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Neuronal stress and injury in C57/BL mice after systemic kainic acid administration

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Abstract

Kainate-induced seizures are widely studied as a model of human temporal lobe epilepsy due to behavioral and pathological similarities. While kainate-induced neuronal injury is well characterized in rats, relatively little data is available on the use of kainate and its consequences in mice. The growing availability of genetically altered mice has focused attention on the need for well characterized mouse seizure models in which the effects of specific genetic manipulations can be examined. We therefore examined the kainate dose–response relationship and the time-course of specific histopathological changes in C57/BL mice, a commonly used founder strain for transgenic technology. Seizures were induced in male C57/BL mice (kainate 10–40 mg/kg i.p.) and animals were sacrificed at various time-points after injection. Seizures were graded using a behavioral scale developed in our laboratory. Neuronal injury was assayed by examining DNA fragmentation using in situ nick translation histochemistry. In parallel experiments, we examined the expression an inducible member of the heat shock protein family, HSP-72, another putative marker of neuronal injury, using a monoclonal antibody. Seizure severity paralleled kainate dosage. At higher doses DNA fragmentation is seen mainly in hippocampus in area CA3, and variably in CA1, thalamus and amygdala within 24 h, is maximal within 72 h, and is largely gone by 7 days after administration of kainate. HSP-72 expression is also highly selective, occurring in limbic structures, and it evolves over a characteristic time-course. HSP-72 is expressed mainly in structures that also manifest DNA fragmentation. Using double-labeling techniques, however, we find essentially no overlap between neurons expressing HSP-72 and DNA fragmentation. These findings indicate that DNA fragmentation and HSP-72 expression are complementary markers of seizure-induced stress and injury, and support the notion that HSP-72 expression is neuroprotective following kainate-induced seizures.

Keywords: Seizure; Kainate; Status epilepticus; Transgenic; In situ nick translation; Apoptosis; Neuronal injury; Heat shock protein; Neuronal stress

1. Introduction

Activation of kainate (KA) receptors causes neuronal depolarization and influx of calcium into neurons, and may result in excitotoxic neuronal injury [4,25]. A single parenteral dose of KA produces *status epilepticus* in rodents accompanied by neuronal damage mainly in limbic structures [3,46]. Hippocampal pyramidal neurons, especially in CA3, are selectively vulnerable to the toxicity of KA, perhaps as a consequence of the anatomical features of CA3 including its direct glutamatergic input from dentate gyrus granule cells [35,41,52]. KA-induced seizures in rodents have been widely used as a model of human temporal lobe epilepsy due to both behavioral and pathological similarities [3,6,36].

Excitotoxic neuronal injury results in cleavage of chromosomal DNA into fragments [25]. Identification of single strand DNA breaks using the in situ nick translation technique offers a convenient assay of injury with resolution at the cellular level. Visualization of DNA fragmentation has been used to study neuronal injury in various neuropathologic conditions including ischemia [47] and epilepsy [57]. While DNA fragmentation has been associated with apoptosis [9,12,15], it does not appear to be specific for any single form of cell death [39,40,57].

Heat shock proteins (HSPs) are highly conserved and the heat shock response is universal across yeast and animals. These polypeptides may be either constitutively

Abbreviations: Standard saline citrate (SSC); Kainate (KA); Phosphate buffered saline (PBS); Heat shock protein (HSP)

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expressed or induced by thermal or metabolic stress [59]. Excitotoxins, ethanol, heavy metals, anoxia, and amino acid analogues all appear to induce HSPs, and interestingly, this response is concomitant with an inhibition in the synthesis of the other proteins [28,54–56]. The HSP-70 family (molecular weights ranging from 66 to 78 kDa) [33] has drawn considerable interest because of its potential importance in the physiology and pathology of the mammalian central nervous system. Recent studies indicate that HSP-72 is highly inducible in central nervous system neurons [59]. HSP-72 is expressed following focal and global ischemia [27,37,51,56] and HSP-72 is expressed at the site of surgical injury in rat brain [5]. HSP-72 immunoreactivity is also observed in rat brain after kainate- or flurothyl-induced seizures [31,55], and HSP-72 mRNA is expressed after electroconvulsive seizure and stereotaxic administration of KA [20,54].

Biochemical studies indicate that HSP-72 is a chaperone protein that appears to regulate protein folding in an ATP-dependent fashion [38,59], suggesting the possibility that HSP expression represents an adaptive response to stress, and in fact several studies have suggested a protective role for HSPs. Cultured cells pretreated with heat sufficient to produce induction of stress proteins are able to survive subsequent hyperthermia that would otherwise be lethal [13,14]. Moreover, in cultured neurons HSP-72 induced by mild heat shock appears to confer effective protection against glutamate- and 1-methyl-4-phenylpyridimum(MPP^+)-associated neurotoxicity [10,30,43]. Pre-induction of HSP-72 by various sub-lethal stressors in vivo may protect retinal neurons against light-induced injury and cerebral neurons against seizure-induced damage [1,21,29,44]. In spite of these studies suggesting a protective role for HSP-72 expression, it remains unclear whether the expression of HSPs in the CNS mediates neuroprotection or is simply a marker of neuronal stress or injury [31]. For example HSP staining is seen in neurons that appear destined to die following limbic seizures [17,48,53], global ischemia [51] and mechanical injury [5].

The growing availability of genetically altered mice has focused attention on the need for well-characterized mouse seizure models in which the effects of specific genetic manipulations can be examined. Although DNA fragmentation after KA-induced seizures is well characterized in rats, relatively little data is available on the use of KA and its consequences in mice. Kokate et al. [22] utilized a behavioral endpoint in their examination of the anticonvulsant action of neuro-active steroids against kainate or pilocarpine-induced seizures in mice but did not examine pathological changes. By contrast, several groups have examined the pathological reaction to kainate seizures in transgenic [23] or knockout mice [34,45], however a lack of normative data describing the effect of kainate in specific strains has made those studies difficult to interpret [45]. Similarly, HSP-72 expression has been examined after seizures in rats, but only rarely in mice. The present studies were therefore designed to examine the dose–response relationship and the time-course of specific histo- and immuno-pathological changes induced by systemic kainic acid in C57/BL mice, a commonly used founder strain for transgenic technology.

2. Materials and methods

2.1. Induction of limbic seizures

Male C57/BL mice (22–25 g, Charles River Laboratories) were used for all experiments. An animal use protocol was approved by the institutional committee on animal care and followed the guidelines of the NIH for the care and use of laboratory animals. Animals were offered free access to food and water. All efforts were made to minimize both animal suffering and the number of animals used. Alternatives to in vivo studies were considered but could not be employed due to the dependence of our endpoint on preserved anatomic connections. Kainic acid (KA) was dissolved in 10 mM phosphate buffered saline (PBS) and pH adjusted to 7.2. For dose–response experiments, animals were injected intraperitoneally with KA at various doses (10, 20, 30 and 40 mg/kg) and sacrificed 72 h later Fig. 1 (Table 1). For time-course studies, all animals were treated with KA (40 mg/kg i.p.) and sacrificed at various time-points (24 h, 48 h, 72 h, 1 week, 2 weeks and 4 weeks after KA injection) (Table 2). Control animals received i.p. injections of an equivalent volume of PBS. The brains were rapidly removed and frozen in isopentane on dry ice, and then stored at $-80°C$.

The behavior of animals was observed following KA administration for 4–5 h continuously. Severity of seizures was classified, using a behavioral scale developed in our laboratory, into five different grades as follows: grade 0—no response; grade I—staring, front- or hind-limb pawing; grade II—staring, rearing, nodding, front- and hind-limb pawing; grade III: staring, rearing, nodding, pawing, jumping, wobbling; falling; grade IV—status epilepticus or death. Animals were graded based on the maximal behavioral response observed within 3 h of treatment. While no formal attempt to determine seizure duration was made, the duration of ictal behavior correlated well with the maximal seizure severity.

2.2. In situ nick translation

DNA fragmentation was examined on mounted sections using in situ nick translation histochemistry as previously described [57]. 11 μm cryostat sections were cut and mounted on gelatin coated slides. Sections were fixed in 4% paraformaldehyde for 10 min, then soaked in 2 × saline sodium citrate (SSC; 1 × SSC contains 150 mM NaCl,

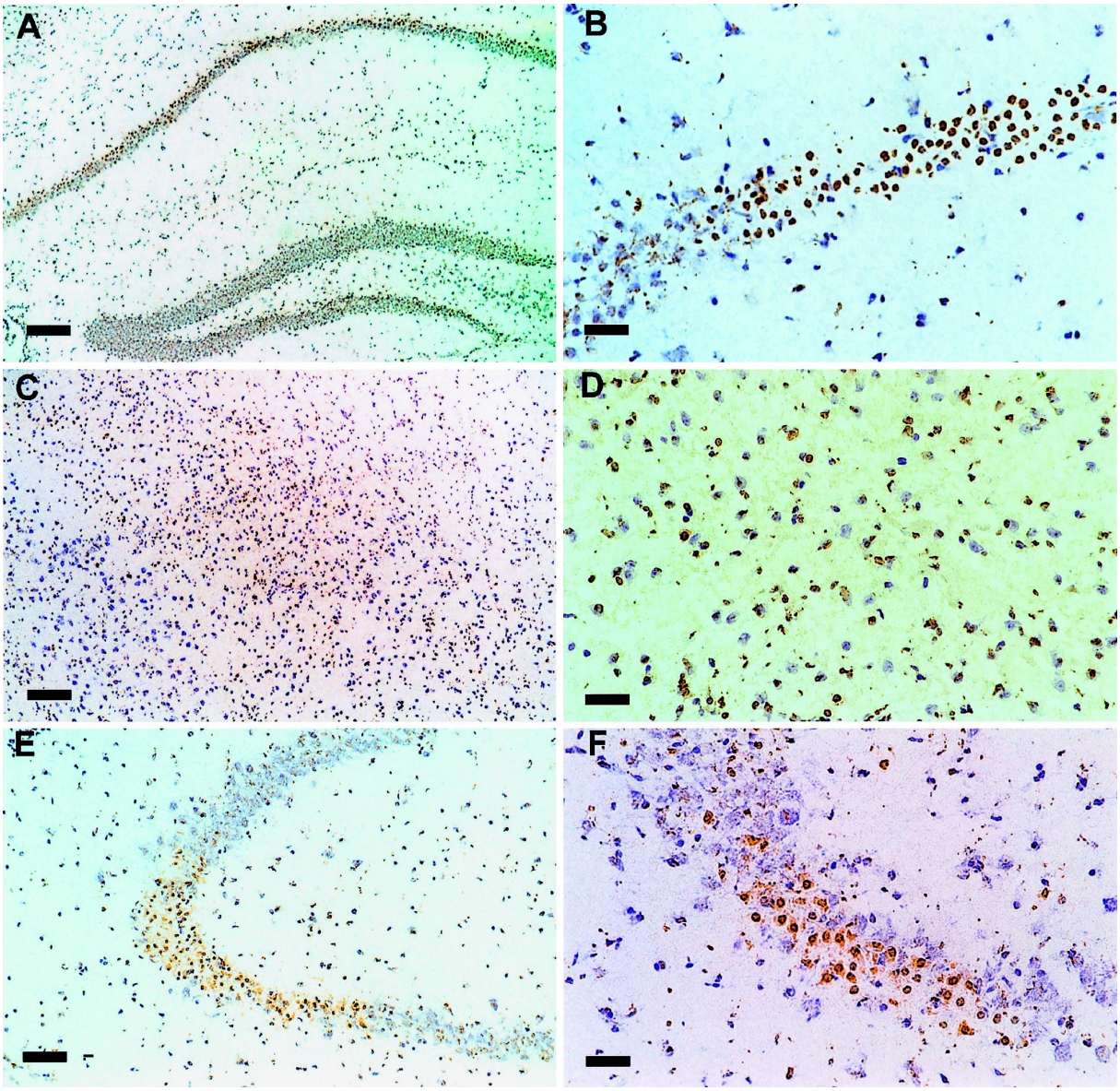

Fig. 1. DNA fragmentation occurs in discrete structures 72 h following systemic kainate-induced seizures (40 mg/kg i.p.) in C57/BL mice. Incorporation of biotinylated dUTP is visualized by avidin–biotin–peroxidase histochemistry as discrete nuclear staining of selected neurons. DNA fragmentation is indicated by deposition of brown pigment. Sections were counterstained with Cresyl violet. Note loss of Nissl staining in affected regions. (A, B) Hippocampal CA1 region; (C–D) lateral amgydala; and (E–F) hippocampal CA3 region. Bar = 150 μm (A, C, E) or 50 μm (B, D, F).

15 mM Na citrate, pH 7.0) at 80°C for 20 min. Sections were treated with pronase (1 μg/ml) (Boehringer Mannheim) for 10 min at room temperature and then transferred into 2% glycine in PBS to stop the digestion for 30 s. Sections were incubated in buffer A containing 50 mM Tris–HCl, pH 7.5, 5 mM MgCl2, 10 mM β-mercaptoethanol and 0.005% bovine serum albumin (BSA) for 5 min and then incubated in 5 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 I was omitted. For positive controls slides were pre-incubated in 0.4 units/ml DNase I (Boehringer Mannheim) in 10 mM Tris–HCl, 10 mM NaCl, 5 mM $MgCl_2$, 0.1 mM $CaCl_2$, 25 mM KCl, pH 7.4 for 15 min before incubation with DNA polymerase I. Sections were rinsed in PBS for 5 min and then switched into 0.1% H_2O_2 in PBS for 15 min followed by three 5 min rinses with PBS. The slides were incubated at room temperature in avidin–biotin–peroxidase complex (1:100) (Avidin–Biotin-Detection Kit, *Elite*, Vector Lab, Burlingame, CA) in PBS containing 1% BSA and 0.5% tween-20 for 30 min. Finally, sections were developed in PBS containing 0.05 mg/ml diaminobenzidine, 10 mM imidazole and 0.03% H_2O_2. Sections were counterstained with Cresyl violet and coverslipped using permount (Fisher).

Table 1
Seizure grade and distribution of DNA fragmentation after systemic administration of kainic acid at different doses

KA (mg/kg)	Animal number	Seizure grade	Hippocampus			Lateral amygdala	Dorsal thalamus	Cortex layer V/VI
			CA1	CA2	CA3			
10	1	0	−	−	−	−	−	−
	2	0	−	−	−	−	−	−
	3	0	−	−	−	−	−	−
	4	0	−	−	−	−	−	−
	5	0	−	−	−	−	−	−
	6	0	−	−	−	−	−	−
20	7	0	−	−	−	−	−	−
	8	0	−	−	−	−	−	−
	9	0	−	−	−	−	−	−
	10	0	−	−	−	−	−	−
	11	II	−	−	+	−	−	−
	12	II	+	−	++	+	−	+
30	13	III	−	−	++	−	−	−
	14	III	−	−	−	−	−	−
	15	III	−	−	+	−	−	+
	16	III	+++	−	+++	+	+	+
40	17	III	−	−	++	−	−	−
	18	III	−	−	++	−	−	−
	19	III	−	−	+	−	−	−
	20	III	+++	−	++	−	−	+
	21	III	+	−	+	++	+	+
	22	III	+	−	++	−	−	+

The succeeding five lines defines the grades for the severity of seizures.
Grade 0: no response.
Grade I: rearing, front or hind pawing, staring.
Grade II: rearing, staring, nodding, bilateral pawing.
Grade III: rearing, staring, nodding, bilateral pawing tonic–clonic, falling, jumping, wobbling.
Grade IV: status epilepticus and death.
The succeeding lines defines each grade of DNA fragmentation.
'−': No neurons positive.
'+': 0–25% of neurons positive.
'++': 25–50% of neurons positive.
'+++': 50–75% of neurons positive.

2.3. HSP-72 immunocytochemistry

Animals were anesthetized with sodium pentobarbital (Abbott Laboratories, Chicago, IL) and sacrificed by transcardiac perfusion with 0.9% NaCl for 10 min followed by periodate–lysine–paraformaldehyde (PLP) solution (pH 7.4) for 10–20 min. Brains were removed and post-fixed in the same solution for 24 h at 4°C and then transferred into 20% glycerol, 2% DMSO in Na_2PO_4 buffer. Free-floating section (50 μm) were cut on a sliding microtome (Zeiss) and stored in 0.1 M Na_2PO_4 buffer at 4°C for immunocytochemistry. The sections were washed in PBS once and immersed in 0.3% H_2O_2 in PBS for 10 min followed by three rinses in PBS. The sections were blocked with 10% goat serum in PBS (GIBCO-BRL) for 1 h and incubated in a mouse monoclonal anti-72 KD heat shock protein antibody (1:300) (RPN1197, Amersham, Chicago) overnight at 4°C. For negative control sections, the primary antibody was omitted. Following three washes in PBS sections were incubated in peroxidase-conjugated goat anti-mouse secondary antibody (1:300) (Boehringer Mannheim) for 3 h and developed with diaminobenzidine in the presence of peroxide. Following several washes in PBS, sections were mounted and coverslipped.

2.4. Double labeling for HSP-72 and DNA fragmentation

Following HSP-72 immunocytochemistry sections were hydrated through graded alcohol and PBS. DNA fragmentation was examined on mounted sections using in situ nick translation histochemistry as previously described. The final detection of biotinylated dUTP was carried out in the presence of 2.5% Nickel (II) sulfate in acetate-imidazole buffer, pH 7.0–7.2, 0.4% diaminobenzidine and 0.005% H_2O_2, to give dark blue staining of nuclear DNA fragmentation.

2.5. Data analysis

The primary means of data analysis was visual inspection of stained sections using light microscopy at 25–400 × magnification. For DNA fragmentation sections from representative levels were assessed for background staining and incorporation of biotinylated nucleotide into nuclear material. Only cells with dense nuclear staining were

Table 2
Seizure grade and distribution of DNA fragmentation after systemic kainic acid administration at different time points

Time point	Animal number	Seizure grade	Hippocampus			Lateral amygdala	Dorsal thalamus	Cortex layer V/VI	Nucleus reticularis
			CA1	CA2	CA3				
24 h	1	II	−	−	−	−	−	−	−
	2	III	−	−	+ +	+	+ +	+	+
	3	III	−	−	+ +	+	+	+	−
	4	III	−	−	+ +	+	−	+	−
	5	III	−	−	+ + +	−	+	−	−
	6	III	−	−	+	−	−	−	−
	7	III	−	−	+ +	−		−	+
48 h	8	II	−	−	+	+	−	−	−
	9	III	+	−	+ +	+ + +	+	+	+
	10	III	−	−	+	+ + +	+	−	+
	11	III	−	−	+ +	−	−	−	−
	12	III	−	−	+ +	+	−	−	−
	13	III	−	−	+ + +	−	−	−	−
72 h	14	II	−	−	−	−	−	−	−
	15	II	−	−	−	−	−	−	−
	16	III	+ +	−	+ + +	+ +	+	+	+
	17	III	+	−	+ +	+	+	+	+
	18	III	−	−	+	+	−	−	−
	19	III	−	−	+	+	−	−	−
	20	III	−	−	+	−	−	−	−
1 W	21	II	−	−	−	−	−	−	−
	22	II	−	−	−	−	−	−	−
	23	II	−	−	+	−	−	−	−
	24	III	−	−	+ + +	−	−	+	+
	25	III	−	−	+ +	−	−	−	−
	26	III	−	−	+	−	−	−	−
2 W	27	II	−	−	−	−	−	−	−
	28	III	−	−	+	+	+	+ +	+
	29	III	−	−	+	−	−	−	−
	30	III	−	−	+	−	−	−	−
4 W	31	II	−	−	−	−	−	−	−
	32	III	−	−	+	−	−	−	−
	33	III	−	−	−	−	−	−	−
	34	III	−	−	−	−	−	−	−
	35	III	−	−	−	−	−	−	−

The succeeding five lines defines the grades for the severity of seizures.
Grade 0: no response.
Grade I: rearing, front or hind pawing, staring.
Grade II: rearing, staring, nodding, bilateral pawing.
Grade III: rearing, staring, nodding, bilateral pawing tonic–clonic, falling, jumping, wobbling.
Grade IV: status epilepticus and death.
The succeeding four lines defines each grade of DNA fragmentation.
'−': no neurons positive.
'+': 0–25% of neurons positive.
'+ +': 25–50% of neurons positive.
'+ + +': 50–75% of neurons positive.

considered positive. Two observers blinded to animal treatment independently rated specific structures on a semi-quantitative scale with respect to severity of DNA fragmentation. As area CA3 was most consistently affected, counts of positively stained neurons in CA3 were made. At least three and as many as nine serial sections were available from each animal. Stained cells in area CA3 were counted in both hemispheres and averaged for each animal to produce a mean value of stained neurons in CA3 for each animal. HSP-72 staining was examined by light microscopy. For double label experiments, we used dark blue nuclear staining as a marker of DNA fragmentation, and brown cytoplasmic staining as a marker of HSP-72 immunoreactivity.

3. Results

3.1. Behavioral observation

KA produced behavioral seizures in a dose-dependent fashion within 15–30 min after systemic injection. Higher

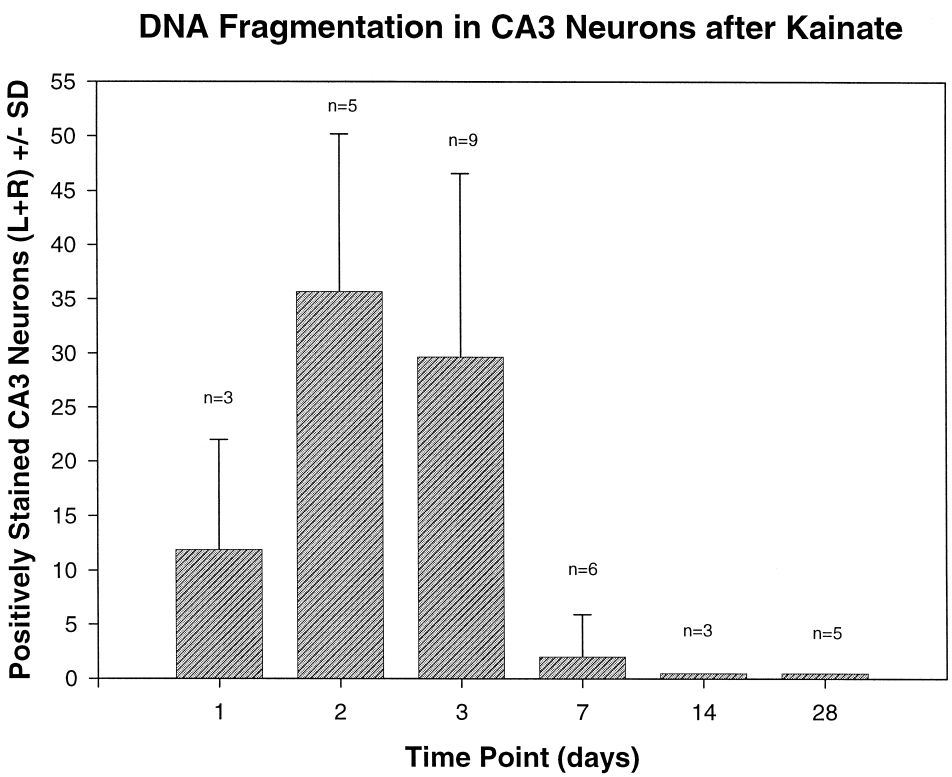

Fig. 2. DNA fragmentation in CA3 is maximal at 48–72 h after treatment. Bars indicate average number of stained neurons in area CA3 in n animals. 24, 48 and 72 h were significantly different from 7, 14 and 28 d ($p < 0.05$: Tukey's test for pairwise multiple comparison procedures). Differences between 24, 48, and 72 h timepoints were not statistically significant.

doses of KA (30 and 40 mg/kg) produced grade II or III seizures with majority of mice having grade III seizures. At 40 mg/kg, 5/18 animals (27.7%) died within 6 h of treatment. No behavioral response to KA was observed at 10 mg/kg, whereas, there was a variable response to 20 mg/kg of KA with approximately 30% of animals showing behavioral abnormality (grade II seizure).

3.2. DNA fragmentation after KA treatment

DNA fragmentation was observed mainly in hippocampus in area CA3, and also distributed in area CA1, dorsomedial and dorsolateral thalamus, nucleus reticularis, the lateral nuclear complex of amygdala, and cortex in layers V/VI following KA systemic treatment. Both the distribution and severity of DNA fragmentation were related not only to the dose of KA but also to the severity of seizure induced by KA (Table 1). There was no DNA fragmentation seen in controls, in any animals treated with 10 mg/kg of KA or in animals without behavioral abnormality. After 20 mg/kg of KA, DNA fragmentation was observed in one out of two animals with grade II seizures. DNA fragmentation was identified in all mice with grade III seizures.

DNA fragmentation appeared and evolved over a characteristic time-course (Table 2). Positively stained neurons were observed prominently in CA3, thalamus, amygdala, and cortex 24 h following injection of KA. Earlier survival times were not studied in the present experiment. No significant difference in the regional distribution of DNA fragmentation was observed between 24, 48 and 72 h, however, staining was maximal 48–72 h after treatment (Fig. 2). At 1 week, DNA fragmentation was occasionally seen to persist in CA3 but was essentially absent elsewhere. Unlike earlier time-points in which DNA fragmentation could be detected in animals with grade II seizures, at longer survival times positively stained neurons were exclusively identified in animals with grade III seizures. DNA fragmentation was still apparent 4 weeks after KA injection in 1/5 animals, but was limited to CA3.

3.3. HSP-72 immunoreactivity after KA treatment

HSP-72 immunoreactivity was observed mainly in area CA3 of hippocampus, cortex, and variably distributed in

Fig. 3. HSP-72 expression after systemic kainate. Immunocytochemical staining for HSP-72 in brain sections from animals 72 h after grade III seizures induced by kainate. Low power view of hippocampus demonstrates staining in CA1, CA3 and dentate hilus (A). High power view of CA1 (B) and CA3 (C). Sparse staining of neurons in amygdala (D). Low (E) and high (F) power views of cortex demonstrate staining in layers I/II and VI. Camera lucida representation of distribution of HSP-72 staining from representative animals (G). Bar = 150 μm (A, E) or 50 μm (B, C, D, F).

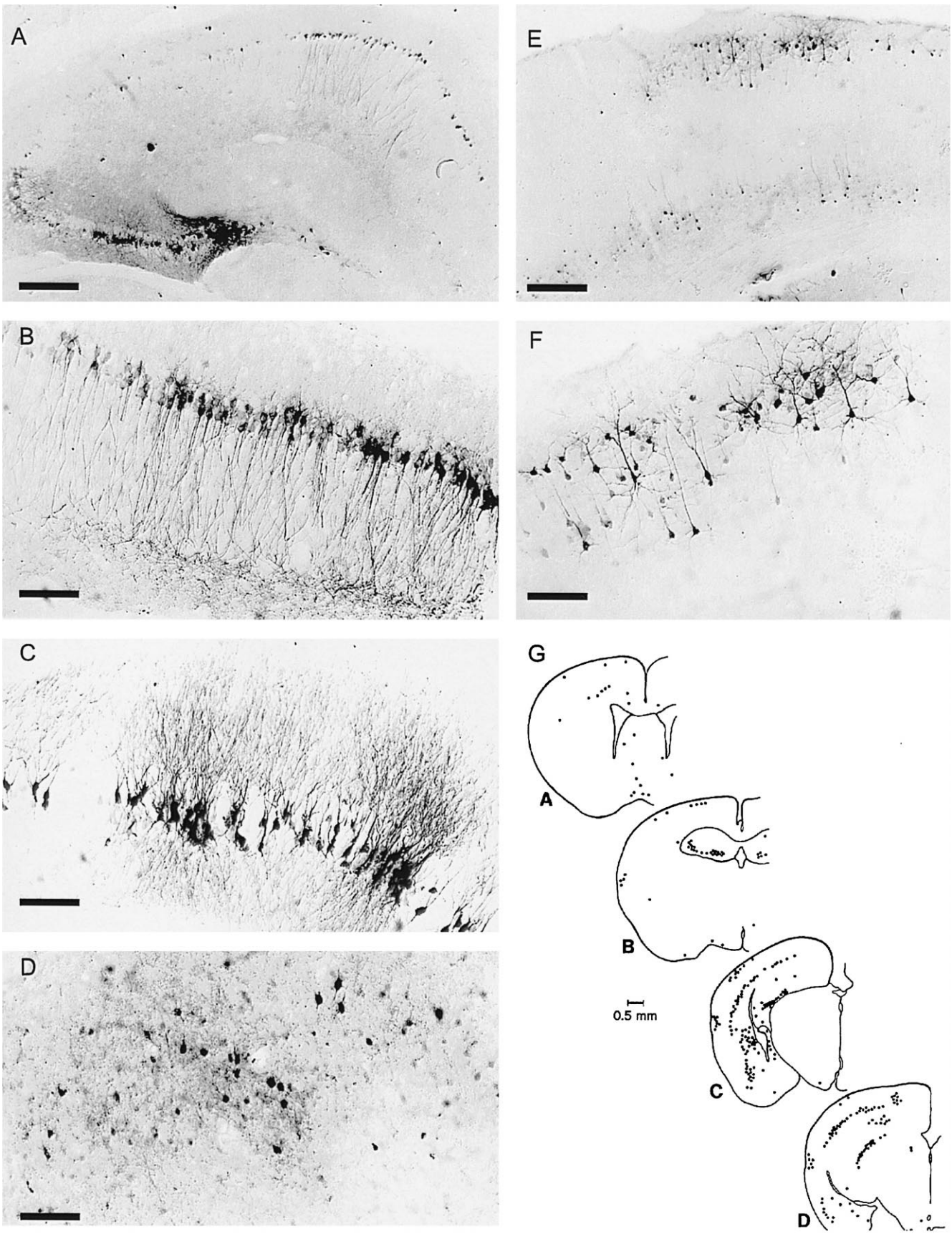
A
E
B
F
C
G
A
B
0.5 mm
C
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Table 3
Seizure grade and immunoreactivity for HSP-72 after systemic kainic acid administration at different time points in C57/BL mice

Time points	Animal number	Seizure grade	Hippocampus				Amygdala	Cortex			Caudate/putamen
			CA1	CA3	Hilus	Dentate		I/II	III/IV	V/VI	
Control	1	–	–	–	–	–	–	–	–	–	–
	2	–	–	–	–	–	–	–	–	–	–
24 h	3	III	++	+++	+	–	+	–	++	–	++
	4	III	–	++	–	–	+	–	+	–	–
	5	III	–	+	–	–	+	–	+	–	+
	6	III	–	+	–	–	–	–	–	–	–
48 h	7	III	–	+	+	–	–	+	–	–	–
	8	III	+++	++	–	–	–	++	–	–	++
	9	III	–	+	–	–	–	+	–	–	–
	10	III	–	+	–	–	–	+	–	–	–
72 h	11	III	+++	++	++	–	–	+	–	++	++
	12	III	–	+	–	–	–	–	–	–	–
	13	III	–	+	–	–	–	–	–	–	–
96 h	14	III	+	+++	+	+	+	++	–	+	+
	15	III	+	+	+	++	+	–	–	++	–

The succeeding five lines defines the grades for the severity of seizures
Grade 0—no response.
Grade I—rearing, front or hind pawing, staring.
Grade II—staring, rearing, nodding, bilateral pawing.
Grade III—staring, rearing, nodding, bilateral pawing, jumping, falling.
Grade IV—*status epilepticus* and death.
The succeeding lines defines each grade of heat shock Protein72KD staining.
'–' no neurons staining.
'+' 0–25% of neurons staining.
'++' 25–50% of neurons staining.
'+++' 50–75% of neurons staining.

area CA1, dentate hilus, amygdala, caudate-putamen, thalamus and rarely, dentate gyrus, following systemic KA administration (Fig. 3). No HSP-72 immunoreactivity was identified in control animals. Positively stained neurons were observed prominently in CA3, amygdala, cortex layer III/IV, caudate-putamen, and hilus 24 h following injection of KA. At 48 h, HSP-72 immunoreactivity appeared in cortex layer I/II and was absent in amygdala. Else-

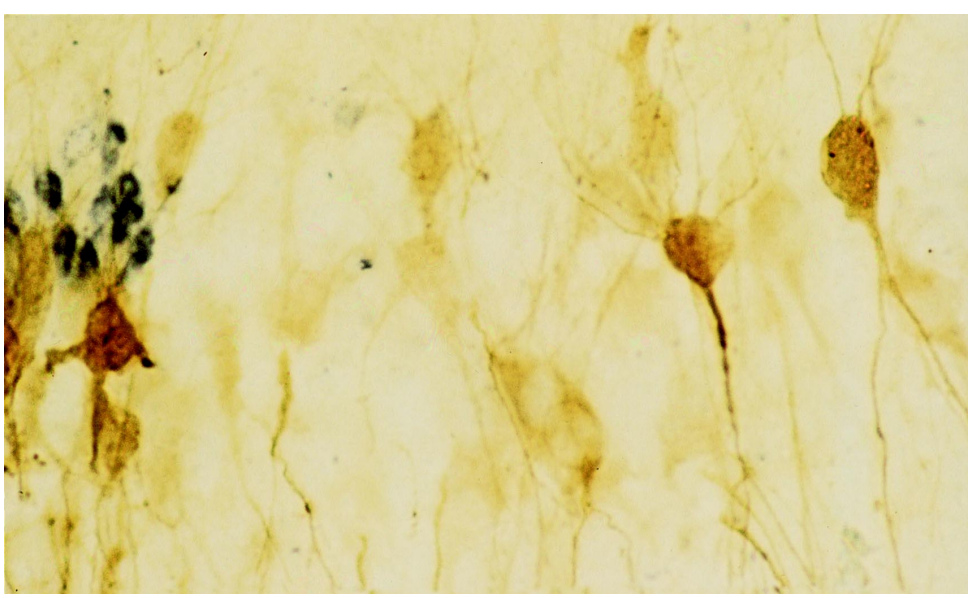

Fig. 4. Double labeling for HSP-72 and DNA fragmentation demonstrates no overlap of expression. High power micrograph of CA3 neurons stained for HSP-72 (brown cytoplasmic staining) and for DNA fragmentation (dark blue nuclear staining). Magnification 400× before reproduction.

where the pattern of HSP-72 expression was unchanged between 24 and 48 h. HSP-72 was expressed in cortex in layers I/II and V/VI 72 h after KA and consistently observed at 96 h survival time. HSP-72 stained neurons were also seen in thalamus at 96 h (Table 3).

3.4. DNA fragmentation and HSP72 expression after KA treatment

Because HSP-72 is expressed in structures known to suffer neuronal loss after KA-induced seizures, and because HSP-72 may have protective functions, we sought to determine whether HSP-72 is expressed in neurons that also manifest DNA fragmentation, a putative marker of irreversible injury. We therefore performed double labeling studies on selected sections found to express high levels of HSP-72. We found that while HSP-72 expression and DNA fragmentation occur with overlapping distributions, the vast majority of neurons expressing HSP-72 do not demonstrate DNA fragmentation (Fig. 4). Visual inspection of 25 sections indicates that less than 5% of neurons expressing HSP-72 are double-labeled.

4. Discussion

The main finding of our study is that a single systemic injection of KA produces limbic seizures in C57/BL mice associated with neuronal injury manifested by DNA fragmentation and neuronal degeneration which appears hours to days later with a highly stereotyped distribution. HSP-72 is also expressed with a characteristic topography after seizures induced by systemic kainate in C57/BL mice, but while many structures containing neurons expressing HSP-72 also manifest DNA fragmentation, HSP-72 and DNA fragmentation occur in essentially non-overlapping neuronal populations. It appears that neurons expressing HSP-72 do not undergo DNA fragmentation. Moreover, cells with HSP-72 staining showed normal morphology by Nissl staining (data not shown). Together these observations support the notion that HSP-72 has a protective role.

Following KA administration at sufficient doses, convulsions start within 15–20 min and seizures last for 2–4 h with some animals developing severe seizures or *status epilepticus.* Detection of neuronal damage using the in situ nick translation technique indicates that regions CA3 and sometimes CA1 in hippocampus, deep layers of cortex, and specific nuclei in thalamus and amygdala are vulnerable to KA with area CA3 being most sensitive to injury. The distribution of DNA fragmentation after systemic administration of KA in C57/BL mice largely parallels that seen in rats [57], however, whereas CA1 is most consistently affected in rats, CA3 is most consistently affected in mice. The pattern of DNA fragmentation is both dose- and time-dependent. Higher doses of KA are required to produce visible neuronal damage in C57/BL mice than those used in rats [57]. DNA fragmentation occurs within 24 h of KA administration and is maximal by 72 h. In general DNA fragmentation in mice is transitory, disappearing by 1 week after treatment. In one animal we noted persistent DNA fragmentation 4 weeks after treatment that may have been associated with un-witnessed seizure recurrence. Because DNA fragmentation can be assayed in situ and quantitated in specific structures, it offers a useful endpoint for the examination of potential exogenous neuroprotective agents. Moreover, assays of DNA fragmentation may be useful to assess the role and importance of specific molecules targeted for either inactivation or overexpression by genetic manipulation.

Our parallel studies of HSP-72 expression indicate that after kainate-induced seizures, C57/BL mice express HSP-72 mainly in CA3, but also in other limbic structures including CA1 and amygdala. Interestingly, HSP-72 expression is also seen in dentate hilus and in caudate-putamen, structures generally considered resistant to kainate-induced injury. Like DNA fragmentation, HSP-72 expression is apparent within 24 h and persists through at least 96 h after seizures. The magnitude of HSP-72 expression is also dependent on seizure severity, with no expression visualized in controls or animals with grade I seizures.

Whereas rats demonstrate a consistent pathological response to kainate-induced seizures, previous studies of the pathological consequences of kainate-induced seizures in mice have yielded variable results. Morrison et al. [34] found that loss of the p53 tumor suppressor gene protected mouse hippocampal neurons from kainate-induced cell death, whereas, Schauwecker and Steward [45] were unable to reproduce that result using mice homozygous for a null mutation in p53 generated from a different strain. Kondo et al. [23] described a pattern of injury induced by kainate in mice overexpressing CuZn-superoxide dismutase similar to that we have seen, but found a different dose response relationship in their strain. These findings, in combination with studies of various inbred strains [16,24] that have noted considerable strain to strain variation in the severity of injury seen in both rats and mice after systemic kainate, emphasize the need for normative data and the importance of controlling for genetic background when using the occurrence and distribution of DNA fragmentation as a phenotypic marker.

4.1. Intracellular calcium may couple excitation with DNA fragmentation

The excitotoxic hypothesis suggests that excessive activation of neurons is an initial pathological event in cell loss [8]. The calcium-permeability of glutamate receptors is dramatically enhanced as a result of excessive and prolonged depolarization of neuronal membranes. Neurotoxicity can be markedly attenuated by removal of extracellular Ca^{2+}, suggesting that the excitotoxicity of glutamate is calcium-dependent [7]. Ca^{2+} influx through volt-

age-operated channels also plays an important role in the propagation and maintenance of epileptiform activity [32]. Kainate receptors demonstrate Ca^{2+}-permeability [4,18], which they share with NMDA-type glutamate receptors. Moreover, injury following KA treatment appears to involve Ca^{2+}-dependent events in insulted cells [49,50]. Interestingly, chromosomal DNA can be cleaved into nucleosome-sized fragments (180–190 base pairs) by Ca^{2+}-dependent endonuclease activity in the presence of glutamate in cultured neuronal cells [25]. Together these observations suggest that increased intracellular Ca^{2+} may couple glutamatergic stimulation to neuronal DNA fragmentation. Intracellular Ca^{2+} is also thought to be necessary for synthesis of HSP-72 under stress conditions. For example, deprivation of Ca^{2+} from medium completely inhibited the synthesis of HSP in hepatoma cells in culture [26]. It therefore appears that increased intracellular Ca^{2+} is required for both the synthesis of heat shock proteins and the induction of DNA fragmentation after KA-induced seizures. Our observation that HSP-72 expression and DNA fragmentation appear over the same time-course supports the notion that both events are triggered by a common stimulus, perhaps intracellular Ca^{2+} flux. It remains unclear why some cells appear to respond to kainate with DNA fragmentation while adjacent neurons appear to express HSP-72 instead.

4.2. CA3 neurons are selectively vulnerable to kainate-induced injury in mice

The selective vulnerability of CA3 pyramidal cells to KA toxicity has been well documented. Mossy fiber terminals in CA3 have been implicated in KA-induced damage since KA fails to cause neuronal death when mossy fiber projections are impaired [35]. Furthermore, there are abundant high-affinity binding sites for KA located on mossy fiber terminals [41] that may mediate increased neurotransmitter release pre-synaptically [11]. Selective sensitivity of CA3 neurons to KA may therefore result from both its anatomical connections and its low threshold for developing epileptiform activity [35].

In the present study, when neuronal injury occurred, CA3 was the most vulnerable and extensively affected brain region and in some cases the only area where DNA fragmentation was detectable. By contrast, in previous studies using rats we invariably noted preferential DNA fragmentation in CA1 neurons, whereas, DNA fragmentation was not universally observed in CA3 [57]. The cause and significance of this species difference is unclear. It is possible that DNA fragmentation is caused in part by local relative ischemia or hypoxemia. Subtle differences in the cerebral circulation between species could then by hypothesized to be responsible for the difference in anatomic distribution of this marker of injury. Morrison et al. found preferential DNA fragmentation in CA1 in their strain of p53 wild type mice [34], suggesting that the pattern of selective vulnerability may be in part genetically determined. Regardless of its origin, as long as DNA fragmentation occurs in a predictable distribution in a specific strain, it can be a useful assay of injury in experiments designed to study pathogenic mechanisms and potential neuroprotective strategies.

4.3. Expression of HSP-72 and DNA fragmentation is mutually exclusive

Our double labeling studies of DNA fragmentation and HSP-72 expression indicate that neurons rarely exhibit both phenomena together, although both occur in apparently overlapping populations. This observation supports the hypothesis that HSP-72 expression is neuroprotective. Several lines of evidence including a number of correlative studies support the notion that HSP-72 is important in recovery from injury. Transient hyperthermia protects against light-induced degeneration in rat retina [1]. Ischemic neuronal damage is significantly attenuated by preconditioning with sub-lethal ischemia sufficient to produce induction of HSP-72 [21,29]. Similarly, ‘epileptic tolerance’ in which injury induced by bicuculline-induced seizures is attenuated by previous seizures sufficient to induce HSP-72 has been described [44]. Glutamate-mediated excitotoxicity in cultured neurons is attenuated by preceding mild heat shock [30,43].

More direct evidence to support a protective role for HSP-72 has emerged from a series of biochemical studies. Cells injected with antibody against HSP-72 fail to survive transient hyperthermia [42]. Enhanced thermo-sensitivity is also demonstrated in cells in which competitive inhibition of HSP-72 gene expression has been established [19]. Although the contribution of the HSP-70 family to recovery of cells from injury is not completely understood, a role for HSPs in regulating protein synthesis has been suggested. Under physiological condition, HSPs are involved in protein folding and assembly [2]. Upon exposure of cells to stressors, HSPs facilitate protein maturation and stabilize proteins that have been denatured [38,58]. Moreover, new protein synthesis appears necessary for HSP-72 regulated cytoprotection as protective actions of the peptide were blocked in the presence of protein and RNA synthesis inhibitor [43].

It is possible that we have missed co-expression of HSP-72 and DNA fragmentation either for technical reasons or due to the time-point we chose to examine. We have considered the possibility that our double labeling technique allows HSP-72 staining to occlude evidence of DNA fragmentation or vice versa, however, the finding of rare neurons positive for both markers makes this possibility unlikely. It is also possible that HSP-72 and DNA fragmentation occur sequentially in neurons and that by sampling at a fixed time-point we have missed successive expression in individual cells. Such a scenario would result in expression of DNA fragmentation and HSP-72 with

successive maxima. Our finding that the maximal expression of both of these markers occurs simultaneously, and that there is essentially no overlap even at that time-point makes it unlikely that we have missed successive expression by significant numbers of individual cells.

In conclusion, the pattern of DNA fragmentation after systemic administration of KA in C57/BL mice is dose- and time-dependent, and the distribution of DNA fragmentation largely parallels findings previously reported in rats. Neurons in region CA3 of the hippocampus are most vulnerable to KA. Our dose-response studies indicate that considerably higher doses of KA are required to produce severe neuronal damage in C57/BL mice as compared with rats. We have also shown that HSP-72 and DNA fragmentation occur in similar structures, mainly limbic areas recruited into the epileptic event. Using double labeling techniques with cellular resolution, however, we find little overlap amongst neurons expressing HSP-72 and DNA fragmentation, suggesting that HSP-72 expression is a marker of resistant cells and supporting the concept that HSP-72 expression is protective.

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