMC) and caspase 6-like (Ac-VEID-AMC) activity in neurons with time following KSD. Values for KSD neurons have been normalised for the background activity from control neurons (KS; typically 1–5% of apoptotic values). Data is expressed as the mean (±S.E.M.) from three independent experiments. Shown beneath is the percentage of cell death assessed using propidium iodide. These percentages are adjusted for the low number of dead cells in normal (KS) cultures (<10%). (**B**) Immunoblot depicting the processing of caspase 6 in CGC extracts with KSD (extracts resolved on 12% SDS–PAGE). A major reactive band (~40 kD) representing zymogen is present in samples from KS (lane 1) and KSD for 1 h (lane 2) and 4 h (lane 3). Prominent bands of processed intermediaries (~30 and 24 kD) are detected only at 4 h KSD. (**C**) Immunoblot depicting the processing of procaspase 3 to yield a 17 kD subunit in KSD neurons but not control neurons (KS). Extracts were resolved on 15% SDS–PAGE. Anti-caspase 3 antibody detects a major band of apparent 32 kD and intermediates of apparent size 20 and 29 kD. Less of the large subunit (17 kD) is present in extracts treated with zVADfmk (80 $\mu$M)

As caspase 6-like activity was detectable at early times with KSD the processing of the caspase 6 zymogen was analyzed using immunoblotted extracts of neurons maintained in KS and those exposed to KSD for 1 and 4 h (Figure 5B). A rodent reactive, anti-caspase 6 polyclonal antibody that recognises zymogen and the large p18 subunit detected bands of approximately 40 kD and 30 kD in all extracts, however the 30 kD band was considerably more intense in the extract prepared from KSD-treated neurons for 4 h. The prominent 40 kD band probably represents the zymogen and the 30 kD band an intermediary. At 4 h of KSD there was an additional reactive band (approximately 24 kD) that was absent from KS samples and may well represent the processed large subunit and prodomain of the enzyme. It is likely that for caspase 6 the fluorogenic assay is more sensitive than immunoblotting with this antibody as the 24 kD band was undetectable in extracts from KSD-treated neurons at 1 h.

Processing and activation of caspase 3 was also confirmed using immunoblotting with an antibody that recognises the zymogen and large subunit. Extracts were prepared from control neurons (KS) and neurons exposed to KSD for 24 h, the peak time of caspase 3 activity. A ~30 kD band comigrated with recombinant human caspase 3 (Figure 5C). Bands at approximately 29 and 20 kD may represent intermediary processed forms of the enzyme. An additional band at 17 kD, which corresponds to the processed large subunit, was only detected however in extracts from KSD neurons or neurons exposed to KSD in the presence of zVADfmk (80 $\mu$M). Clearly the absence of the 17 kD band from the extract from control neurons was not due to under representation on the gel as stripping and reprobing of the same blot with anti-tubulin antibody revealed equivalent protein loading across the extracts. It is possible that zVADfmk partially suppresses caspase 3 processing as extract from neurons exposed to KSD and treated with zVADfmk appeared to show less reactivity for the p17 subunit.

## Inhibition of caspase 6 prevents caspase 3 activation and suppresses DNA fragmentation

Caspases can operate in a branched but co-ordinated cascade in vertebrate cells.[23,24] The capability of active caspases to initiate caspase activation in normal CGC extracts was analyzed using human recombinant, epitope-tagged caspases 3 and 6. Equivalent units of recombinant caspase 3 (0.5 ng) or 6 (1 ng) were incubated with non-apoptotic CGC extracts (200 $\mu$g) and caspase activity monitored using fluorogenic peptide substrates after separation of the exogenous and endogenous caspases. Untreated, control extract from non-apoptotic CGCs had basal levels of caspase 3 and 6 activity (Figure 6A). Recombinant caspase 6 was able to substantially activate CGC caspase 3 and to a lesser degree CGC caspase 6 itself. Analysis of the amount of added caspase 6 units compared with the recovered units of activity, indicated complete recovery of recombinant enzyme using the metal affinity resin (Figure 6A). Recombinant caspase 3 however was far less potent than caspase 6 in activating either endogenous caspase 3 or 6 (Figure 6A). Increasing the units of added recombinant caspase 3 increased the activation of CGC caspase 3 but failed to cause any considerable activation of caspase 6 activity (data not shown).

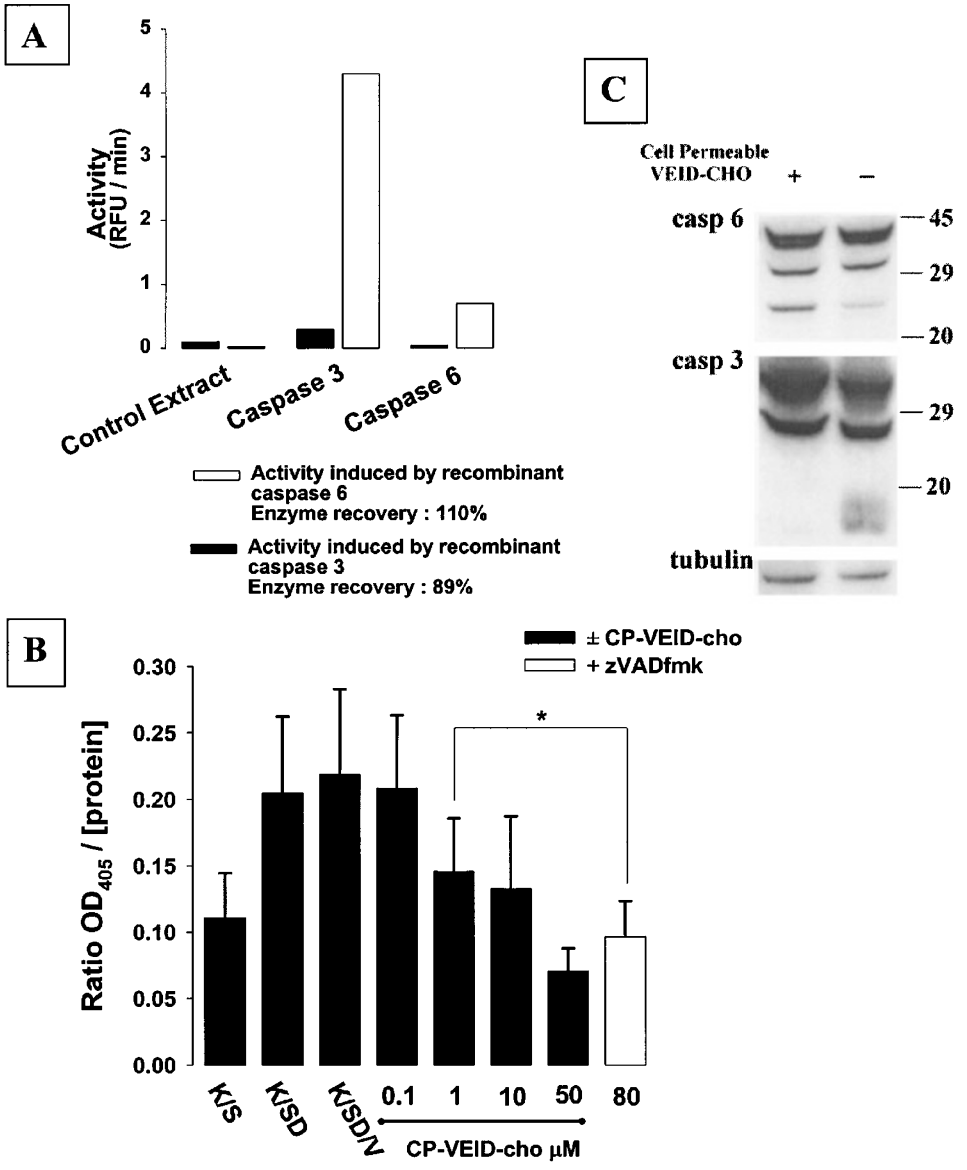


**Figure 6** Caspase 6 processes and activates caspase 3 in apoptotic CGCs. (**A**) Bar chart of a representative experiment (one of three) showing the generation of caspase 3- and caspase 6-like activities in non-apoptotic neurons via treatment of extracts with recombinant caspases. Enzyme recovery is expressed as a percentage of the input and output units of activity. (**B**) Bar chart illustrating the concentration dependent ability of a cell permeable caspase 6 inhibitor (CP-VEID-cho) to prevent histone/DNA fragmentation in neurons after 4 h of KSD. The amount of fragmented histone/DNA (ELISA product absorbance; OD 405 nm) is normalised for the protein concentration of each sample. Results are the means and standard error of the means for three separate experiments (*$P<0.001$ one way ANOVA; Bonferroni test for multiple comparisons with vehicle treatment only (K/SD/V). (**C**) Immunoblot depicts the inhibition of caspase 3 processing with the simultaneous treatment of CGCs with KSD and CP-VEID-cho (at 4 h). Extracts were resolved on 12% SDS–PAGE. Inhibitor treatment (+; 1 $\mu$M) prevents the generation of the large caspase 3 (p17) subunit, but does not affect caspase 6 processing. Caspase 3 and 6 processing occurs in the absence of inhibitor (–)

To assess the involvement of endogenous caspase 6 in initiating caspase 3 activation in CGCs with KSD, a cell permeable, reversible caspase 6 inhibitor (CP-VEID-cho) was firstly tested for its ability to suppress the appearance of apoptotic features. Any inhibitor effect was quantitated using an ELISA method for the detection of histone/DNA fragments, which are released in apoptosis of CGCs stimulated with KSD for 4 h. The previously used MTS assay of viability proved insensitive to detecting differences using the peptide inhibitor for this duration of KSD (data not shown). Caspase 6 inhibitor treatment suppressed DNA fragmentation in a concentration dependent manner (Figure 6B). The effect was statistically significant ($>1$ $\mu$M; Bonferroni test; $P<0.001$, three experiments) and maximal suppression was obtained at an inhibitor concentration of 50 $\mu$M, which was also similar to the effect achieved with exposure of KSD neurons to zVADfmk (80 $\mu$M). However inhibitor treatment for 24 h of KSD failed to suppress DNA fragmentation (data not shown).

Immunoblotting of CGC extracts prepared after 4 h of KSD in the presence of CP-VEID-cho (1 $\mu$M) showed caspase 6 to be processed normally, whereas the large

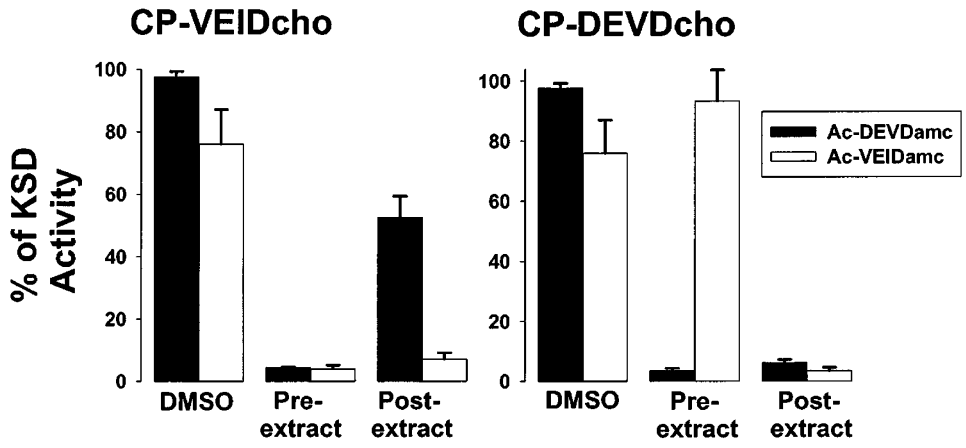


**Figure 7** Inhibition of caspase 6 in deprived CGCs prevents caspase 3 activation. Bar chart of activity for caspase 3- (Ac-DEVDamc) and caspase 6 (Ac-VEIDamc). Neurons were exposed to either KSD (4 h) or a combination of KSD and either cell permeable caspase 6 inhibitor (CP-VEIDcho), caspase 3 inhibitor (CP-DEVDcho) (1 $\mu$M; pre-extracts), or vehicle (DMSO). Activity was then compared with that from prepared KSD extracts subsequently treated with either caspase 6 or 3 inhibitor (1 $\mu$M (post-extract). Values are expressed as percentages of the maximum caspase 3 and 6 activities (240 and 45 RFU/ng protein/min respectively) and represents the mean ($\pm$S.E.M.) from three independent experiments

(p17) caspase 3 subunit was undetectable (Figure 6C). Conversely extracts prepared from KSD-treated neurons maintained in the absence of CP-VEID-cho revealed both caspase 6 and caspase 3 processing. To confirm that endogenous caspase 6 activity initiates caspase 3 activation after 4 h of KSD, CGCs were treated with either CP-VEID-cho or the cell permeable caspase 3 inhibitor, CP-DEVD-cho (1 $\mu$M). Subsequently extracts were analyzed for caspase 3-like (Ac-DEVD-amc) and caspase 6-like (Ac-VEID-amc) activity after the samples had been extensively washed to remove residual inhibitor. Treatment with CP-VEID-cho prior to extraction drastically reduced the level of both caspase 6 and 3 activities, whereas CP-DEVD-cho treatment had no effect on caspase 6 activity but nearly abolished total caspase 3 activity (Figure 7). Conversely, post-extraction treatment of deprived CGCs with CP-VEID-cho substantially suppressed caspase 6 activity but only reduced caspase 3 activity by 50% of that observed in KSD samples lacking inhibitor. The caspase 3 inhibitor CP-DEVD-cho, suppressed both caspase 3 and 6 activities.

To summarise, pre-extraction treatment of CGCs with caspase 6 inhibitor prevents the generation of caspase 3 activity, but only inhibits caspase 3 activity by 50% once it has been previously generated by caspase 6 with KSD for 4 h. Caspase 3 inhibitor treatment at pre-extraction times cannot prevent the generation of caspase 6 activity, but the inhibitor appears to potently suppress both caspase 3 and 6 activities once they have been generated in KSD extracts.

## Discussion

Using rodent cerebellar granule cells (CGCs) we have studied the processing and activation of caspases 3 and 6 during apoptosis induced *in vitro* by withdrawal of trophic support. The cell permeable, caspase 6 inhibitor (CP-VEID-cho) transiently suppressed the appearance of an early apoptotic feature concomitant with inhibition of caspase 3 processing and the generation of caspase 3 activity. This, in combination with the observation that a cell permeable, caspase 3 inhibitor (CP-DEVD-cho) had no effect on the generation of caspase 6 activity, indicates that caspase 6 is required for caspase 3 activation. In a congruous manner recombinant caspase 6 processed and activated procaspase 3 in cellular extracts prepared from normal, non-apoptotic CGCs, whereas recombinant caspase 3 failed to activate caspase 6 in the same extracts. Thus we propose that activated caspase 6 is the significant instigator of the early caspase 3 activity in apoptotic CGCs.

### Effector caspase 3-like activity predominates in CGC apoptosis

These observations on caspase 3 activation in apoptotic CGCs are in accordance with those of Armstrong *et al*,[3] who showed that deprivation from high potassium and serum induced caspase 3 processing. In a related set of studies CGC apoptosis was correlated with caspase 3-*like* activity induced by switching the cells to low potassium (5 mM), high serum-containing medium,[2,4,5,13] although processing of the zymogen was not always observed in these studies. Present findings are compatible with those showing caspase 3 mRNA induction[4] and zymogen processing[13] with serum deprivation alone and it is conceivable that a combination of low potassium and serum deprivation activates other nervous system-expressed, caspase 3-like enzymes (such as caspase 2). A proportion of the caspase 3-like activity in CGCs may therefore be provided by active caspase 2 and/or 7. We have detected the processing of caspase 2 in this model (data not shown). It is likely that active caspase 3 is the predominate caspase in the CGC extracts and is inhibited by zVADfmk, although a contribution from caspase 7 cannot be excluded. Caspase 7 mRNA has been shown to be expressed in embryonic CNS, albeit in low abundance.[25]

Maximum caspase 3-like activity is attained 24 h after KSD and yet immunoblotting with a caspase 3 antibody that recognises the zymogen and large subunit indicates only partial processing of the total cellular caspase 3 complement. In addition the processing of caspase 3 is relatively insensitive to zVADfmk treatment, consistent with this peptide possibly delaying rather than completely suppressing the full features of apoptosis. The apparent incomplete processing of caspase 3 could be due to a contribution of unprocessed caspase 3 retained in 20% of the neurons that, based on our estimates with propidium iodide staining, are still viable after 24 h of deprivation. Alternatively it might indicate that a fraction of caspase 3 remains unprocessed because it is protected from activating signals (other active caspases) that are more readily generated when the neurons are stimulated with KSD.

### Activation of caspase 3 by caspase 6 in apoptotic CGCs

Present throughout the duration of KSD a low amount of Ac-VEID-amc cleavage activity in CGCs is detected that is probably caspase 6 and not caspase 3, consistent with caspase 6 mRNA and protein expression. Caspase 3 itself has an almost exclusive preference for aspartate, rather than valine in the P4 position[26] and is thus unlikely to be utilising the Ac-VEID-amc substrate. In addition the reversible caspase 6 inhibitor, Ac-VEID-cho, was able to inhibit the Ac-VEID-amc cleavage activity and at a concentration which had been shown to have no effect on caspase 3 activity (consistent with the studies of Srinivasula *et al*[15]). Thus caspase activity distinct from that of caspase 3 and probably derived from the processing of procaspase 6 was present at early times of KSD. Caspase 6 in addition to caspases 8, 9 and 10 have been shown to activate caspase 3 in other cell types,[14,15,27,28] consistent with the proposed activation sequence in the caspase 3 zymogen (−IETDS-) being a possible caspase 6 cleavage site. Recombinant caspase 6 can catalyse the activation and processing of caspases 3 and 6 in non-apoptotic CGC extract (Figure 6), but the reciprocal activation of endogenous caspase 6 by recombinant caspase 3 does not occur. Caspase 6-mediated caspase 3 processing and activation in the extracts was totally blocked with the caspase 6 and 8 inhibitors Ac-VEID-cho and Ac-IETD-cho (1 $\mu$M) (data not shown).

We propose that caspase 6 activates caspase 3 in KSD-induced death of CGCs as immunoblotting of extracts prepared from KSD neurons treated with CP-VEID-cho showed caspase 6 but not caspase 3 processing and procaspase 6 is processed concomitantly with the onset of apoptosis following KSD. This proposal is also substantiated by the observation that CP-VEID-cho treatment inhibits the generation of both caspase 6 and 3 activities, whereas caspase 6 activity is unaffected in KSD neurons treated with CP-DEVD-cho. However caspase 6 may not be required for continuous caspase 3 activation as CP-VEID-cho was ineffective at preventing DNA fragmentation at 24 h of deprivation (data not shown). The notion that caspase 6 catalyses caspase 3 activation in KSD-induced neuronal death is also supported by a recent study which showed that caspase 6 co-purifies with and catalyses the Fas-independent processing of caspase 3 in Jurkat cell extracts.[29] To our knowledge this would be the first detailed description of a role for caspase 6 in the activation of a caspase 3-mediated apoptosis in neurons. However the strict conclusion that caspase 6 directly catalyses endogenous caspase 3 activation cannot be made as the peptide inhibitors may interfere with a caspase(s) of unknown identify. Activation of caspase 6 in experimental neuronal apoptosis induced by serum deprivation has been described previously.[30] Caspase 6 has also been shown to catalyse the proteolysis of two other neuronal substrates, the amyloid precursor protein (APP) and tau.[30,31] Caspase 6-mediated APP cleavage generates $\beta$−amyloid-containing fragments by an alternate (non-secretase) pathway in neurons.

We do not know how caspase 6 itself might be activated and whether an upstream caspase catalyses either or both caspase 3 and 6 activation in response to KSD. Recent genetic evidence has indicated that during naturally occurring cell death an apoptosis associated factor (apaf-1) and caspase 9-mediated cascade is required for caspase 3 activation. Analysis of the potential activation of caspase 9 in CGCs failed to detect either the presence of the large, processed subunit or enzyme activity. Estimations using the optimised substrate for recombinant human caspase 9, Ac-LEHD-amc, indicate that the lower limit of detection in the fluorogenic assay is approximately 100 pmoles of active enzyme (data not shown). Although a very low level of caspase 9 activation that is beyond this detection limit is possible, the findings are consistent with the majority of the caspase 9 being expressed in CGCs in the catalytically inactive form under these culture conditions. Cerebellar granule cells express a prominent immunoreactive band of approximate size that is similar to that described for the inactive, short variant of the enzyme.[21,22]

In summary, we have provided evidence for the co-ordinated activation of caspases 3 and 6 in the apoptosis of cerebellar granule cells induced by withdrawal of trophic support. As a result of these findings we propose that caspase 6 acts as the initiator in the activation of a proportion of caspase 3, thereby ensuring the efficient disposal of cerebellar granule cells following death induction. Future studies are aimed at identifying upstream factors regulating the activation of these and other caspases in models of neuronal apoptosis based on neurodevelopment and disease.

## Materials and Methods

### Materials

All general reagents were obtained from Sigma, tissue culture medium from either Gibco BRL or Sigma, zVADfmk and zFAfmk from Enzyme Systems Products (Dublin, CA, USA), CP-VEID-cho from Calbiochem and prepared as recommended as stocks in anhydrous DMSO. Ac-DEVD-amc, Ac-YVAD-amc and Ac-DEVD-cho were obtained from Bachem Feinchemikalien AG (Bachem UK Ltd), Ac-VEID-amc, Ac-

VEID-cho, Ac-IETD-amc and Ac-IETD-cho were obtained from Peptide Institute Inc. (Scientific Marketing Associates, UK). Recombinant caspases 3 and 6 were obtained from Pharmingen. Anti-caspase 3 antibody from Santa Cruz (Autogen Bioclear, UK), anti-caspase 6 antibody from Calbiochem, anti-caspase 9 antibody from Stressgen and other primary antibodies from Sigma. Secondary antibody conjugates were from Amersham-Pharmacia.

## Neuronal cultures

Cultures enriched for cerebellar granule cells were prepared from post-natal day 7 (P7) rats according to established protocols.[1,16] Briefly cerebella were cut into small pieces and incubated with versene (1 : 5000; Life Technologies, Gibco BRL) for 5 min at 37°C. Following titruation dissociated cells were collected by centrifugation and the versene replaced with complete culture medium (Basal medium Eagles (BME), 10% heat-inactivated FCS, 20 mM KCl, 2 mM L-glutamine, penicillin/streptomycin 1 : 100). Cells were plated on 35 mm diameter, 6-well plates previously prepared with poly-D-lysine and at a seeding density of approximately 3000 cells per $mm^2$ in complete culture medium. On the second day *in vitro* (DIV) cytosine arabinoside (10 $\mu$M) was added once to suppress non-neuronal cell proliferation. Cultures were then left to condition the medium for a further 5 DIV.

## Experimental design

Cultures were examined at 7 DIV and only those where the neurons appeared healthy (clusters of phase bright, small cell bodies amongst an extensive neuritic network) were taken for experimentation. At this time estimates of glial contamination, assessed by cells immunopositive for GFAP, was 5% or less (data not shown). Cultures maintained in high potassium and serum containing medium (KS) were induced to die by gently washing once with warm BME without serum and then maintained in complete medium with low potassium but without serum (BME, 2 mM L-glutamine, penicilllin/streptomycin 1 : 100) for the indicated period of time (KSD). The viable neurons maintained in the continued presence of KS were considered as control neurons. Preparations of caspase inhibitors and peptide reagents (diluted from DMSO stocks) were diluted into culture medium and added to the neurons at the time of deprivation.

## Viability assays

Viability was either assessed using the CellTiter 96® $AQ_{ueous}$ One Solution Cell Proliferation (MTS) assay (Promega), the Cell Death ELISA for histone/DNA fragmentation (Boehringer Mannheim) or direct cell counting following propidium iodide staining. At the stated time point the CellTiter 96®$AQ_{ueous}$ reagent was added directly to culture wells containing neurons, incubated for 2 h and then the absorbance of a sample of the supernatant read at 490 nm according to manufacturer's instructions. Diluted peptides in the absence of cells had no effect on the absorbance readings of the MTS compound. In stated experiments the OD readings from KSD neurons were expressed as a percentage of the maximum obtained from control neurons of the same age. For the Cell Death ELISA extracts of treated neurons were diluted (1 : 50) and centrifuged prior to performing the sandwich-based ELISA according to manufacturer's instructions. Absorbance readings (OD 405 nm) were normalised for variations in protein content of each sample by expressing data as a ratio of the ELISA optical densities (405 nm) and protein concentration (protein assay absorbance at 560 nm).

For direct counting of fluorescent viable and dead neurons, cultures were loaded with calcein-AM (acetoxy methyl ester; 5 $\mu$M, Molecular Probes) in serum free medium for 30 min at 37°C. Concomitantly they were stained with propidium iodide (PI; 5 $\mu$g/ml, Sigma). Following washing with warm, fresh medium neurons were visualised for calcein and propidium iodide fluorescence using appropriate excitation and emission filters (calcein; 495 nm $ex_{max}$, 535 nm $em_{max}$) (PI; 540 nm $ex_{max}$, 605 nm $em_{max}$). For each experimental condition three separate, randomly chosen fields of cells were visualised on a microscope and images digitised using a low light level camera (TILL Photonics). The numbers of clearly defined, single cell somas fluorescent at the appropriate wavelength was counted in individual fields. The number of PI-positive cells (defined as dying by loss of membrane integrity) was expressed as a percentage of the combined total number of cells that were fluorescent for PI and calcein. Combined total fluorescent profile counts varied between 300 and 600 per microscope field, consistent with variations in dissociated cell densities within individual wells). Cells possessing large, non-uniform cell somas (macroglia) or those neurons in aggregates where single cell identification was not possible were excluded.

## Fluorogenic peptide substrate assays for caspase activity

The assay for caspase activity in extracts from neurons was based on that described previously for caspase 3-like activity in apoptotic CGCs[3] and further refined to optimise conditions for enzyme activity.[17] At the stated time each tissue culture plate (6 wells; approximately $5 \times 10^6$ viable cells in total) was placed on ice, the cells scraped into the culture medium and all the material collected by centrifugation ($200 \times g$; 10 min). The pellets were washed twice with ice cold PBS and carefully drained pellets extracted in a minimal volume of ice cold buffer A (10 mM HEPES-KOH pH 7.4, 50 mM KCl, 2 mM $MgCl_2$, 5 mM EGTA, 0.5% CHAPS, 1 mM DTT, 1 mM PMSF, 10 $\mu$g/ml of aprotinin, pepstatin A and leupeptin). Extraction was at a given cell to volume ratio of approximately $2-3 \times 10^5$ cells per microlitre.

The cells were permitted to swell and lyse by incubating in buffer A on ice for 20 min. Following a single freeze/thaw cycle from $-70$°C to 4°C, the material was centrifuged for 30 min (4°C, $12\,000 \times g$) to remove nuclei, unlysed cells and membrane material. The protein concentration of the resulting supernatant was estimated using the BCA method (Pierce). The supernatant was then diluted between 5–10-fold with ice cold buffer B (25 mM HEPES-KOH pH 7.4, 50 mM NaCl, 2 mM $MgCl_2$, 5 mM EGTA, 1 mM EDTA, 10% sucrose, 0.1% CHAPS, 10 mM DTT). Caspase activity was determined at 37°C using fluorogenic, amino-methyl coumarin (AMC) coupled tetrapeptide substrates (25 $\mu$M final concentration) in a 96-well plate format ($ex_{max}$ 340 nm; $em_{max}$ 460 nm). Conditions were assessed in quadruplicate. Enzyme activity was either plotted as units of relative fluorescence intensity per microgram of total protein in the neuronal extracts, or as units of activity (relative fluorescent units/microgram protein/minute). For the latter, the rate of activity was calculated from only the linear rate of AMC accumulation, often within the first 30 min of the reaction. The substrates and inhibitors for each of the different caspase activities were: caspase 3, Ac-DEVD-amc, Ac-DEVD-cho; caspase-8, Ac-IETD-amc, Ac-IETD-cho; caspase 6, Ac-VEID-amc, Ac-VEID-cho; caspase-9, Ac-LEHD-amc, Ac-LEHD-cho.

Deprived CGCs that had been previously exposed to cell permeable, peptide inhibitors were extensively washed in PBS prior to subsequent extraction for analysis of caspase activity. In order to confirm that this was sufficient to remove residual inhibitor from KSD extracts, extract from non-apoptotic CGCs (KS) that had been previously exposed to inhibitors was supplemented with a known quantity of recombinant human caspase and the activity compared with recombinant enzyme alone.

## Immunoblotting

At stated treatment times approximately $5 \times 10^6$ cells were collected and washed in ice cold PBS. The cell pellet was immediately extracted in heated SDS–PAGE (2 × Laemlli) sample buffer. The DNA was sheared and the extracts normalised for protein content. Extracts (25–30 $\mu$g) were then resolved on either 10, 12 or 15% SDS–PAGE and transferred to either nitrocellulose (Hybond C, Amersham) or PVDF (Immobilon, Millipore) membranes (0.45 $\mu$m pore size) using standard methods. Membranes were briefly rinsed in either PBS or TBS (pH 7.4; 10 mM Tris-Cl, 100 mM NaCl) and then blocked in either PBS-Tween 20 or TBS-Tween 20 (0.05 or 0.1%) containing 5–10% non-fat milk powder for 1 h (PBS-TM or TBS-TM). Blots were incubated with one of the following primary antibodies in either PBS-T or TBS-T for 1 h. Anti-caspase 3 (1 : 400 Santa Cruz), anti-PARP (1 : 5000 Enzyme Systems Products), anti-caspase 6 (Calbiochem 1 : 50). Blots were then washed extensively and incubated with the appropriate secondary species-specific HRP conjugate (Amersham) diluted (1 : 1500) in PBS-TM or TBS-TM for 1 h. Following extensive washing, blots were rinsed in either PBS or TBS alone and then the immunoreactive bands visualised using ECL (Amersham).

To verify that approximately equivalent amounts of protein were loaded for different extracts on each gel, blots were subsequently stripped of antibodies and reprobed using antibodies for brain tubulin (1 : 2000 Sigma) or actin (1 : 500 Sigma). Membranes were first equilibrated in TBS, and proteins removed by incubation in stripping buffer (0.2 M glycine pH 2.2, 0.1% SDS, 0.1% Tween 20) for 30 min at room temperature. Incubation in ECL reagent confirmed that the antibodies had been totally removed. The blots were then blocked as described previously and incubated with the appropriate primary and secondary antibody conjugates as described.

## Recombinant caspase activation of neuronal extracts

Non-apoptotic neuronal extract was prepared as follows. Cerebellar granule cells previously maintained in KS were collected, washed in ice-cold PBS and extracted in buffer C at a ratio of $2 \times 10^5$ cells per microlitre (buffer C: 10 mM Tris-Cl pH 8.0, 100 mM NaCl, 0.1% CHAPS, 2 mM $MgCl_2$, 1 mM $\beta$-mercaptoethanol, pepstatin A, aprotinin, leupeptin (10 $\mu$g/ml), 1 mM PMSF). Cells were permitted to swell and lyse on ice for 30 min, followed by freeze and thawing. The extract was sonicated ($\mu$-probe sonicator, 3 × 5 s) to shear DNA then centrifuged (5 min, 2000 r.p.m., 4°C) to remove membrane fragments. The supernatant was adjusted to 4–5 mg/ml protein with buffer C. Two-hundred micrograms ($\mu$g) of neuronal extract was mixed with recombinant human (His-tagged) caspase 3 (0.5 ng) or caspase 6 (1 ng) and incubated for 1 h at 37°C. The recombinant enzymes were removed from the extracts by treatment with TALON (Clontech) metal affinity resin and the extracts then analyzed for caspase 3 and 6 activation using the appropriate peptide substrates. This procedure was previously optimised for the total removal of the recombinant caspase from the extract. Enzyme recovery was estimated by eluting the precipitated enzyme from the resin by incubation with EDTA (100 mM), and aliquots of equivalent units of enzyme activity compared with that for the same caspase sampled at the time of addition and preincubation with the extract.

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