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AN ENHANCED EXPRESSION OF THE IMMEDIATE EARLY GENE, Egr-1, IS ASSOCIATED WITH NEURONAL APOPTOSIS IN CULTURE

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Abstract—Cultured cerebellar granule cells grown in medium containing 10 mM K^+ (K10) underwent apoptosis after four to five days *in vitro*, unless they were rescued by the addition of insulin-like growth factor-I. The few GABAergic neurons present in the cultures were more resistant to apoptotic degeneration, as indicated by double fluorescent staining with the chromatin dye Hoechst 33258 and with glutamate decarboxylase-67 antibodies. As compared with sister cultures grown in 25 mM K^+, K10 cultures showed an increased expression of the Egr-1 protein and a reduced expression of the Fos protein. The increase in Egr-1 was more substantial in granule cells than in GABAergic neurons, and was not oberved in K10 cultures chronically exposed to insulin-like growth factor-I. To examine the temporal relationship between the increase in Egr-1 and the development of programmed cell death, we induced apoptosis in K25 cultures at six days *in vitro* by replacing their medium with serum-free K10 medium. A substantial, but transient, increase in Egr-1 expression was observed in granule cells 6 h after switching the medium, a time that preceded the appearance of the phoenotypical markers of apoptotic death. An early reduction in the Fos protein was observed after switching the medium from K25 into serum-free K10, but also after switching the medium into serum-free K25, a condition which was not associated with the development of apoptosis nor with the increase in Egr-1.

We suggest that a transient induction of Egr-1 contributes to the chain of events leading to the execution phase of neuronal apoptosis in culture. © 1999 IBRO. Published by Elsevier Science Ltd.

Key words: cultured granule cells, GABAergic neurons, apoptosis, Egr-1, Fos.

Apoptotic neuronal death occurs both pre- and postnatally, and regulates the shape of the developing CNS by eliminating about 50% of supranumerary neurons.[21] It is generally believed that apoptotic degeneration requires the execution of a death program, which eventually leads to the activation of caspases.[19] The initial nuclear events that trigger the apoptotic program in developing neurons are still unknown, although a number of immediate early genes, including members of the fos and jun families, have been implicated.[7,17] Cultured cerebellar granule cells have been adopted as a model for the study of developmental apoptosis in neurons. These cells are usually grown in media containing high concentrations of K^+ (25 mM; K25), which, by depolarizing granule cell membranes, mimic the trophic input provided by afferent mossy fibers *in vivo*.[1,13] If granule cells are grown in media containing a lower concentration of K^+ (e.g., 5 or 10 mM), they undergo apoptosis spontaneously after four to five days of maturation *in vitro* (DIV), unless they are rescued by exogenous trophic agents, such as *N*-methyl-D-aspartate, metabotropic glutamate receptor agonists or insulin-like growth factor-I (IGF-I).[2,5,8,9,12] Previous studies have shown that cultured granule cells grown in "low-K^+"-containing medium express higher levels of Egr-1 (also named NGFI-A, zif/268 or Krox24) mRNA and lower levels of c-fos mRNA at 5 DIV, a time at which granule cells begin to show the hallmark features of apoptotic death.[6]

EXPERIMENTAL PROCEDURES

Cell culture

Primary cultures of cerebellar granule cells were prepared from eight-day-old Sprague–Dawley rats (Charles River, Calco, Italy), as described previously.[20] Cells were plated on 35-mm dishes (2×10^6/dish) in 2 ml of basal Eagle's medium (Gibco) containing 10% fetal calf serum and 25 or 10 mM K^+ (K25 or K10). Cytosine-D-arabinofuranoside (10 μM) was added 16–18 h after plating to avoid the

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Abbreviations: DIV, days *in vitro*; EDTA, ethylenediaminetetra-acetate; GAD, glutamate decarboxylase; IGF-I, insulin-like growth factor-I; OD, optical density; SC, serum-containing; SF, serum-free; TBS, Tris-buffered saline.

replication of non-neuronal cells. In some experiments, the medium of cultures grown in K25 at 6 DIV was switched into K25 or K10 medium, either in the absence or presence of serum. In the former case, fresh serum-free basal Eagle's medium was used. In the latter, we have used a conditioned medium from sister cultures to avoid the induction of excitotoxic death by the high concentration of glutamate present in fresh serum.[23]

Western blot analysis

Western blot analysis was performed in total protein extracts from cultured granule cells, using Fos or Egr-1 polyclonal antibodies (Santa Cruz; 100 μg/ml, dilution 1:300), as follows. Cultures were harvested in phosphate-buffered saline containing 5 mM EDTA, centrifuged at low speed and the pellets were immediately frozen at −80°C. After thawing, cell pellets were lysed in Tris–HCl buffer (40 mM, pH 6.8) containing 2.5% sodium dodecyl sulfate. Lysates were then sonicated and boiled for 5 min. Sixty micrograms of proteins were loaded per lane on 12% (Fos) or 8% (Egr-1) sodium dodecyl sulfate–polyacrylamide gels. Gels were then electroblotted on Hybond nitrocellulose paper for 2 h at 0.8 mA/cm^2 of gel, and the filters were blocked for 16 h in Tris-buffered saline (TBS; 100 mM Tris, 0.9% NaCl)/1% Tween 20 containing 1% bovine serum albumin. Blots were then incubated for 2 h with primary antibodies and, after washing three times with TBS/1% Tween 20, were incubated with secondary antibodies. Immunostaining was revealed by electrochemiluminescence. The specificity of Fos or Egr-1 antibodies was proven by preabsorbing the antibodies with the respective immunizing peptides (100 μg/ml) purchased from Santa Cruz.

Immunocytochemistry

Cells were fixed with 4% paraformaldehyde/4% sucrose in TBS for 15 min. Permeabilization was performed in TBS with 0.1% Triton X-100 for 5 min. After blocking for 30 min with 4% normal goat serum, incubation with either Fos or Egr-1 antibodies (1:1000) was carried out overnight at 4°C. Then, after washing, biotin-conjugated goat anti-rabbit secondary antibodies (dilution 1:100) were added for 1 h. Following a 45-min incubation with avidin–biotin–peroxidase complex (Vector), staining was developed using 3,3′-diaminobenzidine/H_2O_2. For double-staining experiments, cells were blocked again in 4% normal goat serum for 30 min, and then incubated with glutamate decarboxylase (GAD)-67 antibodies (Chemicon; 1:1000 for 2 h). Cy3-conjugated anti-rabbit secondary antibodies (Vector; 1:200) were used. Incubation with fluorescent secondary antibodies in the absence of GAD-67 primary antibodies showed only a light background staining.

The Egr-1 and Fos expression in granule cells was quantified by computer-assisted densitometry, using the MCID system (Imaging Research, St Catharine's, Ontario, Canada). Cells observed with a ×40 oil immersion objective were visualized on a video monitor connected to a CCD video camera. The same field was also observed under epifluorescent illumination using the rhodamine filter for the identification of GAD-positive neurons. The integrated optical density (OD) was obtained by the software-operated conversion of absolute grey values in arbitrary OD units. This computation was done after obtaining a linear calibration curve generated by the system, attributing the arbitrary value of 0 to the lightest grey value and 10 to the highest value. These values were averaged from several readings in different culture dishes for each experiment. Background values (ranging from 0.5 to 0.8 arbitrary units) did not vary substantially in different experiments, and were always subtracted. The integrated ODs in four random fields/dish were analysed.

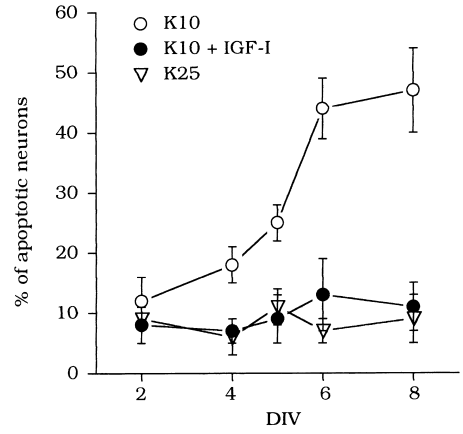


Fig. 1. Percentage of apoptotic neurons in cultures grown in K10, K25 or K10 + IGF-I at different DIV. IGF-I (25 ng/ml) was added daily during the first 4 DIV. Values are expressed as percentage of apoptotic neurons and were calculated from six to nine culture dishes. Neurons were counted from three random microscopic fields/dish (about 85–150 cells per field). Apoptosis was examined after staining the cultures with the fluorescent chromatin dye, Hoechst 33258. Neurons showing fragmentation and/or condensation of nuclear chromatin were scored as apoptotic. $*P < 0.01$ (one-way ANOVA + Fisher PLSD), as compared with the corresponding values obtained from cultures grown in K25 or in K10 + IGF-I.

Assessment of apoptotic death

Chromatin fragmentation and condensation, two hallmark features of apoptotic death, were visualized by staining the cultures with the fluorescent dye Hoechst 33258 (0.4 μg/ml), as described previously.[5] The number of apoptotic neurons was counted from three randomly chosen microscopic fields per dish.

RESULTS

Assessment of apoptosis in cultured cerebellar neurons

Cultured cerebellar granule cells grown in K10 underwent apoptosis after 4–5 DIV, as reported previously.[5] Hoechst chromatin staining revealed that the percentage of neurons bearing the hallmark features of apoptotic degeneration (i.e. chromatin fragmentation and/or condensation) increased linearly between 4 and 6 DIV, reaching a plateau afterwards (Fig. 1). In a few cultures, however, the development of apoptosis was accelerated or delayed for unexplained reasons. In the experiment reported in Table 1, for example, about 50% of cells grown in K10 already showed the phenotypical hallmarks of apoptosis at 4 DIV (not shown). In this particular K10 culture, the total cell number was already reduced by 40% at 5 DIV, as compared with sister cells grown in K25. Apoptotic death progressed more slowly from 5 to 8 DIV (Table 1). We have chosen the experiment reported in Table 1 to describe the different sensitivity of granule cells and GABAergic neurons to "low-K^+"-induced apoptosis. Although granule cells were largely predominant in our cultures (>90% of the total

Table 1. GABAergic neurons are more resistant than granule cells to developmental apoptosis in cultures grown in K10

	K25		K10	
	5 DIV	8 DIV	5 DIV	8 DIV
Total number of neurons	456 ± 30	365 ± 33	284 ± 28*	207 ± 13*
Number of GABAergic neurons	22 ± 2.6	31 ± 3	22 ± 4.5	14 ± 0.7*
Percentage of GABAergic neurons	4.8 ± 0.7	8.4 ± 0.6	7.8 ± 1.2*	6.8 ± 0.8

Values are means ± S.E.M. of six individual determinations. Cell number was counted from three random fields/dish. *$P < 0.01$ (Student's *t*-test), as compared with corresponding values in K25 cultures. In this particular culture, apoptosis developed at earlier times (from 3 to 5 DIV), leading to a substantial reduction in the number of neurons already at 5 DIV in cultures grown in K10. This rapid development of neuronal death was optimal for the examination of the relative resistance of GABAergic neurons to "low-K^+"-induced apoptosis.

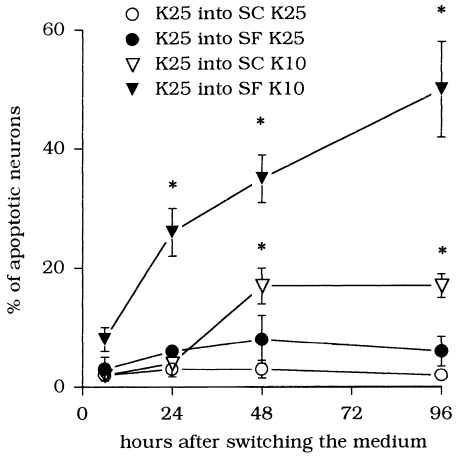


Fig. 2. Development of apoptosis in cultures grown in K25 and switched at 6 DIV into SC K25 (controls), SF K25, SC K10 or SF K10. Values are means ± S.E.M. of six individual determinations. *$P < 0.01$ (one-way ANOVA + Fisher's PLSD), compared with the respective controls.

cell population), 3–4% of GABAergic neurons could be identified by immunostaining with GAD-67 antibodies. Interestingly, the number of GABAergic neurons was not reduced in K10 cultures at 5 DIV in spite of the extensive neuronal loss (Table 1), suggesting that these neurons are more resistant than granule cells to "low-K^+"-induced apoptosis.

Treatment of K10 cultures with IGF-I (25 ng/ml; applied daily from the time of plating up to 4 DIV) rescued neurons from apoptotic death throughout the cultivation time (Fig. 1).

In some experiments, cultures grown in K25 at 6 DIV were acutely switched into serum-free (SF) K10 medium for a rapid induction of apoptosis, and examined at different times after the medium shift. The number of apoptotic neurons did not increase at 6 h, but was markedly enhanced at 24–96 h after switching the medium (Fig. 2). However, GAD-67-positive neurons never showed signs of chromatin fragmentation and/or condensation (Fig. 3A, B), and their relative percentage was increased after switching the medium from K25 into SF K10 (Fig. 3C), confirming the resistance of GABAergic neurons to this particular type of apoptosis. When cultures were switched from K25 into conditioned serum-containing (SC) K10 medium, apoptosis developed, but to a smaller extent as compared to cultures switched into SF K10 medium. Switching from K25 into SF K25 medium did not affect neuronal viability substantially up to 96 h (Fig. 2).

Developmental expression of Fos protein in cultured cerebellar neurons

Western blot analysis with polyclonal Fos antibodies revealed a major band at about 62,000 mol. wt, which corresponds to the deduced molecular size of the Fos protein. The intensity of this band was greater in protein extracts from K25 cultures at 4 DIV than in extracts from sister cultures grown in K10 (Fig. 4). In K25 cultures at 2 or 8 DIV, Fos expression was lower than at 4 DIV, but always greater than in corresponding cultures grown in K10 (not shown).

Immunocytochemical analysis performed in K25 cultures at 4 DIV showed a substantial expression of Fos protein, which was distributed heterogeneously within the cell population and, at the cellular level, was expressed in the nuclear region (Fig. 5A). Fos immunostaining was nearly absent in K10 cultures (Fig. 5B). Double labeling with Fos and GAD-67 antibodies showed that Fos expression was always lower in GABAergic neurons than in granule cells throughout the cultivation time (not shown).

Developmental expression of the Egr-1 protein in cultured cerebellar neurons

Western blot analysis with polyclonal Egr-1 antibodies revealed a major band of mol. wt 80,000 in protein extracts from mouse NHI3T3 cells, but a lower mol. wt band (about 70,000) in extracts from cultured granule cells (Fig. 6). This is consistent with the lower molecular size of rat Egr-1, as compared with mouse or human Egr-1.[14] Egr-1 expression was higher in K10 than in K25 cultures,

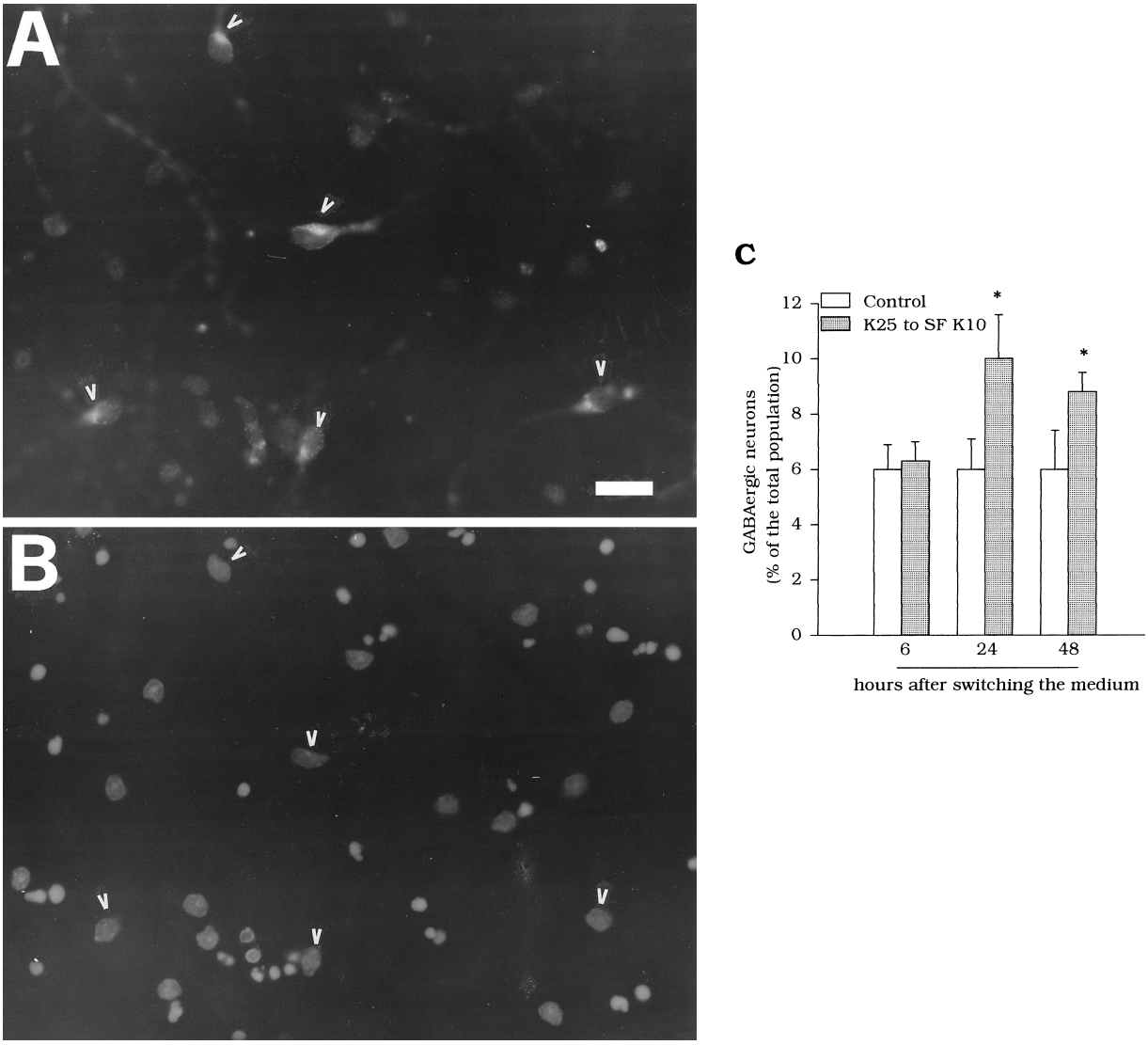


Fig. 3. (A) GAD-67 immunostaining (arrowheads) in cultures grown in K25 and switched at 6 DIV into SF K10 medium. Cultures were examined 48 h after switching the medium. In spite of some light background staining, GABAergic neurons are clearly distinguished from granule cells by their shape, as well as from the intense staining observed near the emergence of the axon. Note that the staining excludes the nuclear region of the cell. Scale bar = 20 μm. (B) The same field is stained with the fluorescent chromatin dye, Hoechst 33528. Note that none of the GABAergic neurons shows any sign of chromatin fragmentation and condensation, in spite of the high percentage of apoptotic neurons in this particular culture. (C) The percentage of GABAergic neurons is increased 24 or 48 h after switching the cultures from K25 into SF K10 medium, confirming that these neurons are more resistant to apoptotic death. Values are means ± S.E.M. of 12 individual determinations. $*P < 0.01$ (Student's *t*-test), compared with corresponding values obtained in control cultures (switched from K25 into SC K25 medium collected from sister cultures).

and was particularly intense at 6 DIV (Fig. 6). This pattern of expression was confirmed by immunocytochemical analysis, which showed a greater immunostaining in K10 than in K25 cultures at 6 DIV (Fig. 7A, B, Table 2). In the few cultures grown in K10, the peak in Egr-1 expression was anticipated or delayed. Interestingly, in these particular cultures, the development of apoptosis was also anticipated or delayed (not shown). Treatment of K10 cultures with IGF-I reduced the percentage of granule cells immunopositive for Egr-1 (Table 2). In K10 cultures at 6 DIV, Egr-1 immunostaining was lower in GABAergic neurons than in granule cells (Fig. 7C, D, Table 2).

Expression of Fos and Egr-1 in cultures switched from K25 into K10 medium for the acute induction of apoptosis

In cultures grown in K25 and acutely switched into SF K10 medium at 6 DIV, the expression of Egr-1 was increased in granule cells as early as 6 h

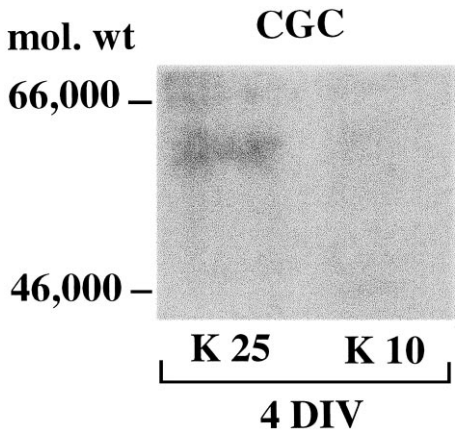


Fig. 4. Representative western blot showing the greater expression of Fos protein in cultured granule cells (CGC) grown in K25 at 4 DIV, as compared with sister cultures grown in K10. No additional bands were present in the immunoblot other than the major band of mol. wt 62,000 and a very light band at a mol. wt of about 45,000.

after the medium shift (Fig. 8). A strong induction of Egr-1 was also observed in the nuclei of the few astrocytes contaminating the cultures, but this effect was possibly related to the physical stress associated with the medium shift, because it was also observed in control cultures switched from K25 into SF K25, a condition in which there was no Egr-1 induction in neurons (Fig. 8A). The higher expression of Egr-1 in granule cells switched from K25 into SF K10 medium had a short half-life, and was less substantial at 24 h (Fig. 8D, E). Egr-1 was induced to a much lesser extent in GABAergic neurons at 6 or 24 h after switching the medium from K25 into SF K10 (Fig. 8E).

Fos expression was rapidly and markedly reduced after switching the medium from K25 into SF K10, but this reduction did not directly correlate with the development of apoptosis, because it was also observed in control cultures switched from K25 into SF K25 medium (Fig. 9, Table 3), in which no clear-cut apoptosis was observed (see Fig. 2). In addition, Fos expression was reduced to the same extent in cultures switched into SC K10 medium (Fig. 9B, Table 3), a condition that was associated with a lesser increase in Egr-1 expression (not shown) and in the percentage of apoptotic neurons (see Fig. 2) than in cultures switched into SF K10 medium.

DISCUSSION

Primary cultures of cerebellar neurons from eight-day-old rats are highly enriched in granule cells, which, at this age, still divide and migrate across the cerebellar cortex.[11] Few GABAergic neurons (mostly basket, stellate or Golgi cells) are present, but they do not exceed 3–5% of the total cell population.[20] These cultures are usually grown in media containing depolarizing concentrations of

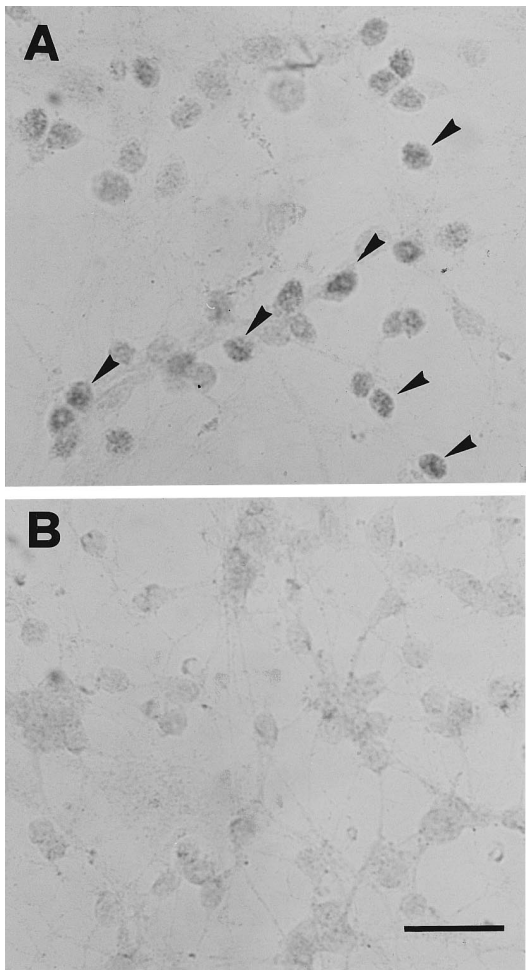


Fig. 5. Fos immunoreactivity in cultures at 4 DIV grown either in K25 (A) or in K10 (B). Note, in A, that immunostaining is distributed heterogeneously within the cell population and is confined to the nuclear regions of granule cells. Scale bar = 30 μm.

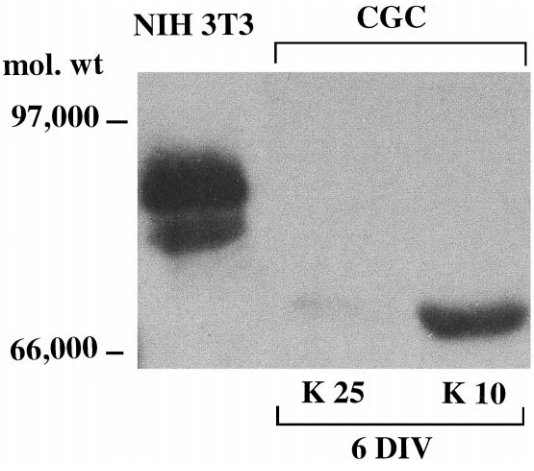


Fig. 6. Representative western blot showing the greater expression of the Egr-1 protein in cultured granule cells (CGC) grown in K10 at 6 DIV, as compared with sister cultures grown in K25. Note that, in mouse NIH3T3 cells, the Egr-1 antibody labeled a doublet of higher molecular weight, in agreement with Huang *et al.*[14] No additional bands were present in the immunoblots.

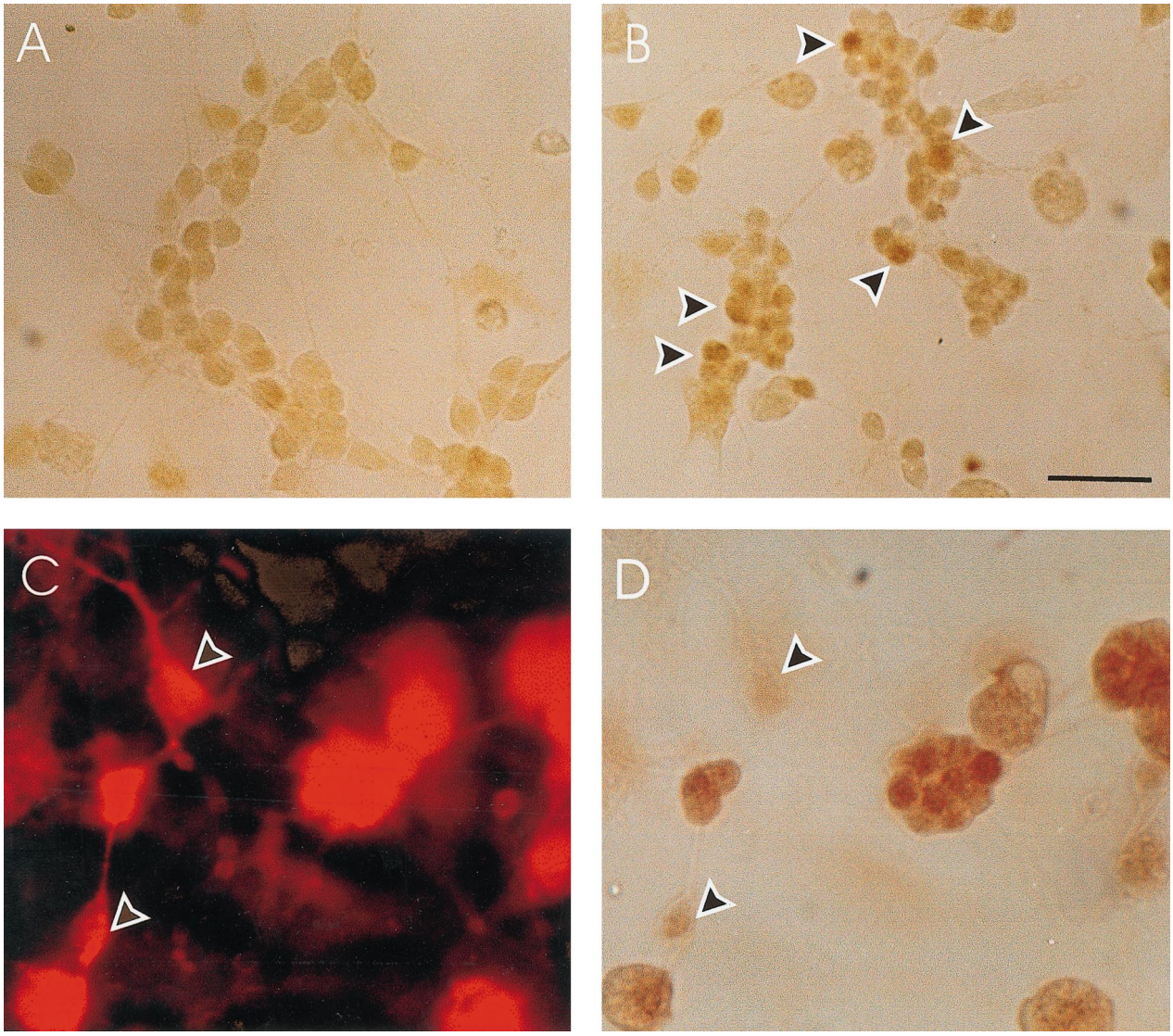
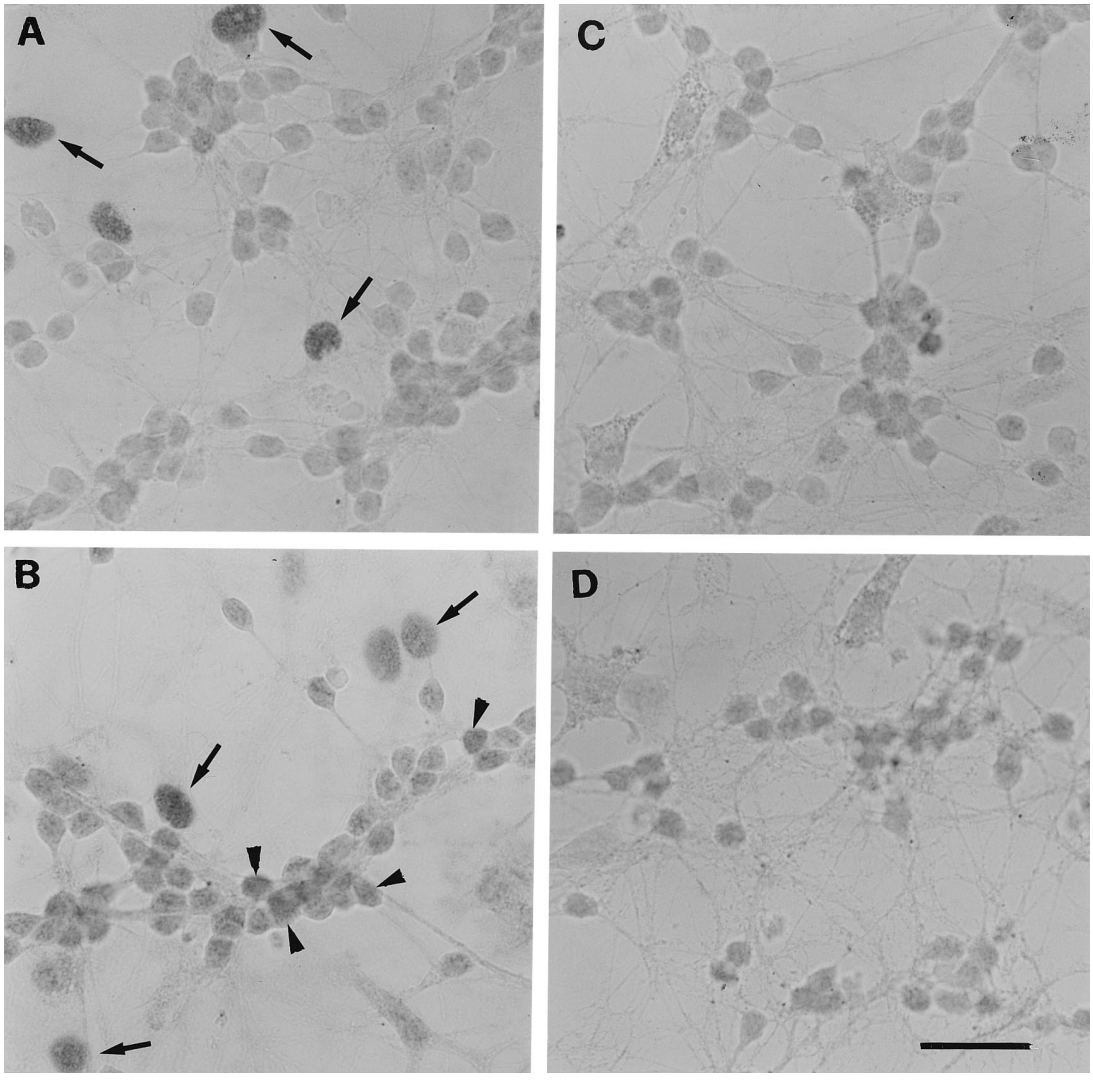


Fig. 7. Egr-1 immunostaining in two representative sister cultures grown in K25 (A) or K10 (B) medium at 6 DIV. Scale bar = 30 μm. In C and D, double immunostaining for GAD-67 (C) and Egr-1 (D) is shown in a representative culture grown in K10 at 8 DIV. In spite of the high background in C, GABAergic neurons were identified by the neuritic staining, as well as by the particular shape of the cell body. Note that Egr-1 immunostaining is lighter in GABAergic neurons (arrowheads) than in granule cells. Scale bar = 15 μm.

K^+ (i.e. K25), which support neuronal survival by producing membrane depolarization, resulting in the opening of voltage-sensitive Ca^{2+} channels.[1,13] It is generally believed that chronic depolarization mimics the excitatory drive provided by the mossy fibers afferent to the granule cell layer in the intact cerebellum.[1] When grown in media containing a lower concentration of K^+ (e.g., K10), cultured cerebellar neurons undergo apoptotic death after the first 4 DIV. Apoptosis may also be induced by acutely switching the medium from K25 into K10 or K5,[8] a procedure that allows the examination of the temporal relationship between specific biochemical changes and the onset of apoptotic degeneration. Interestingly, the present data show that the few GABAergic neurons present in these cultures are more resistant than granule cells to low-K^+-induced apoptosis. This offers an additional instrument for the identification of gene products or other biochemical markers that are related to apoptotic death in a cell-specific manner.

The execution phase of an apoptotic program, leading to cell shrinkage and chromatin fragmentation and condensation, is not cell specific, and is mediated by a cascade of enzymatic reactions that involves caspase activation.[19] The "condemned" phase of apoptosis is instead cell specific, and is triggered either by the lack of trophic inputs, or by extracellular signals that cause a metabolic perturbation or alter cell's adhesion to the extracellular matrix.[10,16] The "linking bridge" between the condemned and the execution phase of neuronal



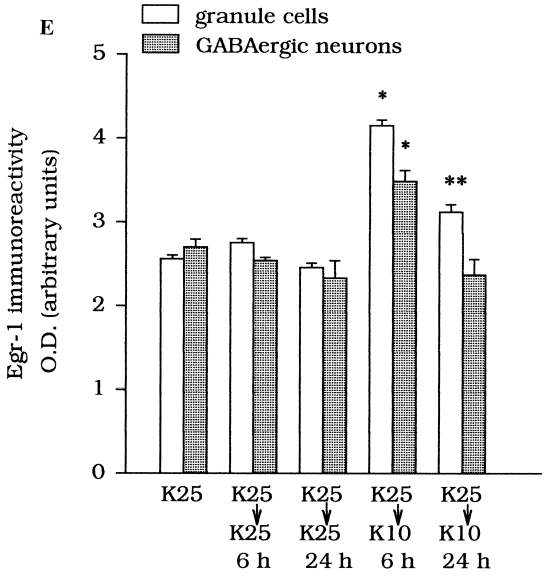


Fig. 8. Transient induction of Egr-1 in cultures grown in K25 and switched at 6 DIV into SF K10 medium. (A) Control cultures switched from K25 into serum-free K25 medium, 6 h after the medium shift. (B) Cultures switched into SF K10 medium, 6 h after the medium shift. (C) As in A, but 24 h after the medium shift. (D) As in B, but 24 h after the medium shift. Note, in B, the induction of Egr-1 in granule cells (arrowheads); in A and B, the non-specific induction of Egr-1 in the nuclear region of glial cells is shown by arrows. (E) Quantitative assessment of Egr-1 immunoreactivity in granule cells and GABAergic neurons in the same conditions as in A–D. Note the lower expression of Egr-1 in GABAergic neurons. $*P < 0.01$ vs the corresponding values obtained under all the other conditions; $**P < 0.01$ vs K25 or K25 switched into K25, 6 or 24 h (one-way ANOVA + Fisher PLSD).

Table 2. Quantitative analysis of Egr-1 immunoreactivity in cultures at six days *in vitro* grown in K25, K10 or K10 + insulin-like growth factor-I

	Egr-1 immunoreactivity OD (arbitrary units)	
	Granule cells	GABAergic neurons
K25	2.26 ± 0.032	2.65 ± 0.28
K10	3.98 ± 0.059*	2.60 ± 0.28
K10 + IGF-I	3.08 ± 0.048**	2.90 ± 0.17

For K25 cultures, $n = 244$ granule cells and 11 GABAergic neurons from randomly chosen microscopic fields from three dishes; for K10 cultures, $n = 422$ granule cells and 33 GABAergic neurons from seven dishes; for K10 + IGF-I cultures, $n = 308$ granule cells and 17 GABAergic neurons from four dishes. IGF-I was applied daily at concentrations of 25 ng/ml during the first 4 DIV. In cultures grown in K10, a large proportion of cells (49.76%) showed OD values higher than 3.98 (13.27% of cells had OD values >5.19), 29% of cells had values ranging from 2.77 to 3.98, whereas the remaining cells (21.3%) had OD values <2.77. Only 13.1% of cells from K25 cultures had OD values >2.77, with only one of 244 cells showing a value >3.98. About half of the cells from cultures grown in K10 + IGF-I had OD values ranging from 2.77 to 3.98, but only 15% had values >3.98 (four of 308 cells had values >5.19). *$P < 0.01$ vs the corresponing values in K25 and K10 + IGF-I; **$P < 0.01$ vs the corresponding values in K25 (one-way ANOVA + Fisher PLSD).

Table 3. Quantitative assessment of Fos immunoreactivity in cultures grown in K25 and switched into serum-containing or serum-free K25 or K10 medium

	Fos immunoreactivity OD (arbitrary units)
K25	2.64 ± 0.021
K25 into SC K25	2.58 ± 0.044
K25 into SF K25	1.22 ± 0.014*
K25 into SC K10	1.35 ± 0.031*
K25 into SF K10	1.11 ± 0.025*

Cultures were treated as described in the legend of Fig. 9; $n = 142–218$ neurons from three dishes. Values are means ± S.E.M. *$P < 0.01$ (one-way ANOVA + Fisher PLSD), compared with K25 cultures which were not switched into K25 or K10 medium.

apoptosis involves a number of genes that have been explored in cultured sympathetic neurons deprived of nerve growth factor[7] or in cultured cerebellar granule cells grown in K25 and acutely switched into SF K5 medium.[17] These models have in common an early induction of c-jun, which precedes the execution phase of apoptosis, whereas c-fos and fosB mRNA levels are reduced in granule cells but increase in sympathetic neurons.[7,17] No attention has been focused on Egr-1, an immediate early gene that encodes a zinc-finger nuclear protein[3,15,24] and is rapidly induced by synaptic activity or by activation of excitatory amino acid receptors in the CNS.[4,22,25,27] Cultured cerebellar neurons grown in K10 at 5 DIV show a dramatic increase in Egr-1 mRNA levels, associated with a reduction of c-fos mRNA levels.[6] We now report a similar increase in the Egr-1 protein that peaks at 6 DIV, when the percentage of apoptotic neurons reached a plateau. Neurons grown in K25 showed a lower expression of Egr-1, an effect that appears to be correlated with cell viability, rather than to chronic depolarization with the ensuing influx of extracellular Ca^{2+}. Accordingly, a reduced expression of Egr-1 was also observed in K10 cultures chronically exposed to IGF-I, which rescues granule cells through the activation of the phosphatidylinositol-3-kinase pathway.[9] The low expression of Egr-1 in GABAergic neurons, which survived better than granule cells in K10 cultures, led us to hypothesize that induction of Egr-1 was causally linked to the development of apoptosis. We therefore examined the temporal relationship between changes in Egr-1 expression and the development of apoptosis in cultures acutely switched from K25 into SF K10 medium. This medium shift was followed by a rapid and transient induction of Egr-1, which preceded the morphological abnormalities of nuclear chromatin, and was absent in GABAergic neurons. It cannot be excluded that the increase in Egr-1 was secondary to the rapid reduction of Fos in the chain of events leading to apoptotic death. However, this seems unlikely because Fos expression was equally reduced in cultures switched from K25 into SF K25 medium, a condition which was not associated with the induction of Egr-1 nor with the development of apoptosis. Thus, we speculate that a reduction in Fos expression is not sufficient for the development of apoptosis, whereas an early induction of Egr-1 may be a harbinger of granule cell death. A causative role for Egr-1 has been demonstrated in melanoma cells, where either negative dominant mutants or Egr-1 antisense oligonucleotides inhibit thapsigargin-induced apoptosis.[18] It will be interesting to examine whether Egr-1 regulates the expression of genes that are involved in the execution of an apoptotic program.

CONCLUSIONS

The present results show that an increased expression of Egr-1 is associated with the development of apoptosis in cerebellar granule cells grown in primary cultures. This raises the question of whether the induction of Egr-1 is causally linked to the execution of the apoptotic program or is rather an epiphenomenon of programmed neuronal death. We favor the former hypothesis because, after switching the culture medium from K25 into SF K10, a transient induction of Egr-1 preceded the morphological demise of granule cells, and because no induction was observed in GABAergic neurons, which exhibited an intrinsic resistance to apoptosis. Studies with antisense oligonucleotides are needed to examine this question more directly.

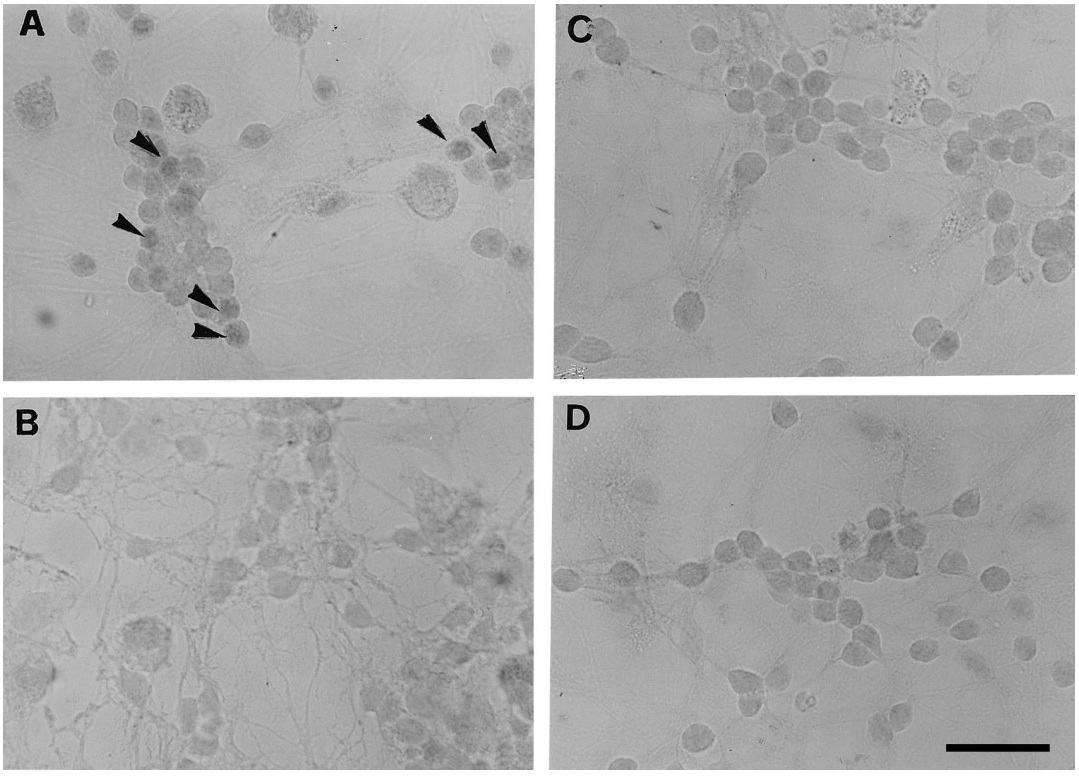


Fig. 9. Fos immunoreactivity in cultures grown in K25 and switched at 6 DIV into SC K25 (A), SC K10 (B), SF K25 (C) or SF K10 (D). Immunostaining was performed 6 h after switching the medium. Note the reduction of Fos immunostaining in B–D. Scale bar = 30 μm.

Egr-1 and c-fos are reported to be co-induced (although with a different threshold and time-course) in response to synaptic activation in CNS neurons.[4,22,25,26] "Low-K^+"-induced apoptosis of cultured granule cells provides a unique example of an opposite regulation of these two immediate early genes in neurons. It is an attractive hypothesis that the balance between c-fos and Egr-1 contributes to regulate neuronal commitment to apoptotic death.

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