

# Up-regulation of lysosomal cathepsin L and autophagy during neuronal death induced by reduced serum and potassium

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## Abstract

Serum and potassium deprivation-induced neuronal death on the primary culture of rat cerebellar granule neurons is being widely used as an *in vitro* model of neurodegeneration and neuronal apoptosis. In our experiments, serum and potassium deprivation for 12 h induced neuronal death in  $\approx 20\%$  of cerebellar granule neurons as demonstrated by Trypan Blue assay. Neuronal death was accompanied by a transient increase in the intralysosomal cathepsin L activity, which preceded neuronal death. During this time, the lysosomal membrane integrity remained preserved and no leakage of cathepsin L into the cytosol was seen. Ultrastructural analysis revealed the appearance of multiple vacuoles and autophagosomes in the cytoplasmic compartment of serum- and potassium-deprived granule neurons. Addition of selective cathepsin L inhibitors or of the autophagy inhibitor 3-methyladenine provided partial protection against serum and potassium deprivation-induced death. Our data also show that combining cathepsin L inhibitors and caspase-3 inhibitors leads to a synergistic neuroprotective effect against serum and potassium deprivation. The results of the current study suggest that activation of the autophagosomal-lysosomal compartment plays an important role in neuronal death induced by serum and potassium deprivation in cultured cerebellar granule cells.

## Introduction

Cerebellar granule cells, the most abundant neuronal type in the mammalian brain, have being widely used in culture-based studies of the mechanisms of neuronal death (Yan & Paul, 1997). The survival of the cerebellar granule cells *in vitro* is dependent on the presence of trophic factors and depolarizing conditions provided by the conditioned serum and high potassium concentrations. Removal of serum and potassium (serum and potassium deprivation; SPD) from the culture medium results in delayed neuronal death with the features of neuronal apoptosis such as nuclear condensation and DNA fragmentation as well as activation of caspase-3 (D'Mello *et al.*, 1993; Miller & Johnson, 1996; Yan & Paul, 1997; Marks *et al.*, 1998; Villalba, 1998). Other studies suggest, however, that SPD also leads to activation of caspase-3-independent death pathways. Indeed, caspase-3 inhibitors demonstrate only partial protection in this model of neuronal death (D'Mello *et al.*, 1998).

In the last decade, accumulating evidence suggests that programmed cell death is not confined to apoptosis but that cells use different pathways for active self-destruction. Characterizing several examples of physiological cell death, Clarke (1990) suggested that programmed cell death might involve classical apoptotic death, which is caspase-dependent (Type 1) and apoptotic-like or autophagic death (Type 2), which recruits lysosomal proteases in the execution of death. Autophagy

is a process that results in the degradation of cytosolic components inside lysosomes (Cuervo, 2004). It involves the formation of double-membrane structures, called autophagosomes or autophagic vacuoles, which fuse with the primary lysosomes where their contents are degraded. Autophagic activity is induced by nutrient starvation and seems to be crucial for cell adaptation and survival under extreme conditions (for review see Cuervo, 2004). Once a certain level of intracellular damage is reached, autophagy might become an effective trigger of the neuronal death (Bursch, 2001). Recent studies have demonstrated that many neurodegenerative disorders such as Parkinson's disease, Alzheimer's disease and Huntington's disease are accompanied by autophagy (for review see Larsen & Sulzer, 2002). An excessive autophagy has also been demonstrated in *in vitro* models of neurotoxicity such as dopamine-induced death in human neuroblastoma cells (Gomez-Santos *et al.*, 2003), nerve growth factor deprivation-induced death in cultured sympathetic neurons (Xue *et al.*, 1999) and methamphetamine-induced degeneration of cultured midbrain dopaminergic neurons (Larsen *et al.*, 2002). Recent studies have demonstrated an abundant overlap between apoptotic and autophagic cell death and suggest that forms of death have common aspects and might precede each other or even coexist in the same cell (Lemasters *et al.*, 1998; Bursch, 2001; Shimizu *et al.*, 2004; Canu *et al.*, 2005; Lum *et al.*, 2005).

In some models of neurodegeneration, activation of autophagy and apoptosis were accompanied by an increase in the activity of lysosomal cathepsins (Cataldo & Nixon, 1990; Bever & Garver, 1995; Uchiyama, 2001; Boland & Campbell, 2004), suggesting the involvement of lysosomal hydrolases in the death pathways.

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In the present study, we provide evidence that SPD induces activation of lysosomal cathepsin L and abundant autophagy, which participate in the execution of neuronal death in this model of neurodegeneration.

## Materials and methods

### *Cerebellar granule cell cultures*

Primary cultures of cerebellar granule cells were prepared according to the method described previously (Gallo *et al.*, 1982) with minor modifications. Wistar rats were obtained from Scanbur (Denmark) and maintained and bred in the local animal facility (12-h light : 12-h dark cycles, temperature 22 °C and humidity 45–55%). Eight-day-old Wistar rats were decapitated and cerebelli were dissected from the brains. Experiments conformed to local (Animal Protection Act 01.07. 2001) and international legislation (European convention for the protection of vertebrate animals used for experimental and other scientific purposes; Council of Europe, European Treaties ETS no. 123, Strasbourg, 18.03. 1986) guidelines on the ethical use of animals, and all efforts were made to minimize the number of animals used.

Cells were dissociated by mild trypsinization (0.025% trypsin) at 35 °C for 15 min, followed by trituration in a 0.004% DNase solution containing 0.05% soybean trypsin inhibitor. The cells were re-suspended in basal Eagle's medium with Earle's salts containing 10% foetal bovine serum, 25 mM KCl, 2 mM glutamine and 100 ng/mL gentamicin. The cell suspension was seeded in 35-mm dishes for toxicity experiments or in 8-well Laboratory-Tek™ II Chambered Coverglass (Nunc, Denmark) for fluorescence imaging, both precoated with 10 µg/mL of poly L-lysine (Sigma, USA). The cells were grown in a basal minimal Eagle's medium containing 25 mM KCl and 10% (v/v) fetal calf serum (Gibco-Invitrogen, Denmark) at a density of  $1.3\text{--}1.4 \times 10^6$  cells/mL for 7 days in a humidified 5% CO<sub>2</sub>–95% air atmosphere at 37 °C. The day after plating, 10 mM cytosine arabinoside was added to the cultures to prevent the proliferation of non-neuronal cells. For SPD, the medium was replaced with fresh basal minimal Eagle's medium with 5.3 mM KCl without serum. At 2, 4, 8 and 12 h following SPD the cells were assessed for cathepsin L activity and the occurrence of neuronal death. All drugs were added to the culture medium at the beginning of SPD.

### *Visualization of lysosomes*

LysoTracker Red DND-99 (Molecular Probes, USA) is a weak basic amine freely permeant to cell membranes that selectively accumulates in cellular compartments with low internal pH. The cells were exposed to SPD and at various times following deprivation the cells were treated with 50 nM LysoTracker Red for 60 min at 37 °C and then examined by confocal microscopy at excitation and emission wavelengths of 568 and 605 nm, respectively. Parallel measurements were made from acridine orange accumulating in acidic cellular compartments, and also staining nuclei. Neurons were treated with 5 µg/mL dye for 10 min at 37 °C then rinsed in a complete culture medium and, 10 min later, were examined with confocal microscopy at excitation and emission wavelengths of 488 and 585 nm.

### *Determination of cathepsin L activity by (CBZ-Phe-Arg)<sub>2</sub>-R110 staining*

Lysosomal cathepsin L activity was measured *in situ* using the dipeptidyl rhodamine diamide substrate (CBZ-Phe-Arg)<sub>2</sub>-R110 (Molecular Probes). This nonfluorescent substrate penetrates lysosomal

membrane where it becomes cleaved by active cathepsin L and cleavage products emit a green fluorescent signal. The substrate is 800-fold more selective for cathepsin L than for cathepsin B, thus allowing a relatively selective detection of cathepsin L activity in the presence of high activity of cathepsin B (Assfalg-Machleidt *et al.*, 1992). Neurons were exposed to 10 µM of (CBZ-Phe-Arg)<sub>2</sub>-R110 for 60 min, rinsed in PBS and examined via confocal laser scanning microscopy on excitation at 488 nm. Emission, at 522 nm from randomly chosen fields (×200 magnification), was recorded and the mean pixel intensity was calculated using LaserSharp 2000 V4 software.

### *Immunohistochemical detection of cathepsin L*

Cells were fixed with 4% paraformaldehyde solution for 20 min at room temperature and washed twice with 0.1 M phosphate-buffered saline (PBS); 0.1% Triton X-100 in PBS was added for 10 min to permeabilize cells. Cells were incubated with mouse monoclonal antibodies recognizing rat cathepsin L (Alexis Corporation, Switzerland; 1 : 250 dilution) for 30 min followed by incubation with biotinylated secondary antimouse IgG (1 : 100 dilution) for 1 h, and were visualized with the streptavidin–biotin peroxidase method (ABC system and diaminobenzidine as chromogen; Vector Laboratories, UK). All antibodies were diluted in PBS containing 3% goat serum, 0.25% Triton X-100 and 0.5% Tween-20. The expression of cathepsin L was evaluated microscopically. The level of cathepsin L expression was determined as average pixel intensity in a single cell using MicroImage 1.0 software (Olympus, Japan).

### *Assays for neuronal death*

The neuronal death was quantitatively assessed using the Trypan Blue exclusion method. For the Trypan Blue assay, the cultures were washed with PBS (145 mM NaCl, 3 mM KCl, 0.42 mM Na<sub>2</sub>HPO<sub>4</sub>, 2.4 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.4), and then incubated with a 0.4% Trypan Blue solution at room temperature for 10 min. At the end of incubation the cells were washed twice with PBS. In some experiments neuronal death was also assayed using the TUNEL method as described earlier (Kaasik *et al.*, 2001) using an ApopTag Plus peroxidase kit (Chemicon International, UK). Briefly, fixed and permeabilized cells were incubated at 37 °C for 60 min in a mixture containing 40 µM biotin–16-dUTP, 0.5 U/µL terminal deoxytransferase, 25 mM Tris HCl, 200 mM sodium cacodylate pH 6.6 and 2.5 mM cobalt chloride. The biotinylated DNA was linked to extravidin–alkaline phosphatase conjugate and visualized with 5-bromo-4-chloro-3-indolyl-phosphate, 4-toluidine salt and nitro blue tetrazolium chloride colour reaction. The cells, with DNA fragmentation, were identified morphologically by the presence of condensed dark blue nuclei.

Trypan Blue- or TUNEL-stained culture dishes were mounted on the motorized microscope stage (BX51 with 20× lenses; Olympus) connected with a video camera and five to eight random fields (305 × 232 µm), each containing 100–200 neurons, were acquired and stored in the computer (final magnification of images was 930×). The images were later analysed by blind observer, using the CAST program (Olympus, Denmark). The number of Trypan Blue- or TUNEL-positive cells and total number of cells were counted in eight randomly chosen fields in each dish (1000–1500 cells per dish). At least three dishes were used for each data point and experiments were performed twice.

The following drugs were tested against neuronal death induced by SPD: cathepsin L inhibitors N-(benzyloxycarbonyl)-L-phenyl-alanyl-L-tyrosinal (FY-CHO; Merck/Calbiochem, Germany)

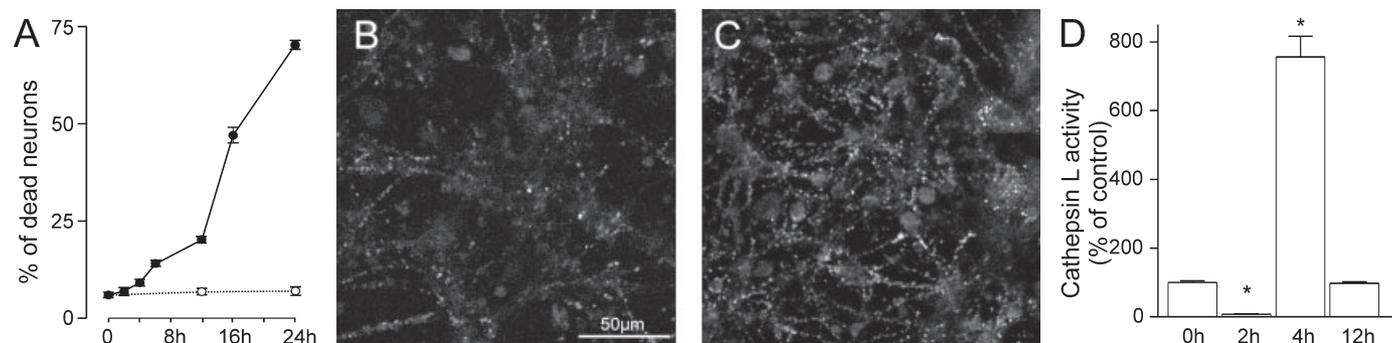


FIG. 1. Time course of neuronal death and cathepsin L activity in SPD-treated cerebellar granule neurons. (A) ●, toxicity in SPD-treated cultures; ○, untreated, control cultures. Cell death or survival was measured using the Trypan Blue assay. The data are mean  $\pm$  SEM and at least three dishes per data point were used in each experiment. (B) Neurons were treated with 10  $\mu$ M of (CBZ-Phe-Arg)<sub>2</sub>-R110 for 60 min and examined with confocal microscopy at excitation and emission wavelengths of 488 and 522 nm. (C) Culture after 4 h of SPD. (D) Mean intensity of fluorescence (mean pixel intensity) reflecting the cathepsin L activity at various times following SPD. The data are mean  $\pm$  SEM of three independent experiments. \* $P$  < 0.05 (one-way ANOVA followed by the Bonferroni test).

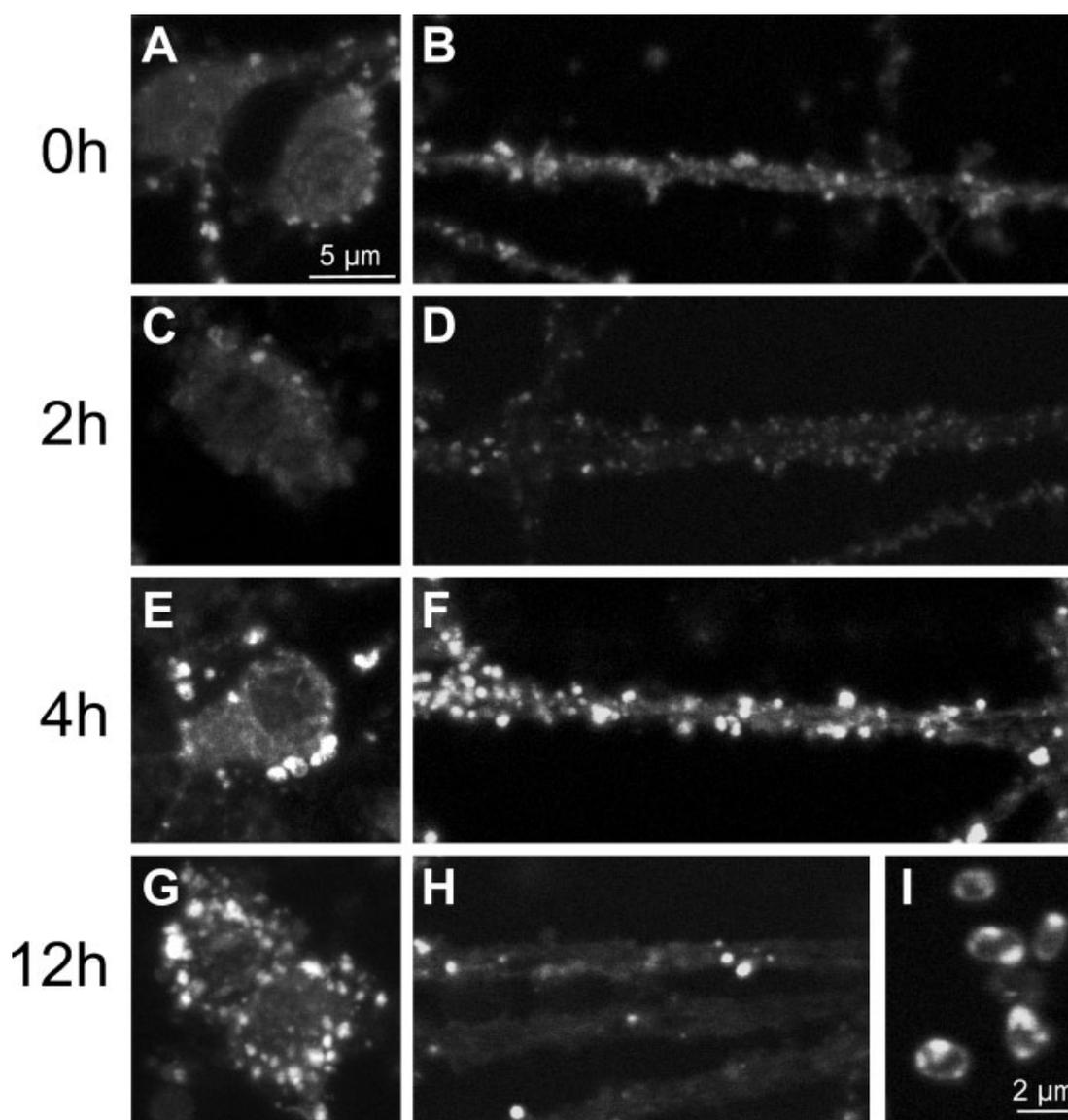


FIG. 2. Representative microphotographs of the localization of cathepsin L activity in (left column: A, C, E and G) perikarya and (right column: B, D, F and H) neurites of neurons exposed to SPD for 0 (control), 2, 4 and 12 h of SPD. (I) Multiple freely floating vacuoles with retained cathepsin L activity found in the medium. The experiments were repeated two to four times with similar results.

and N-Acetyl-L-leucyl-L-leucyl-L-methional (ALLM; Tokris, UK) and the cathepsin B inhibitor *L-trans*-Epoxy succinyl-Ile-Pro-OMe propylamide (CA-074Me; Peptides International, USA), the irreversible caspase-3 inhibitor DEVD-fmk (Merck-Calbiochem, Germany) and the autophagy inhibitor 3-methyladenine (3-MA; Sigma, USA).

#### Electron microscopy

Cultures were fixed for 1 h at 4 °C in 2.5% glutaraldehyde in a cacodylate buffer (pH 7.4). Fixed cells were then scraped off and centrifuged at 300 *g* for 5 min. After washing the pellet in buffer, cells were postfixed in 2% osmium tetroxide for 1 h. The samples were dehydrated in graded alcohol and embedded in Epon 812. The ultrathin sections were stained with lead citrate and uranyl acetate and studied under transmission electron microscopy using a Tecnai 10 (FEI Co., Eindhoven, the Netherlands). For quantitative analysis, randomly photographed cells, at a magnification of 10 000 $\times$  and 20 000 $\times$ , were analysed using Adobe Photoshop; electron-dense vacuoles, containing a heterogeneous material, were identified as autophagosomes. These vacuoles were further examined at higher magnification (32 000 $\times$ ).

#### Statistics

Data were analysed using one-way ANOVA followed by the Bonferroni multiple comparison test. In all instances,  $P < 0.05$  was considered statistically significant.

## Results

### Neuronal death in cerebellar granule cells following SPD

Rat cerebellar granule neurons were cultured for 7 days in a depolarizing concentration of potassium (25 mM) in the presence of serum. Simultaneous lowering of potassium concentrations to 5 mM and removal of serum (e.g. SPD) led to a loss of neuronal viability. The first signs of neuronal death were observed after 4 h of deprivation, following which there was a progressive increase in the number of dead neurons (Fig. 1A). At 12 h of deprivation 20% of neurons were Trypan Blue-positive whereas at 24 h 70% of cells had died. The TUNEL assay, which detects cells with internucleosomal DNA fragmentation (hallmark of apoptosis), showed that at 12 h of SPD 47% of dead neurons had nuclear features of apoptosis.

### Cathepsin L activity in SPD neurons

Our next task was to find out whether SPD-induced neuronal death is accompanied by cathepsin L activation. Cathepsin L activity, in intact neurons, was measured using the dipeptidyl rhodamine diamide substrate (CBZ-Phe-Arg)<sub>2</sub>-R110. The activity of cathepsin L is reflected by the mean pixel intensity of green fluorescence. Control cultures demonstrated a very low activity of cathepsin L, as was demonstrated by the low intensity of green fluorescence (Fig. 1B). SPD induced bi-directional changes in the activity of cathepsin L: a small but significant decrease at 2 h of SPD was followed by a

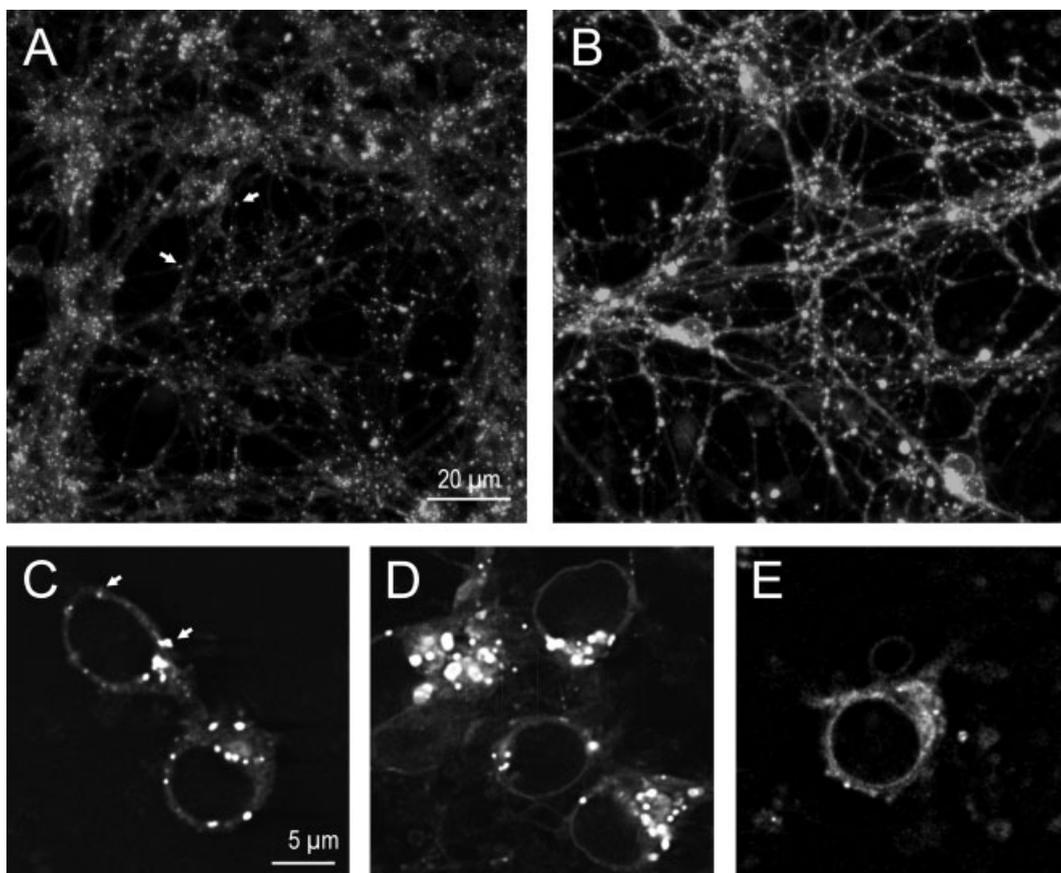


Fig. 3. Representative microphotographs demonstrating lysosomal membrane integrity in (A and C) control neurons and in (B and D) neurons exposed to SPD for 12 h at (A and B) low and (C and D) high magnification. The cells were exposed to SPD and treated with LysoTracker Red for 60 min and visualized using confocal laser scanning microscopy, as given in Materials and Methods. (E) Leakage of dye from lysosomes in neurons treated with a protonophore, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone. Some representative lysosomes are marked with arrows. Experiments were repeated three times with similar results.

significant increase in cathepsin L activity. This initial decrease is not fully understood and could also be explained by slower accumulation of the probe into the lysosomal compartment under SPD conditions. An increased activity of cathepsin L was observed at 4–8 h following SPD (Fig. 1C) while at 12 h of deprivation the activity cathepsin L had declined to control levels (Fig. 1D).

In order to obtain more information about subcellular localization of cathepsin L-related activity, the neurons, from control and SPD cultures, were also examined at higher magnification. Figure 2 shows that a (CBZ-Phe-Arg)<sub>2</sub>-R110-derived increased fluorescence signal was found in lysosomes located in both neurites and perikarya and that the signal was increased considerably in both regions after 4 h. At 12 h of SPD, part of the the cathepsin L activity had translocated from neurites to perikarya: no signal was any longer observed in neurites while the signal in perikarya increased considerably. The disappearance of the cathepsin L activity from neurites at the 12-h time point might be related to the neurite degeneration, whilst neuronal cell bodies remain intact at this time point. At the 12-h time point multiple vesicles with fluorescent signal were found in the medium and probably represent lysosomes released from ruptured neurites (Fig. 2, panel D). However, as neurites are responsible for most of the signal this explains the decreased total intensity demonstrated in Fig. 1. No reliable signal could be seen outside lysosomes at any time point during 12 h of SPD.

#### Lysosomal membrane integrity

Previous studies have demonstrated that active cathepsins could translocate or leak from ruptured lysosomes into the cytosol and

initiate there the cellular death program (Jäättelä & Tschopp, 2003). To determine whether SPD induces damage of lysosomal membranes, leading to leakage of cathepsins into the cytosol, we applied a LysoTracker Red uptake test. Rupture of LysoTracker Red-loaded lysosomes should abolish the proton gradient across the lysosomal membrane and lead to immediate release of dye. Indeed, as shown in Fig. 3E, treatment of lysosomes with a protonophore, carbonylcyanide p-trifluoromethoxyphenylhydrazone, led to leakage of dye from lysosomes within minutes. During 2–12 h of SPD, we failed to find any signs of lysosomal rupture before onset of detectable neuronal death (Fig. 3). On the contrary, SPD-treated cells appeared to have increased staining with LysoTracker Red compared to untreated cells. This could be explained by increased lysosomal activity as LysoTracker Red accumulation depends on lysosomal pH. Only in some neurons, with clear nuclear condensation, had the LysoTracker Red fluorescence signal disappeared from lysosomes, suggesting a rupture of the lysosomal membrane. Similarly, no leakage from the lysosomal compartment was observed when acridine orange was used (data not shown).

#### Immunocytochemistry of cathepsin L

In a separate series of experiments we tested the expression of cathepsin L under SPD conditions. Cultures were stained with antibodies recognizing rat cathepsin L, but not rat procathepsin L or other cathepsins (Weber *et al.*, 1997). A quantitative analysis of cathepsin L immunoreactivity, at the single-cell level, identified two different types of cells: intensely stained cells with normal morphology and 'pale' cells that were shrunken and often had an irregular shape, both typical of cell death (Fig. 4). The fraction of pale cells was negligible in control (3%) and 4-h SDP groups (5%)

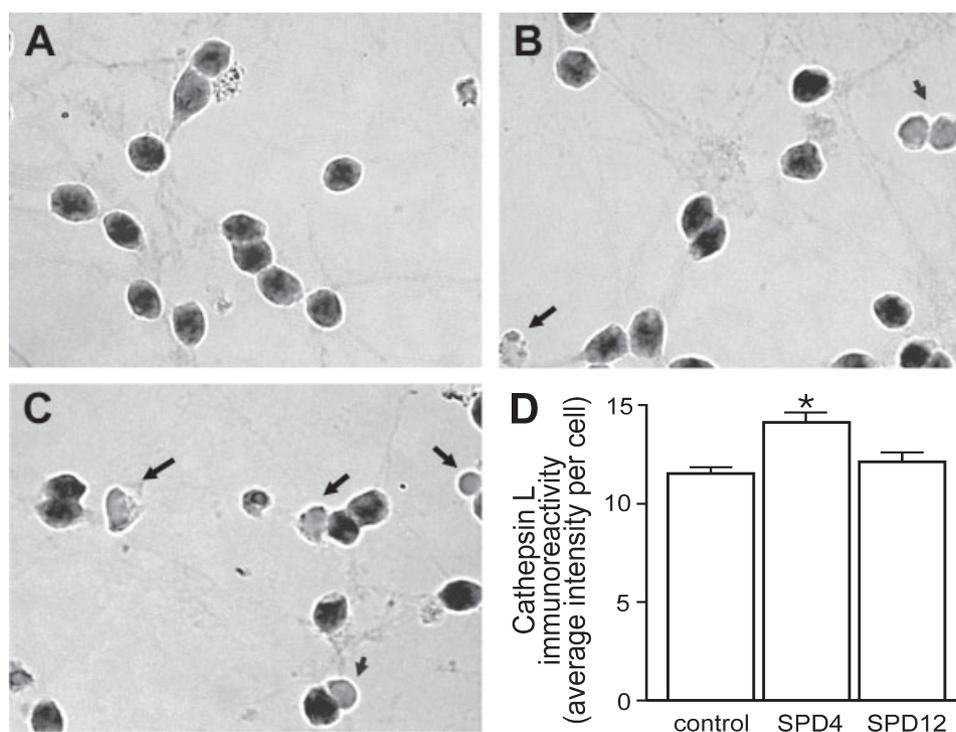


FIG. 4. Cathepsin L-immunoreactive cerebellar granule cells in (A) control cultures and in (B,C) cultures exposed to SPD for (B) 4 and (C) 12 h. (D) A quantitative analysis of cathepsin L immunoreactivity shows the average pixel intensity at the single-cell level ( $n = 100$ –110 cells from three independent experiments). Data are expressed as mean  $\pm$  SEM. \* $P < 0.05$  (one-way ANOVA followed by the Bonferroni test). Pale or dead cells are shown with arrows.

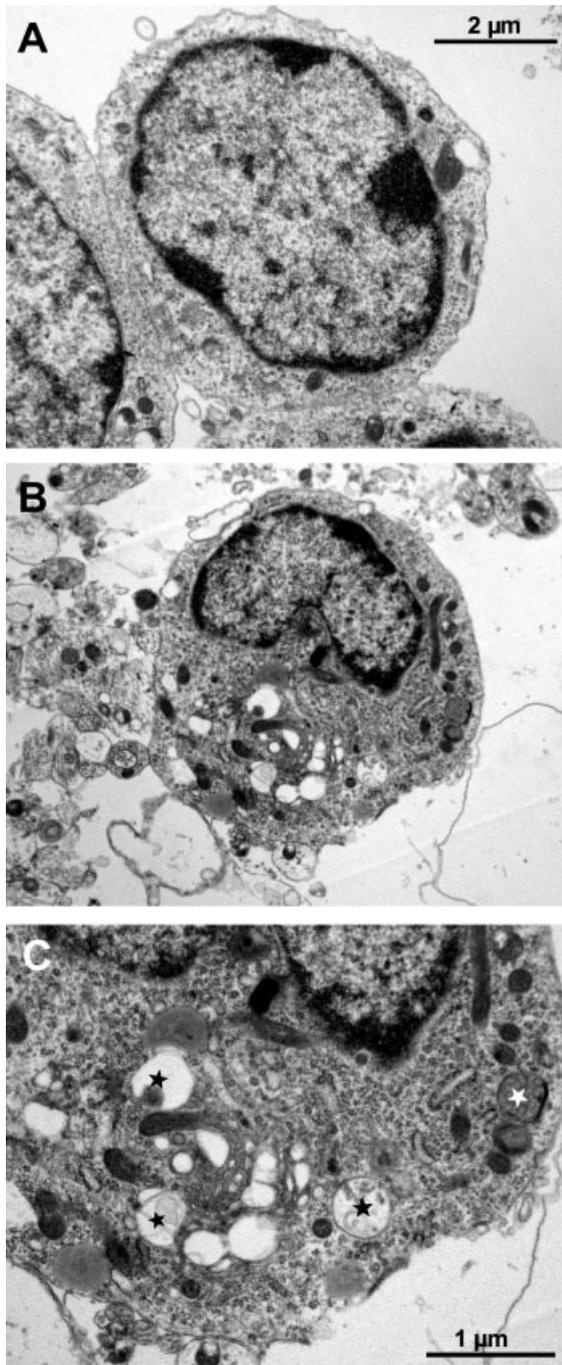


FIG. 5. Electron microscopy of cerebellar granule cells in (A) control cultures and in (B and C) cultures exposed to SPD for 12 h; magnification in C is twice that in B. Autophagic vacuoles, present in cytoplasm of SPD exposed neurons, are marked with asterisks. The experiment was repeated twice with similar results. Scale bar in A also applies to B.

but reached 20% in the 12-h SPD group. These data suggest that dying or dead cells lose their cathepsin L immunoreactivity and therefore we excluded pale cells from further analysis. Analysis of average pixel intensity in the viable population demonstrated a 23% increase in cathepsin L immunoreactivity in 4-h SPD cells when compared with control cells, but no increase after 12-h SPD (Fig. 4D).

#### Ultrastructural analysis of SPD granule neurons

Because activation of lysosomal enzymes is often associated with activation of autophagy, our next step was to examine a possible occurrence of autophagosomes in the neurons under conditions of SPD. A quantitative ultrastructural electron microscopy analysis revealed an increased number and area of autophagic vacuoles in 12-h SPD-treated neurons (Fig. 5, asterisks). The number of autophagic vacuoles increased 3.9-fold and the area of autophagic vacuoles 2.5-fold when compared with control values (Table 1).

#### Effects of autophagy-, caspase- and cathepsin inhibitors on SPD-induced death

Because morphological examination revealed the existence of autophagosomes and biochemical analysis revealed activation of lysosomal cathepsin L, our next task was to test whether activation of autophagy contributes to the neuronal death under SPD. For that purpose we tested the neuroprotective actions of the autophagy inhibitor 3-MA (which inhibits autophagy at the sequestration phase). In low micromolar concentrations, 3-MA failed to afford any neuroprotection. However, when 3-MA was added in millimolar concentrations (5 mM), 59% neuroprotection was observed (Fig. 6). The protective effect of 3-MA cannot be explained solely by the inhibition of apoptotic cell death as the caspase-3 inhibitor DEVD-fmk, 100  $\mu$ M, rescued only 31% of the neurons (Fig. 6).

Next, we tested the efficiency of selective cathepsin L inhibitors in suppressing the SPD-induced intralysosomal cathepsin-like activity. For that purpose the cultures were exposed to SPD for 4 h, to obtain maximal activation, and then exposed for 1 h to selective cathepsin L inhibitors, ALLM or FY-CHO. Both cathepsin L inhibitors concentration dependently inhibited intralysosomal cathepsin L activity as demonstrated in Fig. 7A. This inhibition, however, was not complete: 70% inhibition was observed at 100  $\mu$ M FY-CHO and 53% inhibition at 100  $\mu$ M ALLM. To control for the specificity of the fluorescent signal to cathepsin L, we also tested the effect of the specific inhibitor of another main lysosomal cathepsin, cathepsin B. No changes in cathepsin L-like activity were observed when cells were treated with up to 100  $\mu$ M of the cathepsin B inhibitor Ca074Me.

FY-CHO and ALLM were also partially effective against SPD-induced neuronal death: 49% inhibition of neuronal death was

TABLE 1. Quantification of electron micrographs of cerebellar granule cells in control cultures and in cultures exposed to SPD for 12 h

	Control ( $n = 124$ cells)	SPD ( $n = 100$ cells)
Number of autophagic vacuoles per section in cell	$0.61 \pm 0.07$	$2.36 \pm 0.15^*$
Average area (in pixels) of autophagic vacuoles per section per cell	$4130 \pm 570$	$10470 \pm 830^*$
Area of autophagic vacuoles as fraction of cytoplasm	$0.012 \pm 0.002$	$0.034 \pm 0.002^*$

\* $P < 0.0001$ .

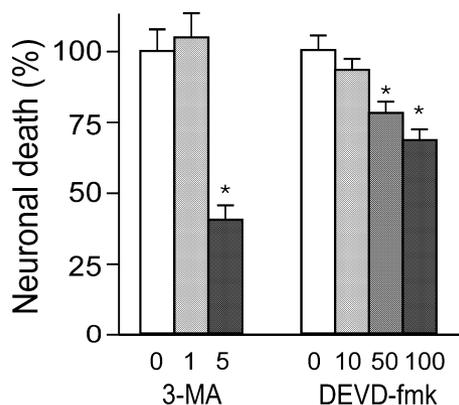


FIG. 6. Effects of the autophagy inhibitor 3-MA (5 mM) and the apoptosis inhibitor DEVD-fmk (100  $\mu$ M) on neuronal death induced by SPD. Drugs were added immediately after initiation of SPD and neuronal survival was determined 12 h later using the Trypan Blue assay. The data are mean  $\pm$  SEM. Similar results were obtained in two independent experiments. \* $P < 0.05$  (one-way ANOVA followed by the Bonferroni test).

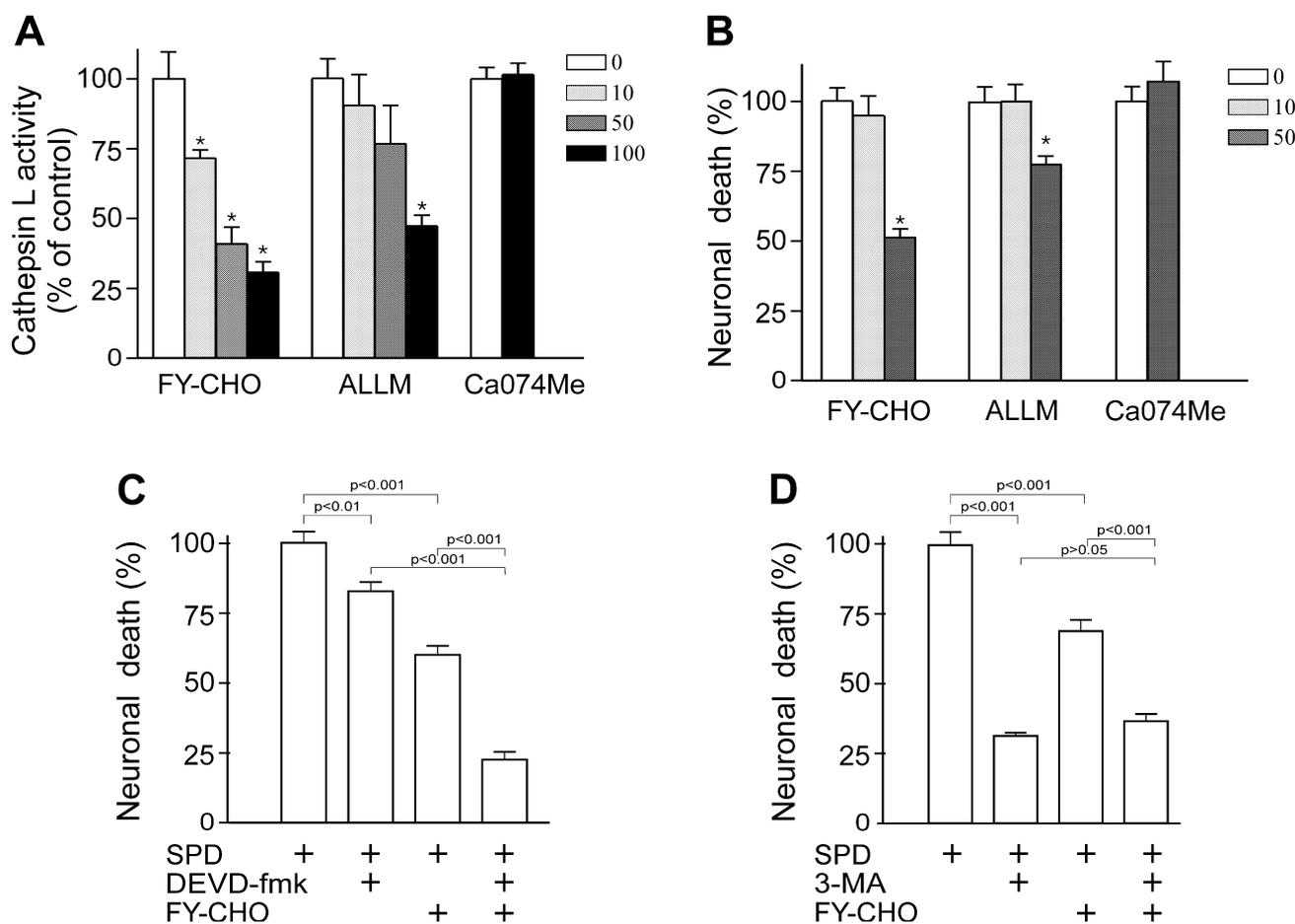


FIG. 7. Effects of cathepsin inhibitors on cathepsin L activity and neuronal survival. (A) Cathepsin L activity in SPD neurons exposed to the cathepsin L inhibitors FY-CHO (10, 50, 100  $\mu$ M) and ALLM (10, 50, 100  $\mu$ M) and the cathepsin B inhibitor CA074Me (100  $\mu$ M). Neurons were treated with 10  $\mu$ M of (CBZ-Phe-Arg)<sub>2</sub>-R110 for 60 min and examined with confocal microscopy at excitation and emission wavelengths of 488 and 522 nm to calculate the mean intensity of fluorescence reflecting the cathepsin L activity. (B) Effects of FY-CHO (10, 50  $\mu$ M), ALLM (10, 50  $\mu$ M) and CA074Me (50  $\mu$ M) on SPD-induced neuronal death. (C and D) Additive effect of FY-CHO and a caspase-3 inhibitor or an autophagy inhibitor. SPD neurons were exposed to the cathepsin L inhibitor FY-CHO (50  $\mu$ M), the caspase-3 inhibitor DEVD-fmk (50  $\mu$ M) or the autophagy inhibitor 3-MA (5 mM). All drugs were added immediately after initiation of SPD and neuronal survival was determined 12 h later using the Trypan Blue assay. Data are mean  $\pm$  SEM. One-way ANOVA, followed by the Bonferroni test, was used to test statistical significance. \* $P < 0.05$ . Similar results were obtained in two or three independent experiments.

observed after treatment with 50  $\mu$ M FY-CHO and 23% inhibition was observed after treatment with 50  $\mu$ M ALLM (Fig. 7B). These numbers match well with the effects of these inhibitors on cathepsin L activity (Fig. 7A): 50  $\mu$ M FY-CHO inhibited cathepsin L activity by 60% and 50  $\mu$ M ALLM by 25% (although the latter did not reach statistical significance). Higher concentrations of cathepsin L inhibitors exerted toxic effects on cultures and were not being tested. No neuroprotection was observed after the addition of the cathepsin B inhibitor Ca074Me at any concentration tested. It should be noted here, however, that Ca074Me delayed the neuronal death: 6 h after starting SPD the toxicity in Ca072Me-treated groups was lower than in the SPD-only groups.

We also tested the hypothesis that combined use of cathepsin L inhibitors and caspase-3 inhibitors increases resistance of cultures to SPD. Indeed, as demonstrated in Fig. 7C and D, combined treatment with a cathepsin L inhibitor (50  $\mu$ M FY-CHO) and a caspase-3 inhibitor (50  $\mu$ M DEVD-fmk) rescued a significantly higher percentage of neurons than FY-CHO or DEVD-fmk given alone. No synergistic effect was observed when cultures were cotreated with FY-CHO and 5 mM 3-MA.

## Discussion

The results of our study demonstrate that SPD is associated with transient activation of lysosomal cathepsin L, with maximum activity at 4–8 h after the initiation of SPD. Cathepsin L inhibitors ALLM and FY-CHO inhibited cathepsin L activity and rescued half of the neurons from neuronal death during the first 12 h of deprivation. Similar concentrations of the cathepsin B inhibitor Ca074Me had no persistent effect. These data suggest that cathepsin L, rather than cathepsin B, is involved in the neuronal death due to SPD.

Increased lysosomal cathepsin L activity, and subsequent neuronal death in SPD-treated neurons, might be related to excessive autophagy. Serum- and potassium-deprived neurons demonstrated an increased number of cytosolic autophagic vacuoles, indicating activation of autophagy. During the process of autophagy, lysosomes are fused with autophagosomes and thereby increased lysosomal cathepsin L activity might be necessary for the execution of efficient autophagy. Simultaneous activation of autophagy and the lysosomal system have been found in several other *in vitro* models of neuronal degeneration (Xue *et al.*, 1999; Kägedal *et al.*, 2001; Larsen *et al.*, 2002; Boland & Campbell, 2004) and in several neurodegenerative disorders (for review see Larsen & Sulzer, 2002). Indeed, ultra-structural analysis revealed an increased number of autophagosomes in the SPD-exposed neurons. Also, the experiments with 3-MA support the involvement of autophagy in SPD-induced neuronal death. Previous studies have reported that 3-MA inhibits class III phosphatidylinositol 3-kinase involved in the formation of autophagosomes at the stage of sequestration (Petiot *et al.*, 2000). In our experiments, this provided 60% neuroprotection against SPD-induced neuronal death.

There are some data which demonstrate that activation of cathepsin L might induce an activation of the apoptotic pathway. This ability of cathepsin L to induce apoptosis, however, has been demonstrated in models where the lysosomal membrane is ruptured and cathepsin L has translocated into the cytoplasmic compartment (Ishisaka *et al.*, 1999; Boland & Campbell, 2004). In our experiments, we were unable to detect any fluorescent signal associated with cathepsin L activity, from the cytosol and cellular compartments other than lysosomes, during the first 12 h of SPD. Similarly, the LysoTracker Red staining failed to demonstrate the rupture of the lysosomal membrane during the initial 12 h of SPD. These data argue against translocation of cathepsin L into the cytosol. It does not exclude, however, the possibility that some cathepsin L could be relocated to the cytosol immediately prior to the execution of death, and this would not be detected by the methods employed here.

Our study also suggests that, in the SPD model, cathepsin L-related neuronal death is at least partially caspase-3-independent. Treatment of neurons with a caspase-3 inhibitor or a cathepsin L inhibitor, separately, rescued only 20–40% of neurons from death induced by SPD. When both cathepsin L and caspase 3 inhibitors were present,  $\approx 80\%$  of neurons were rescued from death. On the other hand, the cathepsin L inhibitor did not augment the neuroprotective effect of the autophagy inhibitor 3-MA. The existence of two cell populations dying, either via apoptosis or autophagy, is unlikely. Most probably both pathways are present in the same population of cells and both pathways, interacting with each other, are activated in parallel. This could provide an explanation of why inhibition of one of these pathways could not provide complete neuroprotection, as another pathway could become over-activated. It is therefore tempting to speculate that inhibition of lysosomal–autophagic pathways, together with apoptotic pathways, could provide more complete neuroprotection in other models of neuronal death that involve lysosomes. However, further investigations are required to explore this hypothesis.

Taken together, the current experiments demonstrate that activation of lysosomal cathepsin L and excessive autophagy are prominent features of SPD-induced neurodegeneration in the primary culture of cerebellar granule cells. Furthermore, the present data demonstrate that inhibition of cathepsin L activity and/or autophagy rescue neurons from SPD-induced neuronal death. Data also demonstrate the synergic effect of cathepsin-L inhibitors and caspase-3 inhibitors against SPD. Thus, the lysosomal–autophagic pathway of neuronal death might be considered an important target for the development of new neuroprotective agents.

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## Abbreviations

3-MA, 3-methyladenine; ALLM, N-Acetyl-L-leucyl-L-leucyl-L-methionine; CA-074Me, 1-trans-Epoxy succinyl-Ile-Pro-OMe propylamide; DEVD-fmk, z-Asp-Glu-Val-Asp-fluoromethylketone; FY-CHO, N-(benzyloxycarbonyl)-L-phenyl-alanyl-L-tyrosinal; PBS, phosphate-buffered saline; SPD, serum and potassium deprivation.

## References

- Assfalg-Machleidt, I., Rothe, G., Klingel, S., Banati, R., Mangel, W.F., Valet, G. & Machleidt, W. (1992) Membrane permeable fluorogenic rhodamine substrates for selective determination of cathepsin L. *Biol. Chem. Hoppe Seyler*, **373**, 433–440.
- Bever, C.T. Jr & Garver, D.W. (1995) Increased cathepsin B activity in multiple sclerosis brain. *J. Neurol. Sci.*, **131**, 71–73.
- Boland, B. & Campbell, V. (2004) A $\beta$ -mediated activation of the apoptotic cascade in cultured cortical neurons: a role for cathepsin L. *Neurobiol. Aging*, **25**, 83–91.
- Bursch, W. (2001) The autophagosomal-lysosomal compartment in programmed cell death. *Cell Death. Differ.*, **8**, 569–581.
- Canu, N., Tufi, R., Serafino, A.L., Amadoro, G., Ciotti, M.T. & Calissano, P. (2005) Role of the autophagic-lysosomal system on low potassium-induced apoptosis in cultured cerebellar granule cells. *J. Neurochem.*, **92**, 1228–1242.
- Cataldo, A.M. & Nixon, R.A. (1990) Enzymatically active lysosomal proteases are associated with amyloid deposits in Alzheimer brain. *Proc. Natl Acad. Sci. USA*, **10**, 3861–3865.
- Clarke, P.G.H. (1990) Developmental cell death: Morphological diversity and multiple mechanisms. *Anat. Embryol.*, **181**, 195–213.
- Cuervo, A.M. (2004) Autophagy: in sickness and in health. *Trends Cell Biol.*, **14**, 70–77.
- D'Mello, S.R., Aglieco, F., Roberts, M.R., Borodezt, K. & Haycock, J.W. (1998) DEVD-inhibited caspase other than CPP32 is involved in the commitment of cerebellar granule neurons to apoptosis induced by K<sup>+</sup> deprivation. *J. Neurochem.*, **70**, 1809–1818.
- D'Mello, S.R., Galli, C., Ciotti, T. & Callisano, P. (1993) Induction of apoptosis in cerebellar granule neurons by lowering of extracellular potassium: inhibition of death by IGF-I and camp. *Proc. Natl. Acad. Sci. USA*, **90**, 10989–10993.
- Gallo, V., Ciotti, M.T., Coletti, A., Aloisi, F. & Levi, G. (1982) Selective release of glutamate from cerebellar granule cells differentiating in culture. *Proc. Natl. Acad. Sci. USA*, **79**, 7919–7923.
- Gomez-Santos, C., Santidrian, F.I., Barrachina, A.F., Gil, J. & Ambrosio, S. (2003) Dopamine induces autophagic cell death and alpha-synuclein increase in huma neuroblastoma SH-SY5Y cells. *J. Neurosci. Res.*, **73**, 341–350.
- Ishisaka, R., Utsumi, T., Kanno, T., Arita, K., Katunuma, N., Akiyama, J. & Utsumi, K. (1999) Participation of a cathepsin 1-type protease in the activation of caspase 3. *Cell Struct. Funct.*, **24**, 465–470.
- Jäättelä, M. & Tschopp, J. (2003) Caspase-independent cell death in T lymphocytes. *Nat. Immunol.*, **4**, 416–423.

- Jia, L., Dourmashkin, R.R., Allen, P.D., Gray, A.B., Newland, A.C. & Kelsey, S.M. (1997) Inhibition of autophagy abrogates tumor necrosis factor- $\alpha$  induced apoptosis in human T-lymphoblastic leukaemic cells. *Br. J. Haematol.*, **98**, 673–685.
- Kaasik, A., Kalda, A., Jaako, K. & Zharkovsky, A. (2001) Dehydroepiandrosterone sulphate prevents oxygen-glucose deprivation-induced injury in cerebellar granule cell culture. *Neuroscience*, **102**, 427–432.
- Kägedal, K., Zhao, M., Svensson, I. & Brunk, U. (2001) Sphingosine-induced apoptosis is dependent on lysosomal proteases. *Biochem. J.*, **359**, 335–343.
- Larsen, K.E., Fon, E.A., Hastings, T.G., Edwards, R.H. & Sulzer, D. (2002) Methamphetamine-induced degeneration of dopaminergic neurons involves autophagy and upregulation of dopamine synthesis. *J. Neurosci.*, **22**, 8951–8960.
- Larsen, K.E. & Sulzer, D. (2002) Autophagy in neurons: a review. *Histol. Histopathol.*, **17**, 1–12.
- Lemasters, J.J., Nieminen, A.L., Qian, T., Trost, L.C., Elmore, S.P. & Nishimura, Y. (1998) The mitochondrial permeability transition in cell death: a common mechanism in necrosis, apoptosis and autophagy. *Biochim. Biophys. Acta*, **1366**, 177–196.
- Lum, J.J., Bauer, D.E., Kong, M., Harris, M.H., Li, C., Lindsten, T. & Thompson, C.B. (2005) Growth factor regulation of autophagy and cell survival in the absence of apoptosis. *Cell*, **120**, 237–248.
- Marks, N., Berg, M.J., Guidotti, A. & Saito, M. (1998) Activation of caspase-3 and apoptosis on cerebellar granule cells. *J. Neurosci. Res.*, **52**, 334–341.
- Miller, T.M. & Johnson, E.M. Jr (1996) Metabolic and genetic analyses of apoptosis in potassium/serum-deprived rat cerebellar granule cells. *J. Neurosci.*, **16**, 7487–7495.
- Petiot, A., Ogier-Denis, E., Blommaert, E.F.C., Meijers, A.J. & Codogno, P. (2000) Distinct classes of phosphatidylinositol 3'-kinases are involved in signalling pathways that control macroautophagy in HT-29 cells. *J. Biol. Chem.*, **275**, 992–998.
- Shimizu, S., Kanaseki, T., Mizushima, N., Mizuta, T., Arakawa-Kobayashi, S., Thompson, C.B., & Tsujimoto, Y. (2004) Role of Bcl-2 family proteins in a non-apoptotic programmed cell death dependent on autophagy genes. *Nat. Cell Biol.*, **6**, 1221–1228.
- Uchiyama, U. (2001) Autophagic cell death and its execution by lysosomal cathepsins. *Arch. Histol. Cytol.*, **64**, 233–246.
- Villalba, M. (1998) Bisindolylmaleimide prevents cerebellar granule cells apoptosis: a possible role for PKC. *Neuroreport*, **9**, 1713–1717.
- Weber, E., Bahn, H. & Gunther, D. (1997) Monoclonal antibodies against cathepsin L and procathepsin L of different species. *Hybridoma*, **16**, 159–166.
- Xue, L., Fletcher, G.C. & Tolkovsky, A.M. (1999) Autophagy is activated by apoptotic signalling in sympathetic neurons. An alternative mechanism of death execution. *Mol. Cell Neurosci.*, **14**, 180–198.
- Yan, G.-M. & Paul, S.M. (1997) Cultured cerebellar granule neurons as a model of neuronal apoptosis. In Poirier, J. (ed.), *Apoptosis Techniques and Protocols, Neuromethods 29*. Humana Press, Totowa, New Jersey, pp. 47–66.