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Both apoptosis and necrosis occur following intrastriatal administration of excitotoxins

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Abstract To learn about the mechanisms of excitotoxic cell death in vivo, three different excitatory amino acid receptor agonists (kainic acid, quinolinic acid or quisqualic acid) were injected in the left striatum of adult rats. Brains were examined at 24 and 48 h after injection. Morphological and biochemical studies were performed using conventional stains, histochemistry, in situ labelling of nuclear DNA fragmentation, and agarose gel electrophoresis of extracted DNA. Large numbers of cells with cytoplasmic shrinkage and nuclear condensation or granular degeneration of the chromatin, and fewer cells with apoptotic morphology were distributed at random in the injured areas of the three groups of treated animals but not in rats injected with vehicle alone. A ladder pattern, typical of internucleosomal DNA fragmentation, was observed 24 h after treatment. This was replaced by a smear pattern, consistent with random DNA breakdown, at 48 h. These morphological and biochemical results suggest that prevailing necrosis together with apoptosis occur following intrastriatal injection of different excitotoxins.

Key words Apoptosis · Necrosis · Kainic acid · Quisqualic acid · Quinolinic acid

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Introduction

It is generally believed that apoptosis and necrosis are different forms of cell death which can be recognized on the basis of their morphological and biochemical properties [19, 39, 44]. Apoptosis is characterized morphologically by early chromatin condensation, extreme nuclear shrinkage and formation of apoptotic bodies, and biochemically by double-strand cleavage of DNA to produce fragments which are multiples of about 180–200 bp (internucleosomal DNA fragmentation). This process is the result of endonuclease activation and is manifested as a ladder pattern on agarose gel electrophoresis of extracted DNA [2, 8, 14, 19, 39, 31, 42, 45]. In contrast, necrosis is characterized by loss of membrane integrity and cytoplasmic damage, followed by nuclear condensation and digestion of the entire chromatin by proteases and endonucleases. This is accompanied by random nuclear breakdown, which is manifested as a diffuse smear on agarose gel electrophoresis of extracted DNA [19, 39, 44].

The examination of morphological and biochemical features of cell death has been the subject of recent studies geared to disclosing the role of apoptosis and necrosis in different models of neurodegeneration.

Excitatory amino acids are the primary neurotransmitters in approximately 50% of the synapses in the mammalian forebrain. These substances participate in a large variety of physiological processes and may be involved in producing brain damage in several neurological disorders [6]. Excitatory amino acids act on the central nervous system through different receptors that are categorized into two subgroups: ionotropic and metabotropic. Ionotropic receptors act on cation-specific ion channels and are classified into three subtypes: *N*-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazole-propionate (AMPA/quisqualate) and kainate receptors [17, 43]. The metabotropic receptor is coupled to G proteins and increases phosphatidyl inositol hydrolysis as a result of the activation of phospholipase C [17, 26, 34]. Glutamate-induced neuronal cell death in cerebellar cultures is not as-

sociated with internucleosomal DNA fragmentation [10]. However, glutamate exposure in cultured cortical cells is associated with internucleosomal DNA cleavage [20]. These results suggest that different mechanisms of cell death can be observed in different cell types, probably depending on the molecular events activated by glutamate receptors stimulation. Recent studies in vivo have shown that internucleosomal DNA cleavage was found following intra-amygdaloid injection of kainic acid [29]. Similarly, a ladder pattern of DNA fragmentation was observed following intraperitoneal (i.p.) administration of this excitotoxin [12]. These findings suggest that cell death induced by kainic acid is mediated by apoptosis. Yet, classic morphological studies have shown that necrosis is the predominant lesion following administration of different excitotoxic agents [4, 9, 23, 25, 27, 28, 35, 36, 38, 41].

Since the different types of excitatory amino acid receptors have been localized in the striatum [3, 5], this nucleus is a good target for studying the effects of excitotoxicy in vivo.

The present work analyzes the effects of intrastriatal injection of different excitatory amino acid receptor agonists, including kainic acid (a non-NMDA kainate receptor agonist), quisqualic acid (a non-NMDA AMPA/quisqualic receptor agonist and metabotropic receptor agonist) and quinolinic acid (a NMDA receptor agonist) in the adult rat, using conventional stains and immunohistochemistry, in situ labelling of nuclear DNA fragmentation, and agarose gel electrophoresis of extracted DNA, to help increase understanding about the nature of excitotoxic cell death in vivo.

Material and methods

Male Sprague-Dawley rats (250–300 g) were anesthetized with thiobarbital (50 mg/Kg, i.p.) and placed in a David Kopf stereotaxic instrument with the incisor bar 5 mm above the interaural line. A microinjection canula was implanted into the left striatum and 1 µl of vehicle, kainic acid (5 nmol), quisqualic acid (50 nmol), or quinolinic acid (34 nmol) was injected (0.2 µl/min) at two different coordinates as previously described [1]. Excitotoxins were obtained from Sigma. Subsequently, the animals were housed separately with food and water ad libitum in a colony room maintained at constant temperature (20–22 °C) and humidity (40–50%) on a 12:12 h light/dark cycle. Animal welfare was conducted according to the policy on the use of animals in neuroscience research published by the Society of Neuroscience (NIH, publication no. 85–23, 1985).

Animals were killed 24 and 48 h after injection. For morphological studies, rats (total $n = 32$; $n = 4$ for each group at each time point) were anestherized with diethylether and perfused through the heart with saline followed by 4% paraformaldehyde in phosphate-buffered saline (PBS). Brains were then removed from the skull and immersed in a similar fresh solution of fixative for 24 h. Following fixation, the brains were washed in PBS and cut with a vibratome for microtubule-associated-protein-2 (MAP-2) immunohistochemistry, or embedded in paraffin, cut with a sliding microtome and stained with hematoxylin and eosin or processed for in situ labelling of nuclear DNA fragmentation [13]. For biochemical studies, rats (total $n = 24$; $n = 3$ for each group at every time point) were decapitated, the brains were rapidly removed from the skull and the left striatum was immediately frozen in liquid nitrogen and stored at –80° C until required.

Serial 40-µm-thick vibratome sections were processed for MAP-2 immunohistochemistry according to the avidin-biotin-peroxidase (ABC) procedure (Vectastatin, Vector). After blocking endogenous peroxidase with 0.3% hydrogen peroxide and 10% methanol, the sections were treated with 3% normal horse serum for 2 h, and then incubated overnight at 4° C with a well-characterized monoclonal antibody against MAP-2 (Sternberger) used at a dilution of 1:2500. Sections were then incubated in rat-adsorbed biotinylated horse anti-mouse IgG antibody (Vector) diluted 1:100 for 1 h, and, finally, with ABC at a dilution of 1:100 for 1 h. The peroxidase reaction was visualized with 0.05% diaminobenzidine and 0.01% hydrogen peroxide. To rule out false-positive reactions, a few sections were incubated without the primary antibody.

Serial 5-µm-thick paraffin sections were dewaxed and stained with hematoxylin and eosin, or processed for in situ labelling of nuclear DNA fragmentation. For the latter, sections were incubated with 20 µg/ml proteinase K for 15 min at room temperature, washed in distilled water and immersed in terminal deoxynucleotidyl transferase (TdT) buffer (25 mM Trizma base at pH 6.6, 200 mM cacodylic acid and 200 mM potassium chloride) for 15 min at room temperature. The sections were then incubated with 12 µl TdT (Boehringer-Mannheim), 36 µl biotin-16-dUTP (Boehringer-Mannheim) in 1000 µl TdT buffer at 37° C for 60 min, and, subsequently, with a solution composed of 0.3 *M* sodium chloride and 0.03 *M* sodium citrate for 15 min. The sections were rinsed in distilled water, covered with 2% aqueous solution of bovine serum albumin for 10 min, rinsed in distilled water, and immersed in PBS for 5 min. Finally, the sections were incubated with the ABC complex, diluted 1:25 in distilled water, at 37° C for 30 min. The peroxidase reaction was visualized with diaminobenzidine and hydrogen peroxide.

For biochemical studies, frozen left striatal nuclei were homogeneized in DNA extraction buffer (10 m*M* EDTA, 10 m*M* NaCl, 2% SDS) using a hand-operated homogenizer and incubated with RNase A (10 mg/ml) for 30 min at 37° C. The lysate was digested overnight with proteinase K (20 mg/ml) at 55° C. The mixture was then sequentially extracted with phenol, phenol:sevag (v/v) and sevag, and precipitated at –20° C in two volumes of absolute ethanol, 0.3 *M* sodium acetate. DNA was pelleted by centrifugation, vacuum-dried and resuspended in TE buffer. Equal amounts of DNA were run in each lane of a 1.5% agarose gel containing ethidium bromide, and electrophoresed at 100 V for 60 min. Gels included molecular weight markers and a positive control for internucleosomal DNA fragmentation (liver of a newborn rat subjected to hypoxia). To improve the detection of DNA fragments, DNA was denatured and transferred to nylon membranes (Hybond-N, Amersham), according to the technique of Southern, and the filters were hybridized with a ^{32}P-labelled genomic DNA probe.

Results

Loss of MAP-2 immunoreactivity was observed 24 and 48 h after intrastriatal injection of kainic acid, quinolinic acid or quisqualic acid at the site of injection. Lesions were most severe with kainic acid and least with quisqualic acid (Fig. 1).

Dewaxed paraffin sections stained with hematoxylin and eosin showed massive cytoplasmic shrinkage and nuclear condensation which involved contiguous cells, together with spongiosis in the ipsilateral striatum following injection with any of the three excitotoxic agents. No similar changes were observed in animals treated with vehicle alone (Fig. 2). At higher magnification, many dying cells showed granular degeneration of the chromatin (Fig. 3). In addition, other cells were characterized by extremely dark, often fragmented, nuclei decorated with small protrusions (Fig. 3). The latter were similar to apoptotic cells in other systems [19]. Necrotic cells and apoptotic cells were distributed at random in the injured areas in rats injected with

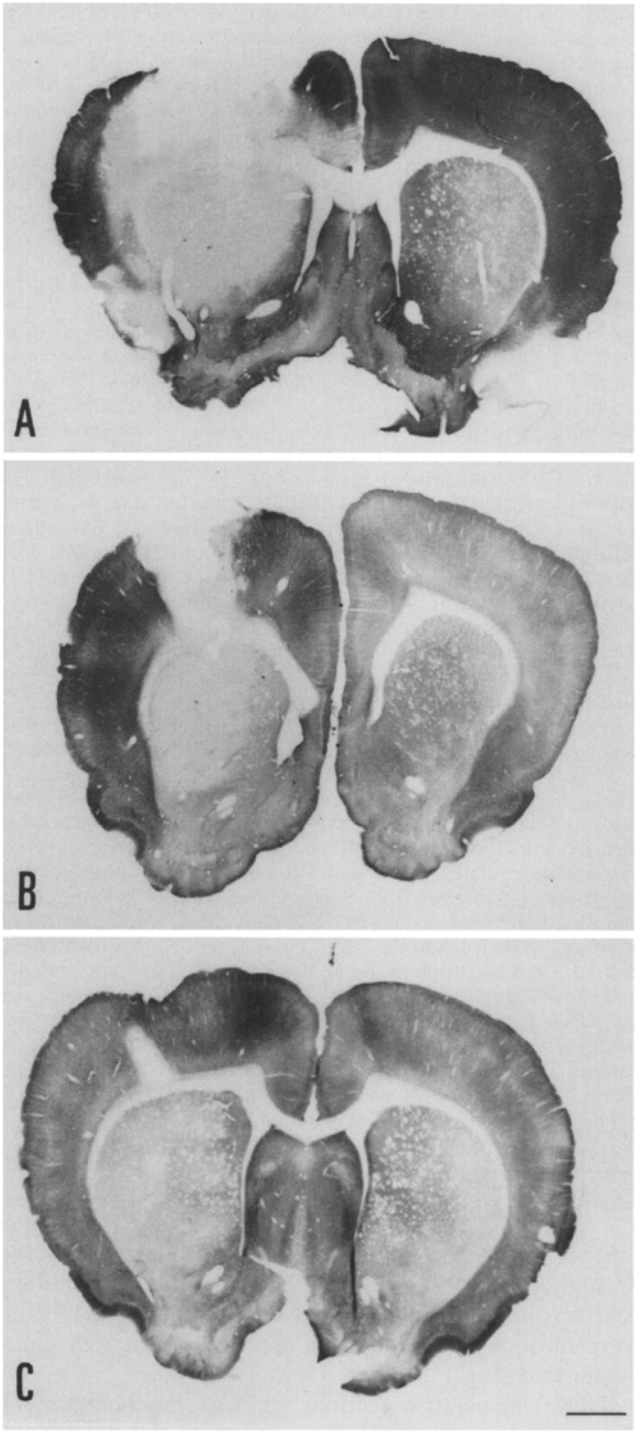

Fig. 1 MAP-2 immunohistochemistry of the rat brain 24 h after injection of kainic acid (**A**), quinolinic acid (**B**) or quisqualic acid (**C**) in the left striatum. Loss of immunoreactivity, although variable depending on the agent, is observed at the site of injection. *Bar* = 1 mm

kainic acid, quinolinic acid or quisqualic acid. Apoptotic cells were less abundant than necrotic cells and represented about 15% of the total number of dying cells. These relative numbers were practically the same at 24 and 48 h after injection in the three groups of treated animals.

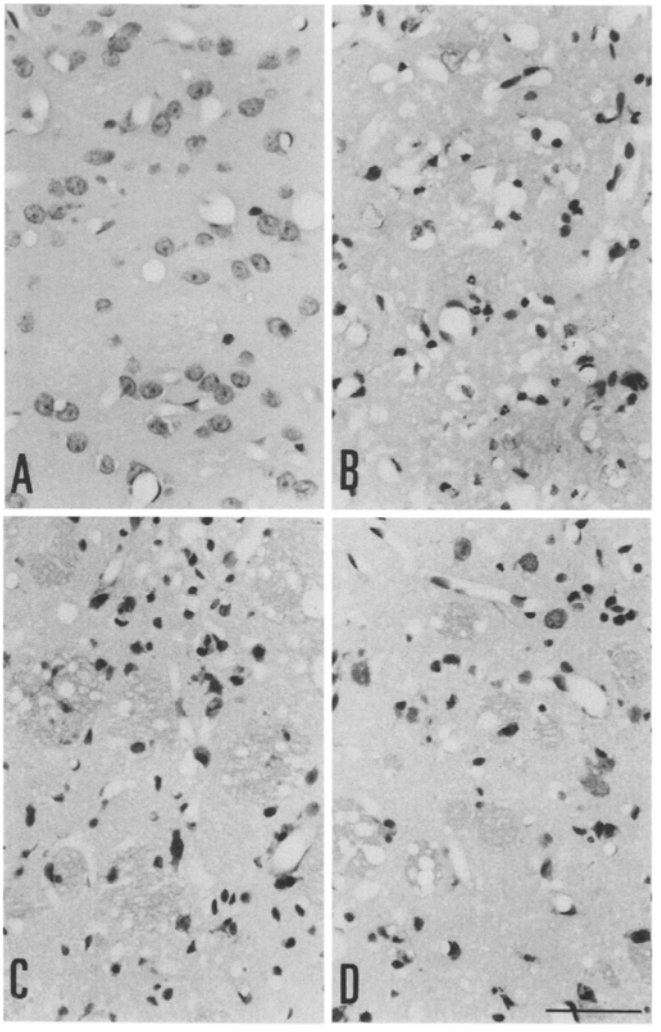

Fig. 2 Left striatum of rats at 48 h after intrastriatal injection of vehicle (**A**), kainic acid (**B**), quinolinic acid (**C**), or quisqualic acid (**D**). Tissue necrosis, characterized by massive involvement of cells with shrunken cytoplasm and condensed nuclei, together with spongiosis of the neuropile, is found in animals treated with excitotoxic agents. Hematoxylin and eosin, *bar* = 50 μm

In situ labelling of nuclear DNA fragmentation demonstrated the presence of cells with either granular degeneration of the chromatin or apoptotic morphology, i.e., cells bearing fragmented DNA (Fig. 4). No cells were stained with this method in the contralateral side of the injection or in animals treated with vehicle alone.

Southern hybridization with ^{32}P-labelled rat genomic DNA following agarose gel electrophoresis of extracted DNA from the left striatum showed a typical ladder pattern of internucleosomal DNA fragmentation at 24 h after injection of kainic acid, quisqualic acid or quinolinic acid. However, this pattern was replaced by a smear pattern, typical of random nuclear breakdown, at 48 h after intrastriatal injection of these excitotoxins (Fig. 5). No DNA fragments were detected in the striatum of rats treated with vehicle alone (data not shown).

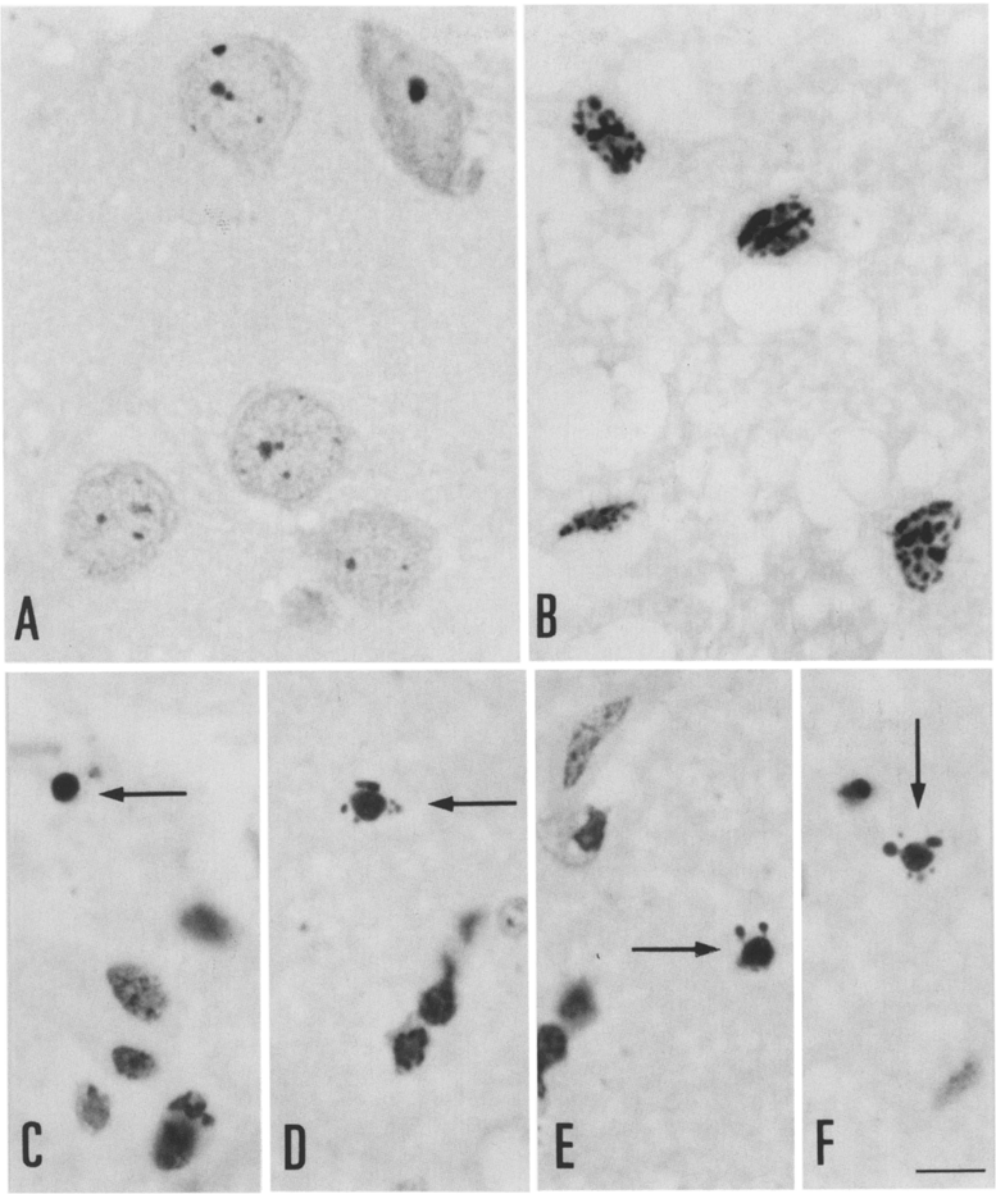

Fig. 3 High magnification of the left striatum in control rats (**A**) and in animals treated with kainic acid (**B–D**), quinolinic acid (**E**), or quisqualic acid (**F**) 48 h after intrastriatal injection, showing granular degeneration of the chromatin (**B**) or apoptotic morphology (*arrows*) (**C–F**) of many dying cells within the areas of striatal injury in rats treated with excitotoxins. Hematoxylin and eosin, *bar* = 10 μm

Discussion

Results using the present experimental design show that, albeit with differences in the intensity depending on the agent, intrastriatal injection of kainic acid, quisqualic acid or quinolinic acid produces cell death with morphological features of necrosis or apoptosis. Necrosis affects massive and contiguous cells, which have shrunken cytoplasm and condensed nuclei reminiscent of established ischemic cell changes [11], and cells with granular degeneration of the chromatin. Apoptosis [19] occurs in dying cells with extremely dark, often fragmented, nuclei which are distributed at random in damaged areas. Apoptosis can be observed using in situ labelling of nuclear DNA fragmentation, which enables the identification of individual cells bearing fragmented DNA. However, the present results show that this method recognizes not only dying cells with the morphological features of apoptosis, but also other forms of cells death with granular degeneration of the chromatin. Programmed cell death in certain in vitro models may be associated with granular degeneration of the chromatin [15, 37]. Similarly, DNA fragmentation, as revealed by in situ labelling, has been observed in cells with granular chromatin condensation in patients with Alzheimer's disease [21].

Agarose gel electrophoresis of extracted DNA shows that a ladder pattern occurs 24 h after kainic acid, quisqualic acid or quinolinic acid administration, and that this pattern is replaced by a smear pattern at 48 h. These biochemical results indicate that both internucleosomal DNA fragmentation and random nuclear breakdown occur following intrastriatal injection of different excitotoxic agents, thus further supporting the concept that intrastriatal injec-

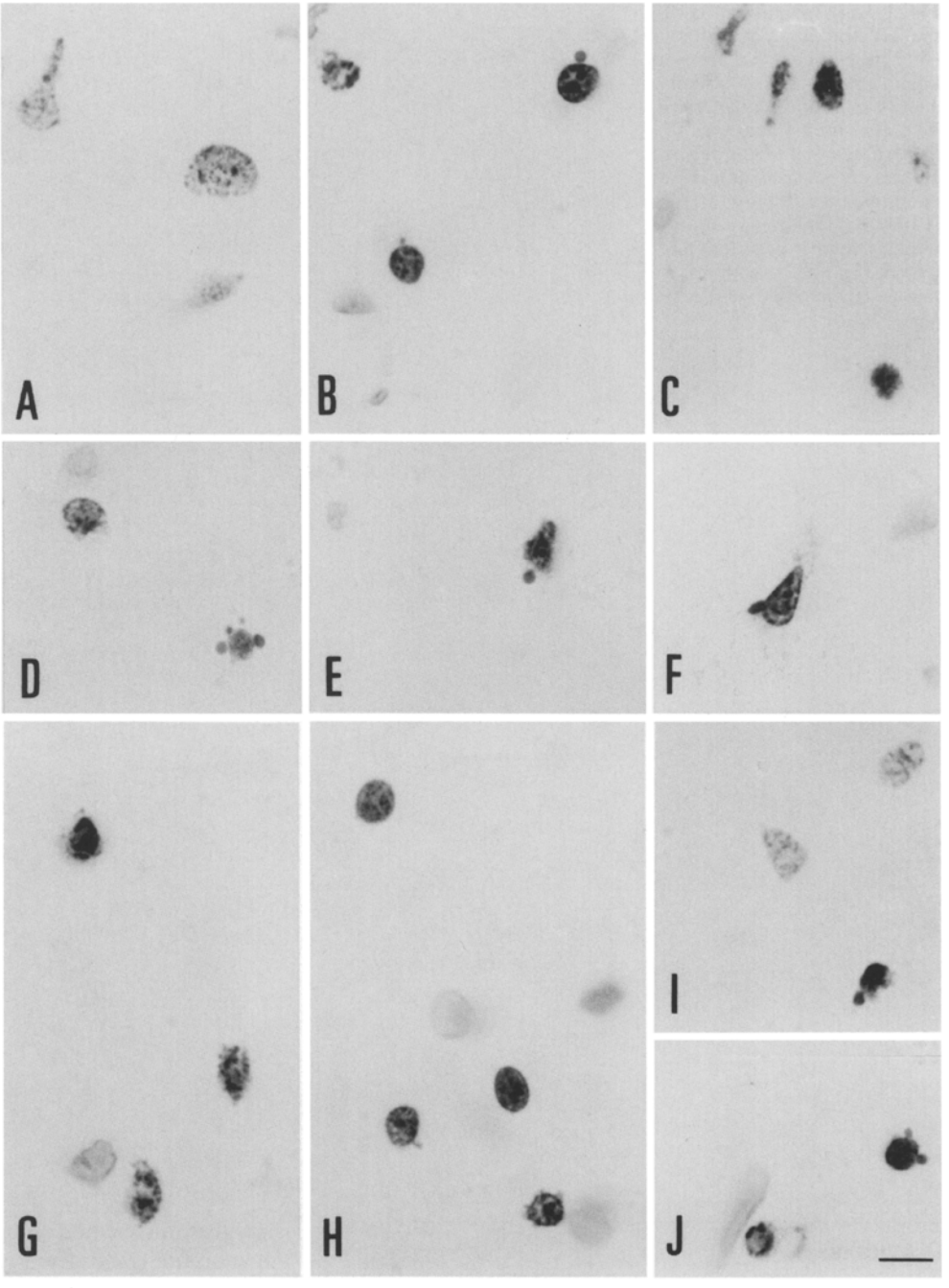

Fig. 4 In situ labelling of nuclear DNA fragmentation in the striatum of rats following intrastriatal injection of kainic acid (**A, D, G**), quinolinic acid (**B, E, H**), or quisqualic acid (**C, F, I, J**) 24 h (**A–F**) and 48 h (**G–J**) postinjection. Cells with either granular degeneration of the chromatin or apoptotic morphology are stained with this method. No counterstaining, *bar* = 10 μm

tion of kainic, quisqualic or quinolinic acids may produce both apoptosis and necrosis. It is likely, however, that at 48 h after excitotoxic treatment, internucleosomal fragments are concealed by massive random DNA breakdown, as cells with apoptotic morphology are still seen at this time.

Previous studies have not shown apoptosis at 8 and 16 h following intracerebral injection of kainic acid or quinolinic acid [18]. Nevertheless, our findings partially agree with some recent studies showing that internucleosomal DNA fragmentation occurs at later times following administration of kainic acid in vivo [12, 29]. Recently, it has been shown that reserpine treatment induces an increase an increase in endogenous glutamate activity which produces apoptosis in striatal neurons. This effect is only partially blocked by the NMDA antagonist ketamine, suggesting the involvement of different excitatory amino acid receptor subtypes [24].

The simultaneous occurrence of apoptosis and necrosis in the central nervous system is not an exceptional event in toxicology since both apoptosis and necrosis are produced in the liver following the administration of dichloroethylene [33], dimethylnitrosamine [30], heliotrine [16] and thioacetamide [22]. Furthermore, endonuclease-dependent nuclear DNA fragmentation precedes random nuclear breakdown following treatment with the hepatotox-

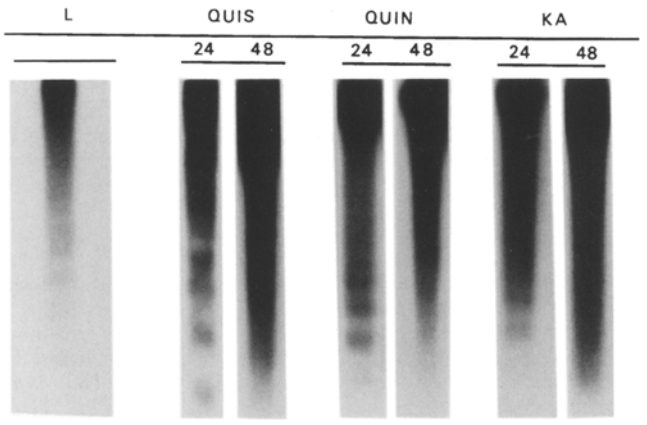

Fig. 5 Southern hybridization with ^{32}P-labelled genomic DNA following agarose gel electrophoresis of extracted DNA of the left striatum. A typical ladder pattern of internucleosomal DNA fragmentation is seen 24 h after intrastriatal injection of kainic acid (*KA*), quisqualic acid (*QUIS*) or quinolinic acid (*QUIN*). This early ladder pattern is, however, replaced 48 h after injection by a smear pattern which characterizes random DNA breakdown. *L* Liver of a newborn rat subjected to hypoxia

ins acetaminophen and dimethylnitrosamine [31, 32, 40]. Finally, the DNA of murine mastocytoma cells shows internucleosomal DNA fragmentation during diethylaminoethyl (DEAE) dextran-induced cell death by necrosis [7]. Therefore, the present results support the concept that toxins may produce cell death simultaneously through different mechanisms, and that apoptosis and necrosis occur following an excitotoxic insult in vivo.

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