Resistance of Immature Hippocampus to Morphologic and Physiologic Alterations Following Status Epilepticus or Kindling

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ABSTRACT: Seizures in adult rats result in long-term deficits in learning and memory, as well as an enhanced susceptibility to further seizures. In contrast, fewer lasting changes have been found following seizures in rats younger than 20 days old. This age-dependency could be due to differing amounts of hippocampal neuronal damage produced by seizures at different ages. To determine if there is an early developmental resistance to seizure-induced hippocampal damage, we compared the effects of kainic acid (KA)-induced status epilepticus and amygdala kindling on hippocampal dentate gyrus anatomy and electrophysiology, in immature (16 day old) and adult rats. In adult rats, KA status epilepticus resulted in numerous silver-stained degenerating dentate hilar neurons, pyramidal cells in fields CA1 and CA3, and marked numerical reductions in CA3c pyramidal neuron counts (−57%) in separate rats. Two weeks following the last kindled seizure, some, but significantly less, CA3c pyramidal cell loss was observed (−26%). Both KA status epilepticus and kindling induced mossy-fiber sprouting, as evidenced by ectopic Timm staining in supragranular layers of the dentate gyrus. In hippocampal slices from adult rats, paired-pulse stimulation of perforant path axons revealed a persistent enhancement of dentate granule-cell inhibition following KA status epilepticus or kindling. While seizures induced by KA or kindling in 16-day-old rats were typically more severe than in adults, the immature hippocampus exhibited markedly less KA-induced cell loss (−22%), no kindling-induced loss, no detectable synaptic rearrangement, and no change in dentate inhibition. These results demonstrate that, in immature rats, neither severe KA-induced seizures nor repeated kindled seizures produce the kind of hippocampal damage and changes associated with even less severe seizures in adults. The lesser magnitude of seizure-induced hippocampal alterations in immature rats may explain their greater resistance to long-term effects of seizures on neuronal function, as well as future seizure susceptibility. Conversely, hippocampal neuron loss and altered synaptic physiology in adults may contribute to increased sensitivity to epileptogenic stimuli, spontaneous seizures, and behavioral deficits. *Hippocampus 2001;11:615–625.*

KEY WORDS: CA1; CA3; kainic acid; seizures; development; epilepsy; dentate gyrus

INTRODUCTION

Severe seizures in both pubescent and adult rats can produce persistent neuronal dysfunction, resulting in deficits in learning and memory (Dos Santos et al., 2000; Sarkisian et al., 1998; Stafstrom et al., 1993; Thurber et al., 1992), and an enhanced susceptibility to further seizures (Holmes, 1991; Okada et al., 1984; Stafstrom et al., 1992). Damage in the hippocampus, a structure implicated in both memory acquisition and seizure expression, may be an important contributor to these effects. Specific populations of hippocampal neurons are highly susceptible to damage from seizures evoked by a wide range of convulsants, including kainic acid (KA) (Sloviter and Damiano, 1981; Franck and Schwartzkroin, 1984; Nitecka et al., 1984; Sperber et al., 1991), kindling (Cavazos and Sutula, 1990), flurothyl (Nevander et al., 1985;

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Sperber et al., 1999), bicuculline (Ben-Ari et al., 1981), pilocarpine (Cavalheiro et al., 1987), and picrotoxin (Olney et al., 1986). Seizure-susceptible neurons include mossy cells and somatostatin/neuropeptide Y-containing interneurons of the dentate hilus, and pyramidal cells in areas CA1 and CA3 (Freund et al., 1992; Sloviter, 1987).

Dentate granule cells, GABAergic hilar interneurons, and CA2 pyramidal cells are comparatively resistant to seizure-induced death (Sloviter, 1991). Although dentate gyrus granule-cell numbers are not reduced following seizures, they do undergo dramatic morphologic alteration. Seizures elicited by systemic KA (Cronin and Dudek, 1988; Sperber et al., 1991; Tauck and Nadler, 1985) or electrical kindling (Sutula et al., 1988) induce granule-cell axons to sprout collaterals which form abnormal ectopic synapses in molecular layers of the dentate. Studies indicate that this new recurrent excitatory pathway terminates on both granule cells (Kotti et al., 1997; Okazaki et al., 1995) and inhibitory interneurons (Kotti et al., 1997), potentially enhancing synaptic excitation and inhibition onto granule cells. Experimentally, examination of seizure-induced granule-cell hyperexcitation requires pharmacologic blockade of synaptic inhibition (Cronin et al., 1992; Tauck and Nadler, 1985), suggesting that hyperexcitability may be present, but masked by a concomitant enhancement of synaptic inhibition (Haas et al., 1996). These seizure-induced morphologic and physiologic alterations are persistent and may not only disrupt normal hippocampal neuronal function, but may also be a focus for future seizure initiation.

A controversial issue in clinical epilepsy is whether significant pathophysiology accompanies seizures occurring early in development. The high incidence of seizures in young infants and children (Gibbs and Gibbs, 1963; Hauser and Kurland, 1975), has raised concerns that these seizures could induce hippocampal alterations predisposing individuals to epilepsy later in life. Indeed, it has been suggested that hippocampal sclerosis associated with temporal lobe epilepsy in adults may arise from seizures which occurred during a seizure-prone period in early development (Falconer et al., 1964), and it has been reported that children with severe epilepsy can exhibit evidence of neuronal loss and synaptic reorganization (Mathern et al., 1994).

To test the validity of this theory, we compared the effects of experimentally induced seizures in 2-week-old and adult rats. Rats in the first 3 postnatal weeks are more sensitive to epileptogenic stimuli (Moshé and Albala, 1982, 1983; Cavalheiro et al., 1987; Okada et al., 1984), and exhibit more severe seizure behavior (Albala et al., 1983; Cavalheiro et al., 1987; Haas et al., 1990) compared to adults, consistent with the heightened incidence of epilepsy in human infants and young children. However, seizures during this developmental window are not obligatorily associated with long-lasting behavioral alterations, such as disruption of learning acquisition (Sarkisian et al., 1998; but see Dos Santos et al., 2000; Lynch et al., 2000), or a generalized enhanced susceptibility to epileptogenic agents (Okada et al., 1984; Stafstrom et al., 1993; but see Dubé et al., 2000). To determine whether seizures early in development produce hippocampal damage correlated with changes in inhibition, we examined hippocampal anatomy and synaptic physiology after systemic KA or amygdala kindling. While marked cell loss, synaptic rearrangement, and altered dentate inhibition were induced by seizures in adults, seizures induced significantly less cell loss, and no change in these other parameters in the hippocampus of immature rats. A portion of these results was reported previously (Sperber et al., 1991).

MATERIALS AND METHODS

Seizure Induction

Kainic acid-induced status epilepticus

Fifteen immature rats (male and female), age postnatal day 16 (P16), and 15 adult rats (male P60), all Sprague-Dawley (Taconic Farms, Germantown, NY), were injected intraperitoneally with kainic acid (KA; 5 mg/kg and 15 mg/kg, respectively). KA (Sigma Chemicals, St. Louis, MO) was prepared in phosphate-buffered saline, pH 7.2. The doses chosen reliably produced long-duration, severe seizures in both age groups (Okada et al., 1984). Animals were monitored continuously for 5 h after injection and then intermittently for 48 h. In order to examine the effects of severe seizures, the few rats which exhibited seizure behavior for less than 30 min were excluded from this study.

Kindling

Ten P14 (male and female) and 10 adult (male, P60) rats were implanted with electrodes in the amygdala on one side of the brain selected at random, and kindled as described previously (Haas et al., 1990). Briefly, adult rats were anesthetized with a combination of xylazine (6 mg/kg) and ketamine (70 mg/kg), while immature rats received a 1:10 dilution of the adult dose. Twisted bipolar electrodes (MS 303/2, Plastics One, Norfolk, VA) were implanted using the following coordinates (referenced to bregma, mouth bar set at −3.5 mm). For P14 rats, coordinates were 1.7 mm anterior, 3.5 mm lateral, and 7.4 mm ventral; for adults, 2.0 mm posterior, 3.9 mm lateral, and 9.5 mm ventral. Electrodes were held in position with skull screws and dental acrylic. Kindling stimuli (1 s, 400 μA, 60 Hz, biphasic current) were initiated 1 day after surgery for immature rats, and 1 week in adults. To avoid problems inherent in the study of developmental epilepsy, such as brain growth and displacement of implanted electrodes with time, stimulation was delivered at 15-min intervals over 2 days. This stimulation paradigm was feasible, since immature rats do not demonstrate kindling-refractory periods when stimulation is delivered at short intervals (Moshé and Albala, 1983). In adult rats, interference between kindling stimuli, due to postictal-refractory periods, was avoided by using interstimulus intervals of 3 h. Kindling seizure behavior in adults was scored using the 5-stage classification scale of Racine (1972). For P16 rats, this scale was modified to include stage 6 (wild running and jumping) and stage 7 (tonus), which are readily evoked in immature rats following relatively few stimulations (Haas et al., 1990). Stimulation was terminated after five

stage 5 seizures in adult rats, and after five stage 5–7 seizures in P16 rats.

Histology

Silver stain

Acute neuronal damage was assessed 24 h following KA-induced status epilepticus using the silver impregnation technique of Gallyas et al. (1980), as modified by Nadler and Evenson (1983), which marks degenerating neurons. KA-treated adult (n = 5) or P16 rats (n = 5), and age-matched controls (n = 5 for each age), were anaesthetized with sodium-pentobarbital and perfused transcardially with ice-cold saline (0.9%), followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Brains were removed and fixed overnight in a 30% sucrose solution to prevent freezing artifacts, and then sectioned 50 μm thick on a cryostat (−18°C). The staining protocol involved washing in distilled H_2O three times for 5 min, followed by pretreatment in 9% NaOH plus 1.2% NH_4NO_3 two times for 5 min, and impregnation for 8 min in 9% NaOH, 16% NH_4NO_3, and 50% $AgNO_3$. The tissue was then rapidly washed (three times for 5 min in 1.2% NH_4NO_3 plus anhydrous Na_2CO_3 in 95% EtOH and dH_2O), and left in developer (1.2% NH_4NO_3, 0.5 g anhydrous citric acid in 37% formalin, 95% EtOH, and 9% NaOH, pH 5.8–6.1) for at least 1 min. Sections were dehydrated in a series of ethanols (70–100%), and mounted onto gelatin-dipped slides. Alternate sections were stained with Timm or thionin stain for cell-loss determination, as described below.

Timm and thionin stain

Two weeks following either KA injection or the last kindled seizure, treated rats and age-matched controls were sacrificed. In KA experiments, half of each brain was used for histology and half for electrophysiology. In each Timm's stain processing run, KA and control sections were processed concomitantly. Techniques for fixing and staining hippocampal tissue with Timm stain have been described in detail elsewhere (Laurberg and Zimmer, 1980; Nadler et al., 1980; Sperber et al., 1991; Tauck and Nadler, 1985). Brains were cut horizontally in 1-mm-thick sections, and slices were submerged in 0.4% sodium sulfide for 20 min, before overnight fixation in a 1% paraformaldehyde, 1.25% glutaraldehyde solution. To prevent freezing artifacts, brains were placed in a 30% sucrose solution overnight. Brains were cut in a cryostat (30 μm thick), and mounted on gelatinized slides. Mounted sections were developed for 45–60 min in the dark, in a solution of 20% (w/v) gum arabic, 5.6% (w/v) hydroquinone, 17 % silver nitrate, and citric acid-sodium citrate buffer. Alternate sections were stained with thionin, and dehydrated in alcohol for examination of cell loss. In kindled rats we initially used the same approach: half of each brain was processed for histology, and half for electrophysiology. For technical reasons, the Timm stain did not work in kindled adult rats or concurrently run controls, so we kindled another group of rats and performed the stains. Therefore, in the kindling experiments, we could not correlate Timm stain data with electrophysiologic data from the same rats.

Stereological Cell Counts

Quantitative estimation of neuronal numbers per unit volume was performed using stereological methods (West and Slomianka, 1998; West, 1999). Two weeks following KA-induced seizures, kindled seizures, or saline injections, rats were euthanized and perfused transcardially with 4% paraformaldehyde, and their brains were dissected out and stored in 4% phosphate-buffered formalin for 6–12 months. Then, both hippocampi were dissected out, mounted in thin celloidin, and cut into 70-μm-thick transverse sections. Sections were stained for 60 s in Cresyl violet, serially rinsed for 5 min each in 2 × 70% EtOH and then 3 × 95% EtOH, and mounted in Permount for microscopic evaluation.

CA3c and CA1 pyramidal cell numbers were estimated stereologically in four representative sections from each rat as follows. Using a Nikon Eclipse E1000 microscope, 4,000× photographs of CA3c or CA1 pyramidal-cell layers were taken by focusing down through the hippocampus in 2-μm steps through the center 50 μm of each section. Twenty-five photos were acquired with a CCD color camera using NIH Image v1.61, and combined into a stack for analysis. In each field of CA3c or CA1, four separate regions of interest (ROI) of approximately equal area completely within the pyramidal-cell body layer were outlined, and each pyramidal neuron completely within the ROI was counted only once at its position of maximal focus. All counts were performed by the same individual, who was blinded to the experimental condition. For each ROI, area was calculated and multiplied by the 50-μm depth to yield volume, and each ROI count was corrected per unit volume before all four per section were averaged. The mean counts per volume for each section were averaged to yield a single pyramidal-cell number per 100 μm^3.

Electrophysiology

The brain halves not used for histochemistry (contralateral to the side stimulated when kindled) were rapidly transferred to an ice-cold bath of artificial cerebrospinal fluid (ACSF). The combined hippocampus and entorhinal cortex were dissected and sliced, 400 μm thick, using a submerged vibratome (Ted Pella, Inc., Redding, CA). Slices were transferred to an interface perfusion chamber (Haas et al., 1979), and bathed in heated (33–34°C) oxygenated ACSF (perfusion rate 2 ml/min) containing in mM: NaCl 126, KCl 5, NaH_2PO_4 1.25, $MgSO_4$ 2, $CaCl_2$ 2, $NaHCO_3$ 26, and glucose 10, pH 7.2. Slices were constantly exposed to humidified 95% O_2/5% CO_2 from above.

We recorded extracellular field potentials using glass micropipettes filled with 2 M NaCl (1–5 MΩ) placed in stratum granulosum of the suprapyramidal blade of the dentate gyrus. Population action potentials from granule cells were elicited by stimulation of the perforant path with a bipolar, Teflon-coated, stainless steel electrode (tip diameters, 25 μm). Stimulation intensity (100–300 μA) was selected to elicit 75% of maximal response. Inhibitory synaptic transmission on granule cells was measured with a paired-pulse paradigm in which two identical stimulations were delivered to the perforant path, with interstimulus intervals ranging from 10 ms to 9 s. The percent change in amplitude of the second population spike compared to the first was used as a measure of net

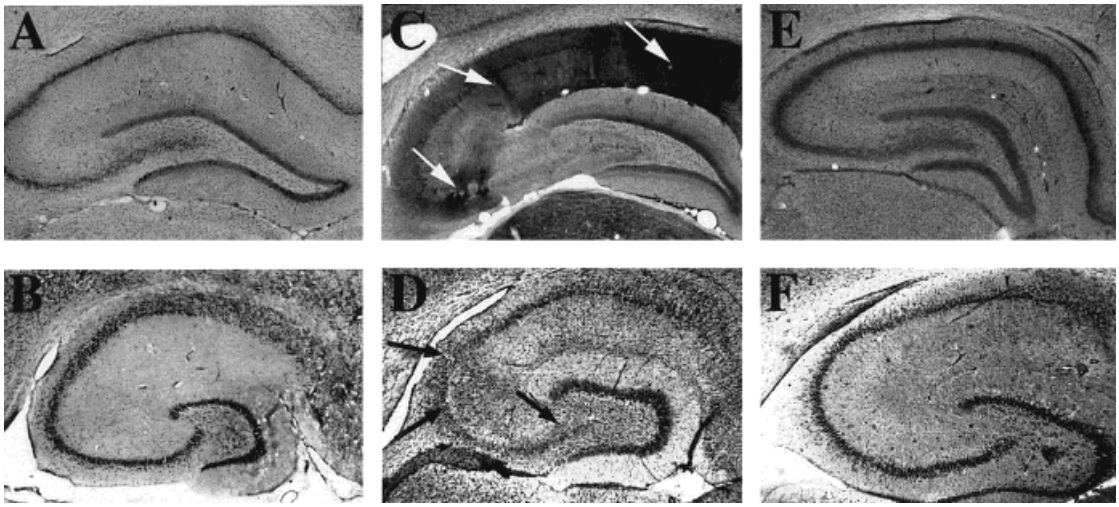

FIGURE 1. Cell loss following kainic acid (KA) status epilepticus in adult, but not P16 rats. A: Silver stain of adult control hippocampus, demonstrating background staining without dark argyrophilic cells. B: Thionin cell stain of adult control, showing pyramidal- and granule-cell body layers. C: Silver impregnation for degenerating cells, darkly stained neurons, and their processes in the adult hilus, CA3, and CA1 24 h after seizures. Arrows point to groups of argyrophilic CA3 and CA1 pyramidal cells. Note large amount of stained processes in stratum radiatum of CA1. D: Thionin in adults 2 weeks post-KA status epilepticus shows large CA3 lesion (arrows). E: Silver impregnation 24 h after KA status epilepticus in P16 rats demonstrates no darkly staining argyrophilic cells, indicating no cellular degeneration. F: No cell loss is apparent with thionin 2 weeks after KA status epilepticus in P16 rats.

inhibition or facilitation. Data were statistically analyzed with a one-way ANOVA and Fisher post hoc tests, with significance set at $P < 0.05$. All values are expressed as mean ± SEM.

RESULTS

Seizures

Systemic KA produced severe seizures in both P16 and adult rats, beginning approximately 30 min postinjection in P16, and 60 min postinjection in adults. Seizure behavior started with intermittent mouth clonus and head nodding, followed by forelimb clonus. As seizures progressed in P16 rats, animals lay on their sides and exhibited continuous fore- and hindlimb clonus. Adults developed continuous forelimb clonus, interspersed with rearing and falling, and occasional jumping. In both age groups, continuous seizures lasted for approximately 1–3 h, after which rats demonstrated a period of quiescence (typically 30–60 min), at times interrupted by brief bouts of seizures.

All rats implanted with electrodes developed fully kindled foci in response to repeated amygdala stimulation. The rate of kindling progression was not significantly different between age groups (20.4 ± 0.8 stimulations to reach five consecutive seizures greater than or equal to stage 5 for P16, and 17.8 ± 1.2 for adult rats, $P > 0.20$, Student's *t*-test). However, kindled seizure behavior was more severe in P16 rats, often progressing from stage 4 directly to stage 6 (wild running and jumping) and stage 7 (tonus). Severe kindled seizures (stages 6 and 7) were not observed in adults.

Seizure-Induced Histological Alterations

Silver-stained degenerating neurons

In adult rats, silver impregnation 24 h post-KA status epilepticus resulted in dark, argyrophilic cells throughout the hippocampus (Fig. 1C). In the dentate hilus, most argyrophilic neurons had morphologies typical of mossy cells (Ribak et al., 1985; Scharfman and Schwartzkroin, 1988). Pyramidal cells in areas CA1 and CA3 were also darkly stained. In CA3, degenerating pyramidal cells tended to appear in closely associated groups, while in CA1, individual argyrophilic cells were dispersed, separated by nonstained cells. Degenerating neuronal processes were also stained in the hilus and stratum radiatum. Silver stain of sections of adult control hippocampus demonstrated no argyrophilic, degenerating cells, or processes (Fig. 1A). Cell stain with thionin 2 weeks after KA status epilepticus demonstrated extensive loss of pyramidal cells, most pronounced in CA3 (Fig. 1D).

Silver stain of sections from immature rats exposed to KA status epilepticus suggest that these seizures produced little or no hippocampal cell death at this age (Fig. 1E). In contrast to the extensive neuronal degeneration seen after KA seizures in adults, no argyrophilic, degenerating cells were observed after KA status epilepticus in P16 rats. No difference was apparent between KA-treated immature rats and age-matched controls. In addition, KA status epilepticus at P16 did not produce any lesions detected with thionin stain 2 weeks after seizures (Fig. 1F).

Stereological neuron counts

Neuronal cell counts were performed using stereological methods (West and Somianka, 1998; West, 1999) in experimental and

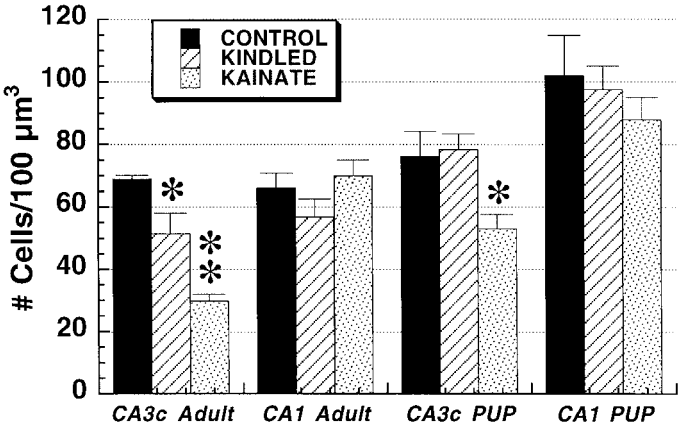

FIGURE 2. Stereological quantification of neuronal density changes in fields CA3c and CA1 following KA and amygdala kindling-induced seizures. Mean ± SEM number of cells per 100 μm³ volume in representative regions of fields CA3c and CA1 pyramidal-cell layers of control (solid bars), kindled (hatched bars), and KA-treated (stippled bars) rats. KA seizures produced marked loss of CA3c pyramidal neurons in adult rats ($P < 0.05$, compared to control CA3c), and a lesser, but significant, loss in field CA3c of P16 rat pups (*$P < 0.05$, compared to both controls and adult KA-treated rats). Kindled seizures produced lesser, but significant, loss of CA3c pyramidal neurons in adult rats (*$P < 0.05$, compared to control CA3c), in contrast to no significant neuron loss in P16 rats.**

control rats in both age groups. The data revealed that seizures in adults produced significant cell loss in the CA3 region, which was more pronounced in the KA-status epilepticus than the kindled group ($F2,9 = 34.9$, $P < 0.0001$). Moreover, cell counts confirmed the relative resistance of 2-week-old rat pups to both KA- and kindling-induced cell loss ($F2,10 = 6.4$, $P < 0.02$). Figure 2 summarizes the results of these cell counts, all of which were performed by the same individual, who was blinded to the experimental treatment. In adult rats, KA treatment that evoked status epilepticus produced a selective, 57% loss of CA3c pyramidal neurons (Fig. 2, stippled bar with two asterisks, $P < 0.05$ compared to controls). In contrast, KA-induced seizures in 2-week-old rat pups was associated with significantly less cell loss (22%) in field CA3c (Fig. 2, stippled bar, single asterisk, $P < 0.05$ compared to both KA-treated adults and controls), and CA1 pyramidal cell numbers were not significantly altered by KA in either group. Similarly, kindled seizures in adult rats produced a selective 26% cell loss of CA3c pyramidal neurons (Fig. 2, hatched bar with asterisk, $P < 0.05$) that was present 2 weeks after the last seizure, while 2-week-old rats pups showed no significant cell loss in any area.

Since hippocampal sections from KA-treated pups and age-matched controls could not be distinguished visually, even though some cell loss was observed with cell counts, we also measured average CA3c cell layer thickness in these two groups. Control pup CA3c pyramidal cell layer thickness was 52.3 ± 3.6 μm, while KA-treated pup cell layer thickness was 17% larger (61 ± 4.5 μm), although this difference did not reach statistical significance.

Timm stain for mossy-fiber reorganization

The Timm stain of mossy-fiber terminals has proven useful for identifying synaptic reorganization in the dentate gyrus associated with loss of granule-cell afferents and mossy-fiber efferents (Haug, 1967). The normal mossy-fiber terminal fields, seen in Timm stain of controls (Fig. 3A), included the hilus and proximal dendrites of CA3 pyramidal cells. As reported previously (Sperber et al., 1991), KA status epilepticus in adult rats results in mossy-fiber synaptic reorganization which can be demonstrated by aberrant Timm staining in dentate supragranular layers 2 weeks following seizures (Fig. 3C). Abnormal supragranular Timm staining following KA status epilepticus in adults was dense and extended from the suprapyramidal and/or infrapyramidal blades completely around the dentate crest (Fig. 3D). Mossy-fiber reorganization was also present, though less pronounced, following kindling in adult rats (Fig. 3E). Timm stain in supragranular layer in sections from amygdala-kindled adult rats was lighter than in KA-treated adults, and concentrated at the ends of the blades of the stratum granulosum. As in KA-treated rats, supragranular Timm staining was present in slices from all kindled adults, but completely absent from adult controls.

Timm stain of hippocampal sections 2 weeks after seizures in immature rats further supported the absence of damage at this age. Neither KA status epilepticus (Fig. 3F,G) nor amygdala kindling (Fig. 3H) in P16 rats produced aberrant Timm staining in supragranular layers of the dentate gyrus. There was no difference in mossy-fiber terminal fields in rats receiving acute KA-severe seizure at P16, rats receiving repeated brief kindled seizures at P15–16, or age-matched controls. These results are consistent with an absence of detectable neuronal loss of a type thought to promote mossy-fiber synaptogenesis after seizures in adults.

Age-Dependent Changes in Perforant Path Paired-Pulse Inhibition

Electrophysiological recordings in hippocampal slices from each group demonstrated age-dependent effects of seizures on paired-pulse inhibition in dentate granule cells. Paired-pulse stimulation of the perforant path in slices from control adults (n = 25 slices from 8 rats) produced a triphasic profile (Oliver and Miller, 1985; Haas et al., 1996) (Fig. 4A, solid circles). An initial inhibitory component at interstimulus intervals (ISIs) ranging from 10–30 ms was followed by facilitation of the second population spike at interval between 30–120 ms. A late, long-lasting inhibition predominated at intervals from 120 ms to 6 s.

As reported previously (Milgram et al., 1991; Sperber et al., 1991; Haas et al., 1996), KA status epilepticus in adult rats (n = 23 slices) produced marked enhancement of paired-pulse inhibition at ISIs ranging from 10–500 ms (Fig. 4A, open circles with asterisk, $P < 0.05$ compared to controls, one-way ANOVA). Amygdala kindling in adult rats also induced enhancement of the early inhibitory components of paired-pulse profiles in slices examined 2 weeks following the last kindled seizure (n = 18 slices, Fig 4B; open circles with asterisk, $P < 0.05$ compared to controls, one-way ANOVA). At paired-pulse ISIs of 20–90 ms and 120 ms, dentate

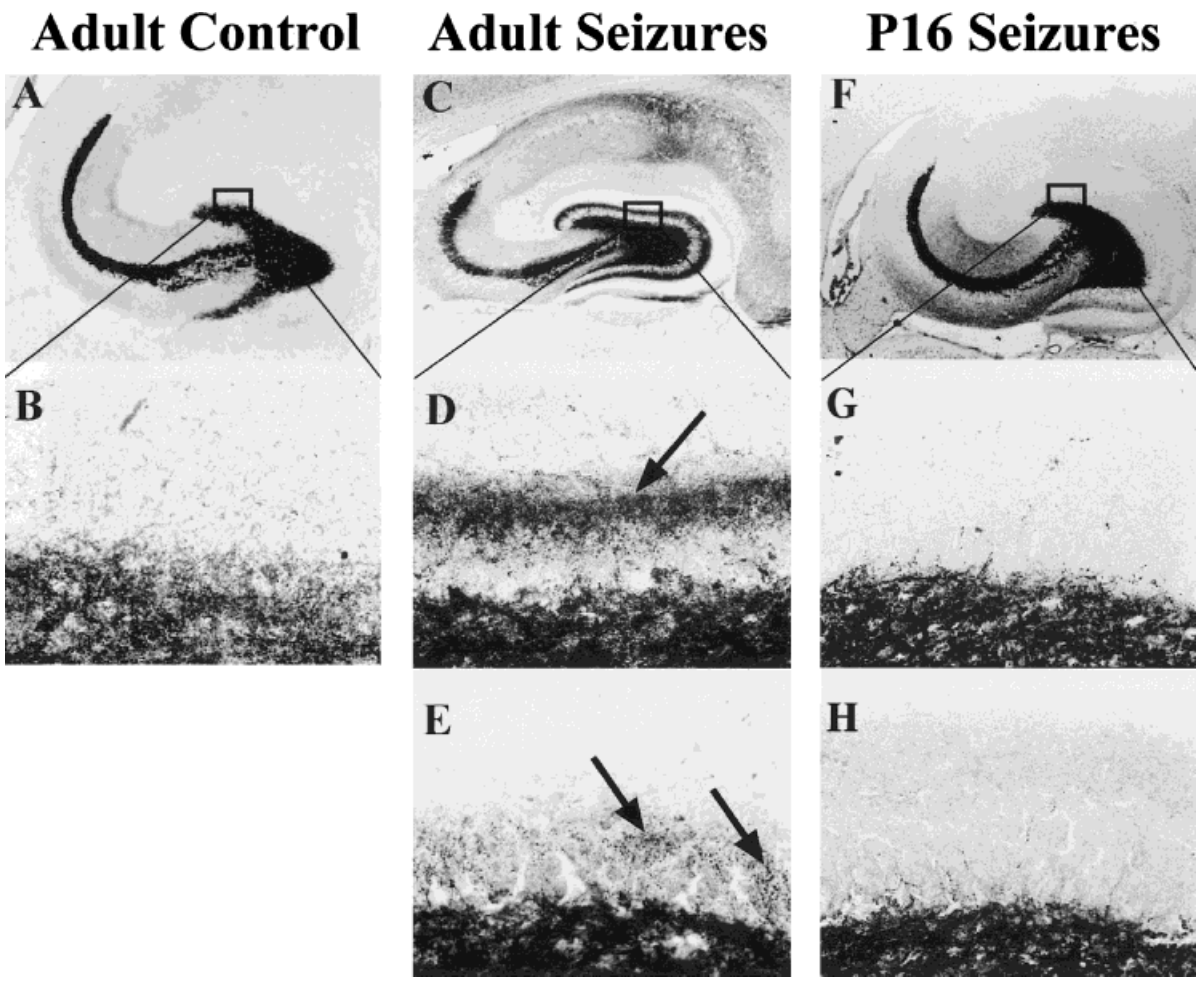

FIGURE 3. Timm silver sulfide stain of zinc in mossy-fiber terminals demonstrates synaptic reorganization 2 weeks post-KA status epilepticus and kindling in adult rats, but not after seizures in P16 rats. A: Timm silver sulfide staining of control adult hippocampus darkly stains mossy-fiber terminals in the hilus and proximal dendrites of CA3 pyramidal cells. B: Enlargement of the hilus and suprapyramidal blade of control adult (region indicated by box in A). C: Timm stain of adult hippocampus 2 weeks post-KA status epilepticus, demonstrating dense supragranular band of ectopic mossy-fiber terminals. D: Enlargement of adult hippocampus following KA status epilepticus, as indicated by box in C, with arrow pointing to dense zone of ectopic supragranular mossy-fiber terminals. E: Timm staining 2 weeks after kindling in an adult rat. Arrows mark patches of sparse supragranular positive Timm stain. F: Timm stain 2 weeks post-KA status epilepticus in P16 rat. Positive Timm stain was restricted to normal mossy-fiber termination zones, including the hilus and CA3 proximal dendrites. G: Enlargement of suprapyramidal blade of the dentate of F. No supragranular staining was observed. H: Timm stain 2 weeks after kindling in P16 rats.

inhibition was significantly greater in slices from kindled adults compared to controls (asterisks, $P < 0.05$, one-way ANOVA). Overall, the profiles of dentate paired-pulse inhibition in slices from KA-treated and kindled adults were biphasic, not triphasic, indicating a complete absence of any facilitatory component.

Paired-pulse profiles from immature control rats (P30) were triphasic, as in slices from adult controls. Neither KA status epilepticus (Fig. 5A, open circles, n = 19 slices) nor amygdala kindling (Fig. 5B, open circles, n = 21 slices) in P16 rats produced any significant alterations in perforant path paired-pulse profiles (one-way ANOVA). This is in sharp contrast to the marked enhancement in inhibition seen in adult slices following severe seizures.

Correlation of Timm's Mossy-Fiber Staining With Changes in Perforant Path Inhibition

While, in general, immature (P16) rats exhibited no detectable changes in supragranular Timm's staining or paired-pulse inhibition, we compared these two phenomena more quantitatively in KA-treated rats (where half of each brain was studied histologically, and the other half electrophysiologically) to determine whether these changes were correlated from animal to animal. We examined two paired-pulse interstimulus intervals, 20 and 500 ms, corresponding to early $GABA_A$ and late $GABA_B$ receptor-mediated inhibition. Timm's staining was quantitated on a 5-point scale modified from Tauck and Nadler (1985), according to the density of terminals in stratum moleculare: 0 = no staining, 1 =

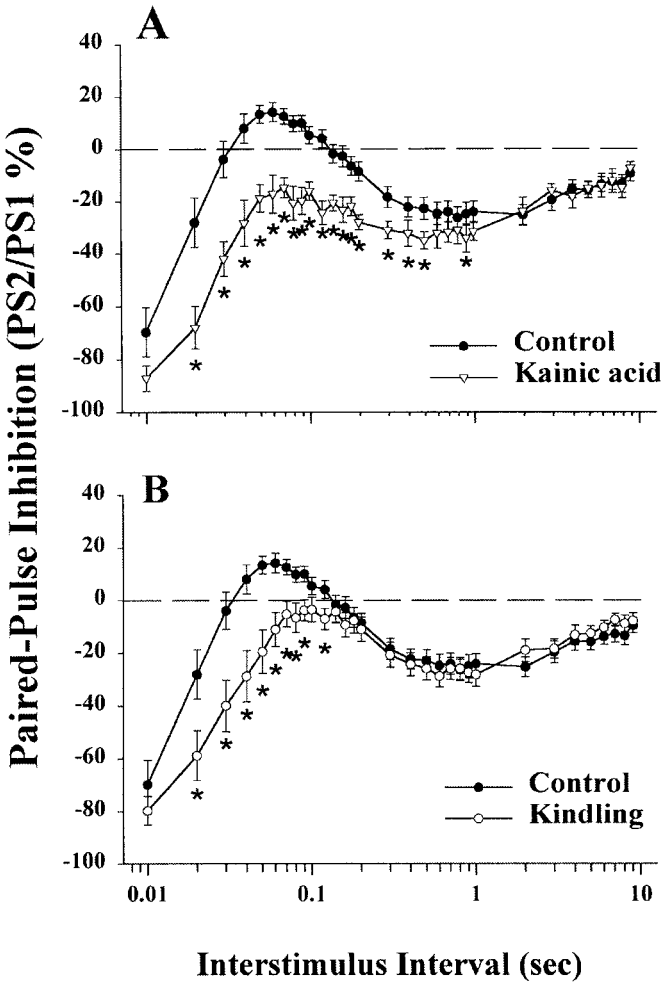

FIGURE 4. Perforant path paired-pulse stimulation of hippocampal slices in vitro demonstrates enhanced dentate inhibition following seizures in adult rats. Each point represents mean ± SEM percent ratio of amplitudes of the second to the first population spike. Values below dotted line represent paired-pulse inhibition, while those above indicate facilitation. *Points of significant difference from controls, $P < 0.05$, one-way ANOVA. A: Control (solid circles) paired-pulse inhibition profile is triphasic, with early and late inhibition separated by facilitation. Both early and late paired-pulse inhibition were enhanced 2 weeks following KA status epilepticus in adult rats (open triangles). B: Selective enhancement of early component of paired-pulse inhibition 2 weeks after amygdala kindling in adults (open circles). Controls (solid circles) are the same as in A.

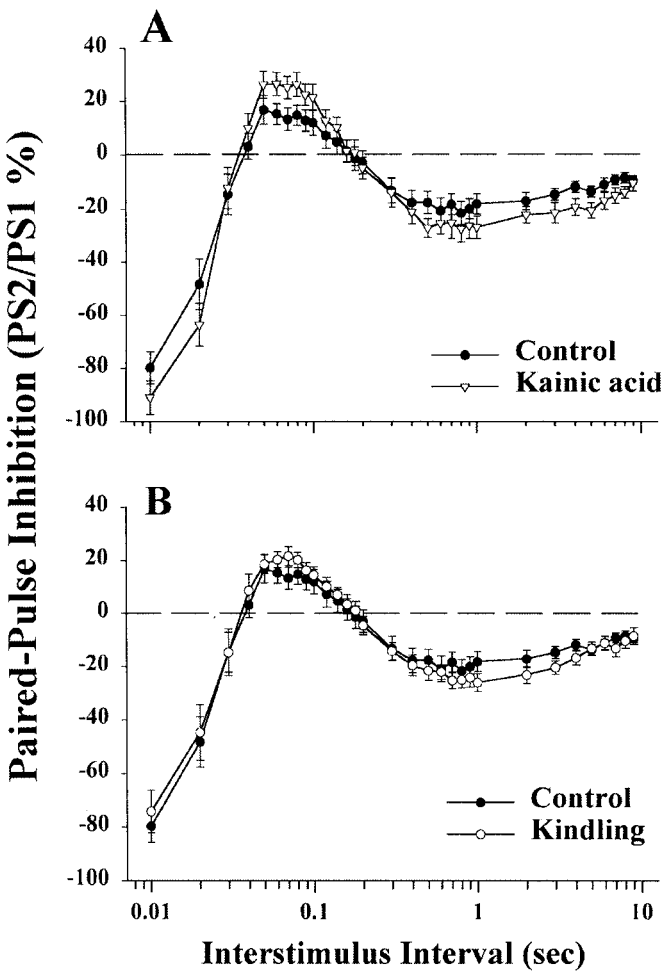

FIGURE 5. Seizures in P16 rats did not produce enhancement of granule-cell paired-pulse inhibition. A: Paired-pulse responses from immature rats 2 weeks after KA status epilepticus (open triangles), and from age-matched controls (solid circles), showed no significant differences. B: Similarly, amygdala kindling in P16 rats (open circles) produced no significant change in paired-pulse inhibition after 2 weeks, compared to control immature rats (solid circles).

occasional faint staining at ends of blades, 2 = sporadic fine or patchy staining, 3 = light but continuous staining in a band, and 4 = dark, clearly defined band of staining. Quantitated in this way, mean Timm's staining severity in adults following KA seizures was 3.07 ± 0.25 (n = 9), and in P16 pups, 0.36 ± 0.12 (n = 8). In matching slices from the same rats, mean paired-pulse inhibition at a 5000-ms interstimulus interval (ppi_{500}) was, in adults, 65.5 ± 3.0%, and in P16 pups, 84.5 ± 4.0% (100% means no inhibition). Calculation of the Spearman correlation coefficient between each slice's change in inhibition and that rat's corresponding Timm's staining showed a significant correlation of $\rho = -0.492$ ($n = 17$, $P < 0.05$, H_0 that Timm and ppi_{500} are independent), with greater staining associated with larger inhibition. In contrast, early paired-pulse inhibition (stimulus interval, 20 ms) was not significantly correlated with Timm's stain density ($\rho = -0.1794$).

DISCUSSION

In adult rats, prolonged seizures produced by either systemic KA, or repeated short-duration seizures evoked by electrical kindling, altered hippocampal anatomy. Severe KA-induced status epilepticus elicited widespread neuronal degeneration throughout the hilus and CA3. Our silver stains of degenerating cells are consistent with previous studies suggesting that certain hippocampal neuronal populations are more susceptible to seizure-induced damage than others (Freund et al., 1992; Sloviter, 1987). Hilar mossy cells, and CA3 and CA1 pyramidal neurons all became

argyrophilic after KA status epilepticus, while dentate granule cells were spared. However, there were dissociations, including the presence of argyrophilic neurons in field CA1 of adults that was not associated with measurable cell loss, and a lesser (22%) CA3c cell loss in the absence of argyrophilic neurons in pups, suggesting either that silver staining is not always a reliable marker of neurons that are destined to die (see also Toth et al., 1998), or that their number may not always correlate well with detectable reductions in cell number.

CA3 pyramidal cells were the most sensitive to KA, with a dramatic 57% loss of CA3c pyramidal cells 2 weeks after KA seizures. In contrast, electrical kindling that induced repeated short-duration seizures caused no gross lesions and a significantly smaller reduction in CA3c pyramidal neuron numbers in adult rats. Other studies using cell counts reported initially subtle cell loss (Spiller and Racine, 1994; Bengzon et al., 1997; Thompson et al., 1998) that eventually reaches nearly 50% in the hilus after 150 evoked seizures (Cavazos and Sutula, 1990). This damage resembles hippocampal sclerosis, the most common lesion observed in patients with temporal lobe epilepsy. The difference in extent of neuronal loss induced by KA status epilepticus and kindling could be due either to differences in their mechanisms of seizure induction, or to differences in seizure severity and duration.

In adults, both KA status epilepticus and kindling induced mossy-fiber sprouting and aberrant synaptogenesis in supragranular layers of the dentate gyrus, although sprouting was much greater after KA treatment. Reactive synaptogenesis is thought to be triggered by granule-cell deafferentation and/or loss of efferent targets, suggesting that neuronal loss may be a feature of both seizure models. Therefore, the greater degree of ectopic Timm staining after KA status epilepticus, compared to kindling, is consistent with the greater extent of neuronal death.

In contrast to the histological evidence of substantial neuronal damage and synaptic reorganization in adult rats following either KA status epilepticus or kindling, immature rats exhibited a remarkable resistance to seizure-induced damage, even when exposed to severe, continuous KA seizures lasting more than 30 min. KA seizures produced no detectable argyrophilic neurons or mossy-fiber sprouting. Although visual analysis did not reveal any overt cell loss, strereologic counting did reveal a significant 22% loss of CA3c pyramidal neurons compared to controls, markedly less than that observed in adults. However, we also observed an increase in cell layer thickness suggestive of dispersion or edema which, though it did not reach statistical significance, was of about the same magnitude (17%) as the observed decrease in cell density. As the counting was performed in a fixed window, an increase in thickness could have contributed to changes in cell density. Until further studies using stereological counting methods are performed, these results should be cautiously interpreted.

A number of studies have reported little or no cell loss following KA treatment in rat pups (Nitecka et al., 1984; Holmes, 1991; Sperber et al., 1991; Ribak and Navetta, 1994; Friedman et al., 1997; Tandon et al., 1999; Lynch et al., 2000), but these studies did not use stereological counting methods. Other studies have suggested that seizures can induce sprouting, even in young rats (Sankar et al., 1998; Holmes et al., 1999; Liu et al., 1999) and children (Mathern et al., 1994). It is possible, especially in the dentate gyrus, that greater neuronal death in immature animals is masked by the birth and migration of new neuronal replacements. However, McCabe et al. (2001) recently reported that 25 fluorothyl seizures in neonatal rats elicited a *reduction* in the number of BrdU-labelled recently differentiated neurons, an effect opposite to that observed following seizures in adults (Bengzon et al., 1997; Parent et al., 1997). Nonetheless, our data clearly show that immature rats are relatively resistant to KA seizure-induced cell death evoked readily in adults, consistent with other seizure models (Nehlig and Pereira de Vasconcelos, 1997; Liu et al., 1999).

This relative resistance was not due to the induction of behaviorally less severe seizures than those in adults. KA status epilepticus in P16 rats involved longer periods of tonus than in adults, and kindling in P16 rats evoked more severe seizure behavior, including wild running and jumping (kindling stage 6), not seen in adults (Holmes and Thompson, 1987; Lee et al., 1989; Veliskova et al., 1994; Johnston, 1996).

The relative resistance to seizure-induced cell death in the hippocampus of P16 rats also cannot be explained by a decreased involvement of the hippocampus in seizures at this age. Studies mapping brain metabolic changes with 2-deoxyglucose utilization during seizures have shown that the hippocampus is highly active at all ages tested in both KA status epilepticus (as early as 3 days old; Tremblay et al., 1984) and kindled seizures (16 days old; Ackermann et al., 1989). Nor is the resistance of P16 rats to KA-induced neuronal damage due to an absence of hippocampal KA receptors (Berger et al., 1984), since direct hippocampal infusion of KA produces local excitotoxic lesions as early as P5–7 (Cook and Crutcher, 1986; Leite et al., 1996). Furthermore, the lack of supragranular Timm staining in immature rats following KA or kindling is not due to an inability of the immature dentate to support mossy-fiber sprouting. Zimmer (1973) demonstrated that, in rats as young as 7 days old, lesions which deafferented dentate granule cells do induce mossy-fiber sprouting. If sprouting is dependent on deafferentation due to neuronal death, then the relative absence of seizure-induced reactive sprouting at P16 suggests that seizures did not induce marked cell loss in these animals.

Electrophysiological measures of changes in synaptic transmission are probably a more sensitive indicator of subtle seizure-induced modifications than cell death or mossy-fiber sprouting. While it is difficult to detect and quantitate cell loss and supragranular mossy-fiber terminals, enhancement of perforant path paired-pulse inhibition has been detected 24 h after even a single kindled seizure (de Jonge and Racine, 1987; Gilbert, 1991). In this study, we observed kindling-induced enhancement of the early component of paired-pulse inhibition 2 weeks after five stage 5 seizures in adult rats. Consistent with the more extensive morphological alterations produced by KA status epilepticus than kindling, granule-cell inhibition was much stronger following KA than after kindling. Interestingly, early, $GABA_A$-mediated paired-pulse inhibition has been shown to be enhanced after both KA and kindling in adults, while enhancement of late, $GABA_B$-mediated inhibition is only observed following KA (Haas et al., 1996). Others have reported that kindling increases both $GABA_A$ and $GABA_B$

components (de Jonge and Racine, 1987; Gilbert, 1991; Tuff et al., 1983), but $GABA_B$ responses return more rapidly to preseizure levels (de Jonge and Racine, 1987). Our current results are consistent with more severe KA seizures producing a longer-lasting enhancement of $GABA_B$ inhibition than kindling.

In contrast to the enhanced dentate inhibition seen after both KA status epilepticus and kindling in adult rats, neither KA nor amygdala kindling in P16 rats caused a change in perforant path paired-pulse profiles. The absence of seizure-induced electrophysiological alterations in P16 rats is consistent with the findings of others that seizures in rats younger than 20 days of age do not produce heightened susceptibility to epileptogenic stimuli (Holmes, 1991; Okada et al., 1984; Stafstrom et al., 1992), induce spontaneous seizures (Stafstrom et al., 1992, 1993), or cause deficits in learning and memory (Dos Santos et al., 2000; Stafstrom et al., 1993; Thurber et al., 1992). In contrast, kindling does produce a permanently enhanced susceptibility to electrical stimulation, irrespective of the age of seizure onset (Moshé and Albala, 1982, 1983). The lack of cell loss and mossy-fiber reorganization we observed after kindling in P16 rats indicates that these histopathologies are not obligatory substrates of kindling.

Although the mechanisms underlying the increased resistance of the immature hippocampus to seizure-induced damage remain unclear, its existence is probably an important defense mechanism capable of protecting the brain during a period when it is highly susceptible to seizures. Our results do not support the hypothesis that epileptic foci in adult human epileptics routinely arise from histopathology produced by seizures early in life. Although there is a high incidence of seizures in human neonates, there is little evidence that these seizures produce pathology or promote seizure occurrence later in life (Holmes, 1991; Holmes et al., 1993; Holmes and Thompson, 1988; Stafstrom et al., 1992, 1993). On the other hand, there is growing evidence that seizures in immature rats can produce more subtle residual effects, such as a rapid loss of (Thompson et al., 1998), and a later enhancement in, presynaptic inhibition (Haas et al., 1996; Chen et al., 1999), EEG alterations and learning impairments (Lynch et al., 2000; Dos Santos et al., 2000), dendritic loss (Swann et al., 2001), and short- and long-term metabolic changes (Nehlig and Pereira de Vasconcelos, 1997). Our study supports the conclusion that seizures early in development should neither be assumed to be totally benign, nor, a priori, to be causes of lifetime brain damage.

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