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Enhanced caspase activity during ethanol-induced apoptosis in rat cerebellar granule cells

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Abstract

The effects of ethanol on cerebellar granule cell death were examined in cultures maintained for either 5 days in vitro (immature) or 8 and 12 days in vitro (mature). Ethanol did not alter cell survival under the usual growth conditions (i.e., 10% serum and 25 mM KCl). However, in mature cultures ethanol enhanced apoptosis induced by either serum withdrawal or incubation in non-depolarizing media. In immature cultures, serum deprivation, but not non-depolarizing media, resulted in granule cell death that was enhanced by ethanol. Serum removal increased both cleavage of the caspase-specific substrate *N*-acetyl-Asp-Glu-Val-Asp-7 amino-4-methylcoumarin (Ac-DEVD-amc) and the amount of active caspase-3. Inclusion of ethanol during the serum deprivation augmented Ac-DEVD-amc cleavage without further increasing the amount of active caspase-3. This study demonstrates that when neurotrophic factors are limiting, ethanol is toxic to cerebellar granule cells regardless of maturation status. The ability of ethanol to promote apoptosis involves an increase in caspase activity, but this does not entail an increase in the proteolytic activation of caspase-3.

Keywords: Apoptosis; Cell death; Ethanol; Fetal alcohol syndrome; Cerebellar granule cell

1. Introduction

During normal development numerous neurons in the central nervous system (CNS) are lost through apoptosis that is regulated, at least in part, by the level of afferent input and the amount of neurotrophic factor support (Oppenheim, 1991; Linden, 1994). In vivo cerebellar granule cell survival appears to depend upon innervation by glutaminergic mossy fibers and the presence of the target Purkinje neurons (Rakic and Sidman, 1973; Chen and Hillman, 1989). Similarly, during the first week in culture cerebellar granule cells acquire the characteristics of mature neurons and become dependent upon the presence of depolarizing concentrations of KCl or *N*-methyl-D-aspartate (NMDA) which presumably mimic the afferent input of the glutaminergic mossy fibers (Burgoyne et al., 1993). In the absence of elevated KCl or NMDA, mature granule cells undergo apoptotic cell death (D'Mello et al., 1993; Yan et al., 1994). Likewise, removal of serum also results in cerebellar granule cells undergoing apoptosis (Atabay et al., 1996; Miller and Johnson, 1996). However, the kinetics and extent of the cell death differ after removal of serum or reducing KCl (Miller and Johnson, 1996) suggesting the possible involvement of different mechanisms.

Exposure to ethanol during development also causes a well-characterized decrease in the number of both cerebellar granule cells and Purkinje neurons (Bauer-Moffett and Altman, 1977; Hamre and West, 1993; Napper and West, 1995). This neurotoxicity, however, is only manifested when the ethanol exposure occurs at specific developmental times (i.e., there are windows of vulnerability) (Hamre and West, 1993). Addition of ethanol to freshly isolated cerebellar cell cultures was reported to increase cell loss (Pantazis et al., 1993; Luo et al., 1997). However, with continued time in culture, the vulnerability of the granule cells to the neurotoxic action of ethanol was reported to diminish. Thus, in 7–8-day-old cultures a 24-h treatment with ethanol was reported not to alter granule cell survival (Pantazis et al., 1993; Castoldi et al., 1998; Zhang et al., 1998). In the neuronal-like PC 12 cell line, ethanol alone did not alter cell viability, but rather ethanol induced apoptosis in the presence of permissive conditions (Oberdoerster et al., 1998). Similarly, when granule cell cul-

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tures were incubated under non-depolarizing conditions, ethanol was reported to enhance granule cell death by increasing apoptosis (Wegelius and Korpi, 1995; Bhave and Hoffman, 1997). In contrast, others failed to observe an ethanol-mediated increase in granule cell death under non-depolarizing conditions (Castoldi et al., 1998; Zhang et al., 1998). Numerous methodological differences, however, exist amongst the few studies investigating the neurotoxic effects of ethanol on granule cells.

The present study was undertaken to investigate the neurotoxic action of ethanol on cerebellar granule cells. The effects of ethanol were systematically studied under the usual growth conditions (i.e., 10% serum + 25 mM KCl), under non-depolarizing conditions (i.e., 10% serum + 5 mM KCl) which mimics the absence of the afferent input from the mossy fibers, and under depolarizing serum-free conditions (i.e., 0% serum + 25 mM KCl) which mimics the lack of neurotrophic factor support. Because mature and immature granule cells display differences including opposite responses to lithium (D'Mello et al., 1994) and inhibitors of protein synthesis (Kharlamov et al., 1995), the effects of ethanol were examined in both mature and immature granule cells. Finally the molecular mechanisms responsible for the neurotoxic actions of ethanol were investigated.

2. Materials and methods

2.1. Materials

Cell culture media and agarose was purchased from Gibco BRL (Grand Island, NY). Fetal calf serum was purchased from HyClone Laboratories (Logan, UT). The ethidium homodimer and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Molecular Probes (Eugene, OR). Proteinase K was purchased from Fisher Biotech (Fair Lawn, NJ) and RNase A from Boehringer-Mannheim (Indianapolis, IN). For caspase activity assays, the *N*-acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin (Ac-DEVD-amc) was purchased from Bachem Bioscience (King of Prussia, PA) and the *N*-acetyl-Asp-Glu-Val-Asp-aldehyde (Ac-DEVD-CHO) from Calbiochem (La Jolla, CA). For immunoblots, anti-caspase-3 immunoglobin G (IgG) was purchased from Upstate Biotechnology (Lake Placid, NY). Secondary antibody (^{125}I-labeled goat anti-rabbit IgG) was purchased from NEN Life Science Products (Boston, MA). All other chemicals were purchased from common commercial suppliers.

2.2. Tissue culture

Primary rat cerebellar granule cell cultures were established as described by Edelman et al. (1992). Briefly, the cerebella from 7-day-old rats were incubated in Dulbecco's modified Eagle medium (DMEM) containing 0.1% trypsin and 0.2% deoxyribonuclease and then filtered through a 150-μm nylon mesh. Cells were pre-plated in a T-flask coated with 50 μg/ml poly-L-lysine for 15 min at 37°C to remove astrocytes and the medium was filtered sequentially through 85- and 25-μm nylon mesh. The cells in DMEM supplemented with 10% fetal calf serum, 50 mM glucose, 24.5 mM KCl, 1 μg/ml *para*-aminobenzoic acid (complete DMEM) were plated at a density of 0.08 cerebellum/cm^2 onto culture dishes coated with 500 μg/ml poly-L-lysine and were maintained at 37°C in a humidified atmosphere containing 95% air–5% CO_2. On days 1 and 5 after isolation, the cells were treated with 10 μM cytosine arabinoside for 24 h to remove any proliferating cells. Subsequently, medium was changed every 3 days. For experiments, granule cells were rinsed once with DMEM and the appropriate experimental conditions added (i.e., complete DMEM; low KCl: 10% serum, 5 mM KCl; serum deprivation: no serum, 25 mM KCl). Ethanol exposure was carried out by including ethanol in the media and incubating the plates at 37°C in plastic desiccators containing an atmosphere of 95% air, 5% CO_2 that was saturated with the appropriate concentration of ethanol. This system has been shown to result in an approximate 20% loss of ethanol from the medium after 2 days (Rabin, 1988).

2.3. Determination of total cell number

The number of granule cells/well was quantified by incubating the cells for 5 min in 0.1 M acetic acid containing 0.1 mg/ml Cresyl violet and counting the number of stained nuclei using a hemocytometer. As an additional measure of total cell number, sister cultures were incubated for 25 min in phosphate-buffered saline (PBS) containing 0.1% Triton X-100 to disrupt the cell membranes and 3 μg/ml of the DNA-binding fluorophore ethidium homodimer. Ethidium fluorescence was measured using a fluorescent plate reader (485 nm excitation, 645 nm emission). Examination of cultures by fluorescence microscopy revealed that 100% of the cells were labeled with ethidium after the treatment with Triton X-100.

2.4. Evaluation of cell viability using MTT

The tetrazolium salt MTT is cleaved to a formazan only by cells containing functional mitochondria (Mosmann, 1983), and thus MTT reduction especially in non-proliferating cells, such as cerebellar granule cell cultures, is an indicator of cell viability (Ankarcrona et al., 1995; Harada and Sugimoto, 1997). Granule cells were incubated in PBS containing 4 μg/ml of the cell-permeable DNA-binding fluorophore Hoechst-33342 and 100 μg/ml MTT. The total DNA content was measured immediately using a fluorescent plate reader (356 nm excitation, 458 nm emission) and salmon sperm DNA as a standard as previously described (Labarca and Paigen, 1980). After a 40-min

incubation at 37°C, the PBS was removed from the wells, and the reduced MTT was extracted in dimethyl sulfoxide (DMSO). The optical density of the MTT was then determined (490 nm minus 630 nm) as previously described (Balázs et al., 1988; Harada and Sugimoto, 1997).

2.5. Evaluation of cell death using ethidium homodimer

Granule cells were incubated at 37°C for 1 h in PBS containing 3 μg/ml of the cell-impermeable DNA-binding fluorophore ethidium homodimer. Ethidium fluorescence was measured using a fluorescent plate reader (485 nm excitation, 645 nm emission).

2.6. Evaluation of internucleosomal DNA cleavage

DNA laddering was evaluated using the method of Oberdoerster et al. (1998). Briefly, granule cells were removed in 1 vol. Hanks' buffered salt solution and stored at −20°C for 24 h in 5 vol. 70% ethanol. After removal of the ethanol, cell pellets were incubated for 40 min at room temperature in phosphate–citrate buffer (192 mM Na_2HPO_4, 4 mM citric acid, pH 7.8). The $1000 \times g$ supernatant was incubated for 30 min at 37°C with 0.03% Nonidet NP-40 and 0.4 mg/ml RNase A, and then incubated for an additional 30 min at 37°C in the presence of 0.4 mg/ml Proteinase K. Samples then were electrophoresed on a 2% agarose gel using a running buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.3) containing 0.5 μg/ml ethidium bromide. The cumulative fluorescence intensity of each of the lower bands (i.e., intensity of the rungs of DNA ladder) was measured using a Bio-Rad Molecular Imaging system.

2.7. Caspase activity assay

Granule cells (1.5 cerebellum) were removed from the culture plate in lysis buffer (25 mM Hepes, 1 mM EGTA, 5 mM EDTA, 5 mM $MgCl_2$, 5 mM dithiothreitol, 0.01% 3-[(3-cholamidopropyl) dimethyl-ammonio]-1-propanesulfonate (CHAPS), 10 μg/ml pepstatin, 10 μg/ml leupeptin, 1 mM phenylmethylsulfonylfluoride, pH 7.4). After a 20-min incubation on ice, an aliquot of lysate was removed for determination of cellular protein and the remaining lysate was centrifuged at $16{,}000 \times g$ for 30 min. The resulting supernatant was combined with 2 vol. assay buffer (25 mM HEPES, 10 mM dithiothreitol, 15% sucrose, 0.15% CHAPS, pH 7.5) containing the caspase substrate Ac-DEVD-amc (100 μM), and caspase activity was determined by measuring the rate of coumarin liberation. Readings were taken every 4 min for 60 min using a fluorescent plate reader (360 nm excitation and 460 nm emission) to confirm that the liberation of coumarin was linear (r^2 ranged from 0.989 to 1.0). Non-specific Ac-DEVD-amc cleavage, which was measured in the presence 2.5 μM Ac-DEVD-CHO, was $9.4 \pm 1.7\%$ of total Ac-DEVD-amc cleavage. The rate of caspase activity was expressed as [(fluorescence/min) − (fluorescence/min in the presence of Ac-DEVD-CHO)]/mg protein.

2.8. Immunoblot analysis

Immunoblot analysis was carried out using a modification of our previously described method (Rabin, 1993). Cells were removed from the tissue culture plates in 25 mM HEPES, 1 mM EGTA, 5 mM EDTA, 5 mM $MgCl_2$, 5 mM dithiothreitol, 1% Triton X-100, 10 μg/ml pepstatin, 10 μg/ml leupeptin, 1 mM phenylmethylsulfonylfluoride (pH 7.5) and incubated on ice for 20 min. Cell lysate was resolved on a 12.5% polyacrylamide–sodium dodecyl sulfate gel following the method of Laemmli (1970) and the proteins electrically transferred (100 V, 90 min, 4°C) to Immobilon-P (Millipore, Bedford, MA). The membranes were incubated overnight at 4°C with 4% non-fat dry milk in PBS to block non-specific binding and then incubated for 2 h at room temperature in PBS containing 3% non-fat dry milk and anti-caspase-3 antibody (1:1000). After extensive washing in PBS, the membranes were incubated for 1 h at room temperature in PBS containing 3% non-fat dry milk and the secondary ^{125}I goat anti-rabbit IgG (1 μCi). Blots were then extensively washed in PBS containing 0.1% Tween-20 and analyzed using a Bio-Rad Phosphor Imager (Bio-Rad Lab., Hercules, CA).

2.9. Total cell protein

For the determination of total cellular protein, cells were incubated in 0.1 N NaOH, and protein content measured using the colorimetric Bio-Rad protein dye binding procedure with bovine serum albumin (fraction V) as a standard.

2.10. Statistical analysis

Initial statistical analysis revealed that data for some studies did not pass the test for normality or homogeneity of variance with α equal to 0.05 and also showed significant skewness and kurtosis. Because all the data did not appear to be drawn from a normally distributed population, non-parametric methods, which do not assume any specific distribution, were used for the statistical analysis. The data were analyzed using the Friedman repeated measures analysis of variance on ranks and the post-hoc Student–Newman–Keuls pairwise comparison procedure using SigmaStat software (Jandel Scientific, San Rafael, CA). Only the a priori comparisons of KCl reduction vs. complete DMEM, serum deprivation vs. complete DMEM, and the effect of ethanol on these conditions are shown. Results

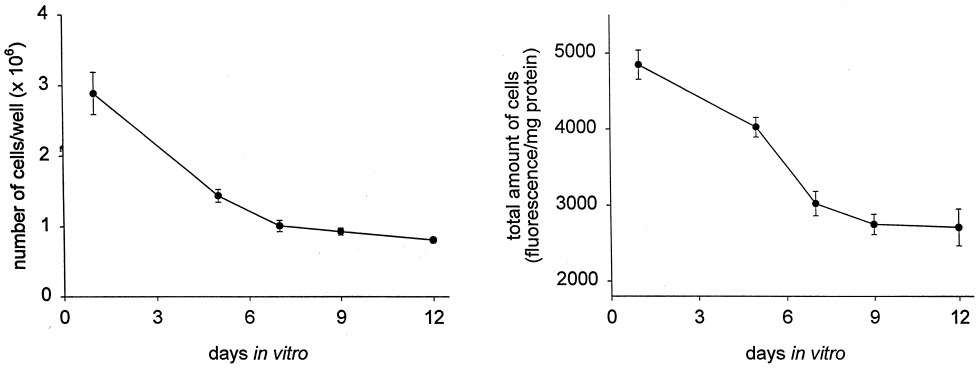

Fig. 1. Decrease in granule cell number during the first week in vitro. Cells were maintained in the presence of complete media containing 25 mM KCl and 10% fetal calf serum for up to 12 days. Left: At 1, 5, 7, 9 and 12 days in vitro the number of cells in duplicate wells of a 24-well plate was counted using a hemocytometer. Right: Sister cultures were treated with 0.1% Triton X-100, and total amount of cells determined using the DNA-binding probe ethidium homodimer. Data are expressed as number of cells/well or as fluorescence/mg protein and are plotted as mean ± S.E.M. ($N = 3$–9 separate litters). Statistical analysis indicated a significant effect of time in culture: $\chi^2_{[4]} = 27.5$ ($P < 0.001$) for the number of cells/well and $\chi^2_{[4]} = 11.5$ ($P < 0.05$) for the total amount of cells.

are presented as mean ± S.E.M. as this is the usual convention for reporting the variance in the data.

3. Results

The number of granule cells/well progressively decreased for the first week in culture after which a stable population was obtained (Fig. 1). Similar results were obtained when the total amount of granule cells was determined by labeling all cells with ethidium homodimer in the presence of 0.1% Triton X-100. Between 7 and 19 days in culture the viability of the granule cells was constant as indicated by the level of MTT reduction (Fig. 2). The initial loss of cells is consistent with previous reports (Fields et al., 1982; Pantazis et al., 1993) and in the present study probably reflects the trauma of isolation as well as the treatment of the cultures with cytosine arabinoside.

Initially, granule cells can be maintained in the absence of elevated K^+, but with continued time in culture the granule cells develop a requirement for depolarizing concentrations of KCl (i.e., 25 mM) or NMDA to survive (Balázs et al., 1988; Copani et al., 1995). This change has been suggested to represent a maturation of the granule cells (Burgoyne et al., 1993; D'Mello et al., 1994; Copani et al., 1995). After 5 days in culture a 24-h exposure to reduced extracellular KCl did not increase the amount of ethidium-labeled cells (Fig. 3) which is consistent with classification of these granule cells as being immature. In

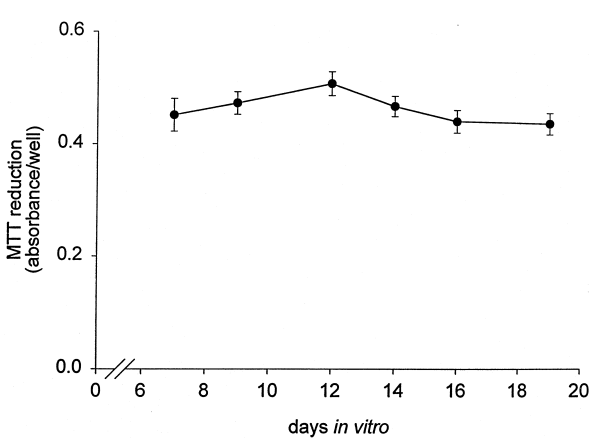

Fig. 2. Granule cell viability remains constant during days 7–19 in vitro. Cells were maintained in the presence of complete media containing 25 mM KCl and 10% fetal calf serum for up to 19 days. Viability was determined by measuring cellular MTT reduction. Data are expressed as reduced MTT/well and are plotted as mean ± S.E.M. ($N = 8$ separate litters). Similar results were obtained when the data were normalized to cellular DNA content (data not shown).

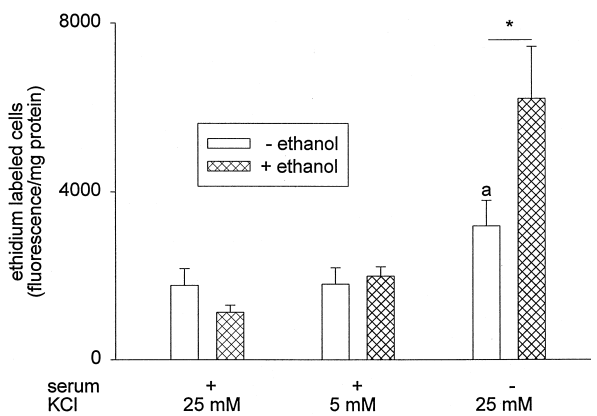

Fig. 3. Ethanol treatment increases the death of immature granule cells after serum deprivation. Granule cells were maintained for 5 days in the presence of complete media and then treated with 100 mM ethanol for 24 h in the absence of serum or in the presence of 5 mM KCl. Cell death was determined using the DNA-binding fluorophore ethidium homodimer. Data are expressed as fluorescence/mg protein and are plotted as mean ± S.E.M. A statistically significant difference was found amongst the groups ($\chi^2_{[5]} = 30.5$, $P < 0.0001$). (a) $P < 0.05$ compared to cells maintained in 10% serum and 25 mM KCl, $^*P < 0.05$ for the specific effects of ethanol, $N = 8$ separate litters.

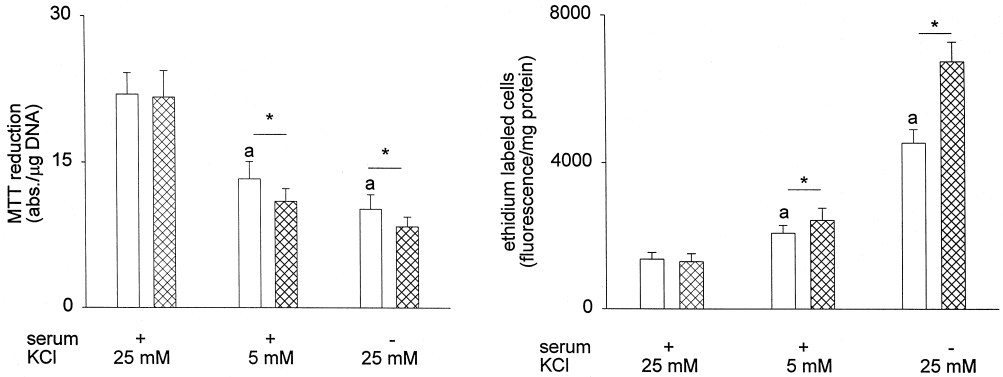

Fig. 4. Ethanol treatment increases death of mature granule cell after serum deprivation and after reduction of extracellular KCl. Granule cells were maintained for 8 days in the presence of complete media (i.e., 25 mM KCl and 10% fetal calf serum) and then treated for 24 h with 100 mM ethanol in either the absence of serum or the presence of 5 mM KCl. Cell viability was assessed by measuring MTT reduction (left panel) and by labeling dead or dying cells with the DNA-binding fluorophore ethidium homodimer (right panel). Data are expressed as reduced MTT/µg DNA or fluorescence/mg protein and are plotted as mean ± S.E.M. A statistically significant difference was observed amongst the groups: $\chi^2_{[5]} = 40.2$ ($P < 0.0001$) for MTT reduction and $\chi^2_{[5]} = 72.3$ ($P < 0.0001$) for ethidium labeling. (a) $P < 0.05$ compared to cells maintained in complete media, $^*P < 0.05$ for the specific effects of ethanol, $N = 8$–19 separate litters. Open bars: cultures incubated in the absence of ethanol. Cross-hatched bars: cultures incubated with 100 mM ethanol.

these cultures removal of serum for 24 h significantly increased cell death even though 25 mM KCl was present. Inclusion of ethanol did not alter cellular viability of the 5-day-old cultures maintained in either complete media or non-depolarizing media. However, the presence of 100 mM ethanol during the serum deprivation significantly enhanced cell death as indicated by the further 2-fold increase in the amount of ethidium-labeled granule cells (Fig. 3).

After 8 days in culture, a 24-h incubation in media containing serum and 5 mM KCl increased the amount of ethidium-labeled cells while the amount of MTT reduction was decreased (Fig. 4). This reduced cell viability in response to non-depolarizing media indicates that the granule cells have undergone maturation. Even in the presence of depolarizing conditions, removal of serum for 24 h also caused a decrease in granule cell survival (Fig. 4). When granule cells were maintained in complete media containing 25 mM KCl and 10% fetal calf serum, a 24 h incubation with 100 mM ethanol did not alter MTT reduction or ethidium homodimer uptake (Fig. 4). However, inclusion of ethanol during serum deprivation significantly reduced survival of the granule cells as the amount of ethidium-labeled cells was enhanced while the amount of MTT reduction was decreased. Similarly, addition of ethanol further reduced the viability of cultures maintained in 5 mM KCl. Ethanol (100 mM) also significantly ($P < 0.05$) enhanced the death of granule cells exposed both to non-depolarizing conditions and to serum withdrawal in cultures maintained for 12 days in vitro (data not shown).

Although changing the culture medium was reported to cause granule cells to undergo excitotoxic cell death (Schramm et al., 1990), the observed increased cell death does not appear to be due simply to changing the culture medium. When granule cell death was measured in 8-day-old cultures using ethidium homodimer uptake, changing the medium had no effect on cell viability; 24 h after exposure to fresh media, ethidium labeling was 361 ± 39 fluorescent units/mg protein, while in sister cells maintained without media change labeling was 346 ± 47 fluorescent units/mg protein ($N = 3$). Further, between 6 and 19 days in vitro the granule cells in the present study underwent five media changes without an apparent loss of viability (Fig. 2).

The potentiation of granule cell death by ethanol does not appear to be a generalized response to the combining of multiple insults. Although granule cells exposed to 0.1 mM H_2O_2 in the presence of complete media containing

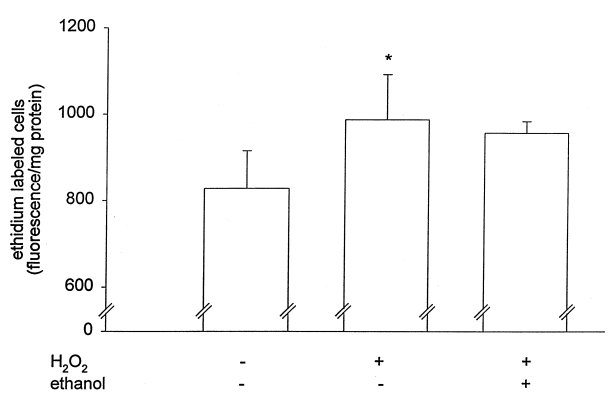

Fig. 5. Ethanol treatment does not enhance H_2O_2-induced granule cell death. Granule cells were maintained in culture for 8 days and then treated for 30 min in the presence of complete media with 0.1 mM H_2O_2 in the absence and presence of 100 mM ethanol. Cell death was determined using the DNA-binding fluorophore ethidium homodimer. Data are expressed as fluorescence/mg protein and are plotted as mean ± S.E.M. A statistically significant difference was observed amongst the groups ($\chi^2_{[2]} = 6.5$, $P < 0.05$). $^*P < 0.05$ compared to untreated cells, $N = 4$ separate litters.

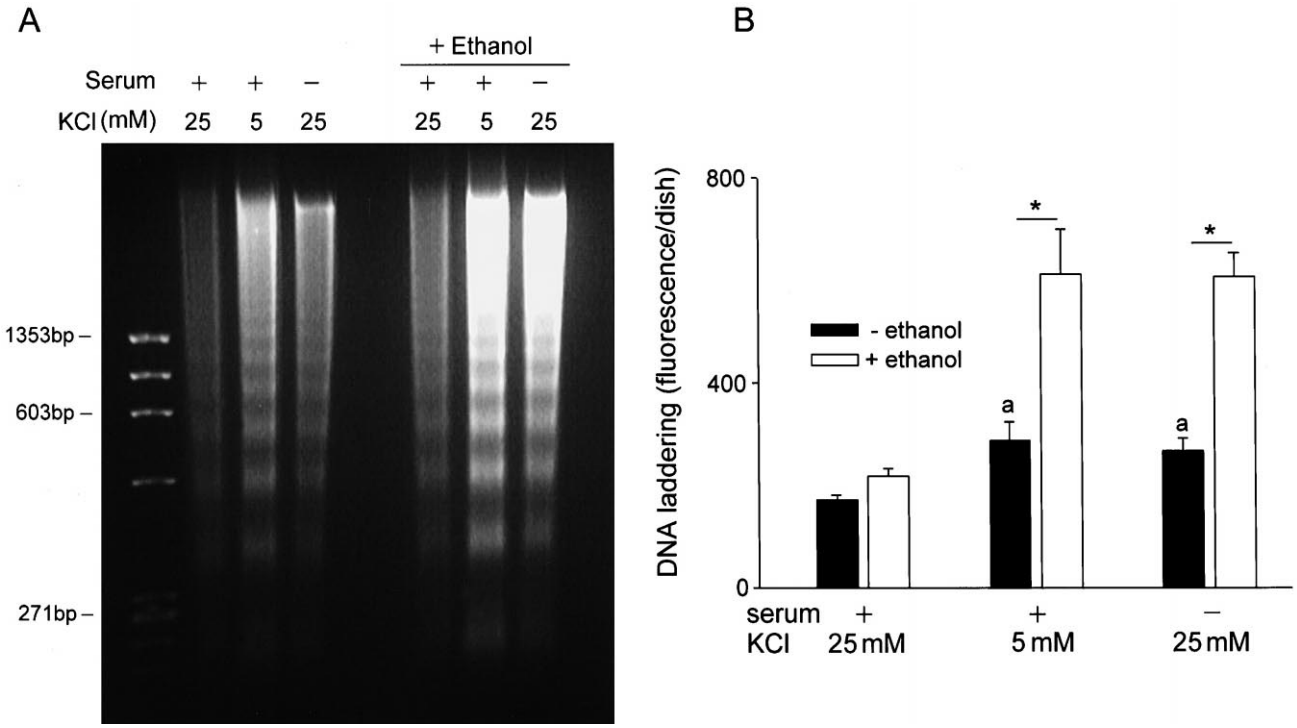

Fig. 6. Ethanol treatment increases internucleosomal DNA cleavage. After 8 days in culture, granule cells were treated with 100 mM ethanol for 24 h in the absence of serum or in the presence of 5 mM KCl. (A) DNA was subjected to electrophoresis and visualized with ethidium bromide and UV trans-illumination. Data are representative of gels from 10–14 separate litters. (B) The extent of DNA laddering was determined by summing the intensity of the lower DNA bands in each lane of the gel. Data are expressed as arbitrary fluorescent units/dish and are plotted as mean ± S.E.M. A statistically significant effect between the groups was observed: $\chi^2_{[3]} = 19.1$ ($P < 0.001$) for the specific comparison of ethanol treatment and the reduction of extracellular KCl; $\chi^2_{[3]} = 15.0$ ($P < 0.002$) for the specific comparison of ethanol treatment and serum deprivation. (a) $P < 0.05$ compared to cells maintained in 10% serum and 25 mM KCl, $^*P < 0.05$ for the effects of ethanol, $N = 6–8$ separate litters.

25 mM KCl and 10% fetal calf serum displayed a significant increase in cell death, inclusion of 100 mM ethanol did not further enhance granule cell death. (Fig. 5). This concentration of H_2O_2 was not maximal as treatment with 0.5 mM H_2O_2 enhanced the cell death an additional 60% (data not shown).

In agreement with previous reports that reducing the extracellular KCl concentration induces granule cells to

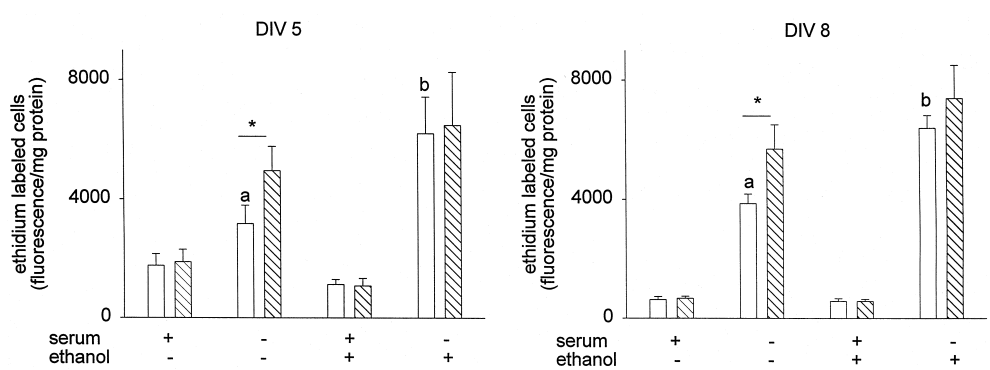

Fig. 7. The ethanol-mediated increase in cell death after serum deprivation does not involve NMDA. Granule cells were maintained in complete media for 5 or 8 days and then treated for 24 h with 100 mM ethanol and 100 μM NMDA in the absence of serum but in the presence of 25 mM KCl. Cell death was determined using the DNA-binding fluorophore ethidium homodimer. Data are expressed as fluorescence/mg protein and are plotted as mean ± S.E.M. A statistically significant effect was found in both DIV 5 cultures ($\chi^2_{[7]} = 48.7$, $P < 0.0001$) and DIV 8 cultures ($\chi^2_{[7]} = 46.3$, $P < 0.0001$). (a) $P < 0.05$ compared to untreated cells in complete media, (b) $P < 0.05$ compared to cells deprived of serum in the absence of ethanol (i.e., effect of ethanol on serum deprivation), $^*P < 0.05$ for the specific effects of NMDA, $N = 8$ separate litters. Open bars: cultures not treated with NMDA. Striped bars: cultures treated with 100 μM NMDA.

undergo apoptosis (D'Mello et al., 1993; Yan et al., 1994), agarose gel electrophoresis of the granule cell DNA revealed that treatment with low KCl resulted in the presence of DNA fragments with molecular weights in multiples of 180–200 bp (i.e., DNA ladder) (Fig. 6). Similarly, serum deprivation also caused the granule cells to undergo apoptosis. Consistent with the observed ethanol-induced increase in granule cell death, ethanol also significantly enhanced the amount of internucleosomal DNA cleavage both after serum deprivation and after incubation in 5 mM KCl (Fig. 6).

Additional studies were undertaken to determine the mechanism by which ethanol enhanced the apoptotic death of the granule cells after serum withdrawal. Because in the presence of non-depolarizing media ethanol-induced toxicity involves antagonism of the trophic activity of NMDA (Wegelius and Korpi, 1995; Bhave and Hoffman, 1997; Castoldi et al., 1998), the role of NMDA in the ethanol-mediated increase in granule cell death in serum deprived cultures was investigated. In both 5- and 8-day-old cultures that were deprived of serum but depolarized with 25 mM KCl, inclusion of 100 μM NMDA significantly increased the amount of ethidium-labeled cells (Fig. 7). The increase in cell death caused by inclusion of ethanol under these conditions was not antagonized by the presence of NMDA. Ethanol, however, appeared to block the ability of NMDA to further increase cell death which is in agreement with other studies (Lustig et al., 1992; Chandler et al., 1993).

The proteolytic activation of caspases, a family of cysteine-containing proteases that cleave after an aspartic acid residue, is an integral step in the apoptotic pathway (Cohen, 1997; Thornberry and Lazebnik, 1998). Possible activation of caspases during ethanol-induced apoptosis was determined by measuring cleavage of the caspase-specific substrate Ac-DEVD-amc. Lysates from granule cells deprived of serum exhibited a 90% increase in Ac-DEVD-amc cleavage (Fig. 8). This Ac-DEVD-amc cleavage activity was further enhanced 2.2-fold by inclusion of 100 mM ethanol during the serum deprivation. The increased Ac-DEVD-amc cleavage activity was not due to a direct effect of ethanol on the caspases, as the in vitro inclusion of 100 mM ethanol during the caspase assay did not alter Ac-DEVD-amc cleavage (data not shown). Immunoblot analysis of granule cell lysates revealed that serum removal resulted in a significant increase in the amount of the active (i.e., 17 kDa) form of caspase-3 (Fig. 9). Ethanol treatment of serum deprived granule cells, however, did not further increase the amount of active caspase-3.

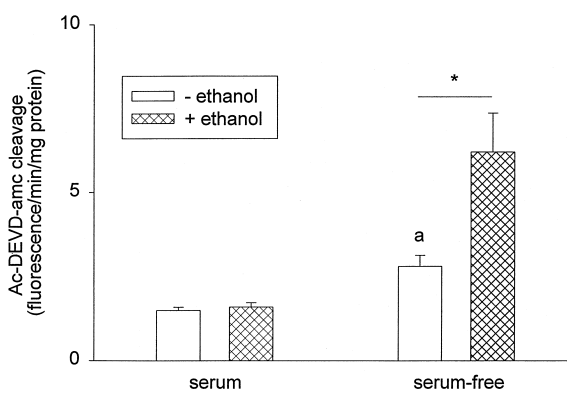

Fig. 8. Ethanol treatment of cerebellar granule cells increases Ac-DEVD-amc cleavage. After 8 days in culture, cells were treated for 24 h with 100 mM ethanol in the presence of complete media containing 10% fetal calf serum and 25 mM KCl or with media containing 25 mM KCl, but no serum. The amount of Ac-DEVD-amc cleavage in granule cell lysates was determined using the tetrapeptide substrate Ac-DEVD-amc (100 μM). The data are expressed as coumarin release/min/mg protein and are plotted as mean ± S.E.M. (a) $P < 0.05$ for the effect of serum withdrawal, $^{*}P < 0.05$ for the specific effect of ethanol, $N = 7$.

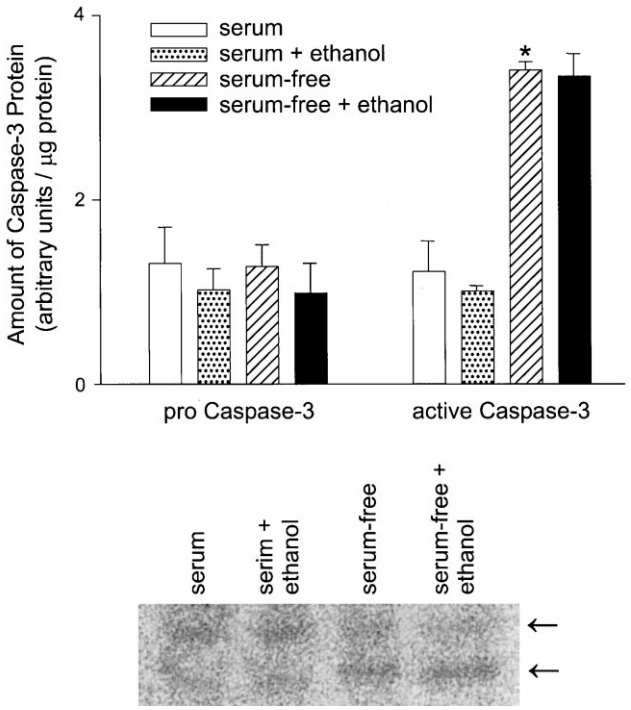

Fig. 9. Ethanol treatment does not increase the amount of active caspase-3 in cerebellar granule cells. After 8 days in culture granule cells were treated for 24 h with 100 mM ethanol in the presence of complete media containing 10% fetal calf serum and 25 mM KCl or with media containing 25 mM KCl, but no serum. The amounts of pro-caspase-3 (32 kDa) and active caspase-3 (17 kDa) were determined by immunoblot analysis. Immunoblot is representative of the results from four separate experiments. Data are expressed as arbitrary units and are plotted as mean ± S.E.M, $^{*}P < 0.05$ for the specific effect of serum withdrawal, $N = 4$.

4. Discussion

In rodents, a blood alcohol concentration of 100 mM or less during postnatal development results in a loss of cerebellar granule cells (Bauer-Moffett and Altman, 1977; Hamre and West, 1993; Napper and West, 1995). The mechanisms responsible for this neuronal loss are unclear.

Ethanol alone does not appear to have a direct neurotoxic action on cerebellar granule cells as a 24 h exposure to ethanol did not alter viability of granule cells cultured under the usual maintenance conditions. The lack of neurotoxicity was not due to the maturation status of the cells as neither immature (5 days in vitro) nor mature (8 and 12 days in vitro) cerebellar granule cells were affected by the ethanol. These findings are in agreement with the report of others (Pantazis et al., 1993; Castoldi et al., 1998; Zhang et al., 1998). Although ethanol alone was reported to significantly decrease the number of cerebellar granule cells in culture, these results were obtained with cells after 1 day in culture and this vulnerability diminished with time in culture (Pantazis et al., 1993; Luo et al., 1997). Because significant granule cell loss occurs for the first several days in culture (Fields et al., 1982; Pantazis et al., 1993; present study), the observed effects of ethanol in freshly isolated cultures may have involved an inhibition of the repair of cells damaged during the isolation.

Although under the usual maintenance conditions ethanol by itself did not alter granule cell viability, ethanol significantly increased the amount of cell death caused by serum withdrawal or by non-depolarizing conditions. The former condition could be considered to mimic the loss of neurotrophic support, whereas the latter mimics the absence of the afferent input from the glutaminergic mossy fibers (D'Mello et al., 1993; Yan et al., 1994); both conditions are required for neuronal survival (Oppenheim, 1991; Linden, 1994). The ethanol-induced increase in granule cell death involves an enhancement in the amount of apoptosis as ethanol increased the amount of DNA laddering. Similarly, ethanol caused a concentration-dependent increase in apoptosis in the neuron-like PC12 cell (Oberdoerster et al., 1998). Although the enhancement in cell death by ethanol after reducing the KCl concentration was observed only with mature cultures, the ethanol-induced increase in apoptotic granule cell death after serum withdrawal was independent of maturation status. Further, the observed increase in granule cell death after serum deprivation or reducing KCl concentration does not represent a generalized response to imposing multiple insults on the granule cells. Rather, the effects of ethanol on granule cell viability appear to display some specificity as ethanol did not alter free radical-induced death. Because under the conditions used H_2O_2 does not induce apoptosis (Oberdoerster et al., 1998), it would appear that ethanol can enhance apoptotic, but not necrotic, granule cell death.

The ethanol-induced enhancement in granule cell death in the presence of non-depolarizing media is in agreement with the findings of Wegelius and Korpi (1995) and Bhave and Hoffman (1997). Conversely, addition of ethanol was reported not to alter granule cell survival in non-depolarzing, serum-free media (Zhang et al., 1998) or in granule cells incubated with 5 mM KCl in the presence of conditioned media (Castoldi et al., 1998). The reason for this discrepancy is unclear, but maybe related to the small sample size used in the latter two studies. Furthermore, in the study by Zhang et al. (1998) the effects of ethanol were determined using serum-free media containing 5 mM KCl which alone resulted in a low percentage of viable granule cells that may have precluded observing any further decrease with ethanol.

In vivo the severity of the neuronal loss after ethanol exposure appears to be a function of the pattern and amount of alcohol consumed as well as the developmental timing of the drinking episodes. Results with primary neuronal cultures indicate that ethanol alone does not exert a direct neurotoxic action on the cerebellar granule cells, but rather permissive conditions (e.g., limiting amounts of neurotrophic factors, absence of depolarizating input) are required for the neurotoxicity to be manifested. This necessity for permissive conditions would explain why ethanol is neurotoxic only at specific times. Thus, the present in vitro results would suggest that an in vivo window of vulnerability to ethanol would occur when the alcohol was present during periods of normal programmed cell death. For rat cerebellar granule cells a window of vulnerability to ethanol occurs around postnatal days 4–5 (Hamre and West, 1993). This is also approximately the time when apoptosis was shown to occur in the external germinal layer and the internal granule layer of the normally developing mouse cerebellum (Wood et al., 1993).

The ethanol-induced increase in death of granule cells incubated with non-depolarizing KCl was suggested to involve an inhibition of the trophic action of endogenous glutamate at the NMDA receptor (Wegelius and Korpi, 1995; Bhave and Hoffman, 1997). A similar mechanism, however, cannot account for the ethanol-induced increase in granule cell apoptosis after serum withdrawal. In agreement with other reports (Cox et al., 1990; Resink et al., 1994) NMDA did not exert a trophic action in the presence of serum-free depolarizing media, but rather NMDA increased granule cell death under these condition. This increase in cell death is probable due to an excessive increase in intracellular Ca^{2+} caused by the stimulation of both the NMDA receptor and voltage-dependent Ca^{2+} channels. Furthermore, addition of NMDA did not prevent the ethanol-induced increase in granule cell death. Because the amounts of ethidium-labeled cells in the presence of ethanol and ethanol + NMDA were comparable, ethanol appears to be antagonizing the effects of NMDA which is consistent with the literature (see Lovinger 1993).

Caspases, a family of cysteine-containing proteases, are integral parts of the apoptotic pathway (Cohen, 1997; Thornberry and Lazebnik, 1998). The caspases exist as inactive zymogens and are activated by proteolytic cleavage. Granule cell death induced by the withdrawal of both KCl and serum was reported to involve the caspases (Armstrong et al., 1997; Eldadah et al., 1997; D'Mello et al., 1998). In the present study the 24 h incubation in serum-free depolarizing media increased cleavage of the caspase-specific substrate, Ac-DEVD-amc, indicating cas-

pase activation. Although Eldadah et al. (1997) did not observe an increase in Ac-DEVD-amc cleavage activity in granule cells exposed to depolarizing serum-free medium, a shorter treatment period (12 vs. 24 h in the present study) was used. Caspase-3 is highly expressed in the CNS, displays high affinity for the substrate Ac-DEVD-amc, and has been implicated in both developmental programmed cell death as well as apoptosis induced by removal of neurotrophic support (Kuida et al., 1996; Stefanis et al., 1996; Keane et al., 1997; Thornberry et al., 1997). In the present study an increase in the amount of the active form of caspase-3 accompanied the enhanced Ac-DEVD-amc cleavage observed after serum deprivation. Conversely, D'Mello et al. (1998) did not observe an increase in the amount of active caspase-3 in granule cells incubated for 8 h in serum-free, non-depolarizing medium. The reason for this discrepancy is unclear, but may be related to the differences in the experimental protocols.

The ethanol-induced increase in granule cell death after serum withdrawal was associated with a further enhancement in Ac-DEVD-amc cleavage activity. This increased enzymatic activity was not due to a direct effect of ethanol on the caspases as inclusion of ethanol in the in vitro assay was without effect. In contrast to the increase in Ac-DEVD-amc cleavage activity, inclusion of ethanol during the serum deprivation did not cause a further increase in the amount of active caspase-3. A similar result was found in PC 12 cells (J. Oberdoerster and R.A. Rabin, unpublished). Because the substrate Ac-DEVD-amc can be cleaved by caspase-2, 3, 6, 7, 8, and 9 (Thornberry et al. 1997), one possible explanation is that ethanol was activating another caspase with affinity for Ac-DEVD-amc. Caspase-2 is also highly expressed in the nervous system (Kumar et al., 1994), but this caspase is not processed to its active form by treatment of cerebellar granule cells with non-depolarizing serum-free media (D'Mello et al., 1998). In addition, ethanol did not alter the amount of active caspase-2 in PC 12 cells (J. Oberdoerster and R.A. Rabin, unpublished). Because the caspase inhibitor Ac-DEVD-CHO blocks the activity of the ethanol-induced caspase (see Materials and methods) and caspase-6 is insensitive to Ac-DEVD-CHO (Srinivasula et al., 1996), this caspase does not appear to have been activated by ethanol. Caspase-7, which has lowest expression in the nervous system, is typically associated with the mitochondrial fraction (Cohen, 1997; Chandler et al., 1998). In the present study, the mitochondrial fraction would have been eliminated during the preparation of cell lysates for the caspase assay, and thus it appears unlikely that caspase-7 is the ethanol-activated caspase. Further as caspase-3 is cleaved to its active form by caspase-8 and -9 (Li et al., 1997; Stennicke et al., 1998), and no ethanol-mediated change in caspase-3 processing was observed, it is unlikely that these two Ac-DEVD-amc cleaving caspases were activated by ethanol. Thus, it appears unlikely that caspase-2, 6, 7, 8, or 9 were activated by ethanol. Although speculative it would appear that rather than increasing the proteolytic processing of the caspases, ethanol may be increasing caspase activity secondary to an action on a modulator such as the inhibitor of apoptosis proteins (IAPs). Additional studies are underway to elucidate the mechanism by which ethanol increases Ac-DEVD-amc cleavage activity.

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References

Ankarcrona, M., Dypbukt, J.M., Bonfoco, E., Zhivotovsky, B., Orrenius, S., Lipton, S.A., Nicotera, P., 1995. Glutamate-induced neuronal death: a succession of necrosis or apoptosis depending on mitochondrial function. Neuron 1, 961–973.

Armstrong, R.C., Aja, T.J., Hoang, K.D., Gaur, S., Bai, X., Alnemri, E.S., Litwack, G., Karanewsky, D.S., Fritz, L.C., Tomaselli, K.J., 1997. Activation of the CED3/ICE-related protease CPP32 in cerebellar granule neurons undergoing apoptosis but not necrosis. J. Neurosci. 17, 553–562.

Atabay, C., Cagnoli, C., Kharlamov, E., Ikonomovic, M., Manev, H., 1996. Removal of serum from primary cultures of cerebellar granule neurons induces oxidative stress and DNA fragmentation: protection with antioxidants and glutamate receptor antagonists. J. Neurosci. Res. 43, 465–475.

Balázs, R., Jørgensen, O., Hack, N., 1988. *N*-methyl-D-aspartate promotes the survival of cerebellar granule cells in culture. Neuroscience 27, 437–451.

Bauer-Moffett, C., Altman, J., 1977. The effect of ethanol chronically administered to preweanling rats on cerebellar development: a morphological study. Brain Res. 119, 249–268.

Bhave, S., Hoffman, P., 1997. Ethanol promotes apoptosis in cerebellar granule cells by inhibiting the trophic effect of NMDA. J. Neurochem. 68, 578–586.

Burgoyne, R., Graham, M., Cambray-Deakin, M., 1993. Neurotrophic effects of NMDA receptor activation on developing cerebellar granule cells. J. Neurocytol. 22, 689–695.

Castoldi, A.F., Barni, S., Randine, G., Costa, L.G., Manzo, L., 1998. Ethanol selectively interferes with the trophic action of NMDA and carbachol on cultured cerebellar granule neurons undergoing apoptosis. Dev. Brain Res. 111, 279–289.

Chandler, J.M., Cohen, G.M., MacFarlane, M., 1998. Different subcellular distribution of caspase-3 and caspase-7 following FAS-induced apoptosis in mouse liver. J. Biol. Chem. 273, 10815–10818.

Chandler, L.J., Sumners, C., Crews, F.T., 1993. Ethanol inhibts NMDA receptor-mediated excitotoxicity in rat primary neuronal cultures. Alcohol.: Clin. Exp. Res. 17, 54–60.

Chen, S., Hillman, D.E., 1989. Regulation of granule cell number by a predetermined number of Purkinje cells in development. Dev. Brain Res. 45, 137–147.

Cohen, G.M., 1997. Caspases: the executioners of apoptosis. Biochem. J. 326, 1–16.

Copani, A., Bruno, V., Barresi, V., Battaglia, G., Condorelli, D., Nicoletti, F., 1995. Activation of metabotropic glutamate receptors prevents neuronal apoptosis in culture. J. Neurochem. 64, 101–108.

Cox, J.A., Felder, C.C., Henneberry, R.C., 1990. Differential expression

of excitatory amino acid receptor subtypes in cultured cerebellar neurons. Neuron 4, 941–947.

D'Mello, S.R., Galli, C., Ciotti, T., Calissano, P., 1993. Induction of apoptosis in cerebellar granule neurons by low potassium: inhibition of death by insulin-like growth factor I and cAMP. Proc. Natl. Acad. Sci. U. S. A. 90, 10989–10993.

D'Mello, S., Anelli, R., Calissano, P., 1994. Lithium induces apoptosis in immature cerebellar granule cells but promotes survival of mature neurons. Exp. Cell Res. 211, 332–338.

D'Mello, S.R., Aglieco, F., Roberts, M.R., Borodezt, K., Haycock, J.W., 1998. A DEVD-inhibited caspase other than CPP32 is involved in the commitment of cerebellar granule neurons to apoptosis induced by K^+ deprivation. J. Neurochem. 70, 1809–1818.

Edelman, A.M., Higgins, D.M., Bowman, C.L., Haber, S.N., Rabin, R.A., Cho-Lee, J., 1992. Myosin light chain kinase is expressed in neurons and glia: immunoblotting and immunocytochemical studies. Mol. Brain Res. 14, 27–34.

Eldadah, B.A., Yakovlev, A.G., Faden, A.I., 1997. The role of CED-3-related cysteine proteases in apoptosis of cerebellar granule cells. J. Neurosci. 17, 6105–6113.

Fields, K.L., Currie, D.N., Dutton, G.R., 1982. Development of THY-1 antigen on cerebellar neurons in culture. J. Neurosci. 2, 663–673.

Hamre, K., West, J., 1993. The effects of the timing of ethanol exposure during the brain growth spurt on the number of cerebellar Purkinje and granule cell nuclear profiles. Alcohol.: Clin. Exp. Res. 17, 610–622.

Harada, J., Sugimoto, M., 1997. Polyamines prevent apoptotic cell death in cultured cerebellar granule neurons. Brain Res. 753, 251–259.

Keane, R.W., Srinivasan, A., Foster, L.M., Testa, M.-P., Ord, T., Nonner, D., Wang, H.-G., Reed, J.C., Bredesen, D.E., Kayalar, C., 1997. Activation of CPP32 during apoptosis of neurons and astrocytes. J. Neurosci. Res. 48, 168–180.

Kharlamov, E., Cagnoli, C.M., Atabay, C., Ikonomovic, S., Grayson, D.R., Manev, H., 1995. Opposite effect of protein synthesis inhibitors on potassium deficiency-induced apoptotic cell death in immature and mature neuronal cultures. J. Neurochem. 65, 1395–1398.

Kuida, K., Zheng, T.S., Na, S., Kuan, C.-Y., Yang, D., Karasuyama, H., Rakic, P., Flavell, R.A., 1996. Decreased apoptosis in the brain and premature lethality in CPP32-deficient mice. Nature 384, 368–372.

Kumar, S., Kinoshita, M., Noda, M., Copeland, N.G., Jenkins, N.A., 1994. Induction of apoptosis by the mouse Nedd2 gene, which encodes a protein similar to the product of the *Caenorhabditis elegans* cell death gene ced-3 and the mammalian IL-1β-converting enzyme. Gene Dev. 8, 1613–1626.

Labarca, C., Paigen, K., 1980. A simple, rapid, and sensitive DNA assay procedure. Anal. Biochem. 102, 344–352.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.

Li, P., Nijhawan, D., Budihardjo, I., Srinivasula, S.M., Ahmad, M., Alnemri, E.S., Wang, X., 1997. Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. Cell 91, 479–489.

Linden, R., 1994. The survival of developing neurons: a review of afferent control. Neuroscience 58, 671–682.

Lovinger, D.M., 1993. Excitotoxicity and alcohol-related brain damage. Alcohol.: Clin. Exp. Res. 17, 19–27.

Luo, J., Wes, J., Pantazis, N., 1997. Nerve growth factor and basic fibroblast growth factor protect rat cerebellar granule cells in culture against ethanol-induced cell death. Alcohol.: Clin. Exp. Res. 21, 1108–1120.

Lustig, H.S., Chan, J., Greenberg, D.A., 1992. Ethanol inhibits excitotoxicity in cerebral cortical cultures. Neurosci. Lett. 135, 259–261.

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

Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: applications to proliferation and cytotoxicity assays. J. Immunol. Methods 65, 55–63.

Napper, R., West, J., 1995. Permanent neuronal loss in the cerebellum of rats exposed to continuous low blood alcohol levels during the brain growth spurt: a stereological investigation. J. Comp. Neurol. 362, 283–292.

Oberdoerster, J., Kamer, A.R., Rabin, R.A., 1998. Differential effect of ethanol on PC12 cell death. J. Pharmacol. Exp. Ther. 287, 359–365.

Oppenheim, R., 1991. Cell death during development of the nervous system. Annu. Rev. Neurosci. 14, 453–501.

Pantazis, N., Dohrman, D., Goodlett, C., Cook, R., West, J., 1993. Vulnerability of cerebellar granule cells to alcohol-induced cell death diminishes with time in culture. Alcohol.: Clin. Exp. Res. 17, 1014–1021.

Rabin, R.A., 1988. Differential response of adenylate cyclase and ATPase activities after chronic ethanol exposure of PC12 cells. J. Neurochem. 51, 1148–1155.

Rabin, R.A., 1993. Ethanol-induced desensitization of adenylate cyclase: role of the adenosine receptor and GTP-binding proteins. J. Pharmacol. Exp. Ther. 264, 977–983.

Rakic, P., Sidman, R.L., 1973. Organization of cerebellar cortex secondary to deficits of granule cells in weaver mutant mice. J. Comp. Neurol 152, 133–162.

Resink, A., Hack, N., Boer, G.J., Balazs, R., 1994. Growth conditions differentially modulate the vulnerability of developing cerebellar granule cells to excitatory amino acids. Brain Res. 655, 222–232.

Schramm, M., Eimerl, A., Costa, E., 1990. Serum and depolarizing agents cause acute neurotoxicity in cultured cerebellar granule cells: role of the glutamate receptor responsive to *N*-methyl-D-aspartate. Proc. Natl. Acad. Sci. U. S. A. 87, 1193–1197.

Srinivasula, S.M., Fernandes-Alnemri, T., Zangrill, J., Robertson, N., Armstrong, R.C., Wang, L., Trapani, J.A., Tomaselli, K.J., Litwack, G., Elnemri, E.S., 1996. The ced-3/interleukin 1β converting enzyme-like homolog Mch6 and the lamin-cleaving enzyme Mch2α are substrates for the apoptotic mediator CPP32. J. Biol. Chem. 271, 27099–27106.

Stefanis, L., Park, D.S., Yan, C.Y.I., Farinelli, S.E., Troy, C.M., Shelanski, M.L., Greene, L.A., 1996. Induction of CPP32-like activity in PC12 cells by withdrawal of trophic support. J. Biol. Chem. 271, 44–52.

Stennicke, H.R., Jurgensmeier, J.M., Shin, H., Deveraux, Q., Wolf, B.B., Yang, X., Zhou, Q., Ellerby, H.M., Ellerby, L.M., Bredesen, D., Green, D.R., Reed, J.C., Froelich, C.J., Salvesen, G.S., 1998. Procaspase-3 is a major physiologic target of caspase-8. J. Biol. Chem. 273, 27084–27090.

Thornberry, N.A., Lazebnik, Y., 1998. Caspases: enemies within. Science 281, 1312–1316.

Thornberry, N.A., Rano, T.A., Peterson, E.P., Rasper, D.M., Timkey, T., Garcia-Calvo, M., Houtzager, V.M., Nordstrom, P.A., Roy, S., Vaillancourt, J.P., Chapman, K.T., Nicholson, D.W., 1997. A combinatorial approach defines specificities of members of the caspase family and granzyme B. J. Biol. Chem. 272, 17907–17911.

Wegelius, K., Korpi, E., 1995. Ethanol inhibits NMDA-induced toxicity and trophism in cultured cerebellar granule cells. Acta Physiol. Scand. 154, 25–34.

Wood, K.A., Dipasquale, B., Youle, R.J., 1993. In situ labeling of granule cell apoptosis-associated DNA fragmentation reveals different mechanisms of cell loss in developing cerebellum. Neuron 11, 621–632.

Yan, G.-M., Ni, B., Weller, M., Wood, K.A., Paul, S.M., 1994. Depolarization or glutamate receptor activation blocks apoptotic cell death of cultured cerebellar granule neurons. Brain Res. 656, 43–51.

Zhang, F.X., Rubin, R., Rooney, T.A., 1998. Ethanol induces apoptosis in cerebellar granule neurons by inhibiting insulin-like growth factor 1 signaling. J. Neurochem. 71, 196–204.