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Induction of tumour-suppressor phosphoprotein p53 in the apoptosis of cultured rat cerebellar neurones triggered by excitatory amino acids

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Abstract

We found that primary cultures of rat cerebellar granule cells, although definitely postmitotic and terminally differentiated, express the tumour-suppressor phosphoprotein p53. In particular, granule cells both expressed significant levels of p53 mRNA and positively reacted to an anti-p53 antibody, from the first day of culturing. During neurone differentiation, p53 mRNA content did not significantly change, at least up to 12 days *in vitro*, while p53 immunoreactivity increased gradually. p53 expression appeared to be further modulable being upregulated after stimulation of glutamate ionotropic receptors by glutamate or kainate. Although qualitatively similar, p53 induction by glutamate and kainate differed in terms of intensity and time-course. The glutamate increase of p53 immunoreactivity appeared within 30 min after the treatment and lasted for at least 2 h. Kainate-induced increase of p53 immunoreactivity was delayed, becoming apparent within 2 h and lasting for at least 8 h. Both kainate- and glutamate-induced increases of p53 immunoreactivity were prevented by the non-competitive NMDA receptor antagonist MK 801. As shown by the electrophoretic mobility shift analysis, both glutamate and kainate induced increases of p53 DNA binding activity. Blockade of p53 induction by a specific p53 antisense oligonucleotide resulted in a partial reduction of excitotoxicity with a complete inhibition of the excitatory amino acids induced apoptosis.

Our data suggest that stimulation of ionotropic glutamate receptors in neurones results in a p53-dependent apoptosis.

Introduction

The tumour-suppressor protein p53 is a cell-cycle checkpoint protein that contributes to the preservation of genetic stability. Particularly, p53 protein originates from a single-copy gene localized on the short arm of chromosome 17 (Isobe *et al*., 1986). Structurally, it can be divided in three segments: the N-terminal portion, involved in transcriptional activation (Lin *et al*., 1994); the central domain which confers sequence-specific DNA binding (Wang *et al*., 1993; Plavletich *et al*., 1993; Bargonetti *et al*., 1993) and the C-terminal domain which contains residues that confer on p53 the ability to oligomerize and bind both single-stranded DNA and RNA (Clore *et al*., 1994; Wu *et al*., 1995). Upon certain conditions, including physical or chemical DNA damage, p53 gene expression can be activated to either arrest cell cycle progression in the late G1 phase, thus allowing the DNA to be repaired before its replication, or induce apoptosis (Lane, 1992). Due to its role, p53 has been defined as ‘safeguard against tumourigenesis’. Indeed, in tumour cells lacking functional p53, the above described pathways are not functional, resulting in inefficient DNA repair and the emergence of genetically unstable cells (Vogelstain & Kinzier, 1992). More recently, it was found that p53 may also play a role in differentiation of pre-B-cells (Shaulsky *et al*., 1991), K562 cells, an acute-phase myelogenous leukaemia cell line (Feinstein *et al*., 1992), oligodendrocytes and neurones (Eizenberg *et al*., 1996).

The mechanism(s) by which p53 can induce cell cycle arrest and/or apoptosis is still largely unknown. Development of transgenic mice deficient for p53 has recently gained further insight on the functional role of p53 (Donehover *et al*., 1992). Interestingly, mice homozygous for p53 null allele appear normal. However, female-associated defects in neural closure were found at high frequency in p53 null mice embryos (Armstrong *et al*., 1995). Thus, at least from these data and with the awareness of the intrinsic limitation of the experimental model, p53 function appears to be dispensable in many apoptotic processes that occur physiologically during the entire life-span in a large variety of organs and systems. However, p53-deficient mice are prone to the spontaneous development of a variety of neoplasms by 6 months of age, suggesting that lack of p53 gene predisposes the animal to neoplastic diseases, although is not obligatory for tumourigenesis. Interestingly, normal development and high risk of tumour are found in family members with dominantly inherited Li–Fraumeni syndrome and this syndrome has been associated with germ line p53 mutation (Srivastava *et al*., 1990).

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Nevertheless, p53 appears to play an important role in promoting apoptosis and this function could have relevant implications for brain function. Indeed, apoptosis of neurones is observed physiologically during development and ageing. Furthermore, apoptosis has been associated, at least in part, with neurodegeneration detectable in various neurological diseases, including Huntington's (Portera-Cailliau *et al*., 1995) and Alzheimer's diseases (Duguid *et al*., 1989).

A series of recent papers has contributed to the unravelling of the role of p53 during a neurodegenerative process (Chopp *et al*., 1992; Li *et al*., 1994; Sakhi *et al*., 1994; Xiang *et al*., 1996). In particular, systemic injection of kainic acid (KA), a potent excitotoxin that produces seizures associated with a defined pattern of neuronal cell loss, induced p53 expression in neurones exhibiting morphological evidence of damage (Sakhi *et al*., 1994). More recently, Morrison *et al*. (1996) found that systemic injection of KA to p53-gene-deficient mice did not induce neuronal cell death. A further indirect, although intriguing, link between excitotoxicity and p53 has been provided by Didier *et al*. (1996) who show accumulation of single-strand DNA damage as an early event in excitotoxicity. This particular DNA damage is indeed capable of inducing p53 expression (Jayaraman & Prives, 1995; Lee *et al*., 1995).

We studied the role of p53 in cultured, genetically unmodified neurones, namely rat cerebellar granule cells, during development *in vitro* and in response to neurotoxicity induced by excitatory amino acid (EAA). Primary cultures of cerebellar granule cells offer not only a morphologically defined system for studying transsynaptic regulation of neuronal gene expression, but also provide the opportunity to analyse the precise temporal sequence of molecular events following stimulation of specific glutamate receptor subtypes. Advantages of this experimental model also include the possibility to study the function of a given gene product using the oligonucleotide antisense technology, thus avoiding redundancy on compensation that may occur in transgenic animal models.

Materials and methods

Cell culture

Primary cultures of cerebellar granule cells were prepared from 8-day-old Sprague–Dawley rat pups as previously described (Pizzi *et al*., 1991). Briefly, cells were plated on to poly-L-lysine-coated dishes and cultured in basal Eagle's medium containing 10% heat-inactivated foetal bovine serum, 2 mM glutamine, 50 μg/mL gentamycin and 25 mM KCl, at the density of 1.5×10^5 cells/cm^2. Cytosine arabinoside (100 μM) was added to the cultures 18 h after seeding to prevent non-neuronal cell proliferation. Usually, experiments were done after culturing the neurones for 12 days *in vitro* (DIV) unless otherwise indicated.

Evaluation of neuronal death

Usually, neurotoxicity was evaluated in cerebellar granule cells at DIV 12 following the exposure of the cells to KA (60 μM) or glutamate (100 μM). The concentration of both glutamate and KA used in the present study were maximally effective in terms of cell death (see below) and were chosen on the bases of the results from a series of previous experiments using different concentrations of both EAA, 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 different concentrations of 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. KA was added to the media for different periods of time, as indicated. At 0.5, 1, 2, 4, and 8 h after the glutamate pulse and at 2, 4, 8, 24 or 30 h following the addition of KA, the culture-conditioned media were collected to measure lactate dehydrogenase (LDH) activity. The results were expressed as percentage of total LDH activity according to Pauwels *et al*. (1989). Total LDH activity was defined as the sum of intracellular and extracellular LDH activity. Extracellular and intracellular LDH activity was measured spectrophotometrically following NADH-oxidation at 340 nm.

Cell viability was also established by a fluorescence method, according to Jones & Senft (1985). Briefly, cells were washed with Locke's solution, stained for 3 min with a mixture of fluorescein diacetate (15 μg/mL) and propidium iodite (80 μg/mL), and examined immediately with a standard epi-illumination fluorescence microscope (450 nm excitation, 520 nm barrier). Fluorescein diacetate crosses the cell membranes and is hydrolysed by intracellular esterases to produce green–yellow staining. This process is reduced by neuronal injury, a condition facilitating propidium iodite penetration and interaction with DNA to yield a red fluorescence. The percentage of cell death in the monolayer was computed by calculating the ratio between the propidium iodite and the fluorescein diacetate plus propidium iodite stainings in photomicrographs of at least three representative fields from each monolayer.

For evaluation of apoptosis, cells were plated on to 100 μg/mL poly-L-lysine coated glass coverslips and cultured as described above. Apoptotic cells were identified by May–Grunwald–Giemsa's stain. Briefly, cells were washed in phosphate-buffered saline (PBS) solution, fixed with May–Grunwald's staining containing methanol for 3 min, then washed with a buffer containing 200 mM NaH_2PO_4/Na_2HPO_4 (pH 7.4) for additional 3 min. Cells were then incubated with Giemsa's staining for 3 min, rapidly washed with three or four changes of water and air dried. The apoptotic cells were evaluated morphologically by the rapid onset of cytoplasmatic blebbing. The percentage of apoptotic neurones was computed by calculating the ratio between blebbing cells and normal cells staining in photomicrographs of at least three representative fields from each dish. Data were presented as the mean ± SEM of at least three separate culture preparations.

The statistical significance of differences between the values was made by one-way analysis of variance followed by Student's *t*-test.

Immunocytochemistry

Experiments were done in cerebellar granule cells at different DIV, as indicated. 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 0.2% Triton X-100 in PBS and incubated for 24 h at 4 °C with the primary antibody. The antibodies used were: a polyclonal antip53 antibody, R19, from Santa Cruz Biotechnology, used at 1 : 500 dilution, and the monoclonal antibody ALZ90, from Boehringer Mannheim, used at 1 : 10 dilution. R19 recognizes the epitope corresponding to amino acid 373–391 mapping at the carboxy terminus of rat p53, and ALZ90 recognizes the sequence corresponding to amino acids 511–689 of Amyloid Precursor Protein (APP). After several rinses, cells were processed using the avidin-biotin complex Kit (ABC Elite Kit, Vector Laboratories). Enzymatic reaction was developed as described by Mattson (1990). In order to verify the specificity of the chromogen reaction, some dishes were processed

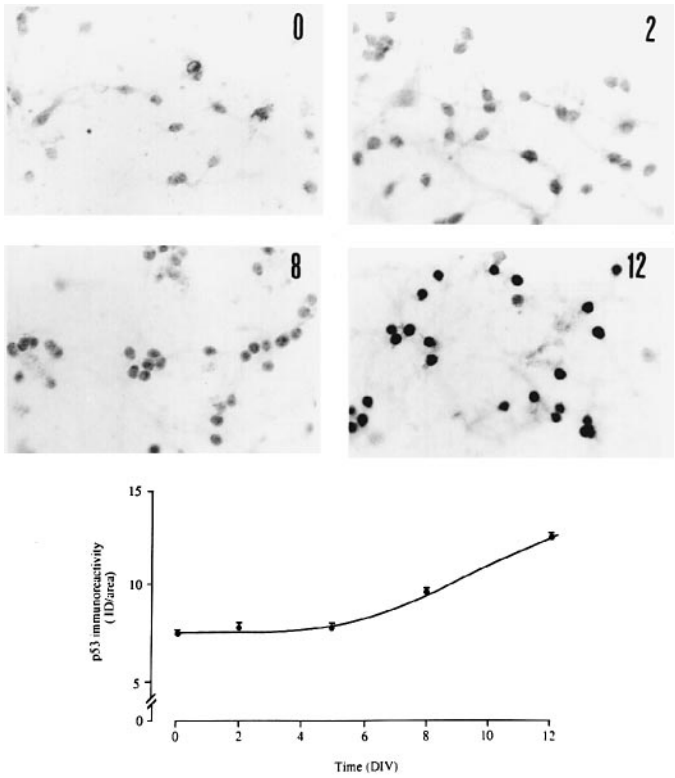

FIG. 1. p53 immunoreactivity in cerebellar granule cells at different stages of neurone maturation. Cells were immunostained with antip53 polyclonal antibody R-19. Upper panel, representative pictures of cultured cells processed for immunocytochemistry after 0, 2, 8, and 12 days *in vitro*. Lower panel, semiquantitative analysis of p53 immunoreactivity expressed as integrated optical density/area. Data represent mean ± SEM of three different preparations and are from three separate cell preparations.

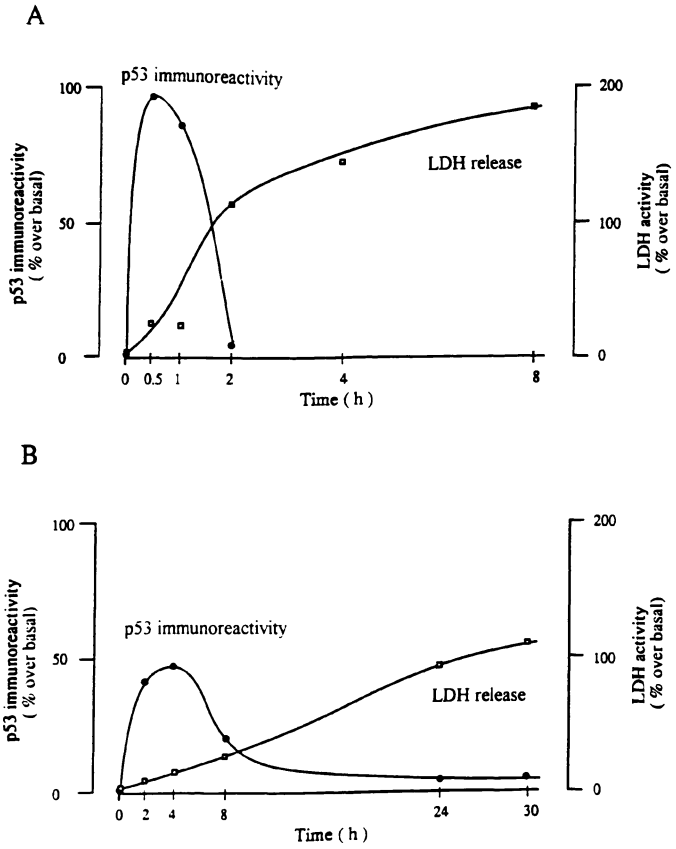

FIG. 2. Time-dependent effects of glutamate (A) and kainic acid (KA) (B) on p53 immunoreactivity and lactate dehydrogenase (LDH) activity in culture medium. Cerebellar granule cells were treated with 100 μM glutamate or 60 μM KA and, at different period of times, as indicated, p53 immunoreactivity, expressed as integrated optical density/area, and cell viability, evaluated as LDH release in the media, were analysed. Data represent mean ± SEM of three separate preparations.

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 neurones 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 us access to the image processing and analysis function of Magiscan. The microdensitometric analysis was performed as previously described (Mize *et al*., 1988) 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 pointset. 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.

Polymerase chain reaction

Messenger RNA was isolated from the cultures using RNAzol (Biotecx Laboratories) procedure, extracted by chloroform–ethanol and reverse-transcribed with Moloney murine leukaemia virus reverse transcriptase in the presence of [^{32}P]dCTP, as recommended by the manufacturer. The resulting cDNA was quantified by determining the amount of radioactivity incorporated into trichloroacetic acid-precipitable nucleic acids. Polymerase chain reaction (PCR) was carried out with *Taq* polymerase (Perkin Elmer Cetus) in 100 μL of standard buffer containing 0.5 ng cDNA, and 1 μM specific primers. Twenty-eight cycles of amplification were performed with a DNA Thermal Cycler (Perkin Elmer Cetus) and a step programme (94 °C, 1 min; 60 °C, 1 min; 72 °C, 2 min), followed by a final 10 min extension at 72 °C. PCR products were separated by electrophoresis and visualized by ethidium bromide staining. In some experiments, bands were cut out and radioactivity incorporated into the bands counted by scintillation spectrometry.

To allow the relative quantification of different mRNA levels by PCR, a series of preliminary experiments was carried out to correlate the effects of different concentrations of cDNA from granule cells with the yield of PCR products. The amount of radioactivity incorporated into the DNA-amplified bands was proportional to the amount of reverse-transcribed mRNA in a range between 0.1 and 2 ng. Thus, for the most accurate quantification, 0.5 ng of cDNA was used for comparing the p53 cDNA amplified products from different cell culture samples.

TABLE 1. Effects of 5 μM MK 801 on glutamate- and kainic acid (KA)-induced increase of p53 immunoreactivity. p53 immunocytochemistry was carried out with the antip53 polyclonal antibody R-19 30 min after glutamate pulse or 4 h after KA exposure. Values are expressed as integrated optical density (ID)/area and represent the mean ± SEM of the results from three independent experiments using three different cell preparations

	Vehicle	MK 801
–	12.6 ± 1.4	11.0 ± 1.0
Glutamate 100 μM	22.5 ± 1.7 *	10.9 ± 1.0
KA 60 μM	19.3 ± 1.0 *	11.5 ± 0.7

*$P < 0.01$ vs. basal values of vehicle treated cells.

Nuclear extracts and electrophoretic mobility shift assay (EMSA)

Nuclear extracts from neuronal primary cultures were prepared according to a small scale protocol as in Andrews & Faller (1991). Protein concentration was assessed by BioRad Bradford assay according to the manufacturer instructions. DNA binding reactions were initiated by combining 2–4 μg of nuclear extracts from cerebellar granule cells with 20 000 c.p.m. (0.1 ng) of γ ^{32}P-labelled oligonucleotides in lipage buffer (10 mM Tris Cl pH 7.5, 50 mM NaCl, 1 mM DTT, 1 mM EDTA, 10% glycerol) containing 0.5 μg of poly(dIdC) in a total volume of 12 μL. In competition experiments, 2 ng (20-fold molar excess) of unlabelled competitor oligonucleotides were added together with ^{32}P-labelled probes. Reactions were carried out for 20 min at room temperature, and protein-DNA complexes were resolved on non-denaturing 4% polyacrylamide gels in Tris-Glycine-EDTA buffer. Gels were then dried and subjected to autoradiography at room temperature.

Synthesis of oligonucleotides

The oligonucleotide sequence containing the p53 DNA binding site (5′-TACAGAACATGTCTAAGCATGCTGGGG-3′) (Kastan *et al*., 1992) together with a mutant isoform with a CATG/TCGC substitution in the p53 binding motif were synthesized using an Applied Biosystem 391 DNA synthesizer, purified by denaturing gel electrophoresis, and annealed to give double-stranded probe. For gel shift analysis the double-stranded oligonucleotide was end-labelled with γ ^{32}P ATP (ICN, specific activity > 7000 Ci/mmol) and T4 polynucleotide kinase (Boehringer Mannheim) to obtain a specific activity of more than 10^8 c.p.m./μg.

Both sense and antisense p53 oligonucleotides were synthesized using phosphoramidite chemistry. Oligonucleotides were purified by reverse-phase chromatography using Oligo-Pak oligonucleotide purification columns following the recommendation of the manufacturer. p53 antisense oligonucleotide sequence was the following: 5′-TAA CTG TCA TGG AGG ATT-3′ corresponding to nucleotide –7 to nucleotide +11 in rat p53 gene sequence. The sense oligonucleotide is the exact inverse complement of the antisense oligonucleotide. The selected target sequence has relatively low homology with any of the other known cDNA sequences found in the GenBank database, as determined by using the GenePro program (Brainbridge Island, WA, USA).

The primers used for PCR analysis were the following: 5′-GGG AAT TCC ATC TAC AAG AAG TCA CAA-3′ corresponding to nucleotides 473–493 and 3′-GCG GAT CCT GAT GAT GGT AAG GAT GGG CCG-5′, corresponding to nucleotides 736–757 of the rat p53 cDNA (Soussi, 1988).

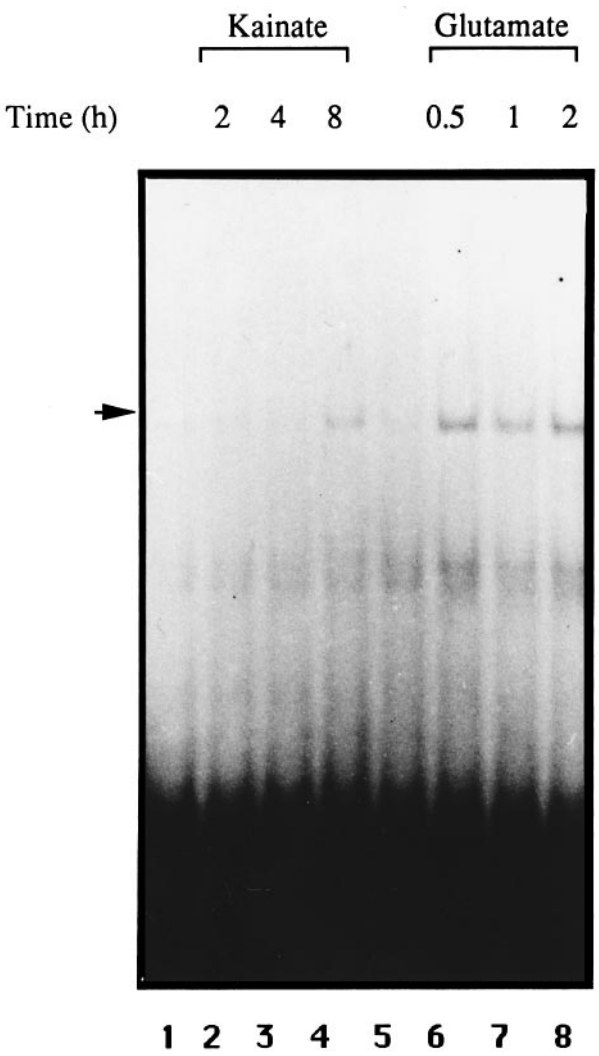

FIG. 3. Time-dependent effects of glutamate and kainic acid (KA) on p53 DNA binding activity in cerebellar granule cells. Gel shift analysis was performed by incubating 4 μg nuclear extracts from either untreated (lanes 1–5) or treated with 60 μM KA (lanes 2, 3, 4) and 100 μM glutamate (lanes 6, 7, 8) cells with γ-^{32}P-labelled p53 oligonucleotide probe. Figure shows a representative experiment. Similar results were obtained in two additional experiments using two different cell preparations.

Results

p53 expression in cerebellar granule cells during development 'in vitro'

p53 expression was investigated in primary cultures of rat cerebellar granule cells at different DIV. Cells were prepared from 8-day-old rat and cultured for as long as DIV 12. During this period neuronal cells undergo maturation with biochemical and morphological changes leading to neurite growth, synaptic formation and expression of several neuronal markers of differentiation (Gallo *et al*., 1987; Peng *et al*., 1991; Pizzi *et al*., 1995a). At DIV 0, 2, 5, 8, and 12, cells were fixed and stained with the antip53 polyclonal antibody R-19. This antibody recognizes the epitope corresponding to amino acid 373–391 mapping at the carboxy terminus of rat p53. p53 immunostaining was found mainly in the nucleus and it gradually increased during the *in vitro* maturation (Fig. 1, upper panel). In particular, as shown in Figure 1, lower panel, semiquantitative analysis of p53

immunoreactivity gave values of 7.1 ± 0.3 ID/area at DIV 0 and it rose very slowly until DIV 5 (ID/area = 7.8 ± 0.4). Then, p53 immunoreactivity increased rapidly and time-dependently, reaching the maximum level at DIV 12 (ID/area = 13 ± 0.3).

p53 mRNA levels were measured during *in vitro* maturation of cerebellar granule cells using a semiquantitative PCR-derived method. PCR performed in the presence of specific p53 primers and cDNAs extracted from granule cells at DIV 0, 2, 5, 8 and 12, revealed a single band of expected size (284 bp) at all stages analysed. Measurement of ^{32}P incorporation into the amplified bands did not show differences between the various experimental samples (data not shown).

Correlation between p53 expression and neurotoxicity

We tested the possibility that p53 could be activated in the cascade of events triggered by EAA leading to neuronal death. In particular, neurotoxicity was induced by exposing the cells to glutamate or KA. Glutamate, in Mg^{2+}-free condition, induces a rapid receptor-mediated neurotoxicity through the activation of the *N*-methyl-D-aspartate (NMDA)-sensitive glutamate receptor subtypes (Choi, 1988). On the contrary, KA, by binding specifically to the non NMDA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA)/KA subtypes of glutamate ionotropic receptors (London & Coyle, 1979), is responsible for a delayed glutamate receptor-mediated cell death (Weiss *et al*., 1990).

Figure 2 shows the time-dependent effects of the exposure of the cerebellar granule cells to glutamate and KA. At each tested time-point, cells were evaluated for p53 immunoreactivity, while the corresponding conditioned media were collected and tested for LDH activity. As shown in Figure 2(A), exposure of granule cells to a 15-min pulse of 100 μM glutamate induced a transient increase of p53 immunoreactivity that reached the maximum after 30 min (+ 96% over basal). Two hours after the glutamate pulse, p53 levels returned to the basal values. In line with previous results, glutamate pulse resulted in a time-dependent loss of cell viability. This effect, measured as LDH release in the medium, in our experimental conditions reached its maximum 8 h after the pulse (180% over basal). Parallel experiments were performed on cerebellar granule cells treated with KA. p53 immunoreactivity and LDH activity were evaluated at 0, 2, 4, 8, 24 and 30 h after exposing the cells to 60 μM KA. As shown in Figure 2(B), KA treatment induced a transient increase of p53 immunoreactivity, associated with a time dependent cell loss. In particular, p53 immunoreactivity values reached the peak after 4 h (+ 48% over basal), then began to decline, returning to basal after 8 h. On the contrary, LDH release increased in a time-dependent manner and at 30 h it was calculated as 100% over the basal.

In a separate set of experiments, granule cells were pretreated for 5 min with 5 μM MK 801, a non-competitive NMDA receptor antagonist (Watkins *et al*., 1990), before being challenged with either 100 μM glutamate or 60 μM KA. Immunocytochemical analysis for p53 was performed 30 min after glutamate pulse and 4 h after KA treatment. As reported in Table 1, MK 801 pretreatment completely prevented both the glutamate- and KA-induced increase of p53 immunoreactivity.

Measurement of p53 mRNA levels using a reverse transcriptase (RT)-PCR method and cDNA extracts from cells exposed to glutamate for 30 min, and 2 h, and to kainate for 4 h and 8 h, revealed that p53 mRNA levels were present at low levels in untreated cells, they increased 30 min after glutamate exposure, and were still present and high 2 h after the glutamate pulse. Moreover, p53 mRNA levels were present and high at 4 h and 8 h after kainate (data not shown).

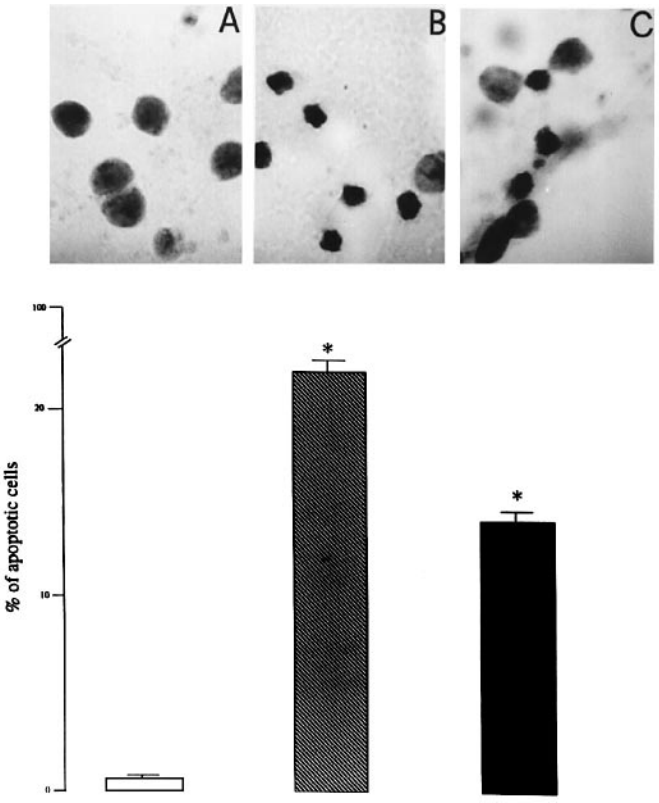

FIG. 4. Effects of saline (open bar) glutamate (hatched bar) or kainic acid (KA) (filled bar) on apoptosis detected by May–Grunwald–Giemsa's staining. Upper panels, representative pictures of May–Grunwald–Giemsa's staining of saline (A), glutamate- (B) and KA- treated cells (C). Lower panel, percentage of apoptosis computed by calculating the ratio between blebbling and total May–Grunwald–Giemsa's staining positive cells. Data represent mean ± SEM of three separate preparations.* $P < 0.01$ vs. control.

Glutamate- and KA-induced p53 DNA binding activity

Nuclear protein extracts from granule cells at different times after glutamate or KA exposure were prepared and incubated with a ^{32}P-labelled oligonucleotide containing a p53 binding site (Kastan *et al*., 1992). Specific DNA protein interaction was then evaluated by gel shift analysis. As shown in Figure 3, very little DNA binding activity was present in control cells. When cells were exposed to 60 μM KA, a detectable DNA binding complex was seen only 8 h after. In contrast, when cells were exposed to 100 μM glutamate, p53 nuclear activity was present already after 30 min and was still detectable at 2 h. Specificity of DNA–protein interaction was assessed by competition analysis with the cold oligonucleotide sequence and with a mutant p53 oligonucleotide, and by comparison with a previously characterized p53 nuclear complex in the human glioblastoma cell line U87MG (data not shown).

Glutamate and ka-induced apoptosis

To obtain further information on the role of p53 on EAA-induced neuronal death, we first analysed the contribution of apoptosis to total neuronal death. Apoptosis was detected morphologically by May–Grunwald–Giemsa's staining. This staining allows detection of intact and apoptotic cells. Indeed, apoptotic cells are easily distinguishable from the living cells by the rapid onset of cytoplasmatic blebbling. Cerebellar granule cells were treated with 100 μM glutamate or 60 μM KA, and stained 8 and 30 h after glutamate and KA exposure, respectively. As shown in Figure 4, both glutamate and KA

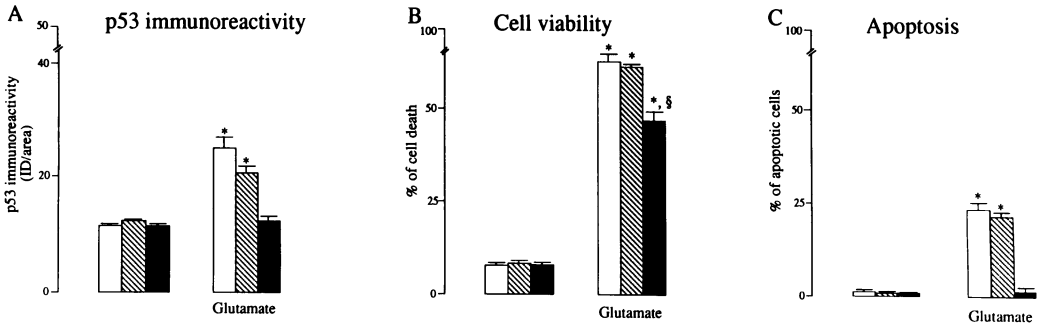

FIG. 5. Effects of glutamate on cerebellar granule cells treated with vehicle (open bars), p53 antisense (filled bars), and p53 sense (hatched bars) oligonucleotide. (A) p53 immunoreactivity, (B) cell viability, (C) apoptosis. p53 immunocytochemistry was carried out with the antip53 polyclonal antibody (R-19) 30 min after glutamate pulse. Cell viability was detected with a fluorescence method 8 h after glutamate exposure. The percentage of cell death was computed by calculating the ratio between propidium iodite stained cells and fluorescein diacetate plus propidium iodite stained cells of at least three representative fields from each monolayer. Apoptosis was evaluated morphologically by a rapid onset of cytoplasmatic blebbing 8 h after glutamate pulse. The percentage of apoptosis was computed by calculating the ratio between blebbling and total May–Grunwald–Giemsa's positive cells. Data represent mean ± SEM of three separate preparations. *$P < 0.01$ vs. the corresponding control values; §$P < 0.05$ vs. values obtained in either vehicle or p53 sense oligonucleotide, glutamate-treated, cells.

induced apoptosis, which was estimated, respectively, as 22% and 14% of total May–Grunwald–Giemsa's positive cells. Figure 4(A–C), show representative pictures of May–Grunwald–Giemsa's staining of untreated (A), glutamate- (B) and KA-treated cells (C). Additional experiments using TUNEL staining method confirmed both qualitatively and quantitatively the induction of apoptosis triggered by glutamate and KA (data not shown).

Effects of p53 antisense oligonucleotide treatment on glutamate-induced p53 expression and cell death

A specific p53 antisense oligonucleotide, or the corresponding sense sequence, were synthesized and added to the culture media at the concentration of 25 μM, 2 h before the glutamate addition. p53 immunoreactivity, cell survival, and percentage of apoptotic cells were evaluated in vehicle, p53 antisense and p53 sense oligonucleotide pretreated cells. As shown in Figure 5(A), basal p53 immunoreactivity, expressed as ID/area, was unchanged in the cells pretreated either with p53 antisense or sense oligonucleotide in comparison with the control. On the contrary, the glutamate-elicited p53 induction was virtually abolished by p53 antisense oligonucleotide treatment (ID/area = 13.0 ± 0.5 in vehicle and ID/area = 0.6 ± 0.2 in p53 antisense oligonucleotide treated cells). The concentration of p53 antisense oligonucleotide used in the present study, 25 μM, was maximally effective in terms of prevention of glutamate-induced increase of p53 immunoreactivity and was chosen on the bases of the results from a series of previous experiments using different oligonucleotide concentrations, ranging from 5 to 50 μM (data not shown).

Pretreatment of granule cells with p53 antisense oligonucleotide for 2 h did not affect the cell viability, however, the response to neurotoxic concentrations of glutamate was significantly changed. As depicted in Figure 5(B), incubation of the cells with 100 μM glutamate resulted in 72% neuronal loss in untreated cells, 70% in 25 μM p53 sense oligonucleotide-treated cells and 47% in 25 μM p53 antisense oligonucleotide-treated cells. Indeed, the p53 antisense oligonucleotide pretreatment prevented by about 30% the glutamate-induced neuronal loss, while sense oligonucleotide treatment did not modify cell death induced by glutamate. Finally, apoptosis, evaluated as percentage of apoptotic cells 8 h after glutamate injury was virtually absent in p53 antisense oligonucleotide-treated cells (24% and 2% of total May–Grunwald–Giemsa's positive cells for vehicle and p53 antisense oligonucleotide-treated cells, respectively).

Effects of p53 antisense oligonucleotide treatment on KA-induced p53 expression and cell death

The effects of p53 sense or antisense oligonucleotide pretreatment was evaluated also in 60 μM KA treated cells. One hour before the KA treatment, 25 μM p53 sense or antisense oligonucleotide was added to the cerebellar neurones. Immunocytochemistry analysis was performed 4 h after exposure of the cells to KA. As shown in Figure 6(A), basal p53 immunoreactivity was not modified by treatment of the cells with p53 sense or antisense oligonucleotide. On the contrary, the KA-elicited p53 induction was virtually abolished by p53 antisense oligonucleotide treatment (ID/area = 6.7 ± 0.3 and ID/area = 0.7 ± 0.1 for vehicle and p53 antisense oligonucleotide-treated cells, respectively).

As shown in Figure 6(B), The degree of cell death induced by KA was similar in vehicle (62%) and p53 sense oligonucleotide-treated cells (60%), and significantly lower in cells treated with the p53 antisense oligonucleotide (44%). Finally, as depicted in Figure 6(C), panel C, apoptosis induced by 60 μM KA was completely prevented by p53 antisense treatment, while p53 sense treatment did not induce any change.

Specificity of p53 antisense oligonucleotide treatment

The specificity of p53 antisense oligonucleotide was assessed by the observation that p53 sense oligonucleotide treatment did not modify p53 immunoreactivity increase induced by both glutamate and KA. Moreover, p53 sense oligonucleotide treatment did not prevent both total cell death and apoptosis induced by the two neurotoxins (see above). A further proof of p53 antisense oligonucleotide specificity was confirmed by measuring the levels of amyloid protein precursor (APP) immunoreactivity. It has been previously shown that the exposure of cerebellar granule cells to glutamate results in APP overexpression (Valerio *et al*., 1995; Grilli *et al*., 1996). In our experimental settings, cells were pretreated with 25 μM p53 antisense oligonucleotide for 2 h before the exposure to 100 μM glutamate. Cultures were fixed 30 min after the glutamate pulse and immunocytochemistry for APP was carried out with ALZ90 antibody. In line with our previous observations, APP immunoreactivity rose by about 40% after glutamate exposure. p53 antisense oligonucleotide pretreatment did not modify the ability of glutamate to increase APP immunoreactivity (Fig. 7).

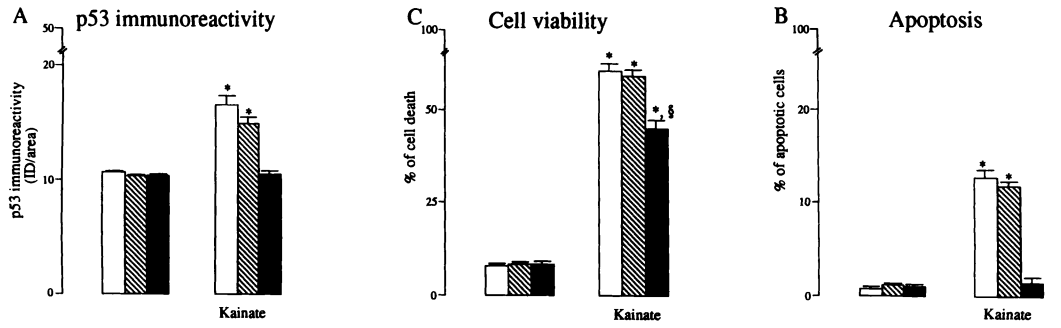

FIG. 6. Effects of kainic acid (KA) on cerebellar granule cells treated with vehicle (open bars), p53 antisense (filled bars), and p53 sense (hatched bars) oligonucleotide. (A) p53 immunoreactivity, (B) cell viability, (C) apoptosis. p53 immunocytochemistry was carried out with the antip53 polyclonal antibody R-19 4 h after KA exposure. Cell viability was detected with a fluorescence method 30 h after KA exposure. The percentage of cell death was computed by calculating the ratio between propidium iodite stained cells and fluorescein diacetate plus propidium iodite stained cells of at least three representative fields from each monolayer. Apoptosis was evaluated morphologically by a rapid onset of cytoplasmic blebbing 30 h after KA treatment. The percentage of apoptosis was computed by calculating the ratio between blebbling and total May–Grunwald–Giemsa's positive cells. Data represent mean ± SEM of three separate preparations. $*P < 0.01$ vs. the corresponding control values; $§P < 0.05$ vs. values obtained in either vehicle or p53 sense oligonucleotide, KA-treated, cells.

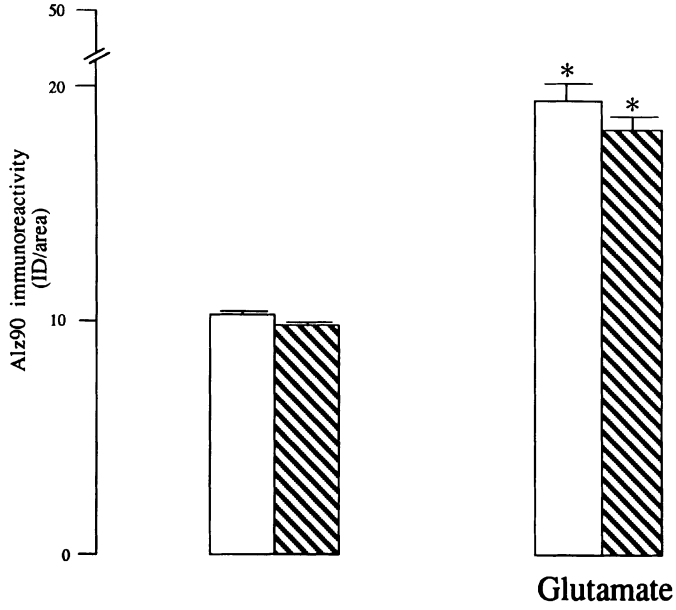

FIG. 7. Effect of p53 antisense oligonucleotide on glutamate-induced increase of APP immunoreactivity. Immunocytochemistry was carried out with the anti-APP monoclonal antibody Alz90. Cells were pretreated with p53 antisense oligonucleotide (hatched bar) or vehicle (open bar) for 2 h before the glutamate pulse. Thirty minutes after, cells were fixed and tested for APP immunostaining. Data represent mean ± SEM of three separate preparations.$*P < 0.01$ vs. the corresponding control values.

Discussion

Although definitely postmitotic and terminally differentiated cells, primary culture of rat cerebellar granule cells express at least one of the crucial proteins controlling cell cycle progression, namely the tumour suppressor phosphoprotein p53. Here we report that p53 is constitutively expressed in the nucleus of cerebellar granule cells, it is upregulated by stimulation of EAA ionotropic receptors and is actively involved in promoting the EAA-induced apoptosis.

Comparing mRNA and protein levels for p53 as a function of *in vitro* cell maturation, our results suggest that p53 expression is a developmentally regulated process that involves post-transcriptional modifications. In fact, primary cultures of cerebellar granule cells both expressed significant levels of p53 mRNA and positively reacted to anti-p53 antibody, from the first day of culturing. However, while p53 mRNA content did not significantly change during the time of culturing, at least up to DIV 12, p53 immunoreactivity increased gradually during neurone differentiation reaching its maximum level at DIV 12. The role of p53 in postmitotic neurones in still unclear. Certainly, these findings further support the view that the function of p53 is not restricted to cell-cycle control and may be related with neurone differentiation. The immunocytochemical studies show that p53 is mainly localized in the nucleus, at least up to DIV 12. However, we cannot exclude the possibility that at longer periods of culturing the subcellular localization of p53 may change, as recently shown in primary cultures of hippocampal neurones (Eizenberg *et al*., 1996).

The expression of p53 in cerebellar granule cells at DIV 12 appears to be further modulable and specifically activated by stimulation of ionotropic glutamate receptor subtypes. In fact, stimulation of glutamate receptors in an experimental paradigm that involves almost exclusively the activation of the ionotropic NMDA-sensitive glutamate receptor subtype (Choi, 1988), or by KA, which involved the AMPA/KA glutamate receptor subtypes (Weiss *et al*., 1990), resulted in a significant, short-lasting increase of p53 immunoreactivity. Previous studies performed in primary neurones have demonstrated that p53 can be induced by DNA damage (Enokido *et al*., 1996) and that its presence is essential for excitotoxicity (Xiang *et al*., 1996), however, to our knowledge, this is the first report showing a direct link between neurotransmitter receptor stimulation and p53 induction. Although qualitatively similar, p53 induction by glutamate and KA differed in terms of intensity and time-course. Direct stimulation of NMDA receptors by glutamate in a Mg^{2+}-free condition resulted in a significant increase of p53 immunoreactivity within 30 min after the treatment and lasted for at least 2 h. KA-induced increase of p53 immunoreactivity was delayed, reaching its maximum within 2 h and lasting for at least 8 h. Both KA- and glutamate-induced increases of p53 immunoreactivity were prevented by the non-competitive NMDA receptor antagonist MK 801, suggesting that EAA-induced enhancement of p53 immunoreactivity results mainly from stimulation of the NMDA glutamate receptor subtype. These data suggest that the KA effects may be mediated by endogenous release of glutamate which in turn activate NMDA receptor subtype. Measurement of p53 mRNA levels by a RT-PCR suggested that treatment with either glutamate or KA results in an increase p53 gene transcription. In this regard, it should be noted that p53 gene in transcriptionally regulated by

NF-κB transcription factors (Wu & Lozano, 1994) and that administration of glutamate to primary cerebellar neurone also results in up-regulation of NF-κB nuclear activity (Grilli *et al*., 1996).

We were then interested in evaluating the possible relationship between EAA-induced p53 expression and cell death. Among the available molecular biological techniques, antisense strategy, particularly oligonucleotides designed to hybridize with specific sequences of the transcripts encoding the protein of interest, are enjoying increasingly wide use. Indeed, use of antisense strategy to interfere with the expression of gene products related with excitotoxicty has been used in a variety of experimental conditions (Caceres & Kosik, 1990; Pizzi *et al*., 1995b; Binsack *et al*., 1996). An oligonucleotide sequence complementary to 18 bases flanking the initiation codon of the p53 gene was synthesized and added to the culture media before treating the cells with glutamate or KA. Blockade of p53 induction, as shown by the prevention of EAA-induced increase of p53 immunoreactivity, resulted in a partial reduction of EAA-induced cell death with a complete inhibition of EAA-induced apoptosis. The specificity of the effects was proven by the lack of efficacy of the sense oligonucleotide and by the observation that the antisense oligonucleotide treatment did not alter the capability of glutamate of increasing APP immunoreactivity. These data suggest that exposure of granule cells to stimulants of ionotropic glutamate receptors triggers different intracellular death pathways, including necrosis and apoptosis. The two fates may not be necessarily distinct. As recently pointed out by Ankarcrona *et al*. (1995), only selective subpopulations of granule cells are prone to apoptosis, possibly depending on their own state of mitochondrial activation. However, it cannot be excluded the possibility that a threshold of glutamate stimulation exists to differentiate the type of cell death. Nevertheless, the apoptosis induced by EAA appeared to be strictly p53-dependent.

As shown by the gel shift analysis, the EAA treatment induced an increase of p53 DNA binding activity. A time course study of the glutamate- and KA-induced effects further evidenced differences in the kinetics. Indeed, the appearance of p53 DNA binding activity after exposure to glutamate was very rapid, being significant already 30 min after the treatment while the effects of KA were detectable at least 6–8 h after the treatment. Intriguingly, p53 immunoreactivity and p53 DNA binding activity were not temporally correlated. Western blot analysis with protein extracts from cells exposed to glutamate for 30 min and 2 h, using the same antibody as in the immunocytochemical studies, revealed that p53 protein is present at low level in untreated cells, it increases 30 min after glutamate, and it is still present and high at 2 h after the glutamate pulse (unpublished data). It may be inferred that the p53 epitope recognized by the antibody used in the present study is protected when p53 is in the tetrameric, DNA binding active form. Discordance between p53 protein levels and its DNA binding activity in response to DNA damage has been found in a variety of cell systems. It is now quite clear that p53 transcriptional activity is a regulated process which may be affected at transcriptional and post-transcriptional level. In this regard, phosphorylation at specific sites may either unravel or repress both antibody-sensitive epitopes and DNA binding sites (Hansen & Oren, 1997).

At the present, the p53 target genes triggered by glutamate receptor stimulation are unknown. Studies in normally or abnormally proliferating cells have shown that several genes are indeed transcriptionally regulated by p53, most of them regulating cell cycle arrest and DNA repair (Kastan *et al*., 1992; El-Deiry *et al*., 1993; Barak *et al*., 1993; Miyashita *et al*. 1994). As an example, one gene regulated by p53 is that coding for the pro-apoptotic bax protein (Myashita & Reed, 1995) which is known to be expressed in the brain and is upregulated after ischaemia (Krajewski *et al*., 1995). Another gene transcriptionally activated by p53 is the so-called MSH2 which is involved in recognizing and repairing mismatch DNA lesions (Palombo *et al*., 1994;Scherer *et al*., 1996). In this regard, we found that cerebellar granule cells contain MSH2 protein and its expression is up-regulated by glutamate injury (unpublished data).

In summary, we found that stimulation of ionotropic glutamate receptors in cerebellar neurones induces p53-dependent apoptosis. The induction of p53 in neurones, similarly to what may happen in other cell phenotypes, may orchestrate the activation of certain genes controlling growth arrest and/or apoptosis. These functions can be applied only partially to neurones. In fact, terminally differentiated neurones do not re-enter the cell cycle, and they cannot be transformed. Incidentally, in no case has there been described a tumour originating from differentiated neurones. Our data may allow different interpretations. One might suggest that EAA-induced apoptosis is a normal physiological response to aberrant signal transduction events occurring in not proliferating cells. Thus, excitotoxicity may be associated with the expression, at least some, cell cycle-related proteins which activate entry into an aberrant mitotic state and lead irreversibly to cellular dissolution.

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Abbreviation

AMPA	α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionate
APP	amyloid precursor protein
DIV	days *in vitro*
DTT	dithiothreitol
EAA	excitatory amino acid
EDTA	ethylenediaminetetraacetic acid
EMSA	electrophoretic mobility shift assay
ID	integrated optical density
KA	kainic acid
LDH	lactate dehydrogenase
MSH2	MutS homologue
NMDA	*N*-Methyl-D-Aspartate
PBS	phosphate-buffered saline
PCR	polymerase chain reaction

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