Early-life seizures in rats increase susceptibility to seizure-induced brain injury in adulthood

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Article abstract—*Background:* Early childhood convulsions have been correlated with the finding of subsequent hippocampal neuronal loss and memory impairment in patients with intractable temporal lobe epilepsy. There is little direct evidence, however, that links early seizures with the later development of epilepsy and selective hippocampal neuronal loss. *Objective:* To study the long-term effect of early seizures on later seizure-induced neuronal damage and behavior. *Methods:* We used a "two hit" rat seizure mode in which systemic kainate was used to induce seizures during the second week of life (P15) and again in adulthood (P45). Memory was subsequently tested using a Morris water maze, and brains were examined for histologic evidence of injury. *Results:* Although the first kainate-induced seizure is not associated with detectable injury or cell death, it predisposes animals to more extensive neuronal injury after kainate-induced seizures in adulthood. Moreover, although early-life kainate-induced seizures cause no impairment of spatial learning, animals that have early-life and adult kainate-induced seizures perform significantly worse than those that have seizures only as adults. *Conclusions:* We concluded that early-life seizures, without causing overt cellular injury, predispose the brain to the damaging effect of seizures later in life. **Key words:** Development—Seizures—Status epilepticus—Kainate—Injury—Memory.

NEUROLOGY 1999;53:915–921

Clinically, temporal lobe epilepsy often starts as an isolated, prolonged convulsion in early life followed by a period of remission after which seizures reemerge and may become intractable.[1] Many retrospective studies have shown a correlation between a history of early childhood convulsions and hippocampal sclerosis.[2-7] These observations suggest that there may be an etiologic association between early-life seizures and later seizure-induced brain damage and that a cerebral insult at a critical time in development may be necessary to produce hippocampal sclerosis in this population. In fact, a recent study of serial MRI examinations of infants with prolonged focal febrile convulsions has provided evidence that prolonged febrile seizures can, in some cases, be causally associated with hippocampal injury.[8] Large prospective epidemiologic studies, however, have been unable to establish a causal link.[9-11] Moreover, clinical and laboratory studies suggest that the immature brain is relatively resistant to seizure-induced brain damage.[12-17] Whether early-life seizures are sufficient to cause epilepsy and hippocampal damage, the molecular and cellular events that accompany early-life seizures may predispose the brain to future seizure-induced damage. This possibility remains largely unexplored.

Kainate-induced seizures have been studied as a model of limbic epilepsy for over a decade. Seizures produced by systemic injections of kainic acid (KA) in adult rats cause cognitive and behavioral deficits[17] and result in a distinctive pattern of neurodegeneration in the hippocampus that resembles human hippocampal sclerosis.[18-20] By contrast, in immature animals, KA does not produce long-term behavioral deficits[17] or neuropathologic changes,[14,15] despite causing status epilepticus at one third of the adult dose per weight.[14,16] Similarly, seizures in immature rats induced by pilocarpine, flurothyl, or kindling also fail to produce any detectable hippocampal damage before postnatal day 20 (P20),[21-24] although several recent studies have begun to challenge this view. For example, after lithium-pilocarpine–induced

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Supported by the Epilepsy Foundation of America (S.K.), The Whitehall Foundation (A.J.C.), and National Institute of Neurological Disorders and Stroke (NS036224) (A.J.C.).

Received January 18, 1999. Accepted in final form June 29, 1999.

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Table *Summary of experimental groups and survival*

Group	Treatment		Survivors at P50
	P15 (n)	P45 (n)	
KK	Kainate (36)	Kainate (32)	26
SK	Saline (16)	Kainate (16)	13
KS	Kainate (6)	Saline (6)	6
SS	Saline (6)	Saline (6)	6
K	Kainate (6)	Killed on P17	—
Total number	70	60	51

seizures or continuous perforant path stimulation, some studies have found evidence of injury using sensitive techniques.[25,26] Even in the absence of histologic evidence of necrosis or gross morphologic changes, status epilepticus induced by flurothyl in early postnatal rats (P4) produces both acute and long-lasting detrimental effects on brain growth and brain DNA synthesis.[27] These animals are not only delayed in acquiring adaptive behaviors but also show increased susceptibility to later flurothyl-induced seizures. Recurrent flurothyl seizures early in life have also recently been associated with long-term detrimental effects on behavior and seizure susceptibility.[28] Thus, although the immature brain is relatively resistant to seizure-induced neuronal death, there may be age-specific effects of seizures on the developing brain.

The aim of the present study was to determine whether early-life limbic seizures, even in the absence of resulting overt morphologic damage, cause heightened susceptibility to later seizures and render the brain more vulnerable to seizure-induced neuronal injury in adulthood. We induced KA seizures on P15, and again on P45. The effect of early-life seizures on later seizure susceptibility was measured by examining seizure onset latency and grade of seizure severity. Seizure-induced spatial memory deficits were assessed using a modified Morris water maze, and neuronal injury was examined using in situ detection of DNA fragmentation and cresyl violet Nissl staining.

Methods. *Experimental design.* Seven litters of Sprague-Dawley rats (Charles River Laboratories, Cambridge, MA) (10 animals in each litter) were divided into five groups, designated K (KA on P15 only), SS (saline on P15 and P45), SK (saline on P15 and KA on P45), KS (KA on P15 and saline on P45), and KK (KA on P15 and P45), as summarized in the table. On P15, 5 to 8 rats from each litter were treated with KA (5 mg/kg intraperitoneally [ip] in phosphate-buffered saline [PBS], pH 7.4). Littermate controls received equal ip volumes of PBS. On P45, animals in the SK and KK groups received KA (15 mg/kg ip), whereas animals in the SS and KS groups received equal volumes of PBS (200 to 250 μL ip). Age-specific doses of KA used in the present study were previously determined to result in 25% to 40% mortality while inducing seizures in over 60% of animals.[16,29] A greater number of animals were assigned to KK group (36) compared with the SK group (16) to allow for the expected higher mortality associated with two KA treatments. On P50, all surviving animals began testing in a modified Morris water maze[30] (see following). Animals were killed on P55 by transcardiac perfusion and brains were processed for histology and for DNA fragmentation assayed by in situ nick translation. Six rats treated with KA on P15 were sacrificed 48 to 72 hours later and processed for histology and DNA fragmentation. We have previously shown that seizure-induced DNA fragmentation is maximal by 48 to 72 hours and remains detectable 10 days after KA-induced seizures.[29]

Behavioral observation and seizure grading. After KA injections, animals were closely observed and their behavior was recorded for 6 hours as previously described.[29] Latency to the first clinical manifestation of seizure activity was recorded. A seizure severity grade was assigned based on the maximal response achieved on a scale from 0 to IV as follows: 0, no response; I, wet dog shake (WDS) and/or behavioral arrest; II, WDS, staring, pawing, and clonic jerks; III, WDS, staring, pawing, clonic jerks, rearing, and falling; IV, continuous grade III seizures for longer than 30 minutes (status epilepticus).

Behavioral testing. A modified Morris water maze[30] was used to assess visuospatial learning and memory.[16] A circular steel tank (117-cm diameter) was filled with water (26 ± 1 °C) to a depth of 25 cm. The water was made opaque by addition of ~100 mL of evaporated milk. The pool was illuminated by overhead fluorescent lights and kept in a permanent location throughout the study. Four points on the rim of the pool were designated as north (N), south (S), east (E), and west (W). On day 1, each rat was placed in the pool for 60 seconds with no platform present for a "free swim." On days 2 through 5, rats were trained for 24 trials, 6 trials per day, to locate and escape onto a plexiglass platform (8 x 8 cm) placed 1.5 cm under the water surface. For each rat, while the quadrant in which the platform was located remained constant, the point of immersion into the pool with the rat held facing the perimeter varied between N, E, S, and W in a quasi-random order for the 24 trials. The latency from immersion into the pool to escape onto the platform was recorded. On mounting the platform, the rat was given a 30-second rest on the platform before the next trial. If a rat did not find the platform in 120 seconds, it was manually placed on the platform for a 30-second rest.

Detection of DNA fragmentation by in situ nick translation histochemistry. Animals were killed by transcardiac perfusion with 50 mL of PBS followed by 200 mL of ice-cold 4% paraformaldehyde in 100 mM PBS, pH 7.4. Brains were removed and post-fixed in the same fixative for 24 hours then cryoprotected in 20% sucrose overnight. Each brain was mounted ventral-side–up onto the stage of a freezing microtome and 50 μm horizontal sections were cut between 9.6 mm and 4.6 mm ventral to bregma.[31] Three horizontal sections through the anterior commissure, located approximately at 6.6, 6.8, and 7.1 mm ventral to bregma were selected and processed for in situ nick translation using a modification of the protocol developed by Wijsman et al.[32] and described in detail previously.[29] Briefly, free-floating sections were incubated in 2X saline sodium citrate (300 mM sodium chloride, 30 mM Na citrate, pH 7.0) at 80 °C for 20 minutes, and then treated for

10 minutes with pronase (1 μg/mL; Boehringer Mannheim). Digestion was stopped in 2% glycine, and the sections were briefly rinsed in H_2O and incubated for 1 hour at room temperature (RT) with 50 μg/mL DNA polymerase I (Promega), 10 μM each of biotin-21-dUTP (Clontech), dCTP, dATP, and dGTP dissolved in buffer A (50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 10 mM β-mercaptoethanol and 0.005% bovine serum albumin). Biotin end labeled DNA fragments were detected using avidin-biotin-peroxidase complex method (Vectastain Elite; Vector Labs, Burlingame, CA) with Nickel (II) sulfate intensification. The sections were mounted onto gelatin coated slides, dehydrated, cleared, and cover slipped in Permount (Fisher Scientific; Fair Lawn, NJ). Adjacent sections from each animal were stained with cresyl violet to allow assessment of neuronal loss.

Cell counts. To quantify DNA fragmentation, sections were analyzed by light microscopy at a final magnification of 100× and at 200×. Using a grid reticule, positively stained cells were counted independently by two observers and verified by a third observer on three horizontal sections located approximately at 6.6, 6.8, and 7.1 mm ventral to bregma.[31] The anterior commissure was used as a specific anatomical landmark to match sections across experiments. At each level of hippocampus, a rectangular grid consisting of 100 50 μm × 50 μm squares was superimposed on each region to be counted. A standard surface area was covered. Counts of positively stained cells per 12,500 μm^2 in each hippocampal subfield, hilus, CA4, CA3, and CA1, were made from both right and left hippocampii and averaged. A total of 204 hippocampal sections were analyzed from 34 animals (three sections per animal, two hippocampii per section).

Statistics. A two-way analysis of variance was used to compare water maze escape latencies over the 24 trials of place learning. For *t*-tests with multiple comparison groups, the Bonferroni correction was applied. The *z*-test or Fisher exact test was used to compare differences in proportion, and the Mann-Whitney rank sum test was used to compare seizure onset latency and number of positively stained hippocampal neurons between two groups. Values are expressed as mean ± SEM, and significance was defined as $p < 0.05$ for all tests.

Results. *KA seizures on P15 and P45.* On P15, KA produced clinically apparent seizures in 48/48 animals less than 20 minutes (seizure onset latency: 18.7 minutes ± 0.8) after IP injections. All but 5 animals (43/48, 90%) had Grade IV seizures that consisted of intense forelimb pawing or scratching, and then falling and continuous trembling of all extremities. One and 4 animals had Grade II or Grade III seizures, respectively. Four animals died in status epilepticus, a mortality rate of 8%. On P45, KA produced clinical seizures in 46/48 animals (seizure onset latency, 30.9 minutes ± 1.8) and 9 animals died in status epilepticus, a mortality rate of 19%. Although the mortality rate was not significantly different between mature and immature animals ($p = 0.309$, *z*-test), seizure onset latency was significantly shorter (18.7 minutes versus 30.9 minutes, $p < 0.001$, Mann-Whitney *U* test) and seizures were more severe in the immature animals. A significantly higher proportion of the immature animals developed status epilepticus (Grade IV) after systemic KA, 90% (43/48) compared with 54% (26/48) in the mature animals ($p < 0.001$, *z*-test).

Seizure susceptibility. To assess the effect of early life seizures on later seizure susceptibility, animals in the KK and SK groups were compared. No statistically significant difference in seizure severity was detected between animals in the SK and KK group ($p = 0.259$, Mann-Whitney Rank Sum Test). Seizure onset latency was comparable between KK and SK groups (KK: 27.6 minutes ± 3.6 versus SK: 30.9 minutes ± 1.8, $p = 0.081$, Mann-Whitney Rank Sum Test), as was mortality rate (KK: 28% (10/36) versus SK: 18% (3/16, $p = 0.673$, *z*-test). When stratified into mildly affected (seizure severity Grades 0, I, and II) and severely affected (seizure severity Grades III and IV), 72% (23/32) of animals subjected to prior KA injection (KK) developed Grade III or IV seizures, compared with 44% (7/16) of control animals (SK). This 28% difference between groups, although not statistically significant ($p = 0.115$, *z*-test), represents a trend indicating that an important difference in seizure severity might be detected if the sample size were higher.

Spatial learning and memory. On P50, 5 days after the second KA or saline injection, all surviving animals were tested in the Morris water maze (total n = 51, see the table). Compared with their littermate controls (SK), as well as to KS and SS animals, animals with prior seizures (KK) performed significantly worse in the Morris water maze after a second KA dose in adulthood (mean latency to escape (seconds) ± SEM: SK (n = 13); 252 ± 23 versus KK (n = 26); 327 ± 16, $p < 0.05$). Both KK and SK animals performed significantly worse than either SS or KS group. Performance of animals in the KS group did not differ significantly from that of SS control (KS [n = 6]; 155.4 ± 34 versus SS [n = 6]; 128.4 ± 34.2, $p > 0.05$) (figure 1).

DNA fragmentation and neuronal loss after KA-induced seizures. No DNA fragmentation was detected within the hippocampus in six of six animals killed 48 to 72 hours after KA-induced seizures on P15 or in KS animals killed on P55 (figures 2g and h). In one animal that had 5.5 hours of limbic status, a few labeled cells were noted in the lateral septum and reticular thalamic nucleus. Ten days after KA-induced seizures on P45, animals with prior KA-induced limbic status (KK) showed increased DNA fragmentation compared with littermates who received KA only as adults (SK). Thus, 92% (24/26) of KK animals showed DNA fragmentation in limbic structures including the amygdala, centromedial thalamus, pyriform cortex, lateral septum, and hippocampus after the second KA administration as compared with 54% (7/13) of SK group ($p < 0.006$) (figures 2e and f). In addition, a distinct pattern of DNA fragmentation within the hippocampus was observed in many KK animals. In 77% of KK animals (17/22) (figures 3B and C) hippocampal pyramidal cells in CA1, CA3, and CA4 (CA3 endfolium) were affected, as were hilar neurons and entorhinal cortical neurons. In contrast, in SK animals where the CA1 subfield showed the most consistent neuronal injury, CA3 neurons were involved to a lesser extent, and hilar neurons were only rarely involved (figure 3A). In adjacent sections stained for Nissl with cresyl violet, hilar neurons were grossly depleted in KK animals but largely spared in SK animals and unaffected in K and KS animals (figures 2a through d). Regional quantification of hippocampal DNA fragmen-

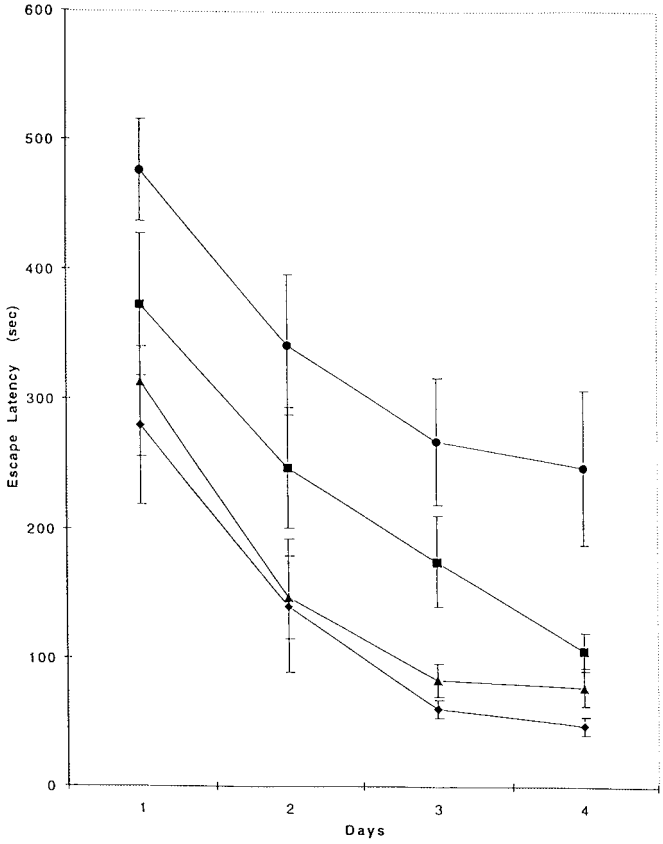

Figure 1. Acquisition of place learning in the Morris water maze. The mean total latency to escape in six trials (seconds ± SEM) is plotted on 4 successive days of testing. There was a significant difference in mean escape latencies between rats treated with kainic acid (KA) on P15 and P45 (KK) and those treated with saline on P15 and KA on P45 (SK). (Two-way analysis of variance with post hoc t-tests with Bonferroni corrections.) Note normal performance of the KS group (KA on P15 and saline on P45), not significantly different from saline controls (SS). N = 26 (KK), 13 (SK), 6 (KS), and 6 (SS). ● = KK; ■ = SK; ▲ = KS; ◆ = SS.

tation was performed on a total of 34 animals (figure 4). Five animals (four KK and one SK) were excluded from the quantitative analysis because of poor section quality in three, and no seizure after KA on P45 in two. The density of DNA fragmentation was significantly different between SK and KK group in hilus (positively stained cells per12,500 μm^2: 5.4 ± 2.5 versus 25.9 ± 5.5, $p = 0.015$) and CA4 (12 ± 7.1 versus 39.9 ± 8.8, $p = 0.045$), but not in CA3 (23.0 ± 7.1 versus 40.5 ± 8.5, $p = 0.207$), or CA1 (49.2 ± 12.9 versus 66.0 ± 8.6, $p = 0.273$).

Discussion. The main finding of our study is that compared with controls, animals subjected to early-life seizures sustained more extensive seizure-induced neuronal injury in adulthood. Moreover, compared with their littermates treated with saline on P15, animals with prior early-life seizures performed significantly worse in the Morris water maze after KA in adulthood. Our anatomic finding parallels the clinical observation that hippocampal sclerosis, assessed either by preoperative MRI or pathologic examination of surgical specimen after temporal lobectomy, is found in only 1% to 5% of patients with adult onset temporal lobe epilepsy, whereas it is found in 50% to 80% of those with antecedent seizures in infancy or early childhood,[1,5-7] while our behavioral result corresponds well with the clinical observation that in patients undergoing temporal lobectomy for intractable seizures, early onset of seizures is correlated with significantly poorer cognitive function.[33] An early initial insult may therefore be a critical factor underlying later seizure-induced hippocampal damage and associated cognitive deficits.

DNA fragmentation, a robust marker of neuronal injury, is noted in hippocampal hilar neurons and CA4 pyramidal neurons, in addition to CA3 and CA1 neurons, in the majority of KK animals with Grade II-IV seizures in adulthood, although it is virtually never seen in hilus or CA4 either in SK animals treated in the present study or in animals treated with KA only as adults.[29] Our examination of Nissl

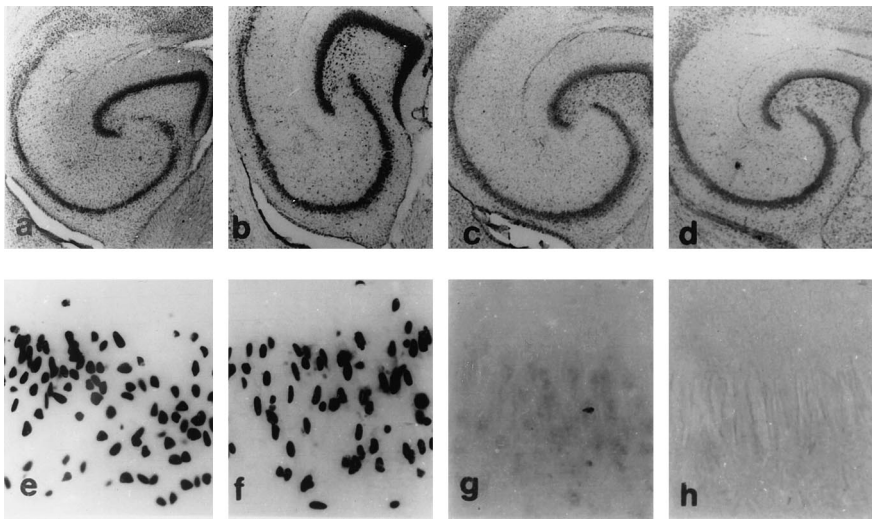

Figure 2. DNA fragmentation occurs only after KA seizures on P45, whereas hilar neuronal loss occurs only after repeated KA seizures on both P15 and P45. Cresyl violet Nissl stain of hippocampal sections (a through d) and DNA fragmentation in CA3 (e through h) in adjacent sections from animals treated with KA on P15 and P45 (KK) (a and e), saline on P15 and KA on P45 (SK) (b and f), KA on P15 and saline on P45(SK) (c and g), or KA on P15 (K) (d and h). Animals were killed on P55. H-E, original magnification × 50 (a through d) or × 200 (e through h) before 19.4% reduction.

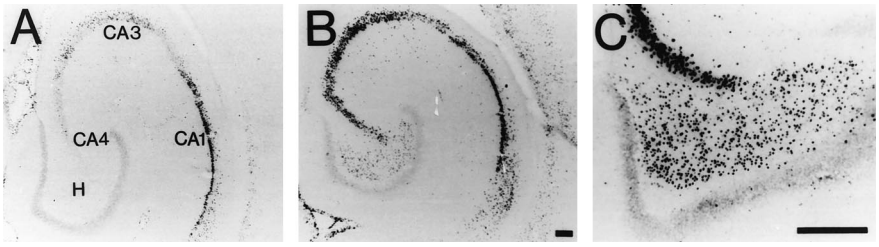

Figure 3. DNA fragmentation in the hippocampus 10 days after KA seizures on P45. (A) Animal treated with saline on P15 and KA on P45 (SK) H indicates dentate hilus. (B) Animal treated with KA on P15 and P45 (KK). (C) Higher magnification view of KK hippocampus from another animal showing extensive hilar and CA4 neuronal injury. Scale bar = 200 μm.

stained sections confirms severe hilar neuronal loss in KK animals, but not in SK or KS animals. Because hippocampal sclerosis in patients with intractable temporal lobe epilepsy invariably involves hilar as well as CA1 and CA3 neurons,[34] animals exposed to early-life seizures appear to simulate the neuropathology of temporal lobe epilepsy more closely than those treated only as adults.

We chose to assess injury using in situ detection of DNA fragmentation. This assay offers cellular resolution, preserves anatomic information, yields reliable information as soon as 24 hours after kainate treatment with a maximal signal at 48 to 72 hours, and can be easily quantitated.[29] Because the technique yields dense nuclear staining, it offers a high signal-to-noise ratio, and both positive and negative controls are readily available. Although DNA fragmentation has been associated with apoptosis, wider use of this technique indicates that the finding of DNA fragmentation is not specific for any particular mechanism of cell death.[29,35,36] It thus remains unclear whether the neuronal injury we have documented in this study is apoptotic, necrotic, or perhaps both.

We considered the possibility that anatomic and behavioral changes seen in KK animals were because of more severe seizures in this group. Although group comparison of all treated animals did not demonstrate a statistically significant difference in seizure severity between our experimental cohorts, a trend toward more severe seizure in adult animals with prior KA on P15 may, at least in part, account for increased neuronal injury and behavioral deficits observed in these KK animals compared with SK controls. We also considered the possibility that increased hippocampal injury after the second KA treatment in animals subjected to KA treatment on P15 (KK) compared with littermates treated with saline on P15 and KA on P45 (SK) was an additive effect from repeated injury, but we rejected this possibility because seizures only on P15 (KS and K groups, see the table) caused neither long-term behavioral deficit nor neuronal injury in our study, as previously shown by others.[14,15,17,37] We also considered but rejected the possibility that spontaneous recurrent seizures occurring after kainate on P15 resulted in increased injury in KK animals. Other groups have emphasized that spontaneous seizures after convulsant treatment before P21 occur rarely if at all.[16,25] Furthermore, if spontaneous seizures were responsible for the differences observed we would have expected to see evidence of injury in KS animals; however, no injury was observed in this group.

Whereas pilocarpine[25] and perforant path stimulation[26] have each been associated with injury in immature animals in some reports, kainate-induced seizures have not been associated with injury before P20 and did not result in detectable injury when

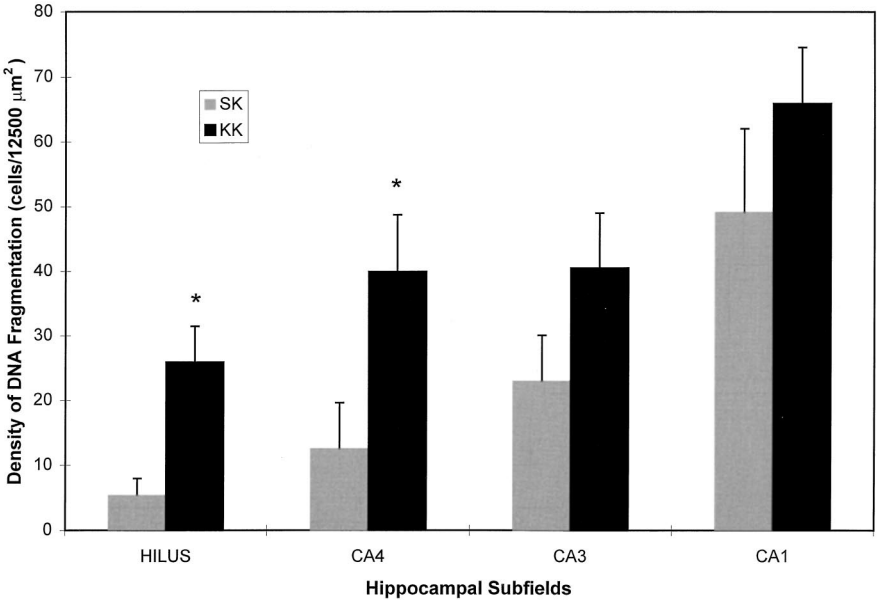

Figure 4. Regional quantification of hippocampal DNA fragmentation (means ± SEM). Density of stained neurons is plotted for indicated regions of hippocampus. The difference between groups was statistically significant in dentate hilus and CA4, but not in CA3 or CA1 (*). n = 26 (KK), 13 (SK).

administered on P15 in our study. It is therefore possible that the kainate model represents a special situation. For example, it is possible that the enhanced effect of KA on P45 in KK animals is because of enhanced penetration of KA into brain or a shift in the concentration-response relationship that somehow results from the initial treatment on P15. Our inability to show a significant behavioral difference in the response to KA in adult animals previously treated on P15 argues against this possibility but does not completely rule it out. Additional studies using other seizure models that do not cause injury on P15 are needed to clarify this issue.

Our finding that detrimental long-term pathologic and behavioral consequences of early KA seizures became evident only when the animals were subjected to another insult later in life highlights the possibility that early KA seizures may alter neuronal connectivity or function, even in the absence of detectable neuronal injury or cell death, in a manner that increases susceptibility to later seizure-induced damage. During normal development, neurons as well as axons and synaptic terminals are overproduced and subsequently eliminated, apparently as a result of a competition between afferents for a limited supply of target-derived substance.[38] In rat CA3 hippocampal pyramidal cells, e.g., an exuberant outgrowth of axons takes place by P10, and axon length, number of varicosities, and axon branch points reach up to 225% of mature levels during the second postnatal week, coincident with the period of enhanced seizure susceptibility.[39] Synaptic activity is critical for the process of refinement of connections between axons and their appropriate targets.[40,41] It is thus conceivable that a prolonged seizure, via intense synchronized neuronal activation, could have a permanent effect at this critical time of afferent-dependent synaptic remodeling. A precedent for this notion can be found in a study in which continuous infusion of penicillin was used to induce epileptiform activity in the visual cortex of rabbits from P5-P21.[42] In penicillin-exposed rabbits, visual callosal projections extended through most of area 17 in a projection pattern characteristic of neonatal rabbits, rather than being restricted to a narrow zone at the lateral border of area 17 as seen in normal mature animals, suggesting that epileptic cortical activity stabilized immature callosal projections that are normally eliminated during development.

Previous studies have shown that in rats with a toxin-induced neuronal migration defect, hyperthermia may result in hippocampal injury independent of seizures.[43] Moreover, animals with neuronal migration defects are more susceptible to kainate and kindling while immature and as adults.[44,45] Together, these studies along with ours suggest that a variety of insults, including but not limited to seizures, during development can increase the susceptibility to seizures in adults and enhance their injurious effect.

Acknowledgment

The authors thank Dr. Gregory Holmes for lending his water maze apparatus, Dr. Zhao Liu for his help with the spatial maze test and data analysis, and Alexander J. Yeats, Jr., for his technical assistance.

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