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ANATOMICAL STUDIES OF DNA FRAGMENTATION IN RAT BRAIN AFTER SYSTEMIC KAINATE ADMINISTRATION

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Abstract––Rats treated systemically with kainate develop stereotyped epileptic seizures involving mainly limbic structures that may last for hours. This model of limbic status epilepticus has been widely studied using classical neuropathological techniques. We used *in situ* nick translation histochemistry to examine patterns of DNA fragmentation in this model. We found a stereotyped and reproducible pattern of neuronal populations that demonstrate evidence of DNA fragmentation from 24 h to one week after kainate treatment. Neither blockade of new protein synthesis nor blockade of the *N*-methyl-D-aspartate-type glutamate receptors significantly altered this response. Moreover, we saw no evidence of the regular internucleosomal cleavage of DNA that produces a characteristic laddered appearance of 180–200 bp DNA fragments after gel electrophoresis in samples obtained from microdissected affected regions. These studies suggest that DNA fragmentation after systemic kainate-induced seizures is not the result of programmed cell death. This assay may be useful for quantitative testing of both neuroprotective agents and mechanistic hypotheses. Copyright © 1996 IBRO. Published by Elsevier Science Ltd.

Key words: seizures, kainate, status epilepticus, DNA fragmentation, *in situ* nick translation, apoptosis.

Single or repeated epileptic seizures may result in long-lasting changes in brain function and structure. Classical neuropathological studies have documented highly stereotyped patterns of gliosis, neuronal loss and, more recently, synaptic reorganization in both animal models of epilepsy[7,8,19,51,52,53,56] and in human *post mortem* and surgical pathological material.[1,54] These studies have relied heavily on staining techniques which are technically demanding, time consuming and capricious.[13,15,56] Mechanistic studies of seizure-induced neuronal damage would be facilitated by the development of an easily quantified measure of cell damage that is technically simple, reproducible, closely correlated with seizure severity, and can be obtained with minimal latency after treatment.

DNA polymerases catalyse the polymerization of nucleotides into duplex DNA in a template-dependent manner. For over a decade these enzymes have been used to assay for the presence of DNA strand breaks induced by exogenous insults.[26,27,28] Several recent studies have utilized DNA polymerases or terminal deoxytransferase to incorporate labeled material into nuclear DNA at free 3′-OH ends *in situ*.[23,35,42,44,62,64] These techniques, sometimes known as *in situ* nick translation histochemistry or TUNEL (terminal transferase-mediated (TdT) dUTP–biotin nick end labeling),[18] appear to be both sensitive and specific for the detection of DNA strand disruption or fragmentation.

For a decade, kainate-induced seizures have been studied as a model of epilepsy and of selective neuronal vulnerability. Administration of kainate into specific brain structures has been used to study both local and distant effects of focal injury, whereas systemic administration of the drug has furnished a robust model of limbic status epilepticus that replicates many of the features of the mesial temporal sclerosis syndrome. We have performed *in situ* nick translation in the presence of biotinylated deoxynucleotides to examine patterns of DNA fragmentation after seizures induced by systemically administered kainate in rats. Because the occurrence of DNA fragmentation has been closely associated with apoptosis,[20,24,62,64,67] a form of active programmed cell death,[45,50,67] we have conducted additional experiments to determine whether the neuronal damage induced by systemic kainate exhibits other characteristics of programmed cell death.

EXPERIMENTAL PROCEDURES

Animal treatments

Male Sprague–Dawley rats (250–300 g; Charles River Labs) were used for all studies. Animal protocols were approved by the institutional committee on animal care and conform to the guidelines of the National Institute of

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Abbreviations: EDTA, ethylenediaminetetra-acetate; MK-801, dizocilpine maleate; NMDA, *N*-methyl-D-aspartate; PBS, phosphate-buffered saline; WDS, wet dog shakes.

Health for the care and use of laboratory animals. Efforts were made throughout the studies to minimize animal suffering and to use the minimum number of animals required. Animals were maintained in a 12-h light–dark cycle, with *ad libitum* access to food and water. For most experiments seizures were induced by intraperitoneal injection of kainate dissolved in phosphate-buffered saline (PBS) and the pH adjusted to 7.2. In preliminary experiments we found that a dosage of 15 mg/kg induced seizures in approximately 75% of animals, with an acute mortality of approximately 10%. Higher doses were too often toxic, while lower doses failed to induce seizures in a sufficient number of animals. Animals were killed by decapitation at appropriate time-points, and brains were rapidly removed and either frozen in an isopentane–dry ice slurry for histochemical analysis or homogenized in the appropriate buffer for DNA or protein analysis.

Behavioral observation and grading

Animals were directly observed following kainate injection for 6 h continuously, and every 6–12 h thereafter. Animals were assigned a seizure severity grade based on the maximal response achieved. In every case, effects of kainate were maximal for each individual within the first 4 h. No effort was made to assess the precise time spent at each grade, but in general progression to maximal severity was rapid, occurring within 30 min of the onset of behavioral alteration. Severity of limbic motor seizure was rated on a scale from 0 to IV as follows: 0, no response; I, wet dog shakes (WDS) and staring; II, WDS, staring, drooling, rearing and pawing; III, WDS, staring, drooling, rearing, pawing and jumping; IV, WDS, staring, drooling, rearing, pawing, jumping and falling, and status epilepticus.

Electroconvulsive treatment

Electrically induced convulsions were produced by standard techniques.[57] Following attachment of saline-soaked earclips, electroshock consisting of a 1-s, 100-Hz, 85-mA stimulus of 0.5-ms square wave pulses was delivered using a UGO Basile ECT unit, Model 7801. At these settings, all animals had generalized tonic–clonic convulsions with hindlimb extension. For chronic electroshock, animals were treated once each day for 10 days. At appropriate time-points following electroshock, animals were killed by decapitation and the brains processed for *in situ* nick translation.

Drug treatments

In some experiments, animals were pretreated 2 h before kainate injection with the non-competitive *N*-methyl-D-aspartate (NMDA)-type glutamate receptor antagonist dizocilpine maleate [(+)-MK-801; 1 mg/kg, i.p.; Research Biochemicals International, Natick, MA, U.S.A.]. For cycloheximide experiments, animals were treated with cycloheximide (Sigma, St Louis, MO, U.S.A.) 4 h before kainate injection (2 mg/kg, s.c.).

In situ *nick translation*

We adapted the protocol developed by Wijsman *et al.*[62] Following treatment, animals were decapitated at appropriate time-points. Brains were harvested and stored at -70°C prior to sectioning. For analysis, 11-μm coronal cryostat sections (Lipshaw) were cut at five predetermined levels and thaw mounted on gelatin-coated slides (chemicals and slides from Fisher). Slides were stored at -70°C until needed.

Slides were warmed to room temperature. They were then immersed in 4% paraformaldehyde for 10 min, transferred into 2 × saline sodium citrate (1 × saline sodium citrate contains 150 mM NaCl, 15 mM sodium citrate, pH 7.0) at 80°C and incubated for 20 min. Sections were treated for 10 min with pronase (1 μg/ml; Boehringer Mannheim) and the digestion process was stopped by immersing the slides in 2% glycine in PBS, pH 7.4, for 30 s. Slides were quickly rinsed in double-distilled H_20 and then incubated in a buffer containing 50 mM Tris–HCl, pH 7.5, 5 mM $MgCl_2$, 10 mM β-mercaptoethanol and 0.005% bovine serum albumin for 5 min. Positive control slides were incubated in 10 mM Tris–HCl, pH 7.4, 10 mM NaCl, 5 mM $MgCl_2$, 0.1 mM $CaCl_2$, 25 mM KCl, with 0.4 units/ml DNase I (Boehringer Mannheim) at 37°C for 15 min. Slides were allowed to air dry and then incubated in a humidified chamber in the presence of 50 μg/ml DNA polymerase I (Promega), 10 μM each of dCTP, dATP, dGTP (Promega) and biotin-21–dUTP (Clontech) at room temperature for 60 min. For negative controls, DNA polymerase 1 was omitted. Sections were rinsed for 5 min in PBS, transferred into PBS with 0.1% H_2O_2 for 15 min, and then rinsed two more times for 5 min in PBS. To detect the incorporation of biotinylated dUTP, slides were incubated in a humidified chamber in 1:100 horseradish peroxidase-conjugated avidin in PBS containing 1% bovine serum albumin and 0.5% Tween–20. Following two rinses in PBS, sections were developed in PBS containing 0.5 mg/ml diaminobenzidine, 10 mM imidazole and 0.03% H_2O_2. After a final rinse in double-distilled H_2O, sections were counterstained with Thionin, dehydrated and coverslipped.

DNA isolation

Seventy-two hours after kainate administration, animals were killed by decapitation and brains were rapidly dissected on ice. One hemisphere was taken for *in situ* DNA fragmentation analysis, while the other was microdissected on a cold plate for regional DNA isolation. Blocks of tissue of 2 mm^3 were dissected from the hippocampus, centromedial thalamus, amygdala and frontal suprarhinal cortex. Samples were homogenized using a hand-held dounce in 500 μl of buffer A (10 mM Tris–HCl, pH 8.0, 1 mM EDTA, pH 7.4). Two volumes of buffer B (10 mM Tris–HCl, pH 7.4, 10 mM EDTA, 0.5% (w/v) sodium dodecyl sulfate, 10 μg/ml RNase A) were added and the mixture was incubated at 37°C for 15 min. Proteinase K (20 μg/ml) was added and samples were incubated overnight at 50°C. Following two phenol extractions and a chloroform–isoamyl alcohol (24:1, v/v) extraction, DNA was precipitated with 0.1 volumes of 3 M sodium acetate, pH 5.2, and 2 volumes of absolute ethanol. Pellets were resuspended in Tris–EDTA and optical densities were measured to estimate yield. On average, 10–15 μg of DNA were isolated from each sample.

DNA analysis

For analysis, either 10 or 20 μg of DNA was loaded on to a 1.2% agarose gel containing ethidium bromide (10 μg/ml) and electrophoresed in Tris–acetate–EDTA at 125 V for 3–4 h. DNA was visualized under ultraviolet light and photographed.

Data analysis

The primary tool for data analysis was microscopic examination of stained tissue sections. Sections were examined for the presence or absence of horseradish peroxidase reaction product, and the degree of staining was graded on a four-plus scale. Only dense nuclear staining was interpreted as positive. The anatomical distribution of staining was recorded using the atlas of Paxinos and Watson[38] as a guide. In some experiments, counts of stained cells were made on comparable tissue sections in specific regions of interest. In these cases, a grid reticule (each square = 10,000 μm^2) was used to count 10 separate squares at a final magnification of ×100. Cell counts were averaged and standardized to yield an estimate of stained cells/0.1 mm^2. This figure was used for statistical analysis. To examine treatment effects, cell counts from specific structures were

Table 1. Behavioral responses to systemic kainate

Seizure severity grade	Naive n	Naive %	Pretreatment MK-801 (1 mg/kg) n	Pretreatment MK-801 (1 mg/kg) %	Cycloheximide (2 mg/kg) n	Cycloheximide (2 mg/kg) %
0	24	24	9	47	11	33
I	17	17	4	21	16	48
II	22	22	2	11	2	6
III	29	29	3	16	3	9
IV	9	9	1	5	1	3
Total	101	100	19	100	33	100

Seizure severity grade represents the maximal response observed during the 6 h following kainate injection (15 mg/kg, i.p.). Grade 0, no response; Grade I, WDS only; Grade II, WDS and rearing; Grade III, WDS, rearing and jumping; Grade IV, status epilepticus.

Note: 11 cycloheximide-treated animals died without having seizures.

obtained from groups of animals with similar seizure severity scores. These were compared using a *t*-test with $P = 0.05$ considered significant.

RESULTS

Behavioral effects

Systemic kainate (15 mg/kg, i.p.) produced clinically apparent seizures in 120 of 153 animals. As shown in Table 1, the majority of animals had Grade II or III seizures; however, there was considerable animal to animal variability in the response to kainate.

DNA fragmentation after kainate seizures

In initial experiments, we examined the distribution of DNA fragmentation after seizures induced by systemic kainate. Animals not exhibiting clinical seizure activity (Grade 0) and animals with only WDS (Grade I) had no evidence of DNA fragmentation at any time-point examined. In animals that exhibited grade II or greater seizures following injection of kainate, evidence of DNA fragmentation was seen in lateral septal nuclei, dorsolateral thalamus, centromedial thalamus, amygdala, endorhinal cortex and the CA1 region of the hippocampus (Fig. 1). In animals that reached Stage 3 or greater seizures, there was occasional staining of the CA4 and CA3 regions. Modest staining in the cerebral cortex, especially the cingulate cortex, mainly in layer II, was also observed. In control experiments, when DNA polymerase was omitted from the incubation with biotinylated dUTP, there was no staining. By contrast, when slides were pretreated with DNase I to generate widespread DNA damage, there was nuclear staining over virtually every cell.

We systematically examined 43 discrete brain structures and semi-quantitatively recorded severity of staining (Table 2). Staining is typically localized directly over cell nuclei. Detailed microscopic analysis shows that stained structures are highly circumscribed and specific. Although the pattern of affected areas varies slightly from one animal to another, a

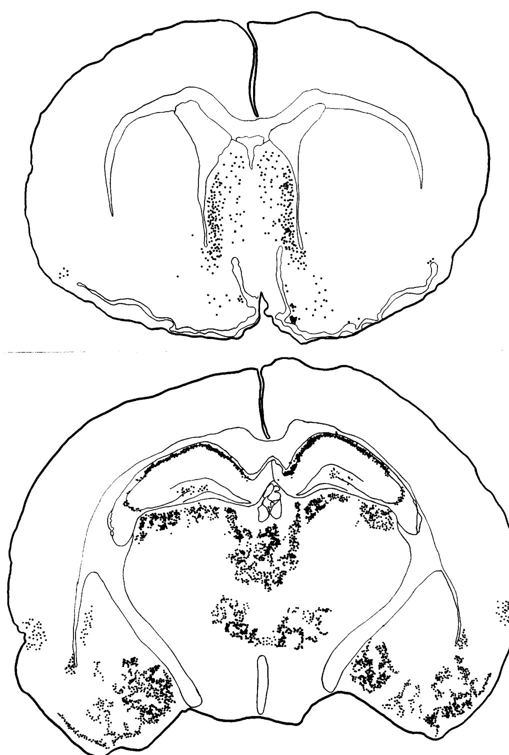

Fig. 2. Distribution of DNA fragmentation following systemic kainate-induced seizures. Camera lucida drawings made from representative sections from an animal killed 72 h after a Grade III seizure are shown at the level of the lateral septum (A) and the dorsal hippocampus (B).

relatively consistent distribution of damaged regions following i.p. kainate can be described (Fig. 2). This pattern is characterized by the parallel degeneration of groups of structures with extensive axonal connections. Cellular degeneration consistently occurs within CA1, mediodorsal and lateral dorsal thalamus, nucleus reuniens, piriform and endorhinal cortex, bed nucleus of the stria terminalis, dorsal endopiriform nucleus, and amygdaloid complex. Within the amygdala, the medial and central nuclei appear to be particularly vulnerable and are always affected, whereas the incidence and severity of the

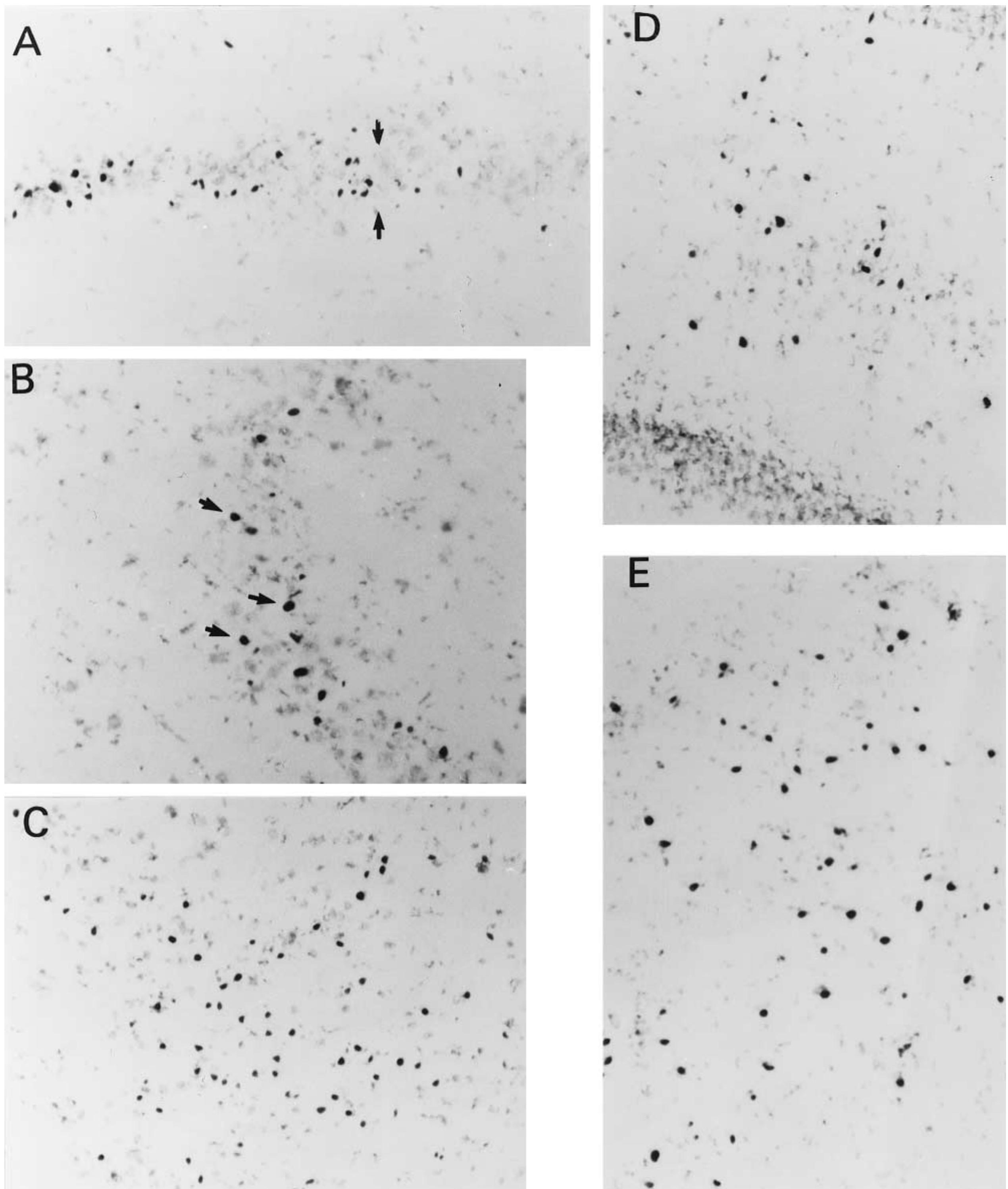

Fig. 1. DNA fragmentation in discrete structures following systemic kainate-induced seizures. Incorporation of biotinylated dUTP is visualized by avidin–biotin–peroxidase histochemistry as discrete nuclear staining of selected neurons. (A) The CA1–CA2 junction is indicated by arrowheads. Note sparing of CA2 (right). (B) Isolated neurons in the genu of stratum pyramidale in CA3. (C) Dorsal lateral geniculate. (D) Isolated interneurons in the dentate hilus. (E) Neurons in the nucleus reuniens of the thalamus. Original magnification ×125.

Table 2. Anatomical distribution of kainate-induced fragmentation

	Seizure severity grade													
	Grade I		Grade II						Grade III					
	n = 7 24 h	n = 3 48 h	n = 4 5 h	n = 3 12 h	n = 4 24 h	n = 4 48 h	n = 4 72 h	n = 2 1 week	n = 3 5 h	n = 2 12 h	n = 3 24 h	n = 5 48 h	n = 5 72 h	n = 5 1 week
Hippocampus														
CA1	–	–	–	–	–	++	+++	++	–	+	+++	++	+++	+++
CA2	–	–	–	–	–	–	–	–	–	–	–	–	–	–
CA3	–	–	–	–	–	–	–	–	–	–	+	–	+	+
CA4	–	–	–	–	–	–	–	–	–	–	+	+	+	–
Thalamus														
Intermediodorsal	–	–	–	–	–	–	–	–	–	–	+	++	++	++
Mediodorsal central	–	–	–	+	++	++	++	+	–	–	–	–	+	++
Mediodorsal medial	–	–	–	–	–	–	–	–	–	+	+	++	++	–
Lateral posterior mediorostral	–	–	–	–	–	–	–	–	–	–	+	+	+	+
Lateral dorsal ventrolateral	–	–	–	–	–	–	–	–	–	+	+	+	++	–
Ventromedial	–	–		–	–	–	–	–	–	–	–	–	–	–
Centromedial	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Centrolateral	–	–	–		–	–	–	–	–	–	–	–	–	–
Paraventricular	–	–	–	–	–		–		–	–	–	–	–	–
Reuniens	–	–	–	+	++	++	++	+	–	+	++	++	++	++
Lat.sep.dor.	–	*	*	+	+	*	*	*	–	*	+	++	++	+
Lat.sep.dor.vent.	–	*	*	*	+	*	*	*	–	*	+	+	++	+
Lat.sep.dor.int.	–	*	*	*	+	*	*	*	–	*	+	+	++	+
Accumbens	–	*	*	*	–	*	*	*	–	*	–	–	++	+
Dor.lat.gen.	–	*	*	*	–	*	*	*	–	*	–	–	+	–
Amygdala														
Med.dor.	–	–	–	+	++	++	++	++	–	++	++	++	++	+
Central	–	–	–	+	++	++	++	+	–	++	++	++	++	++
Basolat.post.	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Basomedial	–	–	–	–	–	+	+	–	–	–	–	–	–	–
Lat.ven.med.	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Lat.dor.lat.	–	–	–	–	–	+	+	–	–	–	–	–	–	–
Post.med.cor.a.	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Amygdalahippoc.	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Bed nuc.st.	–	–	–	+	++	++	++	+	–	++	++	++	++	+
Dor.endopir.	–	–	–	+	++	++	++	+	–	++	+++	+++	++	+
Piriform	–	–	–	+	++	+	++	+	–	++	++	+++	++	+
Caudate/put.	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Claustrum	–	–	–	–	++	–	–	–	–	–	+	++	++	–
Dor.med.hip.	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Cortex														
Cortex/Cing	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Frontal	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Parietal	–	–	–	–		–	–	–	–	–	–	–	–	–
Temporal	–	*	*	*	*	*	*	*	*	*	*	*	*	*
Perirhinal	–	–	–	–	–	++	++	++	–	–	–	+	++	–
Endorhinal	–	–	–	–	–	+	+	–	–	–	–	–	+	–
Hindlimb.	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Forelimb.	–	–	–	–	–	–	–	–	–	–	–	–	–	–

Each cell represents the median score assigned by a blinded observer to sections examined from n animals. + = 0–25% of neurons positive; ++ = 25–50% of neurons positive; +++ = 50–75% of neurons positive; ++++ = 75–100% of neurons positive; – = no neurons positive; * = nucleus not available for analysis in more than one animal.

lesions are less in the lateral and basal nuclei. DNA fragmentation is also prominent in the hippocampus at all time-points examined. CA1 is almost universally affected, whereas only small populations of neurons in CA3 and CA4 show DNA fragmentation. In particular, the genu of the pyramidal cell layer, sometimes known as CA3a,[30,34] seems particularly vulnerable among CA3 neurons. CA2 pyramidal cells are entirely spared. Rare dentate granule cells show DNA fragmentation. The lateral septal nucleus, which receives axonal projections from CA1 and CA3 as well as the subiculum, is damaged by systemic kainate administration. Within the thalamus, the mediodorsal medial and intermediodorsal nuclei are consistently affected. Similarly, cellular degeneration usually occurs in the lateral dorsal and lateral posterior nuclei. Nucleus reuniens is particularly sensitive to kainate-induced damage and is consistently affected.

To examine the time-course of this phenomenon, we killed animals at time-points from 5 h to one week after administration of systemic kainate. Reliable staining is seen earliest at 12 h after kainate treatment, and appears maximal between 48 and 72 h after injection. At early time-points and lower grades of seizure severity (Grade II), there is a tendency for staining to be localized mainly to dorsolateral thalamus, while at later time-points involvement of the medial thalamus and hippocampus is impressive. CA3 staining, when it occurs, is a late phenomenon seen mainly in 72-h and one-week animals.

We recorded behavioral effects of kainate and correlated severity of seizures with extent of staining. As shown in Table 2, animals with higher seizure severity scores invariably had more profound evidence of DNA fragmentation, affecting more structures and more cells within each structure. In animals with Grade III seizures, staining was seen in all affected structures within 24 h. Animals with Grade IV seizures invariably died within 12 h, prior to the appearance of DNA fragmentation.

To test the hypothesis that DNA fragmentation is the direct result of seizures *per se*, we examined brains of animals obtained at 1, 4, 24, 48, 72 h and one week after either a single maximal electroconvulsive seizure ($n = 15$) or after chronic daily maximal electroconvulsive seizures administered for 10 days ($n = 18$). We found no evidence of DNA fragmentation at any time-point examined.

Systemic kainate may act directly on vulnerable neurons to cause DNA fragmentation, or it may act indirectly, perhaps stimulating polysynaptic pathways that impinge on vulnerable populations. The highest concentration of NMDA-type glutamate receptors in the brain is found in area CA1 of the hippocampus, a region that is particularly vulnerable to DNA fragmentation after systemic kainate. We therefore tested the effect of the non-competitive NMDA-type glutamate receptor antagonist MK-801 on kainate-induced DNA fragmentation. Nineteen animals were pretreated with MK-801 (1 mg/kg, i.p.) 2 h prior to kainate injection. Following kainate, a reduced percentage of animals achieved Stage 2 or 3 seizures, consistent with the known anticonvulsant properties of that agent[11] (see Table 1); however, in comparison with animals treated with kainate alone, animals achieving the same seizure severity grade showed no significant difference in the severity or distribution of DNA fragmentation.

Previous studies have suggested that DNA fragmentation is a hallmark of apoptosis.[20,41,64,67] To examine further whether kainate-induced DNA damage is mediated by apoptosis or some other form of programmed cell death, we conducted two types of experiments. First, we pretreated animals with the protein synthesis inhibitor cycloheximide at doses used to block translation *in vivo*[22,48] and then subjected them to kainate-induced seizures. These animals tended to have less severe seizures and a higher mortality rate (Table 1). To evaluate the protective effect of cycloheximide, we counted the labeled cells in various structures and compared them with counts made in sections from animals treated with kainate alone. We examined only animals that achieved Grade II or III seizures, and as there was no significant difference between Grade II and III animals in their cell counts, the groups were combined for statistical analysis. Comparisons were made based on average labeled cell density in specific structures. No statistically significant reduction in average labeled cell density was seen in animals treated with cycloheximide and kainate compared to those treated with kainate alone (Table 3).

In another set of experiments, we examined DNA isolated from specific regions of brains after kainate-induced seizures, looking for the pattern of ''laddering'' of 180–200 bp fragments seen after intranucleosomal endonuclease-mediated DNA cleavage and associated with apoptosis and programmed cell death.[14,23,25] As shown in Fig. 3, laddering was not visualized in DNA isolated from regions that were heavily stained ($n = 3$ animals), including the hippocampus, thalamus and amygdala/entorhinal cortex, nor from regions that are typically unaffected by systemic kainate treatment (lateral frontal cortex). For these studies we examined the contralateral hemisphere of each animal utilized for DNA gel analysis to confirm that DNA fragmentation was in fact present in those specific animals (Fig. 3B).

DISCUSSION

The main result of our study is that systemic administration of kainate results in apparent DNA fragmentation in a precise and predictable anatomical distribution that is correlated with seizure severity. DNA fragmentation is a delayed effect of kainate. By inspection, it appears that most, if not all, affected cells are neurons; however, we cannot rule out the possibility that non-neuronal cells may also

Table 3. Effect of cycloheximide pretreatment on DNA fragmentation after kainate-induced seizures (data indicate stained cells/0.1 mm^2)

	Treatment				
	Cycloheximide + kainate		Kainate		
	Mean ($n = 4$)	S.D.	Mean ($n = 7$)	S.D.	P value
Hippocampus					
CA1	69	61	80	59	0.41
CA3	3	1	2	1	0.15
Thalamus					
Intermediodorsal	33	8	36	15	0.40
Mediodorsal medial	48	19	53	29	0.39
Lateral dorsal ventrolateral	45	29	33	8	0.31
Reuniens	127	27	153	61	0.24
Amygdala					
Mediodorsal	77	26	99	4	0.18
Central	122	76	143	95	0.42
Bed nucleus of stria terminalis	172	74	162	71	0.44
Dorsal endorpiriform nucleus	98	41	103	32	0.43
Piriform	97	10	80	30	0.26

Neurons demonstrating nuclear staining for DNA fragmentation were counted using a grid reticule at a final magnification of ×125. Groups were compared using an unpaired t-test.

undergo DNA fragmentation. Many of the structures previously demonstrated to suffer neuronal loss after systemic kainate[3,49] are affected by this process, but DNA fragmentation does not appear to occur in all affected neuronal populations. Specifically, there appears to be less DNA fragmentation in area CA3 than in area CA1 of the stratum pyramidale, whereas CA3 repeatedly shows more argyrophyllia and neuronal loss than CA1 in classical studies of kainate-induced neuropathology, regardless of the route of administration.[3] This disparity suggests that there is heterogeneity in the mechanisms of neuronal damage after kainate administration.We found a close correlation between the severity of kainate-induced seizures and the magnitude of DNA fragmentation. Agents that attenuate kainate-induced seizure severity reduce the severity of DNA fragmentation, indicating that DNA fragmentation is dependent on the epileptic response to kainate, and not simply a direct effect of kainate independent of its convulsant properties. It is possible that the duration of epileptic activity rather than its severity is critical; however, we were unable to assess these seizure characteristics independently. Our observation that ECT, even if repeated daily, failed to produce DNA fragmentation controls for the effect of brief massive convulsive activity, but does not allow us to distinguish whether the duration or the method of induction of seizure activity or both are critical to induce DNA fragmentation. Additional studies with other seizure models, such as the systemic pilocarpine model,[37] may clarify this issue.

Previous studies have shown convincingly that DNA polymerase- mediated incorporation of labeled nucleotide is template dependent and occurs only at unprotected 3′-OH ends.[62,64] Our negative controls confirm that incorporation is dependent on the presence of DNA polymerase, eliminating the possibility that nuclear staining is artifactual. It is therefore clear that our assay is detecting DNA fragmentation. Previous studies have suggested that DNA fragmentation is associated with apoptosis and other forms of programmed cell death, but DNA fragmentation is not seen exclusively during programmed cell death.[6,12] It is possible that in certain situations DNA fragmentation may be a reversible phenomenon with maintained cell viability after a reparative process;[47] however, comparison of our results with those of classical neuropathological studies suggests that most neurons manifesting DNA fragmentation in this system are destined to die. We did not attempt to quantify or even estimate cell loss after systemic kainate, in part because cell loss occurs on an extended time scale in comparison to DNA fragmentation. Definitive information on the fate of neurons that manifest DNA fragmentation will require careful morphometric and stereological studies comparing groups of animals killed at different time-points.

While other groups have analysed DNA fragmentation after kainate-induced seizures, those studies have either not offered cellular resolution[14,25] or they have utilized local injection of kainate.[42] This study is the first, to our knowledge, to examine the anatomical distribution of DNA fragmentation throughout the forebrain following systemic kainate treatment with resolution at the single cell level.

The anatomical distribution of kainate-induced DNA fragmentation is striking. While numerous, widely dispersed structures are affected, the pattern is highly stereotyped and specific. In some nuclei it is possible to see selected cell populations affected dramatically, while directly adjacent populations are entirely spared. We have compared the affected structures with reports of both the patterns of expression of various kainate, NMDA and metabotropic type

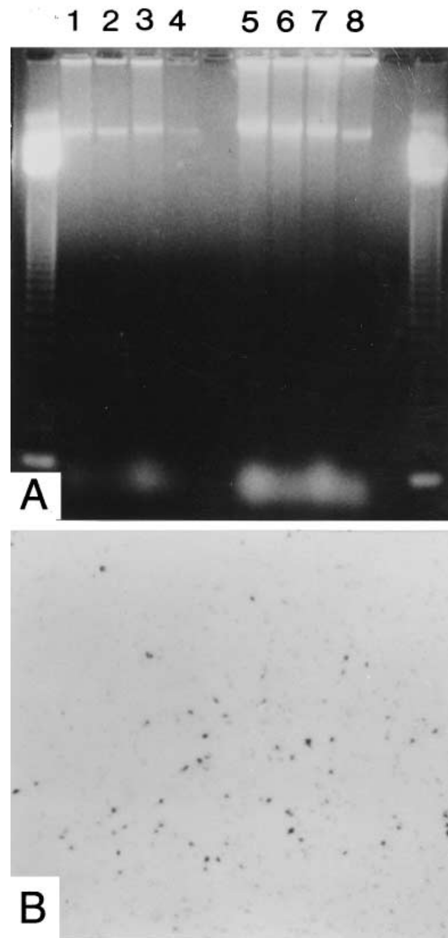

Fig. 3. Ethidium-stained agarose gel analysis of DNA extracted from microdissected structures that demonstrate abundant DNA fragmentation. (A) Stained gel demonstrates no evidence of "laddering" of DNA extracted from the hippocampus (lanes 1 and 5), dorsomedial thalamus (lanes 2 and 6), amygdala (lanes 3 and 7) and suprarhinal frontal cortex (lanes 4 and 8). Left- and right-hand lanes were loaded with 10 μg of 123 molecular weight markers. Lanes 1–8 were loaded with approximately 20 μg DNA each. Lanes 1–4 and 5–8 represent two separate experiments. Animals were killed 72 h after Grade III seizures. (B) Positive evidence of DNA fragmentation in the contralateral thalamus of the animal whose DNA was loaded in lanes 5–8. Original magnification ×60.

glutamate receptor subunits[16,17,21,40,59,63] and the distribution of neuropathological damage seen after specific activation of various subtypes of glutamate receptors,[32,46] and can find no precise overlap. For example, although the distribution of kainate-induced DNA fragmentation in the hippocampus has some similarity to the pattern of cell loss seen after direct intrahippocampal injection of the metabotropic glutamate receptor agonist (1*S*,3*R*)-1-aminocyclopentane-1,3-dicarboxylic acid,[46] dentate granule cells are severely affected in the (1*S*,3*R*)-1-aminocyclopentane-1,3-dicarboxylic acid model, whereas they are largely spared in the kainate model. It therefore appears that the affected structures are related to each other as parts of discrete synaptic pathways. Similar conclusions have been drawn by others with respect to the classical neuropathological changes seen after systemic and intracerebral administration of kainate.[4,5,31,36,49,60] We cannot exclude the possibility that affected populations share some characteristic that confers upon them selective vulnerability to systemic kainate. Similarly, it is possible that spared populations share some protective phenotype. For example, *in situ* hybridization studies of the distribution of various metabotropic glutamate receptor subunits in the hippocampus indicate that subunit 1 is relatively underexpressed in CA1,[16] the most vulnerable region in our model.

It has long been recognized that systemic kainate produces a pattern of neuronal toxicity that differs from that induced by local intracerebral injections of kainate into specific brain structures such as the amygdala.[3,49] Whereas local intra-amygdaloid injection of kainate offers a model of limbic epilepsy with both local and distant sites of pathology, systemic administration makes it more difficult to determine the relevant sites of action, but may more closely mimic seizures induced by circulating substances or metabolic abnormalities. As such, systemic kainate administration has been widely used as a model of convulsive status epilepticus.[2,14,25,29,49,65,66]

Recent reports of epidemic human intoxication with the neurotoxin domoate,[9,39,58] a potent kainate analog, emphasize the relevance of the systemic kainate model to human disease. In animal studies, systemic domoate causes profound neuronal damage in the hippocampus.[55] Several patients affected by domoate intoxication have come to autopsy, and neuropathological examination of those cases reveals striking similarities between the distribution of cell loss in those brains to the pattern of DNA fragmentation observed in this study.[9,61]

Previous reports have suggested that kainate-induced damage to the CA1 may be mediated by secondary activation of NMDA-type glutamate receptors that are particularly abundant in that region.[33] By contrast, high-affinity kainate receptors, which are abundant in CA3, have long been implicated in mediating kainate-induced cell loss in that region.[3,5,33,34] Our study calls these concepts into question. Pretreatment with MK-801, a specific irreversible use-dependent antagonist of NMDA receptors, conferred no protection from DNA fragmentation on CA1 neurons, while CA3 neurons demonstrated relatively little DNA fragmentation in spite of presumed direct activation by kainate. These results suggest first that selective vulnerability of CA1 neurons may be multifactorial, and second that DNA fragmentation is not seen in all populations destined to undergo degenerative changes following kainate treatment. With regard to CA1, it is possible that kainate-induced DNA fragmentation is associated with activation of a specific, and perhaps undiscovered, kainate receptor subunit. *In situ*

hybridization and immunohistochemical studies raise the possibility that the glutamate receptor subunit 5 may be selectively involved in this process.[21,63] With regard to CA3, it is possible that DNA fragmentation does occur in that region, but may have been missed in this study due to either temporal dispersion or unfortunate timing of our samples. Alternatively, CA3 damage in the systemic kainate model may involve predominantly cytoplasmic changes without induction of nuclear DNA cleavage.

Previous studies have suggested that direct intracerebral administration of kainate causes apoptosis, a form of programmed cell death.[41] Those authors based their conclusion on the morphological features of degenerating cells, the observation of the supposedly characteristic pattern of DNA laddering on electrophoretic analysis of isolated DNA and on the occurrence of nuclear labeling after *in situ* end labeling using terminal deoxynucleotide transferase to identify free 3′-OH ends. We performed two kinds of experiments to address this issue in the systemic kainate model. In general, programmed cell death is dependent on new protein synthesis, and can be blocked by administration of protein synthesis inhibitors. We therefore examined the effect of pretreatment with cycloheximide on the occurrence of DNA fragmentation. Cycloheximide did not appear to reduce the severity of DNA fragmentation in our hands; however, it tended to reduce the severity of seizures in treated animals, making interpretation of the experiment problematic. This may explain the differences between our results and those of Schreiber *et al.*,[48] who found that cycloheximide attenuated kainate-induced neuropathological changes as assayed by Nissl staining. In addition, some studies have suggested that the duration of cycloheximide action is less than 18 h.[10,22,44] We were unable to dose animals repeatedly due to almost 100% mortality, thereby leaving open the possibility that prolonged protein synthesis blockade could be protective in this model. In a second experiment, we analysed DNA extracted from microdissected regions of brains harvested after kainate treatment. We analysed the contralateral hemisphere histochemically to be certain that kainate treatment resulted in a similar degree of DNA fragmentation to that usually seen. We found no convincing evidence of internucleosomal DNA cleavage in tissue isolated from affected brain structures, and using the contralateral homologous region we confirmed that DNA was indeed obtained from affected animals. It is possible that evidence of DNA laddering is obscured by the abundance of unaffected cells. It is also possible that laddering may occur at an earlier time-point, and then evolve into smearing associated with necrosis by 72 h.[43] None the less, at face value these experiments argue against the notion that kainate-mediated DNA fragmentation is the result of apoptosis or some other form of programmed cell death.

CONCLUSIONS

These studies characterize an early and robust anatomical and biochemical marker of neuronal injury associated with kainate-induced seizures that can be visualized with cellular and even subcellular resolution and can be quantitated using simple anatomical techniques. This assay suggests that there is considerable heterogeneity in the mechanisms of kainate-induced neuronal toxicity, and it offers an easily measured endpoint to test mechanistic models and neuroprotective agents.

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