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DNA Strand Breaks Induced by Sustained Glutamate Excitotoxicity in Primary Neuronal Cultures

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We developed a new approach to study single- and double-stranded DNA breaks during chronic, moderate excitotoxicity resulting from the inhibiton of the glutamate transporter in cerebellar granule cell primary cultures. A 24 hr treatment of 2-week-old cultures with L-α-amino adipate (LAA), an inhibitor of the cerebellar glutamate uptake transporter, caused a gradual extracellular accumulation of endogenous glutamate that induced reversible morphological change of granule neurons but no neuronal cell death despite sustained, but moderate, elevations of the free intracellular calcium concentrations. Nick translation experiments on isolated nuclei or cells from cerebellar cultures chronically exposed to LAA revealed increased radioactive nucleotide incorporation indicative of DNA nicking. This LAA effect was dose-dependent and suppressed by NMDA receptor antagonists. Cultures treated for 24 hr with LAA and subjected to *in situ* nick translation showed an intense nuclear labeling of neurons but not glia, which could be abolished by MK801. A similar labeling was also observed in altered nuclei of granule neurons acutely exposed to high glutamate concentrations or undergoing an apoptotic cell death. Although the TUNEL labeling method detected no DNA double-strand breaks in LAA-treated cerebellar cultures, it displayed clear evidence of DNA damage during acute glutamate excitotoxicity or during apoptosis. However, Southern blot analysis of nuclear DNA revealed a DNA laddering only in apoptotic cell death. Our results demonstrate that DNA damage, characterized by DNA single-strand breaks, is an early event in chronic, moderate excitotoxicity. This type of DNA degradation, which appears before any nuclear morphological changes, is distinct from the massive DNA single- and/or double-strand damages observed during acute glutamate excitotoxicity or apoptosis.

Key words: excitotoxicity; DNA damage; necrosis; apoptosis; NMDA receptor; glutamate transporter; cerebellar granule cells; primary cultures; nick translation; TUNEL labeling

Acute glutamate receptor overactivation can induce delayed neuronal cell death via calcium-dependent intracellular pathways (Choi, 1987; Coyle and Puttfarcken, 1993; Meldrum, 1993). Although previous work has identified several cytoplasmic processes mediating acute excitotoxicity, less attention has been focused on chronic excitotoxic mechanisms, particularly the effects on the nuclear DNA. Recent studies have implicated excitotoxins in the pathogenesis of several neurodegenerative diseases (Meldrum and Grathwaite, 1990; Beal, 1992; Coyle and Puttfarcken, 1993; Meldrum, 1993). Cumulative DNA damage induced by a sustained glutamate receptor stimulation may contribute to the slow neuronal cell death seen in such chronic conditions. Evidence for early DNA damage during acute excitotoxicity has been obtained by studying the activation of the poly(ADP-ribose) polymerase (PARP) in neuronal primary cultures (Cosi et al., 1994; Zhang et al., 1994). However, no direct investigations have addressed the characteristics of this DNA damage during early stages of glutamate excitotoxicity.

In considering possible excitotoxic DNA damage, many workers use two broad categories: apoptotic and nonapoptotic. Apoptotic DNA degradation results from a specific molecular and biochemical program mediating cell death without the cytoplasmic early manifestations of cell necrosis such as swelling and lysosomal activation (Bursch et al., 1992). Although nonapoptotic DNA damage lacks easily observable changes such as the nuclear morphological transformations and DNA laddering of apoptosis, it generally represents highly specific chemical events such as the strand breaks or chemical adducts mediated by electromagnetic radiation, free radicals, or other chemical species (Nose and Okamoto, 1983; Manoharan et al., 1987; Maehara et al., 1990; Masuk et al., 1990; Wink et al., 1991; Nguyen et al., 1992). Interestingly, after ischemic–hypoxic injuries, a nonspecific random DNA fragmentation pattern coexists with internucleosomal DNA cleavage in rat cortex (Tominaga et al., 1993; Beilharz et al., 1995). Some neuropathological phenomena related to acute toxicity by glutamatergic agonists also produce nonspecific DNA strand breaks (Héron et al., 1993; MacManus et al., 1993; Tominaga et al., 1993; Portera-Cailliau et al., 1995). Whereas a complete understanding of apoptosis and other types of DNA damage in the nervous system has yet to emerge, we believe that one potential importance of glutamate-mediated DNA alteration is that such damage might occur during an early and reversible

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period of the neurodegenerative processes. We therefore decided to apply multiple *in vitro* approaches to quantify and visualize the effects of moderate, but not complete, glutamate toxicity on the neuronal genome.

To obtain gradual, moderate, and reversible excitotoxicity, we treated cerebellar granule cells in primary culture with a competitive blocker of cerebellar glutamate transport, L-α-amino adipate (LAA) (Ferkany and Coyle, 1986; Fletcher and Johnston, 1991; Robinson et al., 1991). This treatment chronically increased extracellular endogenous glutamate to a concentration known to activate the NMDA receptor, without immediately affecting neuronal survival. In this model, we investigated nuclear DNA integrity with nick translation repair and *in situ* terminal deoxynucleotidyl transferase (TdT) labeling. Nick translation experiments using DNA polymerase I without exogenous DNase have measured and visualized nicked DNA successfully in non-neuronal cells (Nose and Okamoto, 1983; Iseki and Mori, 1985; Iseki, 1986; Manoharan et al., 1987; Maehara et al., 1989, 1990; Masuck et al., 1990). In contrast, TdT can, in the presence of particular cations, predominantly catalyze the addition of deoxynucleotides to the protruding 3′-hydroxyl termini of double-stranded degraded DNA. Because polymerase I could end-label some double-stranded DNA and TdT may also detect DNA nicking with low sensitivity, we performed nick translation and TdT labeling in parallel to distinguish single from double-strand breaks of nuclear DNA in cerebellar cultures. We compared the nature of DNA breaks obtained after LAA treatment to that after acute glutamate excitotoxicity or the apoptotic DNA fragmentation.

MATERIALS AND METHODS

Primary cultures of cerebellar granule cells

Cerebellar cultures were performed as described previously (Van-Vliet et al., 1989; Didier et al., 1992). In brief, cerebella from 7-d-old mice (Charles River, Wilmington, MA) were dissociated mechanically in serum-free medium after incubation in an EDTA medium (Versene 1:5000, Gibco, Grand Island, NY). Cells were plated ($1.4–1.5 \times 10^6$ cells/ml, 2 ml per well) in poly-L-ornithine-coated 35-mm-diameter culture dishes. Culture medium was composed of a 1:1 mixture of DMEM and F-12 nutrient (Gibco), supplemented with glucose (0.6%), sodium bicarbonate (3 mM), HEPES buffer (5 mM), KCl (25 mM), horse and fetal calf serum (5% each), penicillin (100 U/ml), and streptomycin (100 μg/ml). To prevent glial cell multiplication, 20 μM cytosine arabinoside was added to the medium 48 hr after plating. After 12–14 d *in vitro*, various concentrations of LAA were added to the culture medium. To induce apoptosis, culture medium was changed 6 d after plating to Neurobasal medium (Gibco), which lacked excitatory amino acids. No serum or potassium was added (D'Mello et al., 1993; Galli et al., 1995).

Determination of amino acid concentrations

Glutamate and aspartate concentrations in the culture medium were determined after HPLC separation of their orthophthaldialdehyde derivatives and fluorescence detection at 455 nm (Kratos FS 950 Fluoromat). Aliquots of 60 μl of culture medium were mixed with 150 μl of orthophthaldialdehyde/2-mercaptoethanol derivatizing solution and injected 1 min later onto a reverse-phase column (4.6 × 250 mm, 5 μm particular diameter, Beckman Ultrasphere ODS) using a Waters automatic sample injector (model 710 B). Gradient elution was performed with buffer A (100 mM sodium acetate, pH 7.2) and buffer B (methanol) at a flow rate of 1.2 ml/min. The areas of the glutamate and aspartate peaks were determined and compared with those of external standards.

Light microscopy and Hoffman interference microscopy

Cell and nuclear morphology were evaluated using light and Hoffman interference microscopy. Cultured granule neurons on coverslips were fixed with 4% paraformaldehyde and 0.5% glutaraldehyde for 10 min and then washed with PBS. Cells were mounted in PBS-glycerol containing bisbenzimide.

Double labeling of living and dead neuronal cells to assess cell viability

Cerebellar cultures were washed in Locke's buffer (Tris-HEPES buffer) and incubated with fluorescein diacetate (FDA; 15 μg/ml) and propidium iodide (PI; 4.5 μg/mi). PI exclusion and FDA uptake and metabolism were used to determine cell viability (Didier et al., 1990). Cells were observed immediately with an inverted phase-contrast fluorescent microscope with the interference filters set for fluorescein or rhodamine detection. Living cells were labeled with FDA and observed with the fluorescein filter; nuclei of dead cells were stained by PI and revealed with the rhodamine filter.

Determination of the cytoplasmic concentration of free intracellular calcium ($[Ca^{2+}]_i$) with single-cell digital videomicroscopy

Glass coverslips (D glass, diameter 25 mm) with attached cells were first washed twice with 1 ml of Locke solution. Cells were loaded with the fluorescent calcium indicator Fura-2 PE3/AM (Vorndran et al., 1995) (Teflabs, Austin, TX) by incubating with 1 μM Fura-2 PE3/AM + 0.03% (vol/vol) Pluronic F-127 (Molecular Probes, Eugene, OR) in Locke solution for 60 min at 37°C in a tissue culture incubator. Fura-2 PE3/AM was prepared as 1 mM stock solution in dimethylsulfoxide (DMSO) and Pluronic F-127 as 20% (w/v) stock in DMSO. Cultures loaded with the calcium indicator were washed again twice with 1 ml of Locke solution and placed into a microscope stage incubator (Medical Systems, Greenvale, NY). Cells were visualized with an inverted epifluorescence microscope (Olympus IMT-2, 40× oil immersion objective, 1.3 NA). An intensified charge-coupled device camera (IonOptix, Milton, MA) recorded the emitted image at 510 nm while the cells were alternatively excited with 340 and 380 nm light (computer-controlled filter wheel). All data acquisition and subsequent image processing were done on an 80486-based computer with the software developed by IonOptix. Emitted light was recorded from an area typically containing at least 50 neurons. Images were taken every 10 sec. For each image, five frames were averaged for each of the two excitation wavelengths. Background was recorded from an area of the coverslip that did not contain any cells. After background subtraction, the ratio (R) of the intensities of emitted light at each of the two excitation wavelengths was calculated and converted pixel-to-pixel to $[Ca^{2+}]_i$ based on equation $[Ca^{2+}]i = [(R - R_{min})/(R_{max} - R)] \times K_d \times \beta$ (Grynkiewicz et al., 1985). R_{min} is the 340/380 ratio of Fura-2 free of calcium, R_{max} is the ratio of Fura-2 saturated with calcium, and β is the ratio of fluorescence intensities of the 380 nm wavelength free of calcium and saturated with calcium, respectively. K_d was taken as 224 nM. Limiting ratios R_{min}, R_{max}, and β were determined by an *in vitro* calibration method. Fura-2 PE3/K5 0.5 μM (1 mM stock solution in water) was added to a solution resembling the intracellular ionic environment (in mM: KCl 110, NaCl 10, $MgCl_2$ 1, HEPES 20, pH 7) supplemented with either 1 mM $CaCl_2$ for R_{max} or 1 mM EGTA for R_{min}. $R_{min} = 0.22 \pm 0.01$, $R_{max} = 3.35 \pm 0.21$, and $\beta = 7.49 \pm 0.44$ ($n = 5$ for all). Recordings of $[Ca^{2+}]_i$ changes over time for an individual cell were obtained by placing a small rectangle around the cell soma at the beginning of each experiment and averaging $[Ca^{2+}]_i$ within that area. All experiments were performed at room temperature. During an experiment, the cells in a microscope-stage incubator were continuously perfused (Ismatec variable speed peristaltic pump, Cole-Palmer, Niles, IL) with Locke solution at the rate of 1 ml/min. Drugs were delivered through a separate inflow channel with an increased flow rate to 2 ml/min.

Detection of DNA strand breaks by nick translation

Isolation of nuclei and cerebellar cells. Nick translation experiments were performed on both isolated nuclei and permeabilized cerebellar granule cells. Nuclei were isolated as described previously (Didier et al., 1992). In brief, cerebellar neurons were scraped twice in ice-cold PBS. The pellet obtained after a 5 min centrifugation at 300 × *g* was lysed for 5 min on ice with 0.5% (v/v) Nonidet P-40 (in 10 mM Tris HCl, pH 7.4, 10 mM NaCl, and 3 mM $MgCl_2$). Nuclei were observed and counted with a phase-contrast microscope. To permeabilize cells, they were scraped in ice-cold PBS and suspended in 0.25 M sucrose, 10 mM $MgCl_2$, and 0.5 mM dithiothreitol in 0.1 M Tris-HCl, pH 7.9. Lysolecithin was added to a final concentration of 100 μg/ml. The cell suspension was kept on ice for 5 min and spun down at 400 × *g* for 5 min.

Nick translation assays. The nick translation mixture contained 5 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 50 μg/ml bovine serum albumin (BSA), 30 μM each of dATP, dGTP, and dTTP, 1 μM dCTP, and 3000

Ci/mmol [^{32}P]dCTP in 50 mM Tris-HCl, pH 7.9. *Escherichia Coli* DNA polymerase I was added at 40 U/ml. Reactions were carried out at 20°C for 20 min and terminated by the addition of cold 5% trichloroacetic acid (TCA). Samples were filtered through GF/C filters and washed several times with 5 ml of cold 5% TCA. The radioactivity retained on filters was measured by liquid scintillation counting.

In situ *nick translation experiments.* Cultured cells grown on coverslips were fixed in 2% paraformaldehyde-methanol/acetic acid (20:1 v/v) for 15 min at room temperature, washed four times with 50 mM Tris-HCl, pH 7.5, and 5 mM $MgCl_2$, and incubated with the nick translation reaction mixture. The mixture contained the same buffer as described above under nick translation assays except that 30 μM dCTP, biotinylated dATP, and 200 U/ml DNA polymerase I were added. After incubation for 1 hr at room temperature, cells were washed four times with ice-cold PBS then permeabilized with 0.1% Triton X-100 for 2 min. After washing twice with PBS and once with PBS–BSA (2 mg/ml), cells were incubated with streptavidin–horseradish peroxidase (HRP) in PBS–BSA for 1 hr at room temperature. Coverslips were washed four times with PBS and once with 50 mM Tris-HCl, 5 mM $MgCl_2$. Detection was carried out by incubation with 6 mg/ml diaminobenzidine (DAB) in 50 mM Tris-HCl, pH 7.5, and 0.05% H_2O_2. To determine the nonspecific labeling, one coverslip from each experiment was treated without DNA polymerase I.

Apotag procedure: in situ *DNA double-strand break staining*

Cultured granule cells on coverslips were fixed with 4% paraformaldehyde and washed three times with PBS. We used a modified terminal transferase dUTP nick end labeling (TUNEL) method (Gavrieli et al., 1992) by means of an Apoptag *in situ* apoptosis detection kit (Oncor, Gaithersburg, MD). Cells, after preincubation in the equilibration buffer for 10 min at room temperature, were exposed to TdT and digoxigenin dUTP for 1 hr at 37°C. After washing in PBS, cells were incubated with the anti-digoxigenin antibodies coupled to peroxidase. The 3′-OH DNA end tailing was revealed by incubating coverslips with DAB/H_2O_2 solution in TBS. Positive controls were obtained by pretreating fixed cerebellar cells with 1 μg/ml DNase I during 15 min at 37°C. For each experiment performed, the nonspecific labeling was investigated by omitting TdT during the first step of the labeling procedure.

TdT labeling of isolated nuclear DNA and electrophoretic detection of DNA fragmentation

A quantity (6×10^6) of granule neurons for each condition, collected in cold PBS, was centrifuged at $1000 \times g$ for 5 min, and the pellet was lysed in 10 mM Tris-HCl, 10 mM EDTA, and 0.2% Triton X-100, pH 7.5. After 15 min on ice, the lysate was centrifuged at 12,000 rpm for 10 min at 4°C. The supernatant (which contained RNA and fragmented DNA but not intact chromatin) was extracted with phenol and then with phenol chloroform/isoamyl alcohol (24:1). Nucleic acids were precipitated with 1 vol of isopropanol overnight after addition of sodium acetate (300 mM). The pellet was rinsed with 70% ethanol, air-dried, and dissolved in 20 μl of 10 mM Tris-HCl/1 mM EDTA, pH 7.5. After RNase A treatment (0.6 mg/ml at 37°C for 30 min), the sample was electrophoresed in 2% agarose gel containing ethidium bromide. In some experiments, isolated DNA was subjected to terminal transferase labeling before its electrophoretic separation: after RNase A treatment, DNA was extracted and precipitated as indicated above. The final DNA pellet was resuspended in 20 μl of TdT buffer (0.1 mM potassium cacodylate, 2 mM $CaCl_2$, 0.2 mM dithiothreitol, pH 7.2). The TdT reaction was performed at 37°C for 30 min in the presence of 15 μCi of [α-^{32}P]dATP and 15 μM TdT. The reaction was stoped by adding EDTA and DNA precipitated as explained above. The sample was finally electrophoresed in 2% agarose gel. After drying, the gel was exposed to a X-OMAT autoradiography film (Kodak, Rochester, NY).

RESULTS

Effect of LAA treatment on extracellular glutamate concentration

Cerebellar granule cells differentiating in primary culture establish excitatory synaptic contact (Van-Vliet et al., 1989; Didier et al., 1992, 1993). A calcium-dependent glutamate release may be evoked by K^+ depolarization, which reaches a maximum after 11 d of culture (Van-Vliet et al., 1989; Didier et al., 1993). We measured endogenous glutamate and aspartate accumulation in media from normal cultures (control) or after 24 hr treatment with 500 μM LAA without or with 1 μM MK801 (Fig. 1). A previous report showed that the initial high glutamate concentration from a fresh DMEM-F12 medium decreased rapidly during the first 2 d of the cerebellar culture (Didier et al., 1990). In our present experiments, the basal extracellular glutamate concentration was ~5 μM after 14 d *in vitro* (Fig. 1). After 24 hr LAA treatment, glutamate levels had increased to over 10 μM. Inhibiting NMDA receptor activation with the noncompetitive antagonist MK801 did not reduce this increase. The extracellular concentration of aspartate, another endogenous excitatory amino acid released by K^+ depolarization in cerebellar cultures, was not affected significantly (Fig. 1).

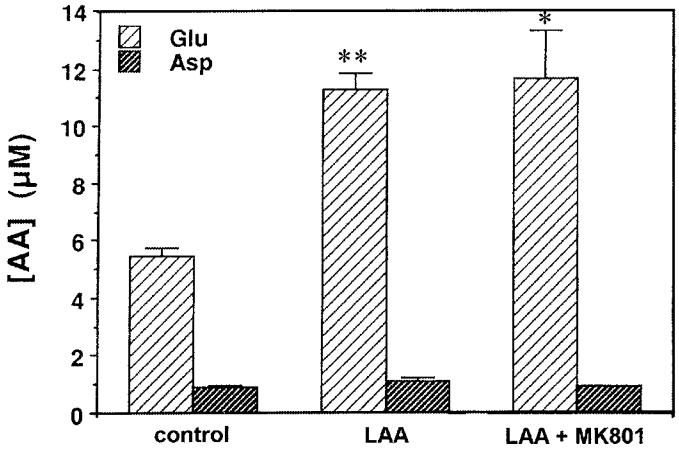

Figure 1. Measurement of glutamate (*Glu*) and aspartate (*Asp*) concentration in cerebellar granule cell culture media after LAA treatment. Extracellular concentrations of amino acids [*AA*] from culture media were determined by HPLC in control cultures (*control*) or after 24 hr treatment with 500 μM LAA without (*LAA*) or with 1 μM MK801 (*LAA + MK801*). Results represent mean ± SEM of three determinations. Levels of glutamate were increased after LAA treatment. Exposure to the NMDA antagonist MK801 did not reduce the endogenous glutamate accumulation resulting from αAA treatment. Levels of aspartate were not affected dramatically. $^{**}p < 0.01$, $^{*}p < 0.05$ compared with glutamate levels measured in control cultures (Bonferroni multiple *t* test).

Excitotoxic effects of LAA treatment on cerebellar granular cells

Treatment with 500 μM LAA for 18–24 hr resulted in a swelling of granule neurons (Fig. 2*A,B*). By 36 hr, a few pyknotic nuclei were evident, suggesting that some cerebellar cells had started to die (Fig. 2*C*). Interestingly, the LAA-induced morphological alterations of granule neurons were reversible after 18–24 hr of incubation in a normal culture medium (Fig. 2*D*). A longer exposure to 500 μM LAA induced cell death, readily visualized with Hoffman optics (Fig. 2*G*). Enzymatic digestion of extracellular glutamate by exogenous glutamate pyruvate transaminase (GPT) or chronic inhibition of NMDA receptors by MK801 added to the medium protected granular cells from LAA-mediated toxicity (Fig. 2*H,I*).

Neuronal viability during LAA treatment was assessed using the double FDA/PI staining method (Table 1). A 24 hr exposure to 500 μM LAA induced a slight neuronal swelling without affecting the survival of granular cells; however, addition of 50 μM glutamate resulted in extensive cell death. LAA, up to a concentration of 500 μM, did not reduce neuronal viability after 24 hr, but caused significant cell death by 48 hr, which was completely blocked by MK801 or GPT. Removal of the LAA-containing

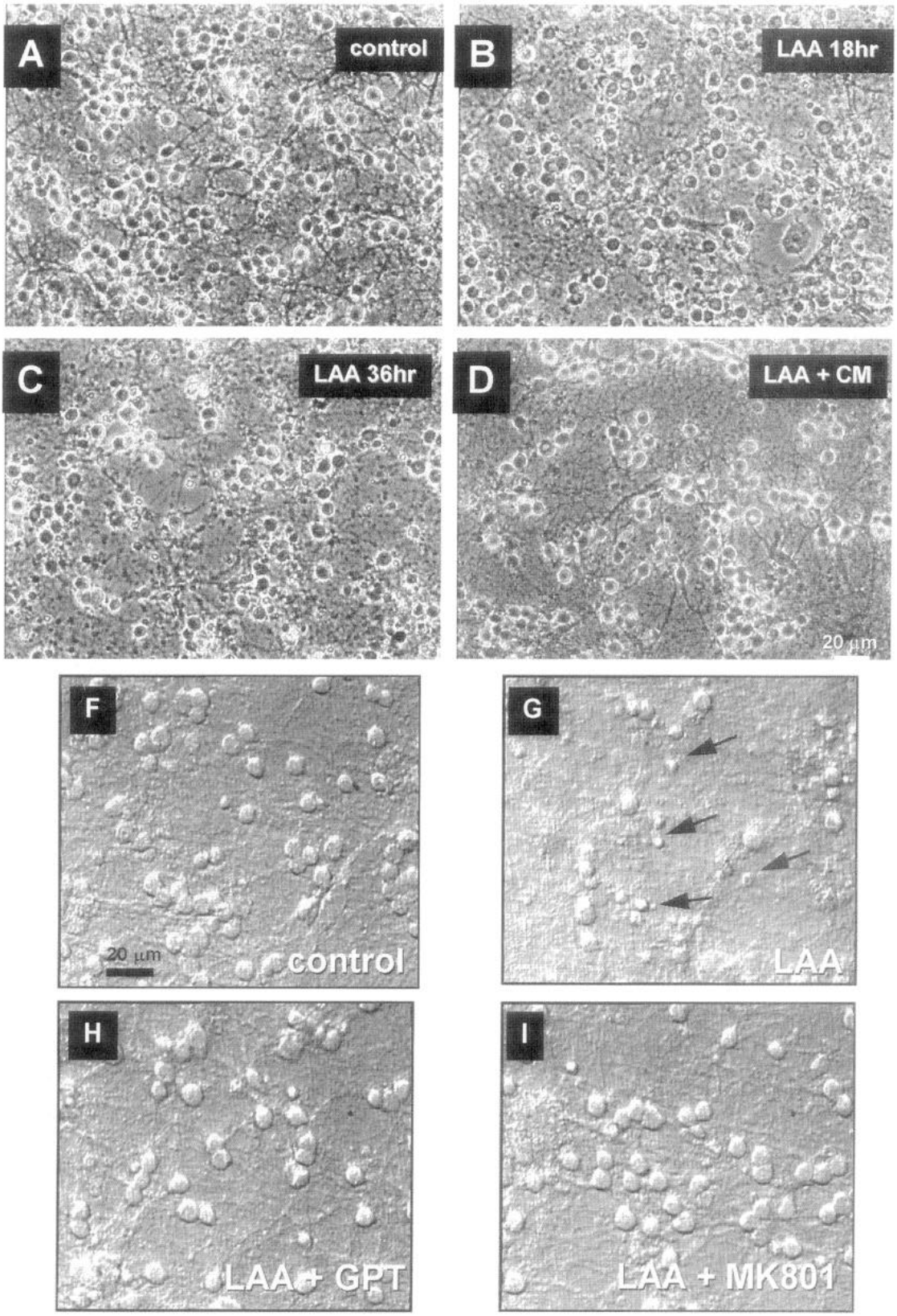

Figure 2. Morphological alterations of cerebellar granule cells during LAA treatment: protection by an NMDA receptor antagonist and removal of extracellular glutamate. Figures in the *top panel* (*A–D*) show representative phase-contrast microscopic images of cerebellar granule cells exposed to normal culture medium (*control*) or to 500 μM *LAA*. After 18 hr LAA incubation, morphological modifications of granule neurons were clearly detected (*B*). After 36 hr exposure, a complete neuronal degeneration was observed for some granular cells as characterized by the presence of pyknotic nuclei. However, when cells were exposed to LAA for 18 hr and then incubated with normal culture medium (*CM*), morphological changes induced by LAA were reversed (*D*). Moreover, these cells did not undergo degeneration typically observed after 36 hr LAA incubation. Figures in the *bottom panel* (*F–I*) taken with Hoffman interference optics show neuronal toxicity observed after 72 hr incubation with 500 μM LAA (*G*). *Arrows* point to shrunken cells. The LAA-mediated neuronal death was prevented by treatment with the glutamate pyruvate transaminase (*H*) or with MK801 (*I*).

Table 1. Determination of the neuronal survival after LAA treatment

Conditions	Dead cells (%)
Control	14.5 ± 1.0
100 μM LAA, 24 hr	11.3 ± 1.0
500 μM LAA, 24 hr	14.2 ± 0.7
500 μM LAA, 48 hr	44.7 ± 4.4*
500 μM LAA + 1 μM MK801, 48 hr	14.7 ± 1.0
500 μM LAA + GPT, 48 hr	12.7 ± 2.4
500 μM LAA, 24 hr + normal CM, 24 hr	15.9 ± 1.5
50 μM glutamate, 24 hr	77.2 ± 7.2**

Living and dead neuronal cells were counted after FDA/PI staining. LAA, at concentrations as high as 500 μM, did not reduce the neuronal viability after 24 hr treatment. GPT, Glutamate pyruvate transaminase; normal CM, normal culture medium that does not contain LAA. Results are means ± SE of three independent determinations. $^{*}p < 0.05$; $^{**}p < 0.01$ when compared to control values (multiple Bonferroni t test).

Table 2. Concentrations of intracellular free calcium in cultured granule cell during LAA treatment

Conditions	Ca^{2+} concentration (nM)
Control	71.1 ± 1.4
500 μM LAA, 24 hr	115.7 ± 12.7**
500 μM LAA, 48 hr	251.9 ± 71.6
500 μM LAA + 1 μM MK801, 48 hr	76.8 ± 1.55
500 μM LAA, 24 hr + normal CM, 24 hr	88.1 ± 7.1*

Basal free calcium levels were determined by Fura-2 video imaging as described in Materials and Methods. Normal CM, normal culture medium that does not contain LAA. Results are means ± SE of at least 32 determinations. $^{*}p < 0.05$; $^{**}p < 0.01$ when compared to control values (multiple Bonferroni t test).

culture medium after 24 hr also eliminated the toxicity observed at 48 hr, indicating that neuronal cells were not injured irreversibly after 24 hr LAA exposure.

Adipic acid itself is an NMDA receptor ligand, although only the D-isomer, not the L-isomer that we use, seems to have pharmacological effects on NMDA receptors (Watkins, 1989). LAA-mediated neuronal depolarization was not evident after measuring intracellular calcium concentration (Fig. 3*A*). By contrast, addition of glutamate resulted in a rapid and dramatic increase of intraneuronal free calcium level in the same granular cells (Fig. 3*A*). The effect of chronic LAA treatment on intracellular calcium concentration was measured by Fura-2-video imaging analysis (Fig. 3*B*). After 24 hr treatment, the resting concentration of cytoplasmic free calcium rose in almost every granule cell an average of 60% (Fig. 3*Bb*, Table 2). These elevations were inhibited by the NMDA receptor antagonist (Fig. 3*Bc*). The removal of LAA after 24 hr led to a minor reduction of the cytoplasmic calcium level 24 hr later (Fig. 3*Be*). Moreover, calcium levels rose 2.5-fold after 48 hr of LAA exposure (Fig. 3*Bd*); however, the frequency distribution of these values revealed that only one-third of granule neurons achieved the very high intracellular calcium concentrations of >140 nM.

Detection of DNA damage in cerebellar cultures

Nick translation radiolabeling on isolated nuclei and neuronal cells

In initial experiments, we assessed DNA nicking in cerebellar cultures by performing nick translation on isolated nuclei using DNA polymerase I. DNA polymerase I is regularly used to label DNA in conjunction with DNase I, an enzyme that catalyzes production of single-strand nicks. Using polymerase I without the DNase provides a sensitive measure of the number of nicks present in the DNA, an index of DNA damage (Nose and Okamoto, 1983; Iseki and Mori, 1985; Iseki, 1986; Manoharan et al., 1987; Maehara et al., 1989, 1990). We monitored the extent of nick repair by using [^{32}P]dCTP and liquid scintillation counting (Fig. 4*A*,*B*). After exposure to 500 μM LAA (24 hr), cells were lysed and nuclei purified (Fig. 4*A*). As alternative treatment, a 10 mJ dose of ultraviolet irradiation served as a positive control for the induction of DNA nicking. Either treatment induced a significant elevation in the reaction level within nuclei. In a second series of experiments, nick translation also was assessed on permeabilized cerebellar granule cells (Fig. 4*B*). After 24 hr treatment with either 300 or 500 μM LAA, incorporation significantly increased over the basal level observed in nontreated cells (Fig. 4*B*). Noncompetitive (MK801) or competitive (CPP) NMDA antagonists reduced the level of nick translation labeling, demonstrating that inhibition of NMDA receptors during LAA treatment protected cells from DNA damage. Finally, LAA mediated DNA damage in a dose-dependent manner (Fig. 5). Significant effects were observed with an LAA concentration >100 μM.

In situ *nick translation labeling in cerebellar cultures*

We developed *in situ* nick translation labeling to visualize the cellular subtypes that displayed DNA damage after LAA exposure (Fig. 6). In these experiments, radiolabeled dCTP was replaced by a digoxigenin-linked nucleotide. The nucleotide incorporation into nuclear DNA was then revealed with a biotinylated antibody followed by streptavidin HRP/DAB staining. We also investigated the reactivity of apoptotic nuclei containing double-strand DNA breaks to nick translation labeling. As already described, apoptotic cell death can be induced by exposing 6-d-old cerebellar cultures to low-K^+ medium without serum (D'Mello et al., 1993; Galli et al., 1995). In control cultures, a light and uniform nonspecific labeling is seen (Fig. 6*A*). When culture medium was replaced with a nondepolarizing, chemically defined medium in 6-d-old cultures, a strong nuclear signal was observed in damaged cerebellar neurons (Fig. 6*B*). Granule cells without the phase-contrast microscopic morphological characteristics of apoptosis were negative for *in situ* nick translation labeling (data not shown). Most cerebellar cells in cultures treated with 100 μM LAA for 24 hr displayed a detectable but moderate nuclear signal (Fig. 6*C*) that increased at higher LAA concentrations (Fig. 6*D*) but completely disappeared when NMDA receptor activation was inhibited by MK801 (Fig. 6*E*). Interestingly, glial cells did not exhibit a positive labeling when incubated for 24 hr with LAA (Fig. 6*D*) or when neuronal cells were irreversibly injured by acute glutamate excitotoxicity (Fig. 6*F*). After 48 hr of LAA treatment, a light nuclear labeling was observed occasionally in some glial cells. In the presence of high exogenous glutamate concentrations, neuronal survival was reduced dramatically and all neurons displayed a positive signal for the nick translation reaction (Fig. 6*F*). When LAA treatment was conducted for 48 hr, nuclear staining was intense (Fig. 6*G*). Finally, 24 hr after removing 500 μM LAA from the medium in culture exposed for 48 hr, the nuclear nick translation signal was reduced, but higher than that observed after 24 hr exposure to 100 μM LAA (Fig. 6*H*).

Detection of double-strand DNA breaks by modified TUNEL labeling method

Results in Figure 6 suggest strongly that the nick translation reaction may label single- and/or double-strand DNA breaks

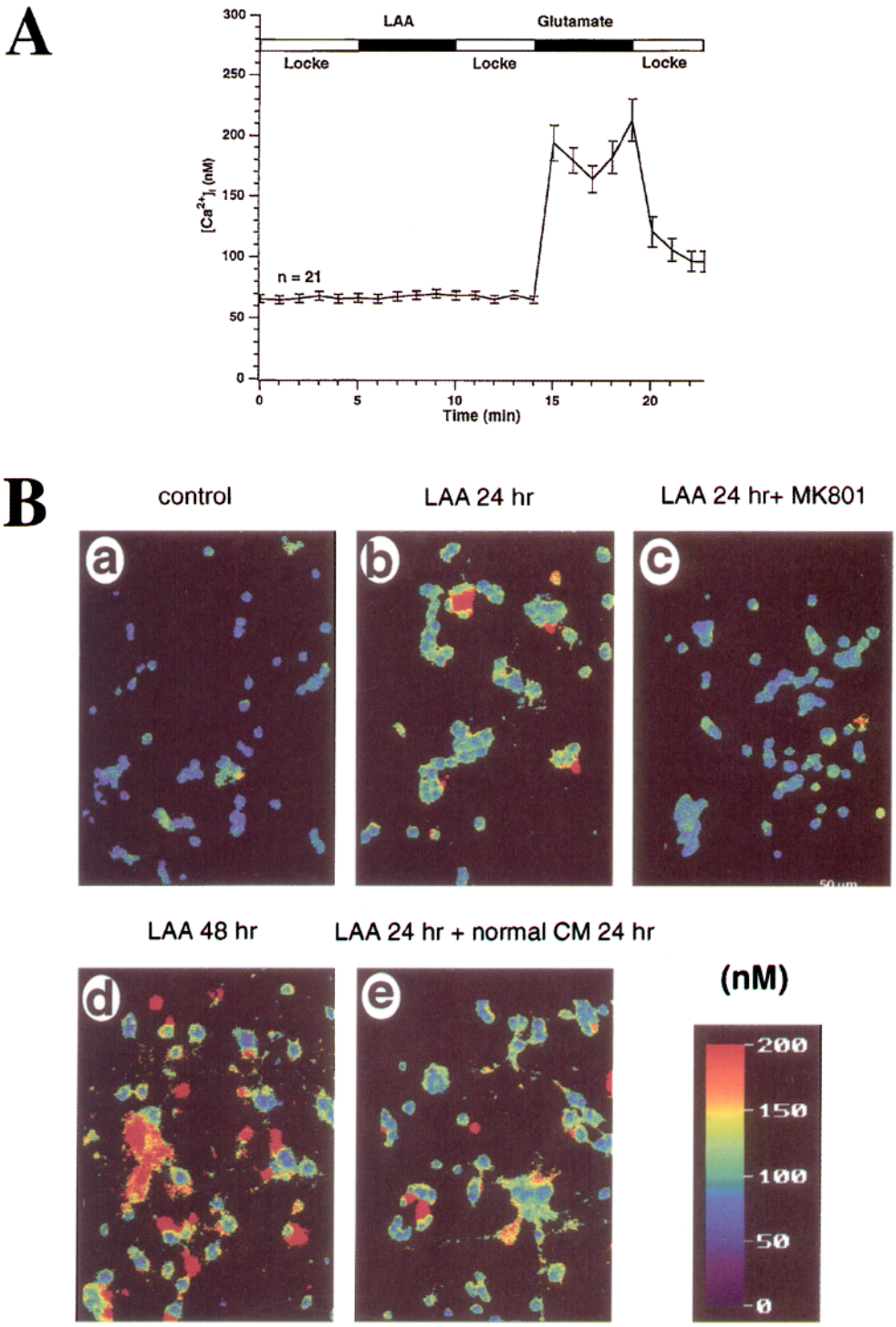

Figure 3. Measurement of intracellular free calcium levels during acute and chronic LAA treatments of cerebellar granule cells. *A,* Intracellular calcium concentration in cerebellar neurons was recorded after treatment with LAA and glutamate in Locke's buffer. A 500 μM concentration of LAA did not affect acutely the cytoplasmic free calcium in cultured granule cells. In contrast, 100 μM L-glutamate rapidly elevated intracellular calcium levels threefold in the same cerebellar neurons. Each point is the mean ± SEM of 21 values recorded in different granule cells. *B,* The resting concentration of free cytoplasmic calcium was measured by a Fura-2 videomicroscopy imaging method in normal cerebellar cultures (*a*) or after 24 hr (*b*) or 48 hr (*d*) treatment with 500 μM LAA. Such LAA exposure increased intracellular calcium resting levels. Addition of MK801 prevented the intracellular calcium increase (*c*). In some experiments, the culture medium containing LAA was replaced 24 hr later with a normal culture medium (*CM*; *e*).

under our experimental conditions. To distinguish further these types of DNA damage, we applied a modified TUNEL method of DNA double-strand breaks labeling using an Apoptag staining (Fig. 7). OH-3′ ends of fragmentated DNA, which appear mainly in apoptotic cells, were labeled preferentially using *in situ* TdT reaction. This method labeled pyknotic nuclei of the small fraction of granular cells dying during maturation under normal culture conditions (data not shown). In addition, apoptosis or

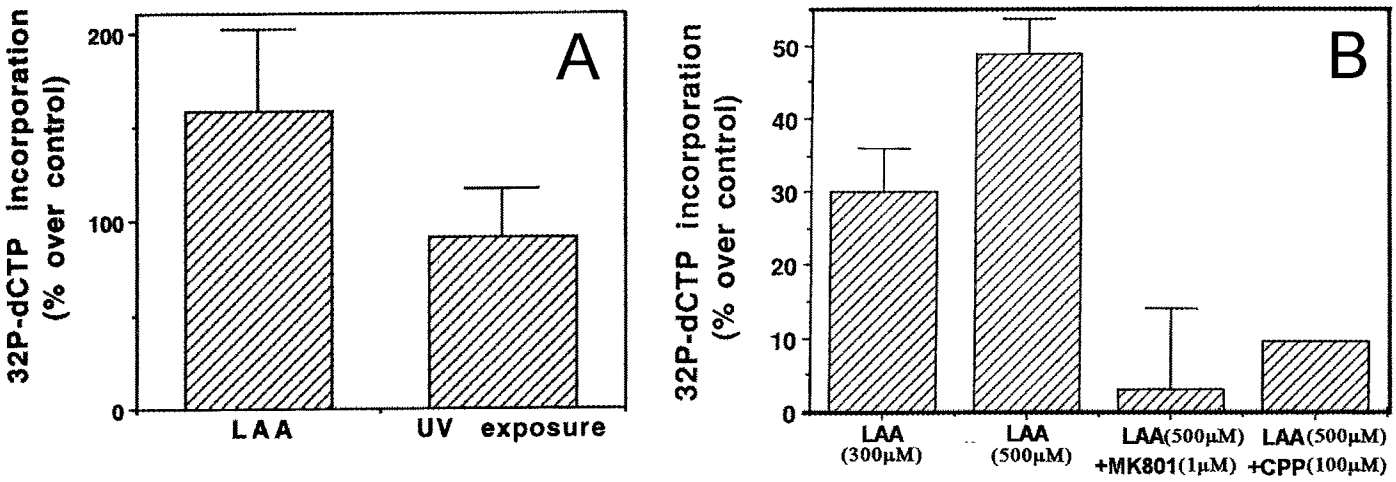

Figure 4. Detection of LAA-induced DNA breaks by nick translation and effect of NMDA receptor antagonists. *A*, A nick translation reaction was performed in presence of [^{32}P]dCTP on isolated nuclei from primary neuronal cultures. Cells were lysed and nuclei purified after exposure to 500 μM LAA (24 hr) or 10 mJ ultraviolet irradiation. The radioactivity retained on filters was measured. Results are expressed as percentages over control that correspond to values obtained in culture cells without any treatment. Mean ± SEM of three (LAA conditions) or two (ultraviolet exposure) independent experiments. Either treatments with LAA and ultraviolet induced a significant difference in the amount of nuclear reaction ($p < 0.05$ compared with control values, Bonferroni t test). *B*, Nick translation experiments were performed on permeabilized cerebellar granule cells after 24 hr treatment with either 300 or 500 μM LAA. Noncompetitive (1 μM MK801) or competitive (100 μM CPP) NMDA antagonists were added during LAA exposure. Results are expressed as radioactive dCTP incorporation increases over control, which correspond to values observed in absence of any LAA treatments. The inhibition of the NMDA receptor during LAA treatment decreased the level of DNA breaks; 300 and 500 μM LAA significantly increased nick translation reaction levels over control values ($p < 0.05$ and 0.01, respectively; Bonferroni multiple t test).

acute excitotoxicity is associated with dramatic changes in the nuclear morphology of affected cells. Thus, it was also interesting to determine whether nuclear transformations occur during LAA treatment or whether DNA damage can be detected before nuclear morphology is altered. We compared in parallel the nuclear morphology and staining obtained by nick translation or Apoptag methods in cultures treated with LAA or glutamate or in cells undergoing apoptosis (Fig. 7). In normal cultures, nuclei have regular contours, are round, and represent ~80% of cellular volume (Fig. 7) (Van-Vliet et al., 1989; Didier et al., 1992). Interestingly, after 24 hr exposure to 500 μM LAA, the nuclear morphology was not affected, yet these nuclei exhibited a specific signal for nick translation but not Apoptag labeling. By contrast, high glutamate concentrations induced either shrunken, irregularly shaped nuclei displaying a light bisbenzimide staining or small, degraded nuclei with aggregation and fragmentation of chromatin. Nuclei displaying either pattern were labeled intensely in nick translation experiments. In particular, staining of condensed chromatin in pyknotic nuclei was extremely dense by both nick translation and Apoptag reactions. Apoptotic cell death resulted in a similar initial nuclear degradation, but a spherical condensation of chromatin with a perinuclear distribution ultimately was observed. In these nuclei, strong signals were detected with either of the DNA-labeling methods.

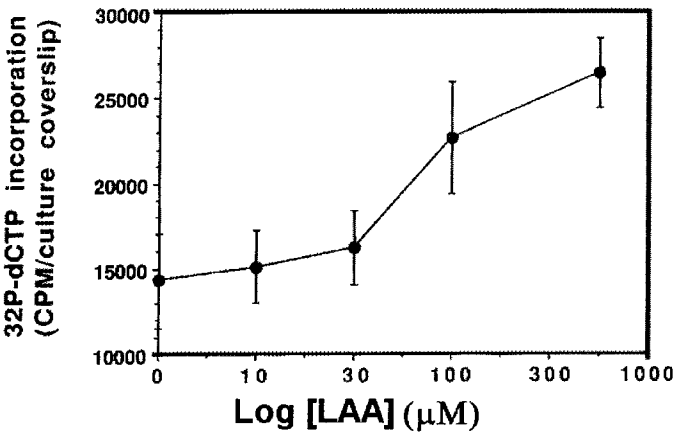

Figure 5. Dose response of LAA on the induced DNA damage. Cerebellar granule cell cultures were exposed to various concentrations of LAA for 24 hr. Cells grown on coverslips then were permeabilized and subjected to nick translation experiments. Results are expressed as the total [^{32}P]dCTP incorporation per coverslip and represent the mean of two determinations. A significant effect was observed with an LAA concentration ≥100 μM LAA.

Electrophoretic analysis of nuclear DNA

Nuclear DNA fragmentation was studied by electrophoretic separation on agarose gels (Fig. 8). In a first set of experiments, we treated cerebellar primary cultures with LAA or induced an apoptotic cell death and then assessed nuclear DNA integrity on ethidium bromide-stained agarose gels (Fig. 8*A*). Internucleosomal cleavage of DNA evident by DNA laddering was observed in cultures undergoing apoptosis, but not after 24 hr of 500 μM LAA treatment. To detect double-stranded internucleosomal fragments more sensitively, we labeled isolated nuclear DNA with radioactive deoxythymidine using TdT, transferred it to a nylon membrane, and detected the label by autoradiography (Fig. 8*B*). A laddered DNA pattern indicative of apoptosis was revealed in cerebellar cells exposed to a nondepolarizing, chemically defined culture medium. No detectable laddering was present in nuclear DNA from cells treated with LAA or high glutamate concentrations.

DISCUSSION

In cerebellar primary cultures, 24 hr treatment with LAA raised extracellular endogenous glutamate that apparently activated NMDA receptors to mediate reversible morphological alterations of granular cells. Concomitantly, a sustained elevation of the free

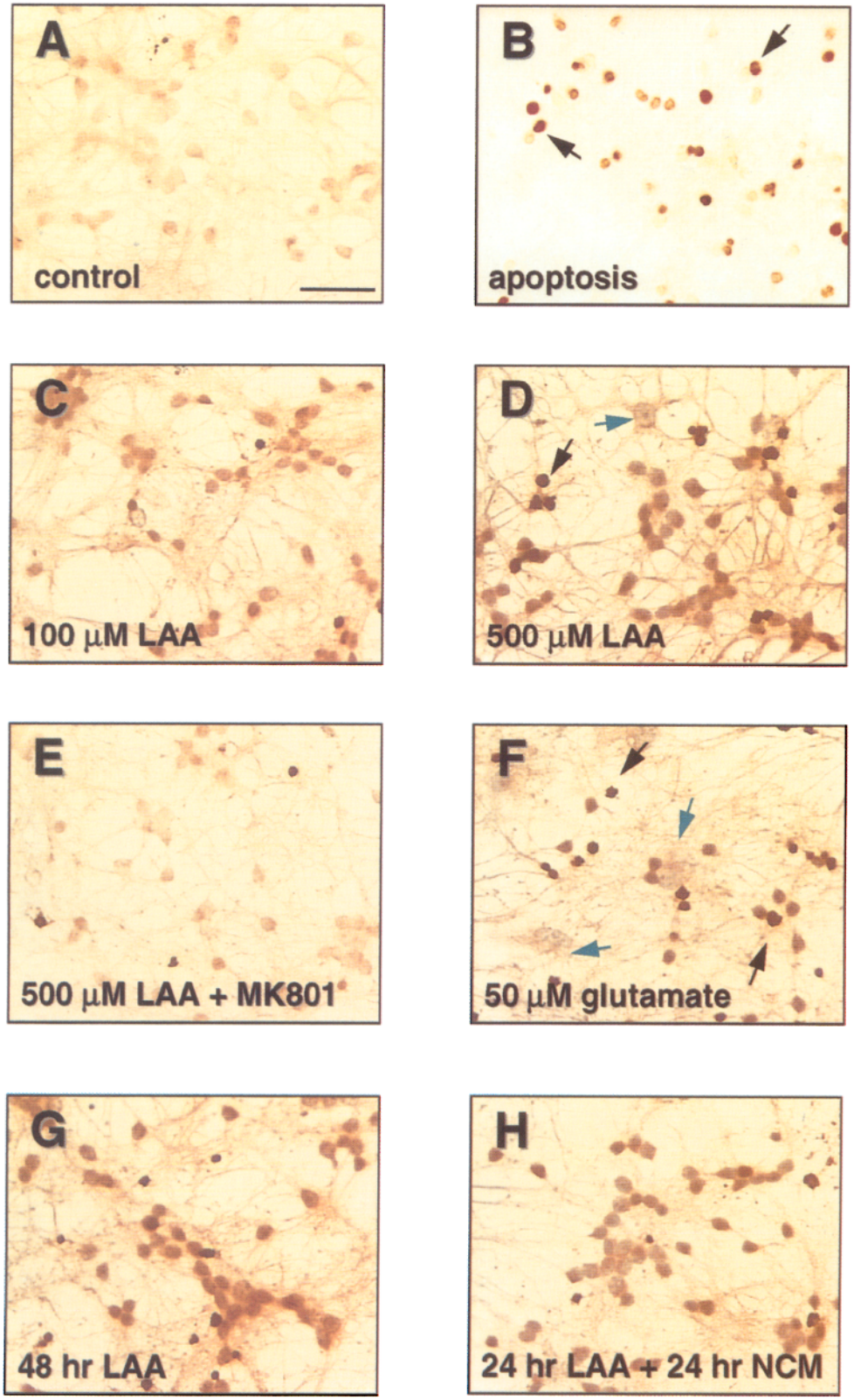

Figure 6. DNA strand breaks detected by *in situ* nick translation after LAA treatment. After exposure to various treatments, cells were fixed with a mixture of paraformaldehyde–methanol–acetic acid. *In situ* nick translation was performed using biotinylated dATP and detected with streptavidin–HRP using DAB as substrate. In control (*A*), neuronal cells were not exposed to LAA. Apoptosis was induced by exposing 6-d-old cerebellar cultures to low-K^+ medium without serum (*B*). Most neuronal cells exhibited a positive nuclear signal after 100 (*C*) or 500 μM (*D*) LAA exposure, and this staining was supressed by the addition of NMDA receptor antagonist during the LAA treatment (*E*). Excitotoxic granular cells after glutamate exposure displayed strong nuclear signals (*F*). Positive nuclear labeling was also observed when cells were treated for 48 hr with 500 μM LAA (*G*) or 24 hr after removal of LAA from the culture medium (*H*). *Black arrows* indicated intense positive staining; *green arrows* show unlabeled nuclei from glial cells. *NCM*, Normal culture medium. Scale bar, 50 μm.

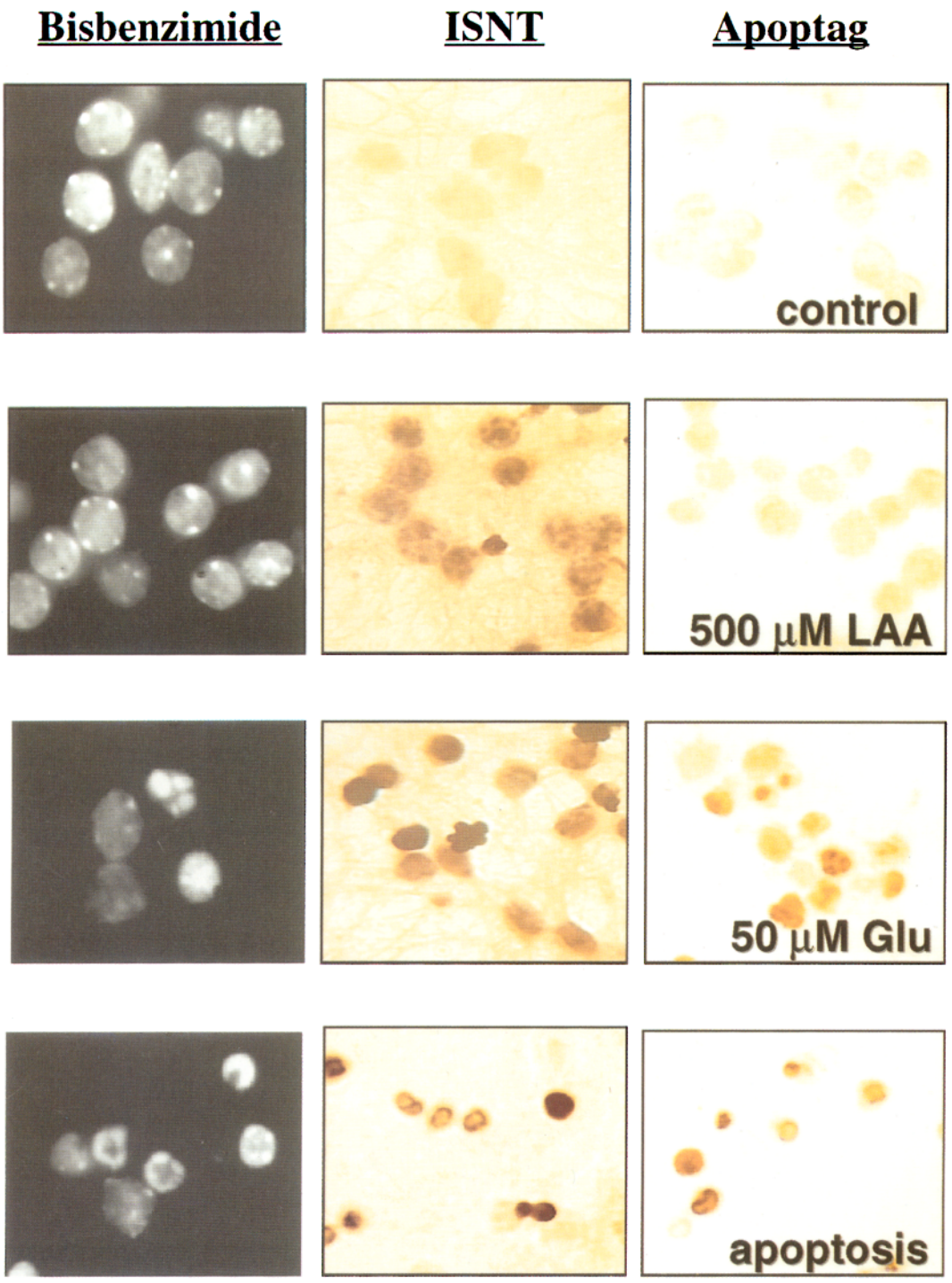

Figure 7. Comparison of nuclear morphology changes, nick translation, and Apoptag labelings in normal cerebellar cells and cultures treated with LAA or glutamate or undergoing apoptosis. Pictures represent cells observed under the fluorescent microscope after DNA staining by *Bisbenzimide*. In normal cultures, nuclei are regular, round, and represent ~80% of cellular volume. LAA treatment did not induce visible transformations of the nuclear morphology. However, DNA damage was observed with *in situ* nick translation (*ISNT*) labeling but not with *Apoptag*, which detects predominantly DNA double-strand breaks. Acute glutamate-induced excitotoxicity resulted in a nuclear degradation characterized by spherical condensation of chromatin without a perinuclear distribution. Nuclei were positive to both nick translation and Apoptag labelings. Apoptosis was observed 18 hr after switch of 6-d-old cultures to new culture medium containing low K^+ concentration (5 mM) and lacking glutamate and serum. After the change of culture media, nuclei began to shrink and a peripheral chromatin ring appeared, typical of apoptosis. Condensed chromatin was stained intensely by both nick translation and Apoptag labeling.

cytoplasmic calcium concentration was observed in these affected neurons. Although no nuclear morphological transformations were noticed, DNA breaks were detected after LAA incubation as evidenced by nick translation repair assays but not by a modified TUNEL method that labels predominantly DNA double-strand breaks (Gavrieli et al., 1992). In contrast, acute glutamate excitotoxic and apoptotic neuronal cell death revealed DNA damage by both nick translation and TUNEL labeling methods.

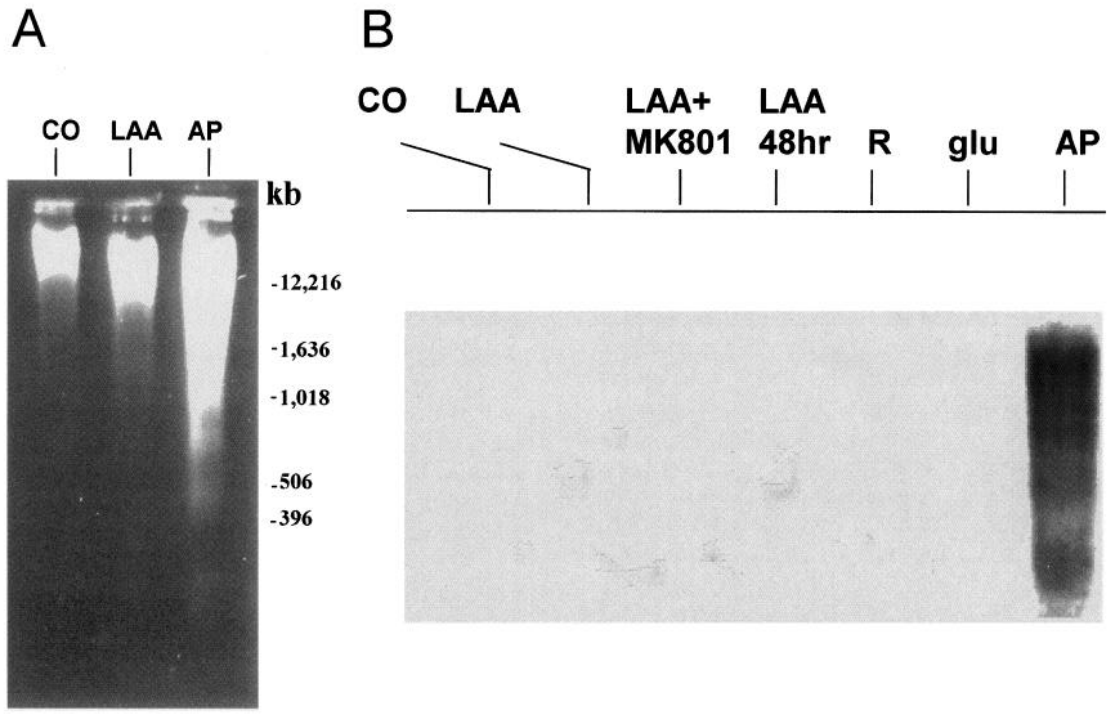

Figure 8. Gel analysis of DNA from cerebellar cultures exposed to LAA or low-K^+ chemically defined culture medium. *A*, Granular cells were treated 24 hr with 500 μM LA (*LAA*) or serum-free culture medium (*AP*) and then lysed. After isolation and RNase treatment, DNA was electrophoretically separated on 2% agarose gel containing ethidium bromide. Only apoptotic cells displayed a typical DNA ladder. *CO*, DNA isolated from control cultures. *B*, After radiolabeling of free 3′-OH DNA ends, only DNA extracted from apoptotic cultures revealed a specific DNA laddering. *R*, 24 hr LAA + 24 hr normal culture medium; *AP*, apoptosis.

Excitotoxicity mediated by endogenous glutamate during LAA treatment

Several sodium-dependent transporters for excitatory amino acids have been distinguished pharmacologically, and the cloning of different forms for these proteins has been reported (Kainai et al., 1993; Danbolt, 1994; Danbolt et al., 1994). Cerebellar high-affinity glutamate transport is characterized by its high sensitivity to LAA and its insensitivity to dihydrokainate (Ferkany and Coyle, 1986; Fletcher and Johnston, 1991; Robinson et al., 1991). Three different molecular forms of glutamate transporter may occur in cerebellar cells and LAA treatment might affect both neuronal and glial cell glutamate uptake (Kainai et al., 1993; Danbolt, 1994; Danbolt et al., 1994; Fairman et al., 1995). Differentiated granule cells *in vitro* may predominantly use glutamate as their synaptic excitatory neurotransmiter (Van-Vliet et al., 1989; Rogers et al., 1991; Didier et al., 1993). LAA treatment mainly upregulates the concentration of glutamate that presumably is released at synaptic contacts of cultured granular neurons. Such treatment increases the total concentration of glutamate in the culture medium by only two- to threefold, but it is important to note that such values reflect the total accumulation of glutamate that diffused from synaptic clefts to the extracellular space. The local glutamate concentration at the synaptic contact probably is much higher, and a sustained inhibition of glutamate transporters causes prolonged activation of synaptic glutamate receptors in neuronal cultures (Tong and Jahr, 1995). Granule cells probably also have presynaptic glutamate transporters because synaptosomal preparations from cerebellum display high-affinity glutamate uptake (Ferkany and Coyle, 1986; Fletcher and Johnston, 1991; Robinson et al., 1991). Glutamate that accumulates in the extracellullar compartment during LAA exposure mediated excitotoxicity by the activation of NMDA receptors in our culture model. LAA neither activated glutamate receptors nor depolarized granular cells and, thus, cannot directly account for the excitotoxic cell death. In contrast, LAA neurotoxic properties were blocked by the enzymatic removal of glutamate. Moreover, a noncompetitive inhibitor of NMDA receptors also supressed the LAA-induced neurotoxicity even though this NMDA antagonist did not affect the LAA-dependent elevation of the glutamate extracellular concentration. Finally, the granular cell morphological alterations produced by 24 hr LAA treatment (i.e., swelling) were reversible after removal of the culture medium. Such a swelling may depend on sustained entry of Na^+, Cl^-, and H_2O which, in contrast to the calcium homeostasis deregulation, can be reversed by the removal of glutamatergic agonists from the culture medium (Choi et al., 1987). Although the final glutamate concentration measured in the culture medium after 24 hr LAA exposure can induce neuronal cell death acutely in cerebellar cultures (Manev et al., 1991), we find no significant neuronal losses 24 hr after LAA addition. The gradual increase in glutamate extracellular concentration may initiate regulatory processes, leading to some granule cell resistance to glutamate (Chuang et al., 1992; Didier et al., 1994). In neurodegenerative diseases that display a late-onset profile of neuronal degeneration, particularly those related to an impairment of glutamate transporter activities, these compensatory processes may extend the neuronal survival significantly.

Single- and double-strand DNA damage during excitotoxicity

Until recently, investigations of neuronal cell death mechanisms focused principally upon pathological modifications in the cytoplasmic compartment of dying neurons. However, the discovery of apo-

ptotic phenomena during *in vivo* neuronal development motivated a search for DNA damage during neuronal injuries, directing increased attention toward DNA degradation processes. For apoptotic death, the detection of internucleosomal DNA cleavage, represented by a DNA ladder on gel electrophoresis, has frequently served as a general hallmark. In contrast, a nonspecific random DNA fragmentation, seen as a smear on agarose gel electrophoresis, has been associated exclusively with a necrotic lysis of neurons. Reported profiles of DNA degradation, however, often have included both random damage and specific internucleosomal alterations. Indeed, in some ischemic and kainate-induced brain insults, these two DNA damage patterns may coexist and occur in the same neuronal population (Tominaga et al., 1993; Beilharz et al., 1995; Portera-Cailliau et al., 1995). In addition, necrotic and apoptotic morphologies could be visualized simultaneously in dying striatal neurons after an acute glutamate receptor activation (Portera-Cailliau et al., 1995). Non-neuronal necrotic cells also have been shown to produce DNA laddering type degradation in some circumstances (Collins et al., 1992; Fukuda et al., 1993). The boundary between necrosis and apoptosis has become less distinct, not only during glutamate neurotoxic processes but also in non-neuronal cell pathological phenomena (for review, see Farber, 1994; Columbano, 1995). Our study reveals that several types of DNA damage can be detected directly in necrotic neuronal nuclei depending on the severity of excitotoxicity. In cultured neurons exposed to LAA and undergoing a moderate and still reversible glutamate excitotoxicity, nick translation repair, using either radioactive or digoxigenin nucleoside triphosphates, displayed a positive signal even without apparent alterations of the nuclear structure. In the absence of exogenous DNase, this method allows a quantitative determination of DNA damage on isolated cells or semiquantitative *in situ* detection of DNA nicks (Nose and Okamoto, 1983; Iseki and Mori, 1985; Iseki, 1986; Manoharan et al., 1987; Maehara et al., 1989, 1990; Masuck et al., 1990). But double-stranded DNA scission could be detected at lower efficiency by nick translation, so we used modified TUNEL labeling to verify that this type of DNA degradation did not appear during LAA treatment. Taken together, these results demonstrate that single-strand DNA degradation can occur during an early stage of glutamate neurotoxicity. It is also probable that excision repair mechanisms of damaged DNA that produce large gaps in DNA strands may enhance the nick translation reaction observed during LAA treatment (Sanzar, 1994). When cerebellar cultures were treated acutely with exogenous glutamate, most neurons exhibited acute cell death and displayed modifications in their nuclear morphology; some showed moderate nuclear shrinkage, but others had more severe nuclear damage, such as pyknosis and karyorrhexis. The first type of damaged nuclei was stained intensely by nick translation reaction and lightly by Apoptag, suggesting that massive nicks of single-strand DNA and/or moderate double-strand DNA degradation appear. In contrast, nuclei that undergo dramatic morphological transformations were intensely labeled by both the methods used, supporting the conclusion that both massive single- and double-strand DNA breaks were present. However, the cleavage of double-stranded DNA was not internucleosomal as judged by our electrophoretic gel analysis. Thus, a progressive DNA degradation may appear during the time course of excitotoxic phenomena.

Does a progressive DNA degradation occur during excitotoxicity?

In our model, intracellular calcium concentrations may correlate with the level of DNA damage. LAA-mediated sustained elevation of cytoplasmic free calcium, even minimally abnormal levels, could initiate a biochemical cascade leading to DNA degradation. Indeed, free radicals and endonucleases have been shown to alter directly the nuclear DNA integrity, and their production or stimulation can be induced by submicromolar concentration of calcium (Jones et al., 1989; Knowles et al., 1989). Reactive oxygen species such as nitric oxide may induce nuclear DNA alterations of different natures such as deamination, breakage, and mutation (Wink et al., 1991; Nguyen et al., 1992). The potential role of nitric oxide in DNA damage during excitotoxicity is supported by the NO-dependent stimulation of the PARP during the early period of acute and irreversible glutamate neurotoxicity (Zhang et al., 1994). Beside NO, arachidonic acid, produced by NMDA receptor agonists, also generates superoxide species in cerebellar primary cultures and thus may contribute to nuclear DNA damage (Lafon-Cazal et al., 1993). In addition, nuclear endonucleases activated by excessive glutamate receptor stimulation could participate in the degradative mechanisms. Their roles have been studied mostly in apoptotic cell death, in which DNA fragmentation is a two-stage process: first, endonucleolytic cleaveage of DNA into large fragments causing chromatin collapse (Walker et al., 1994), and second, the activation of calcium–magnesium-dependent endonucleases (possibly DNase I) producing oligonucleosomes without chromatin condensation (Peitsch et al., 1993; Dexter et al., 1994; Walker et al., 1994). Functional endogeneous endonucleases have been isolated from nuclear preparations of the mammalian brain (Tominaga et al., 1993). Activation of these enzymes, in combination with the stimulation of particular proteases, may determine a massive random or internucleosomal cleavage of nuclear DNA in acute excitoxicity (Tominaga et al., 1993; Portera-Cailliau et al., 1995). In our model, such an advanced DNA degradation was not observed even after exogenous stimulation of glutamate receptors produced dramatic morphological transformations of neuronal nuclei. Thus, it is likely that double-strand DNA breaks observed with the TUNEL method during acute excitotoxicity result from a particular endonucleolytic process that can generate large DNA fragments, too large to visualize by regular electrophoretic gel analysis. A high molecular weight DNA cleavage occurs during an early stage of apoptosis, and it is associated with a chromatin condensation that is apparently sufficient to induce the cell death (Walker et al., 1994). Interestingly, our experiments show double-strand DNA breaks only in nuclei that displayed an evident chromatin condensation and an irreversible excitotoxic damage. Based on these observations, additional studies using pulse-field electrophoresis conditions are required to identify a potential production of high molecular weight DNA fragments during early steps of excitotoxicity (Walker et al., 1994). Such a DNA degradation also may be present during initial periods of the neuronal degeneration occurring after hypoxic–ischemic injuries or excitotoxicity *in vivo*. In several studies, TUNEL-positive neurons and glia were observed clearly before electrophoretic detection of nuclear DNA cleavage (Héron et al., 1993; Beilharz et al., 1995; Portera-Cailliau et al., 1995; Nitatori et al., 1995). In fact, advanced DNA degradation probably requires protease activation. In apoptosis, proteolytic activities facilitate the DNA cleavage into nucleosomal fragments (Walker et al., 1994). During excitotoxic phenomena *in vivo*, the release of proteases from disrupted lysosomes could digest histone proteins uncovering nuclear DNA and consequently allowing its random digestion by endonucleases (Tominaga et al., 1993; Portera-Cailliau et al., 1995). A longer period of glutamate exposure could be required in our culture model to obtain higher level

protease activity sufficient to produce DNA degradation observable by gel electrophoresis.

Potential importance of single-strand DNA damage during excitotoxicity

Single-stranded DNA breaks were detected in cerebellar cultures before an irreversible cell death occurred, suggesting that this type of DNA damage does not affect the neuronal survival immediately. In contrast to double-strand DNA cleavage, DNA nicks should be repaired easily in mammalian cells (Sanzar, 1994). Therefore, a sustained overactivation of glutamate receptors may lead to a long-term accumulation of single-strand DNA nicks that could contribute to a delayed neuronal death. For example, single-stranded DNA damage may account for an early appearance of DNA double-strand cleavage observed during apoptosis (Tomei et al., 1993). In addition, a sustained activation of PARP by nicked DNA can deplete NAD^+ and ATP and ultimately reduce the cellular energy (Cosi et al., 1994; De Murcia and Nenissier De Murcia, 1994; Zhang et al., 1994; Heller et al., 1995). Finally, accumulated and unrepaired DNA damage in nuclei could initiate long-term macromolecule damage and thus chronically affect cellular metabolism. Thus, single-stranded DNA damage could constitute an early marker of neuronal dysfunction in the brain. Indeed, the TUNEL method widely used by several laboratories appears to visualize nuclear degradation connected with the final stages of cell death. The nick translation repair method could allow the quantitative determination as well as the localization of damaged nuclei during the initial period of neurodegenerative processes *in vivo*.

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