PERGAMON

Int. J. Devl Neuroscience 18 (2000) 447–454

INTERNATIONAL JOURNAL of DEVELOPMENTAL NEUROSCIENCE

www.elsevier.com/locate/ijdevneu

Contribution of NF-κB and p53 in the glutamate-induced apoptosis

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Received 26 March 1999; received in revised form 10 May 1999; accepted 15 May 1999

Abstract

Exposure of primary cultures of cerebellar granule cells for 15 min to micromolar concentrations of glutamate results in cell death of both necrotic and apoptotic types. Among the intracellular events triggered by glutamate, we identified two transcriptional factors: the p50 member of the NF-κB family and the tumor suppressor phosphoprotein p53. Pretreatment of the cultures with aspirin, which inhibits NF-κB activation, or with specific p53 antisense oligonucleotide, which inhibits p53 transcription, resulted in a complete prevention of glutamate-induced p53 induction and apoptosis. These findings suggest the existence of a transcriptional program activated by glutamate receptor stimulation in which p50 and p53 play a relevant role. Then, we studied the expression of two p53 downstream genes that could participate in the glutamate-induced pro-apoptotic pathway: p21, which codes for an inhibitor of different cyclin dependent kinases, and MSH2, which codes for a protein involved in the recognition and repair of DNA mismatches. We found that primary cerebellar neurons expressed p21 and MSH2 at very low levels in basal conditions. However, very soon after a brief exposure of the cells to glutamate, the expression of both proteins was dramatically enhanced.

On these bases, we propose NF-κB, p53, p21 and MSH2 as relevant contributors of the glutamate-induced pro-apoptotic pathway. Understanding this cascade of nuclear events may unravel specific targets for pharmacological intervention for those neurological diseases in which excitatory amino acid-induced apoptosis plays a relevant role.

Keywords: Cerebellar neurons; Cyclin-dependent kinase; Excitotoxicity; Glutamate; Neurodegenerative diseases; Tumour suppressor gene; Aging

1. Introduction

Brain aging is a physiological event characterized by a slow and irreversible loss of neurons [6,31,43]. Although this process has been known for decades, the mechanisms underlying the different cell phenotype vulnerability, the age-related extracellular signals triggering cell death, and the intracellular pathways responsible for translating these signals into cell-death programs, are still a matter of extensive investigation. Neuronal death is also involved in various chronic and progressive neurodegenerative diseases, including Alzheimer's and Parkinson's disease [16,37,46,53,55]. The border zone separating physiological and pathological neuronal death is often undetectable. Since the lack of significant signs of inflammatory response, neuronal death occurring during aging is believed to be associated, at least in part, with apoptosis, a process characterized by cell shrinkage, chromatin condensation and internucleosomal DNA cleavage [52,59]. Apoptotic neuronal death also occurs under other physiopatholo-

Abbreviations: cdk, cyclin-dependent kinase; CNS, Central Nervous System; EAA, Excitatory Amino Acids; MSH2, MutS Homolog 2; NF-κB, Nuclear Factor-kappa B.

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0736-5748/00/$20.00
PII: S0736-5748(00)00018-6

gical and experimental conditions, including development [10], neurothrophic factor deprivation [35,47,49], exposure to β-amyloid [22,36], transient ischemia [7,28], and exposure to hydrogen peroxide [33,56,57]. Although a great amount of information is known about the intracellular contributors promoting apoptosis of proliferating cells, little is known about the molecular mechanisms underlying apoptosis in post-mitotic, terminally differentiated cells like neurons.

We previously demonstrated that exposure of primary cultures of cerebellar granule neurons to neurotoxic concentrations of glutamate results in the activation of NF-κB/rel proteins [18]. Functional significance of such effects has been suggested by the finding that under the same experimental conditions, salicylates prevent glutamate-induced neuronal death and that at the same doses, they inhibit the glutamate-induced activation of NF-κB DNA binding. A better understanding of the contribution of NF-κB mediated transcription to neuronal death in neurological diseases represents a recently raised debate with important pharmacological implications [3,19]. To study the pro-apoptotic pathways triggered by NF-κB activation in response to a specific deleterious stimulus it will be crucial to identify the nature of the genes that are under the control of NF-κB/rel factors in the CNS. We have focused our interest on a specific NF-κB modulated gene, the one encoding the tumor suppressor protein p53 [58]. p53 is a cell cycle checkpoint protein that contributes to the preservation of genetic stability [30,54]. In recent years, several groups have contributed to unravel an active contribution of p53 to neurodegeneration [9,15,32,50,64]. On our side, we worked on the hypothesis that NF-κB may contribute to cell death triggered by glutamate by switching on pro-apoptotic target genes among which is p53 [20]. In fact, upon exposure of cerebellar granule cells to a 15-min-pulse of a micromolar concentration of glutamate, results in a significant, short-lasting increase of p53 expression [50]. Measurement of p53 mRNA levels suggested that treatment of the cells with glutamate results, at least in part, in an increased p53 gene transcription. Furthermore, p53 over-expression was associated with increased p53 DNA binding activity, suggesting an enhanced p53 transcriptional activity.

We hypothesized that NF-κB and p53 could be sequential participants in a common intracellular pathway responsible for the induction and progression of neuronal apoptosis. In addition, our effort was to identified p53 downstream genes, that could be activated in the cascade of an event triggered by glutamate leading to neuronal apoptosis. We took into consideration two cell-cycle-related proteins, the cyclin-dependent kinase (cdk) inhibitor p21, which is a well characterized transcriptional target of p53 [14], and the DNA mismatch repair protein MSH2, which is one of the most relevant proteins involved in the recognition and repair of a specific type of DNA damage [41,45].

2. Experimental procedures

2.1. Cell culture

Primary cultures of cerebellar granule cells were prepared from 8-day-old Sprague-Dawley rat puts as previously described [50]. Briefly, cells were plated onto poly-l-lysine-coated dishes and cultured for 12 days in vitro (DIV) in basal Eagle's medium containing 10% heat-inactivated foetal bovine serum, 2 mM glutamine, 50 g/ml gentamicin and 25 mM KCl, at the density of 1.5×10^5 cells/cm^2. Cytosine arabinoside (100 M) was added to the cultures 18 h after seeding to prevent non-neuronal cell proliferation.

2.2. Evaluation of neuronal death

Neurotoxicity was evaluated in cerebellar granule cells at DIV 12 following the exposure of the cells to glutamate (100 μM). The concentrations of glutamate used in the present study were maximally effective in terms of cell death and were chosen on the basis of the results from a series of previous experiments using different concentrations of glutamate, ranging from 5 μM to 100 μM (data not shown). In particular, the culture-conditioned media of cerebellar granule cells were collected and the cells were washed once with Locke's solution (154 mM NaCl, 5.6 mM KCl, 3.6 mM $NaHCO_3$, 2.3 mM $CaCl_2$, 5.6 mM glucose, 5 mM Hepes, pH 7.4) and exposed to glutamate for 15 min. After this period, cells were washed three times with Locke's solution containing 1 mM $MgSO_4$ and returned to the original culture-conditioned media. The apoptotic cells were evaluated morphologically by the rapid onset of pyknotic and shrinking nuclei detected by DAPI fluorescent strain [51]. Briefly, 8 h after glutamate pulse cells were fixed in 4% paraformaldehyde, and permeabilized by a 5-min exposure to 0.02% Triton X-100 in PBS. After permeabilization, cells were incubated for 15 min with (5 mg/ml) DAPI.

When indicated, aspirin (ASA) was present in the culture media at the final concentration of 3 mM, 5 min before and during glutamate exposure. The specific p53 antisense oligonucleotide was synthetized according to Uberti et al. [50], and added to the cultures at the concentration of 25 μM, 2 h before glutamate exposure.

2.3. Immunocytochemistry

Cultures were fixed for 30 min in 50 mM PBS containing 4% paraformaldehyde. Following several rinses

in PBS, cells were incubated for 20 min in Tris-buffered (pH 7.4) saline containing 0.5% hydrogen peroxide to block endogenous peroxidase staining. Immunostaining was performed as follows: cells were permeabilized by a 5-min exposure to 0.02% Triton X-100 in PBS and incubated for 24 h at 4°C with the primary antibody. The antibodies used were: (a) a polyclonal anti-p53 antibody (R19), from Santa Cruz Biotechnology, used at 1:500 dilution, that recognizes the epitope corresponding to amino acid 373–391 mapping at the carboxy terminus of rat p53; (b) a polyclonal anti-MSH2 antibody (provided by Dr J. Jiricny) used at 1:500 dilution, raised against the C-terminal domain of human MSH2; and (c) a polyclonal anti-p50, used at 1:200 dilution [17]. After several rinses, cells were processed using the avidin–biotin complex Kit (ABC Elite Kit, Vector Laboratories). Enzymatic reaction was developed as previously described [50]. In order to verify the specificity of the chromogen reaction, some dishes were processed identically except that cells were incubated with the primary antibody solvent. In these conditions, no immunostaining was generated.

A blind analysis of immunoreactive cells was performed in all dishes. A minimum of 100 neurons were counted in at least three fields from three different culture dishes. A semiquantitative study of immunoreactivity was carried out using the Magiscan Image Analysis System, designed and made by Joyce-Loebl Ltd (London, UK). The General Image Analysis Software (Genias) provided by Joyce-Loebl as standard with Magiscan gave access to the image processing and analysis function of Magiscan. The microdensitometric analysis was performed as previously described [38] by measuring the following parameters in each cell body: (i) integrated optical density (ID), i.e. the sum of optical density for each pixel in the cell area considered; and (ii) area, i.e. the sum of pixels over the image of the cell point set. This evaluation allows subtraction of background to normalize values from different samples. The ratio ID/area from each cell was processed for the statistical analysis. The statistical significance of differences between the values was made by one-way analysis of variance followed by Student's *t*-test. Data are presented as the mean ± SEM of at least three experiments performed in triplicate.

2.4. Western blot

Cells were harvested in 100 μl of lysis buffer containing 50 mM Tris, pH 7.6, 150 mM NaCl, 5 mM EDTA, 1 mM phenyl methyl suphonyl fluoride, 0.5 g/l leupeptin, 5 μg/l aprotinin, and 1 μg/ml pepstatin. The samples were sonicated and centrifuged at 15,000 *g* for 30 min at 4°C. The resulting surnatants were isolated and protein content determined by a conventional method (BCA protein assay Kit, Pierce, Rockford, IL). A total of 30 μg of total proteins were electrophoresed on 12% SDS-PAGE, and transferred to nitrocellulose paper (Schleicher and Schuell, Dassel, Germany). Filters were incubated at 4°C overnight with the polyclonal anti-p21 antibody or the polyclonal anti-p53 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) in 3% non-fat dried milk (Sigma). The secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) and a chemiluminescence blotting substrate kit (Boehringer, Mannheim, Germany) were used for immunodetection.

3. Results

3.1. Involvement of p50 and p53 in glutamate-induced cell death

Previous work has suggested the functional contribution of p53 and NF-κB proteins to the cascade of events triggered by excitatory aminoacids and leading to cell death in primary cultures of rat cerebellar granule neurons [18,20,50]. This study then investigated the role of a specific member of the NF-κB family, NF-κB1 or p50 subunit. By means of a specific antibody, p50 appeared to be homogenously present in cerebellar granule cells. To evaluate the induction of this protein by neurotoxic concentrations of glutamate, cells were treated with 100 μM glutamate for 15 min and 30 min later processed for immunocytochemical analysis. This experimental neurotoxic paradigm results in cell death that can be easily detected 18–24 h after the lesion by a variety of techniques [8]. Fig. 1 depicted the semiquantitative analysis of p50 immunoreactivity, evaluated by different gray levels (see methods section). Exposure of the cells to glutamate induced an enhancement of p50 immunoreactivity which was already significant at 30 min after the lesion, while very low staining of the protein was observed in untreated cerebellar neurons (Fig. 1). Pretreatment of the cells with 3 mM ASA completely prevented the glutamate-induced increase of p50 immunoreactivity. In the same experimental conditions, we also studied the expression of p53. Cerebellar granule cells constitutively expressed p53, as demonstrated previously by Western blot and immunocytochemistry analysis. A significant increase of the protein levels was observed 30 min following glutamate exposure (Fig. 2, panel A). Pretreatment of the cells with 3 mM ASA prior glutamate exposure resulted in a complete prevention of the glutamate-induced enhancement of p53 immunoreactivity (Fig. 2, panel B). According to previous data [50], similar results were obtained by pre-incubating sister dishes with a specific p53 antisense oligonucleotide (Fig. 2, panel B).

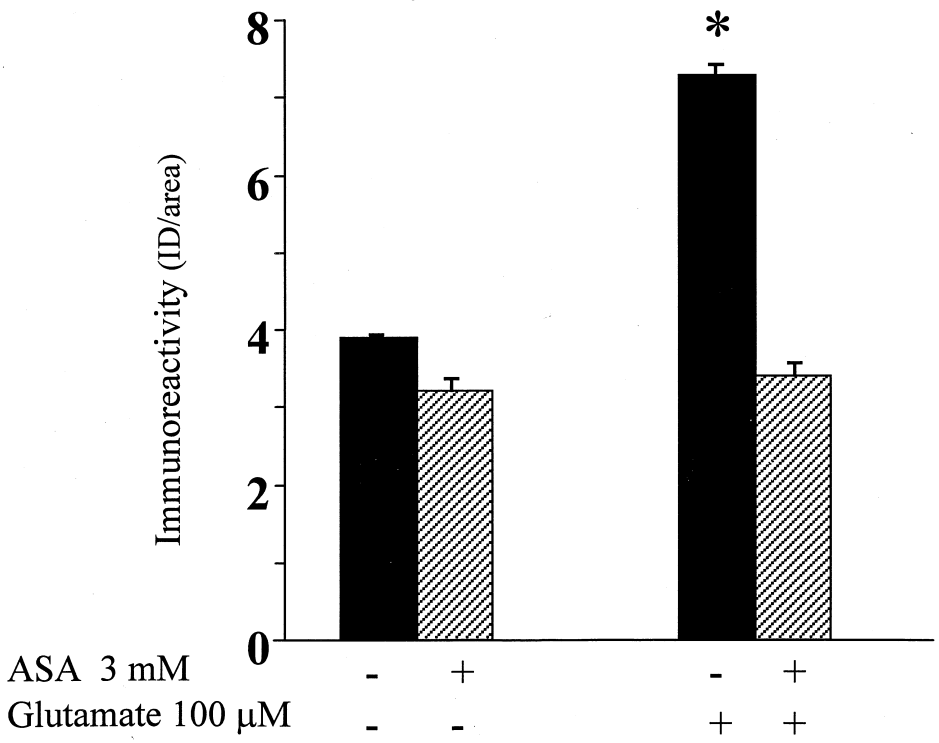

Fig. 1. p50 immunoreactivity in cerebellar granule cells. Cells were either untreated or treated with different drugs as indicated in the bottom of the picture. Values are expressed as ID/area (see methods for details). Each point represent the mean ± SEM of three independent experiments performed with three different cell preparations. $^{*}P < 0.01$ vs the corresponding values of untreated cells.

Since under the same experimental conditions upregulation of p53 is associated with an increased DNA binding activity [50], we evaluated the possibility that in this paradigm, p53 induction could result in the transcription of specific downstream p53-dependent genes. Two well characterized p53 genes were taken into consideration: p21 and MSH2 [14,45].

As shown in a representative Western blot (Fig. 3), cerebellar granule cells constitutively expressed p21 protein, even if at low levels. A significant increase of p21 protein levels become apparent 2 h following glutamate exposure (Fig. 3). Untreated cerebellar neurons were also found immunoreactive to MSH2 antibody with staining restricted to the nucleus (Fig. 4A). As shown in Fig. 4B, a marked and homogenous increase

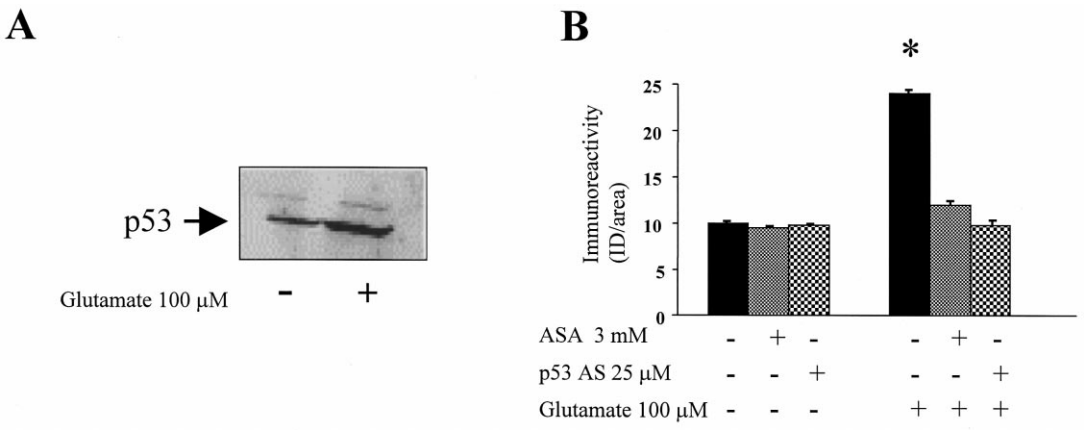

Fig. 2. Western blot analysis (A) and immunoreactivity (B) of p53 in cerebellar granule cells. Cells were either untreated or treated with different drugs as indicated in the bottom of the Figures. ASA, aspirin; AS, oligonucleotide antisense. In (B), values are expressed as ID/area (see methods for details). Each point represent the mean ± SEM of three independent experiments performed with three different cell preparations. $^{*}P < 0.01$ vs the corresponding values of untreated cells.

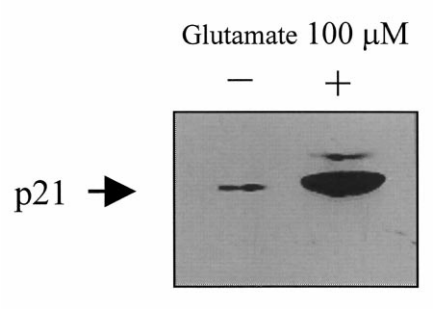

Fig. 3. Western blot analysis with p21 antibody of protein extracts from cerebellar granule cells in basal condition (−) and after exposure to 100 μM glutamate (+) as indicated in the top of the picture. Protein extracts were prepared 2 h after the addition of the neurotoxic agent. Data are from a representative experiment. Similar results were obtained using protein extracts from three different cell preparations.

of anti-MSH2 immunoreactivity was observed 2 h after the exposure of the cells to glutamate.

3.2. Glutamate-induced apoptosis

To investigate the possible contribution of p53 and NF-κB to glutamate-induced apoptosis, cerebellar granule cell cultures were pretreated either with ASA or with a specific p53 antisense oligonucleotide before the neurotoxic insult. An amount of 3 mM ASA was added to the cultures 5 min before glutamate addition, while 25 μM p53 antisense was added 2 h prior the neurotoxic insult.

Eight hours following glutamate exposure, pyknotic and shrinking nuclei suggestive of apoptotic cell death were detected, as evidentiated by DAPI staining (Fig. 5, panel D). Interestingly, ASA or p53 antisense completely prevented glutamate induced apoptosis (panels F and E, respectively), while cells treated with ASA or p53 antisense alone exhibited a normal morphology that was undistinguishable from untreated cells (panel C, B, and A, respectively).

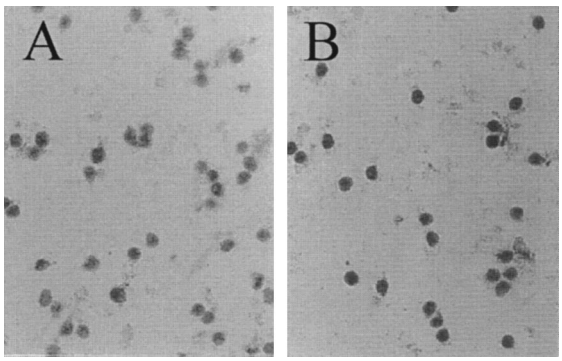

Fig. 4. MSH2 immunoreactivity in cerebellar granule cells in basal condition (A) and after exposure to 100 μM glutamate (B). Staining was localized to the nuclei and was characterized by differences in intensity from cell to cell. The addition of glutamate resulted in a homogenous increase of immunoreactivity. Cells were fixed 2 h after the addition of the neurotoxic agent. Data are from a representative experiment. Similar results were found in cultures from three different cell preparations.

4. Discussion

In recent years, we have focused our interest on the role of proteins known to be involved both in cell cycle regulation and in promoting degeneration and apoptosis of neuronal cells. Most of our studies were carried out in primary cerebellar neurons. This in vitro model offers a morphologically defined system for studying transsynaptic regulation of neuronal gene expression and analyzing the precise temporal sequence of molecular events following stimulation of specific glutamate receptor subtypes. Exposure of these cells for a brief period of time (15 min) to relatively high concentrations of glutamate (micromolar range) results in cell death of both necrotic and apoptotic types [8,40]. Among the intracellular events triggered by neurotoxic concentrations of glutamate, we identified two transcriptional factors: NF-κB/rel and tumor suppressor phosphoprotein p53. Immunocytochemistry and Western analysis demonstrated that glutamate upregulates p50 member of the NF-κB family and p53. Under the same experimental conditions producing cell death, pretreatment of the cultures with ASA, which inhibits NF-κB/rel activation [63], or with specific p53 antisense oligonucleotide resulted in a complete prevention of glutamate-induced increase of p53 immunoreactivity. Furthermore, ASA and p53 antisense treatments prevented apoptosis induced by glutamate. These findings suggest the existence of a transcriptional program activated by glutamate receptor stimulation in which p53 and specific members of the NF-κB family are active contributors.

Numerous in vitro studies have demonstrated that diverse neurotoxic and pro-apoptotic stimuli including high concentrations of glutamate [17], amyloid [26], cytokines [27,47], glycated tau [60], H_2O_2 [57], and glucose deprivation [48], are potent activators of NF-κB in neuronal cells [1,21,34]. Moreover, activated NF-κB has been shown in the brains of patients affected by a number of neurological diseases in which apoptosis play a relevant role [2,12,24,61]. NF-κB activity was also found induced in animal models of neurodegeneration like ischemia [11], head trauma [62], Huntington's disease [42], and experimental allergic encephalitis [25].

Similarly, a series of recent papers have contributed to unravel the active contribution of p53 to neurodegeneration [9,13,32,44,50,51,64]. In particular, systemic injection of kainic acid, a potent excitotoxin that produces seizures associated with a defined pattern of

neuronal cell loss, induces p53 expression in neurons exhibiting morphological signs of damage [44]. Moreover, Morrison et al. [39] found that systemic injection of kainic acid to p53 gene deficient mice does not result in neuronal cell death. Hirata and Cadet [23] have further corroborated the relevance of p53 in promoting neuronal cell death program showing that homozygous p53-knockout mice are protected against neurotoxicity induced by methamphetamine. Our contribution was the demonstration of a direct activation of NF-κB and p53 in response to a pro-apoptotic stimulus.

Then, we analysed the expression of two p53 downstream target genes: p21, which codes for an inhibitor of cdk complexes, and MSH2, which codes for a protein involved in the recognition and repair of a specific type of DNA damage [41]. We found that primary cerebellar neurons expressed p21 at very low levels in basal conditions. However, very soon after a brief exposure of the cells to glutamate, p21 expression was dramatically enhanced. Exposure of cerebellar granule cells to neurotoxic concentrations of glutamate resulted also in a marked increase of MSH2 immunoreactivity. Up to date, very little is known about the role of MSH2 in the brain [4,5]. It could be speculated that MSH2 may act as sensor of DNA integrity. DNA repair is in fact intimately linked with cell cycle progression, and apoptosis is recognized as a physiological response to DNA damage.

In conclusion, we suggest that glutamate, possibly by increasing intracellular calcium concentration and/or oxygen free radicals production, may activate a restricted number of transcription factors which in turn amplify the signal by recruiting other genes to dictate specific transcriptional programs. A hierarchy of intervention is likely to occur. We propose that NF-κB proteins are among the initial orchestrators of the glutamate-induced apoptotic program. Downstream NF-κB-activation is the transcription factor p53. One of the consequence of the increased transcriptional activity of p53 is the up-regulation of p21 and MSH2. On these bases, we propose that NF-κB, p53, p21 and MSH2 could be relevant contributors to the glutamate-induced neuronal apoptosis. The fact that these proteins are also involved in cell cycle regulation supports the hypothesis that aberrant expression of mitotic proteins participates in the neuronal cell death program.

The glutamate-induced apoptosis of primary neuronal cultures was characterized as a strictly p53-dependent program [39,44,50,65] but this is not a generalized phenomenon. As an example, apoptosis of cerebellar neurons in vitro, induced by serum deprivation or low potassium, is p53-independent [13]. Moreover, different genotoxic treatments may cause distinct phosphorylation of p53, which may account for the activation of unique pro-apoptotic pathways [29]. These data support the view that different intracellular programs leading to neuronal apoptosis may exist. It could be inferred that the apoptosis that occurs physiologically

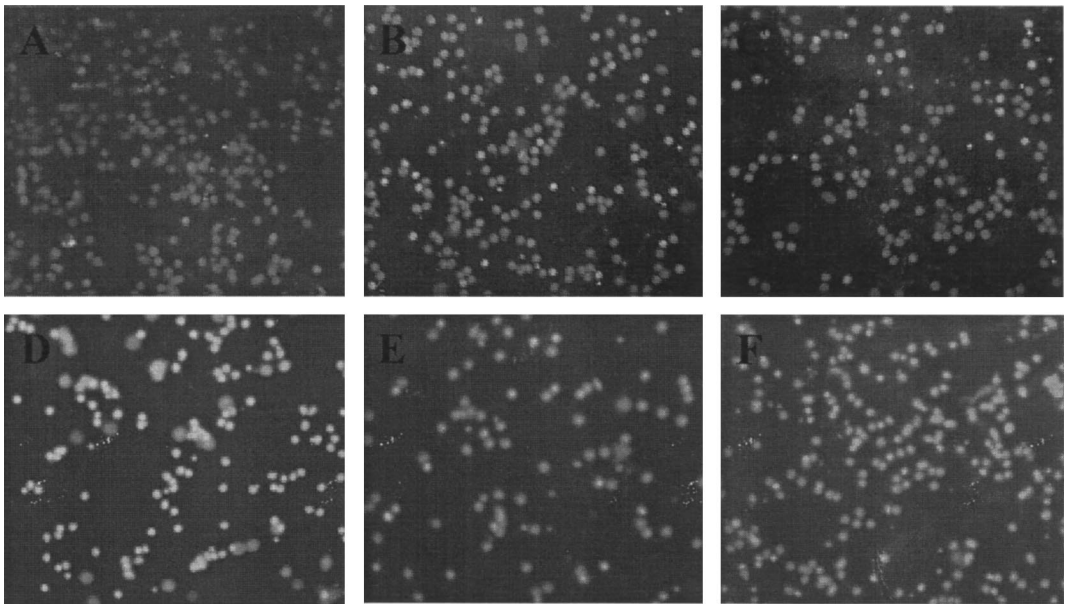

Fig. 5. Detection of glutamate-induced apoptosis in cerebellar granule cells by DAPI fluorescence stain. Cells were either untreated (A) or exposed to 100 μM glutamate alone (D). 3 mM ASA was added to the culture media alone (B) or 5 min before the glutamate pulse (E). The p53 oligonucleotide antisense (25 μM) was added to the culture media alone (C) or 2 h before the glutamate pulse (F). Cells were fixed 8 h after the addition of the neurotoxic agent. Data are from a representative experiment. Similar results were found in cultures from three different cell preparations.

(i.e. during brain development and aging) and the apoptosis that is related with neurodegenerative diseases may depend on different molecular participants. This particular topic definitely requires much attention in the development of anti-neurodegenerative drugs acting at transcriptional level. Understanding this cascade of nuclear events may indeed unravel specific targets for pharmacological intervention for those neurological diseases in which specific types of apoptosis play a relevant role.

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