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Kainic acid-induced recurrent mossy fiber innervation of dentate gyrus GABAergic interneurons:

a possible anatomical substrate of granule cell hyperinhibition in chronically epileptic rats

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Abstract

Kainic acid-induced neuron loss in the hippocampal dentate gyrus may cause epileptogenic hyperexcitability by triggering the formation of recurrent excitatory connections among normally unconnected granule cells. We tested this hypothesis by assessing granule cell excitability repeatedly within the same awake rats at different stages of the synaptic reorganization process initiated by kainate-induced status epilepticus (SE). Granule cells were maximally hyperexcitable to afferent stimulation immediately after SE, and became gradually less excitable during the first month post-SE. The chronic epileptic state was characterized by granule cell hyperinhibition, i.e. abnormally increased paired-pulse suppression and an abnormally high resistance to generating epileptiform discharges in response to afferent stimulation. Focal application of the GABA_A receptor antagonist bicuculline methiodide within the dentate gyrus abolished the abnormally increased paired-pulse suppression recorded in chronically hyperinhibited rats. Combined Timm staining and parvalbumin immunocytochemistry revealed dense innervation of dentate inhibitory interneurons by newly-formed, Timm-positive, mossy fiber terminals. Ultrastructural analysis by conventional- and post-embedding GABA immunocytochemical electron microscopy confirmed that abnormal mossy fiber terminals of the dentate inner molecular layer formed frequent asymmetrical synapses with inhibitory interneurons and with GABA-immunopositive dendrites, as well as with GABA-immunonegative dendrites of presumed granule cells. These results in chronically epileptic rats demonstrate that dentate granule cells are maximally hyperexcitable immediately after SE, prior to mossy fiber sprouting, and that synaptic reorganization following kainate-induced injury is temporally associated with GABA_A receptor-dependent granule cell hyperinhibition, rather than an hypothesized progressive hyperexcitability. The anatomical data provide evidence of a possible anatomical substrate for the chronically hyperinhibited state.

Keywords

epilepsy; hippocampus; mossy fiber sprouting; basket cells; status epilepticus; synaptic reorganization

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INTRODUCTION

Injury-induced hippocampal neuron loss and synaptic reorganization are hypothesized to underlie the epileptogenic process that results in acquired temporal lobe epilepsy (Chang and Lowenstein, 2003). In the hippocampal formation, which is one likely source of seizures in human patients (Falconer and Taylor, 1968; Spencer, 2002), vulnerable dentate hilar mossy cells normally innervate both dentate granule cells (Buckmaster et al., 1996; Wenzel et al., 1997) and inhibitory neurons (Seress and Ribak, 1984; Deller et al., 1994). The death of mossy cells (Sloviter, 1987; Sloviter et al., 2003), or their axotomy (Blackstad, 1956), triggers a process of synaptic reorganization called mossy fiber sprouting (Nadler et al., 1980; Laurberg and Zimmer, 1981; Frotscher and Zimmer, 1983). The pathophysiological effects of synaptic reorganization are unclear (Parent and Lowenstein, 1997), but are presumably mediated by the abnormal synaptic connections that granule cells newly form with surviving mossy cell target neurons that were partially deafferented by mossy cell degeneration. Thus, hypotheses have focused on abnormal granule cell-granule cell connections (“excitatory” sprouting; Tauck and Nadler, 1985), and abnormal granule cell-interneuron connections (“inhibitory” sprouting; Sloviter, 1992; Kotti et al., 1997; Harvey and Sloviter, 2005).

In vivo and *in vitro* studies of animals subjected to prolonged status epilepticus (SE) indicate that granule cell hyperexcitability is the main pathophysiological feature observed during the early period post-SE (Sloviter and Damiano, 1981; Sloviter, 1983; 1987; 1992; Tauck and Nadler, 1985; Klitgaard et al., 2002). Conversely, abnormally increased granule cell inhibition is consistently found during the chronic epileptic state (Sloviter, 1992; Buckmaster and Dudek, 1997; Wu and Leung, 2001; Gorter et al., 2002; Harvey and Sloviter, 2005). In contrast with the *in vivo* studies cited above, most *in vitro* studies have focused on the pathophysiological features of hippocampal slices revealed by incubation of slices in bicuculline (Cronin et al., 1992; Wuarin and Dudek, 1996; Molnar and Nadler, 1999) or elevated potassium (Patrylo and Dudek, 1998; Hardison et al., 2000). The results of these studies have been widely interpreted as indicating that mossy fiber sprouting is essentially excitatory and epileptogenic, even though hippocampal slices from epileptic rats that were incubated in normal medium exhibited intact granule cell inhibition (Cronin et al., 1992). Given the interpretive problems inherent in using anesthesia for *in vivo* analyses, or bicuculline or non-physiological media for *in vitro* studies, hippocampal granule cell excitability remains to be assessed sequentially *in vivo* within the same awake, epileptic rats at different stages of the process of injury and synaptic reorganization initiated by kainate-induced SE.

Unresolved anatomical issues are also evident. Ultrastructural studies of kainate-treated rats have reported the formation of new synaptic connections between aberrant mossy fibers and both granule cells (Represa et al., 1993; Okazaki et al., 1995; Wenzel et al., 2000; Buckmaster et al., 2002; Cavazos et al., 2003), and inhibitory interneurons (Kotti et al., 1997; Wenzel et al., 2000; Buckmaster et al., 2002; Cavazos et al., 2003). However, one recent study of the synaptic connections formed by individual granule cell axons concluded that the relative rarity with which each aberrant granule cell axon contacted an inhibitory interneuron indicated that aberrant connections between granule cells and inhibitory interneurons are probably physiologically inconsequential (Buckmaster et al., 2002). However, many individual granule cell axons undoubtedly converge upon the relatively few inhibitory interneurons present, thereby producing the dense Timm-positive innervation of inhibitory interneurons that is evident at the light microscopic level (Sloviter, 1992; Kotti et al., 1997). Because a convergent input of many aberrant granule cell axons to inhibitory interneurons would be unavoidably missed by counting only the number of synaptic connections formed by individual granule cell axons (Buckmaster et al., 2002), we

hypothesized that a dense convergent input to inhibitory interneurons might be confirmed ultrastructurally if inhibitory interneurons were first identified, and their synaptic inputs were then analyzed.

This study has addressed these two unresolved issues. First, we assessed granule cell excitability repeatedly within the same awake, chronically implanted epileptic rats at successive stages of the cell death/ synaptic reorganization process initiated by kainate-induced SE, and determined the time course of the changes in excitability. Second, we identified inhibitory interneurons in chronically epileptic, mossy fiber-sprouted rats by conventional- and post-embedding GABA immunocytochemical electron microscopy, and determined whether aberrant mossy fiber terminals formed frequent synaptic contacts with GABAergic interneurons, as well as with granule cells.

MATERIALS and METHODS

Animal treatment

Fifty-seven male Sprague-Dawley rats (12 saline-treated- and 45 kainate-treated-rats), weighing 350-450g, were used in this study and were treated in accordance with the guidelines of the National Institutes of Health for the humane treatment of animals, and those of the University of Arizona Institutional Animal Care and Use Committee.

Implantation of chronic stimulating and recording electrodes for awake recording—Under buprenorphine (0.05 mg/kg sc) analgesia and urethane (1.25 g/Kg sc) anesthesia, the surgical area was shaved and cleaned with povidone-iodine. A midline incision exposed the skull, into which six stainless steel screws were inserted. Bipolar stainless steel stimulating electrodes (Rhodes Medical Instruments, Summerland, CA) were placed bilaterally in the angular bundles of the perforant pathway (~4.5 mm lateral from the midline suture and immediately rostral to the lambdoid suture). Unipolar recording electrodes, fabricated from Teflon coated 0.003" diameter stainless steel microwires, (#7910; A-M Systems, Inc., Carlsborg, WA) were lowered into the brain bilaterally (~2mm lateral from the midline, ~3mm caudal to bregma, and 3.5mm below the brain surface). Final tip locations in the granule cell layer were reached by optimizing the potentials evoked by perforant pathway stimulation (Andersen et al., 1966). A layer of dental acrylic cement attached the electrodes to the screws and skull. Plastic connectors (Ginder Scientific, Ottawa, Canada) were fitted to the electrodes and then embedded in acrylic cement to form a mechanically stable cap. Anti-bacterial gel was applied at the surgical margin, and animals were kept warm until ambulatory. Experiments began at least one week post-implantation. Electrophysiological testing of awake animals utilized stimuli (0.1 msec duration) generated by a Master 8 stimulator and stimulus isolation unit (Armon MicroProcessor Instruments, Jerusalem, Israel). Potentials were amplified and recorded digitally at a 10 kHz sampling rate (AD Instruments, Mountain View, CA) on microcomputers (Apple Computer Inc., Cupertino, CA).

Status epilepticus (SE) induced by kainic acid (KA) injection—Chronically implanted rats were briefly anesthetized with ether, and a small incision was made in the skin overlying the saphenous vein, into which KA was injected (9 mg/kg; 10 mg/ml saline; Ocean Produce International, Shelburne, Canada). A single stainless steel staple closed the incision. These chronically implanted animals were used to assess granule cell excitability at different periods after SE, and for the light microscopic studies of hilar cell loss and Timm staining in epileptic rats. Initially naive, unimplanted rats were used for the ultrastructural studies (to avoid the physical damage produced by chronically implanted electrodes), and these rats received kainate (12 mg/kg) subcutaneously. Rectal temperature was monitored

intermittently during SE, and hyperthermia was prevented using fans. All kainate-treated rats allowed to survive more than one month after SE were observed to exhibit spontaneous behavioral seizures, although monitoring was not performed systematically.

Several previous ultrastructural studies have failed to find abnormally large mossy fiber terminals in the inner molecular layer of the dentate gyrus post-SE (Okazaki et al., 1995; Wenzel et al., 2000; Buckmaster et al., 2002; Cavazos et al., 2003), possibly as a result of less than dense mossy fiber sprouting in kainate-treated rats (as illustrated in Cavazos et al., 2003) caused by incomplete hilar mossy cell loss, which is the presumed trigger for mossy fiber sprouting. We regarded the presence of abnormally large presynaptic terminals in the inner molecular layer (Represa et al., 1993; Zhang and Houser, 1999) to be a required criterion for concluding that presynaptic elements were aberrant mossy fiber terminals. Therefore, KA-treated animals were assessed electrophysiologically 3 days post-SE, as previously described (Sloviter, 1992). This was done to identify and select rats that exhibited granule cell hyperexcitability, i.e. multiple granule cell spiking and decreased paired-pulse suppression (as shown in Figure 2, B1 of Sloviter, 1992), because these acute electrophysiological features are reliable indicators of extensive hilar neuron loss (Zappone and Sloviter, 2004), as well as predictors of dense mossy fiber sprouting (Sloviter, 1992).

Focal application of bicuculline in the dentate granule cell layer—To determine whether abnormally increased paired-pulse suppression in granule cells of chronically epileptic rats was GABA_A receptor-dependent, we utilized a glass recording microelectrode containing 10 mM bicuculline methiodide (BMI; Sigma Chemical Co., St. Louis, MO) dissolved in saline, as described in detail previously (Zappone and Sloviter, 2004). In this method, BMI diffuses passively from the electrode tip located within the dentate granule cell layer of urethane-anesthetized rats, and disinhibits a small region of the granule cell layer, as indicated by c-Fos expression (Zappone and Sloviter, 2004).

Perfusion-fixation and tissue treatment—Rats were anesthetized with urethane and perfused through the aorta by gravity-feed using one of two fixation protocols. One protocol, which preserves peptide immunoreactivity optimally, and precipitates synaptic zinc for subsequent Timm staining (Sloviter, 1982), involved perfusion with saline for 2 min followed by 0.1% sodium sulphide (Sigma) in 0.1M phosphate buffer, pH 7.4, for 1 min, followed by saline for 1 min, followed by 4% paraformaldehyde (Sigma) in 0.1M phosphate buffer, pH 7.4, for 10 min. The other fixation protocol, optimal for preserving GABA immunoreactivity and structure for electron microscopy, consisted of saline for 2 min followed by 1% paraformaldehyde/ 1.5% purified glutaraldehyde (Ladd Research, Williston, VT) in 0.1M phosphate buffer, pH 7.4, for 10 min. After storage of the intact rats overnight at 4°C, brains were removed from the skull and placed in perfusate. Sectioning was always performed on the day of removal of the brain from the skull.

For routine histological analysis and immunocytochemistry, 40 :m-thick coronal sections were cut in 0.1M Tris (hydroxymethyl) aminomethane buffer, pH 7.6, using a Vibratome. Regularly spaced sections were mounted on subbed slides for subsequent Nissl (1% cresyl violet) staining. After staining, all slides were dehydrated in graded ethanols and xylene, and then coverslipped with Permount.

Immunocytochemistry protocol—Sections were mounted on Superfrost Plus slides, air dried, and placed in 0.1M TRIS buffer, pH 7.6 (TRIS). Slides were then immersed in 85-87°C TRIS for 1 min, washed in TRIS, and placed in TRIS containing 0.25% bovine serum albumin (BSA; fraction V; Sigma) and 0.1% Triton X-100. Slides were incubated overnight in the following primary antibody solutions: 1) rabbit anti-glutamate receptor subunit 2 (diluted 1:2000; AB1768; Lot number 22070361; Chemicon, Temecula, CA). This

antiserum was raised against the peptide sequence VAKNPQNINPSSSQNS (amino acids 827-842 of the rat GluR2 subunit), which was conjugated to BSA through a cysteine added to the C-terminus of the peptide (Petralia et al., 1997). This antiserum was previously shown to be specific for the GluR2 subunit, and its staining characteristics were prevented by adsorption with the synthetic peptide against which it was raised (Petralia et al., 1997); 2) mouse anti-NeuN (diluted 1:10,000; MAB377; Lot number 22021251; Chemicon). Anti-NeuN was raised against purified cell nuclei from mouse brain, and recognizes 2-3 bands in Western blots in the 46-48 kDa range, and possibly another band at approximately 66 kDa, which constitutes unknown nuclear proteins; 3) mouse anti-parvalbumin (diluted 1:1,000,000; P3171; Lot number 68F4806; Sigma). This antiserum was raised against parvalbumin purified from carp muscle, and reacts specifically with parvalbumin of cultured nerve cells and tissue originating from human, monkey, rat, mouse, chicken, and fish. Immunocytochemical controls included omission of primary antibodies, which eliminated all specific immunostaining.

All antisera were diluted in the same TRIS-BSA-Triton X buffer. After primary antibody incubation, slides were washed in TRIS-BSA-Triton X buffer, pH 7.6 (2×5 min minimum). Slides were incubated in biotinylated secondary antibody solution (1:2000 dilution of goat anti-mouse; Vector Labs, Burlingame, CA) in TRIS-BSA-Triton X buffer for 2 hr, washed in the same buffer, and then incubated for 2 hr in avidin-biotin-HRP complex (Vector Labs Elite kit diluted 1:1000 in TRIS-BSA-Triton X buffer). Slides were then washed in TRIS (3×5 min minimum) and incubated in a hydrogen peroxide-generating DAB solution (100ml TRIS containing 50 mg DAB, 40 mg ammonium chloride, 0.3 mg glucose oxidase, and 200 mg β -D-glucose). After incubation in DAB solution (20-30 min), slides were rinsed in TRIS, dehydrated in graded ethanols and xylene, and coverslipped with Permount.

Light microscopic imaging methods—Brightfield images were acquired digitally on a Nikon E800M microscope with a Hamamatsu C5180 camera. Adobe Photoshop 7.0 was used to acquire images and optimize contrast and brightness, but not to enhance or change the image content. For comparison photographs, all tissue sections were photographed under identical conditions of exposure, and any changes made in brightness or contrast were applied uniformly to all photographs.

Electron microscopic methods—For conventional electron microscopy, 800 μ m-thick sections (2 or 3 per animal) from the dorsal hippocampal region between sections 33-41 in the atlas of Paxinos and Watson (1998) were cut with a Vibratome in 0.1M TRIS buffer, pH 7.6, rinsed in 0.1M cacodylate buffer pH 7.4, post-fixed in 1% osmium tetroxide in 0.1M cacodylate buffer for 90 min, rinsed in 0.1M cacodylate buffer for 10 min, rinsed in distilled H₂O for 10 min, and stained en bloc in 1% uranyl acetate for 90 min. Sections were dehydrated in ethanol, cleared with propylene oxide, infiltrated in 1:1, then 1:3 propylene oxide/LX112 Epon (Ladd Research) for 2 hr each before going into 100% LX112 for 3 changes over 2 days. Polymerization took place in a 60E oven for 60 hr. Blocks were trimmed, sectioned at a thickness of 0.5 μ m, and stained with toluidine blue for light microscopy. Ultrathin sections of 60 to 90 nm were cut on a Reichert Ultracut microtome and picked up on parlodion coated, carbon shadowed, 1 mm $\times$ 2 mm slot grids. Grids were stored in a Hiroaka grid holder before post-staining with 4% uranyl acetate in 40% ethanol and Reynold's lead citrate. Sections were examined and photographed in a JEOL 100SX electron microscope at an accelerating voltage of 80 kV.

All preceding methods were performed in the laboratory of the first author (RSS), and the results are illustrated in Figures 1-6. For an additional blinded analysis by conventional electron microscopy, and the experiments involving post-embedding GABA immunocytochemical electron microscopy, the same control and experimental tissue blocks

were sent to the last author (MF), in whose laboratory the methods described below were conducted. These results are presented in Figure 7.

Post-embedding immunogold labeling for GABA—Post-embedding immunogold labeling for GABA was performed by using a modified protocol of the procedure described by Somogyi and Hodgson (1985). Briefly, thin sections (80-90nm) were cut on a Reichert Ultracut S microtome and picked up on nickel grids (1 mm × 2 mm slot grids). Immunostaining was done with droplets of Millipore-filtered solutions in humid Petri dishes. Grids were immersed in a solution of 0.05M glycine and 0.1% NaBH₄ for 10 min, pre-incubated in 2% human serum albumin (HSA; Sigma) in 0.05 M TRIS-buffered saline (TBS; pH 7.6) containing 0.1% Triton X-100 (TBST; pH 7.6), and then incubated in rabbit anti-GABA diluted 1:5,000 in 2% HSA for 2 hr (Product number A-2052, Lot number 021H4803, Sigma). This antiserum was raised in rabbit using GABA conjugated to bovine serum albumin as the immunogen, followed by affinity purification. After rinsing in TBST, and incubating in 2% HSA in TBST for 10 min, the grids were placed in secondary antibody for 2 hr (goat anti-rabbit IgG-coated colloidal gold, 10nm, diluted 1:20 in TBST; Aurion, Wageningen, The Netherlands). The grids were then washed in distilled water and stained with 1% uranyl acetate and Reynold's lead citrate. In control experiments, the primary GABA antibody was omitted; no labeling occurred under these conditions. Sections were viewed in a Zeiss 109 electron microscope and photographed using Macophot Ort 25 film (70 mm).

RESULTS

Experiment 1: Granule cell behavior during and after kainate-induced status epilepticus

Fourteen rats were used in this experiment (4 saline-treated- and 10 kainate-treated rats). We repeatedly assessed granule cell responses to afferent (perforant pathway) stimulation at different periods (1-7, 14, 21, and 28 days) after kainate-induced SE in the same awake, chronically implanted animals. All 14 rats tested prior to saline or kainate injection exhibited single population spikes and intact paired-pulse suppression in response to perforant pathway stimulation at 0.1 Hz (Fig. 1, A1). Granule cell epileptiform discharges during KA-induced behavioral SE were highly variable; rats that exhibited only occasional granule cell epileptiform discharges (n=5) were eliminated from the study because a previous analysis of KA-treated rats had shown that rats that generated granule cell epileptiform discharges intermittently during SE subsequently exhibited minimal granule cell hyperexcitability, as well as minimal hilar neuron loss (Sloviter et al., 2003). Conversely, the 5 kainate-treated rats that exhibited granule cell epileptiform discharges during >~65% of the 4 hr recording period during SE subsequently exhibited multiple population spikes and minimal paired-pulse suppression in response to afferent stimuli throughout the first week after SE (Fig. 1, A2-A3). Over the subsequent weeks, the same 5 awake rats exhibited a gradual restoration of paired-pulse suppression, and a reduction in the number of population spikes evoked by identical 0.1 Hz perforant pathway stimuli (Fig. 1, A4). The graph in Figure 1,B presents the quantitative data from the paired-pulse stimulation tests from 1-28 days post-SE in all 5 kainate-treated rats tested, compared to 4 saline controls. These data show that the initially diminished paired-pulse suppression evident at a constant 40 msec interpulse interval partially recovered gradually between 7 and 28 days post-SE.

Experiment 2: Granule cell excitability >2months post-SE

Next, we assessed granule cell excitability >2 months post-SE in different kainate-treated rats that exhibited acute granule cell hyperexcitability under urethane anesthesia 3 days post-SE, as previously described (Sloviter, 1992), and all of which became chronically epileptic. These animals (3 saline-treated- and 4 kainate-treated rats) were allowed to survive for 2

months after saline or kainate treatment, prior to chronic implantation. The early post-SE evaluation of granule cell excitability was made acutely using glass microelectrodes to identify rats that would subsequently exhibit dense mossy fiber sprouting, and to avoid the presence of chronically implanted electrodes throughout the post-SE period, which might influence chronic granule cell excitability or the epileptogenic process. Compared to responses in the 3 saline-treated controls, two characteristics of granule cell excitability were consistently altered in all 4 kainate-treated rats. These were: 1) increased paired-pulse suppression of the granule cell population spike evoked by perforant path stimulation, and; 2) an elevated seizure discharge threshold in response to increasing frequencies of afferent stimulation. In all awake, saline-treated control animals tested, the first of two identical afferent stimuli delivered at 0.1 Hz evoked large amplitude population spikes, and second responses that exhibited fully suppressed population spikes at an interpulse interval of 20 msec (ratios of second-to-first spike amplitudes = 0). At interpulse intervals of 40 and 60 msec, the average suppression of the second population spike in control animals stimulated at 0.1 Hz was 94% (40 msec) and 30% (60 msec), respectively (Fig. 1C). At longer interpulse intervals (80 and 100 msec), second population spike amplitudes in controls exhibited potentiation (Fig. 1C). In contrast to the normal responses observed in saline-treated controls, all 4 kainate-treated rats exhibited abnormally elevated paired-pulse suppression at all interpulse intervals (20-100 msec) tested in response to 0.1 Hz perforant pathway stimulation (Fig. 1C).

Finally, the threshold for generating granule cell seizure discharges in awake animals was determined by recording the responses of granule cells to progressively increasing afferent stimulus frequencies. All 3 control animals exhibited a normal collapse of paired-pulse suppression and conversion to granule cell epileptiform behavior at a stimulus frequency of 1 Hz, at 60-100 msec interpulse intervals. In contrast, all 4 awake kainate-treated rats failed to generate epileptiform discharges in response to afferent stimulation at 1 or 2 Hz, and did not exhibit the transition from normally suppressed responses to epileptiform granule cell discharges until the stimulus frequency was increased to 3 Hz (n=2) or 5 Hz (n=2). Thus, awake, chronically epileptic, kainate-treated rats consistently exhibited abnormally increased and prolonged granule cell paired-pulse suppression and abnormally increased seizure discharge thresholds in response to orthodromic afferent stimulation. These results indicating chronic granule cell hyperinhibition in awake kainate-treated rats are consistent with previous *in vivo* findings in anesthetized kainate-treated rats (Sloviter, 1992; Buckmaster and Dudek, 1997; Wu and Leung, 2001) and awake pilocarpine-treated rats (Harvey and Sloviter, 2005). These *in vivo* data are also consistent with *in vitro* results indicating that granule cell inhibition is intact when hippocampal slices from chronically epileptic, kainate-treated rats are incubated in normal medium (Cronin et al., 1992).

Experiment 3: Focal application of bicuculline in hyperinhibited, chronically epileptic rats

This experiment utilized 3 saline-treated- and 8 kainate-treated rats. The dependence of the observed granule cell hyperinhibition on GABA_A receptor activation was addressed in a separate group of 8 chronically epileptic, unimplanted kainate-treated rats by utilizing passive diffusion of the GABA_A receptor antagonist bicuculline from the tip of a glass microelectrode (Zappone and Sloviter, 2004). Abnormally increased paired-pulse suppression and increased seizure discharge thresholds (described above), were verified in 8 kainate-treated rats 8-9 weeks post-SE using a saline-filled recording electrode. Replacement of the saline-filled electrode with a similar glass electrode containing 10mM bicuculline methiodide (Sloviter and Brisman, 1995; Zappone and Sloviter, 2004) rapidly reversed the paired-pulse suppression produced by afferent stimulation at 0.1 and 1.0 Hz, at all interpulse intervals tested (40-100 msec), and in all rats tested. Within seconds of insertion, the loss of paired-pulse suppression was followed by multiple population spikes

and epileptiform discharges in response to a stimulus frequency (1 Hz) that was always subthreshold for evoking epileptiform discharges in chronically epileptic, kainate-treated rats. The transition from inhibited to disinhibited responses in these chronically epileptic kainate-treated rats was as illustrated and described previously, in a study in which the identical experiment was performed in kainate-treated rats tested 3 days post-SE (shown in Figure 4B of Zappone and Sloviter, 2004). Thus, both the residual paired-pulse suppression present 3 days post-SE, and the abnormally increased paired-pulse inhibition present during the chronic epileptic state, were abolished by passive, focal diffusion of bicuculline directly within the region of the granule cell layer from which the hyperinhibited responses to afferent stimulation were recorded.

Experiment 4: Combined Timm staining and parvalbumin immunocytochemistry in kainate-treated rats

This experiment involved anatomical analysis of 4 saline-treated rats, 2 kainate-treated, chronically implanted rats from Experiments 1 and 2 described above, plus an additional 9 unimplanted kainate-treated rats (3 perfusion-fixed 7 days post-SE and 6 perfusion-fixed 17-27 days post-SE). Histological analysis confirmed that frequent granule cell epileptiform discharges during SE were consistently associated with extensive hilar neuron loss (Fig. 2, A,B), as previously described (Sloviter et al., 2003). Timm staining revealed that sparse mossy fiber sprouting was detectable 7 days post-SE (arrows in Fig. 2, C1), and that this was consistently less dense than that observed 28 days post-SE (arrows in Fig. 2, B3). The sparse mossy fiber sprouting observed 7 days post-SE was exploited for a qualitative analysis of presumed mossy fiber target cells because the dense mossy fiber sprouting present >1 month post-SE obscured the field. In control animals perfused, sectioned, and Timm-stained in parallel with the kainate-treated rats, the somata and proximal dendrites of granule cell layer interneurons were prominently outlined by Timm-positive fibers (Fig. 2, C2), as previously described (Ribak and Peterson, 1991; Blasco-Ibanez et al., 2000). Few Timm-positive elements were observed to extend into the inner molecular layer of saline-treated control animals (Fig. 2, C2; n=4). Conversely, kainate-treated rats (n=5; 2 physiologically monitored rats from the group of 5 rats described above, and 3 additional unimplanted, kainate-treated rats) exhibited a consistently increased density of Timm-positive elements in the inner molecular layer 7 days post-SE, some of which prominently outlined interneuron dendrites (arrows in Fig. 2, C3) to an extent not seen in the saline controls (Fig. 2, C2).

Six unimplanted kainate-treated animals were perfusion-fixed during the early post-SE period (17-27 days post-SE). Combined parvalbumin immunocytochemistry and Timm staining confirmed that the abnormal Timm-stained elements in the dentate inner molecular layer surrounded and outlined the somata and dendrites of parvalbumin-positive inhibitory interneurons of the granule cell and molecular layers (Fig. 3). In these animals with submaximal mossy fiber sprouting, apparent termination of Timm-positive terminals on PV-positive inhibitory interneurons was a consistent and prominent feature. In addition, at the outer border of the inner- and middle molecular layers, the few Timm-positive elements present often appeared to contact PV-positive inhibitory interneurons exclusively, in that the only visible Timm-positive terminals present at the border were in apparent contact with PV-positive dendrites (arrows in Fig. 3, A2, E, G, and I). The two most obvious examples are shown in Figure 3, A2 and E (arrows), in which the Timm-positive terminals appear to leave the main mossy fiber terminal lamina in the inner molecular layer and terminate on PV-positive dendrites at the outer margins of the main mossy fiber termination zone.

Experiment 5: Ultrastructural analysis of kainate-treated rats

To avoid the pathology produced by chronically implanted electrodes, we used 16 unimplanted rats for the ultrastructural studies (2 saline-treated- and 14 KA-treated rats).

Four of the 14 kainate-treated rats assessed physiologically using glass microelectrodes under urethane anesthesia 3 days post-SE, exhibited acute granule cell hyperexcitability in response to perforant pathway stimulation (multiple population spikes and decreased paired-pulse suppression, as shown in Figure 1, A2). These 4 kainate-treated rats and 2 saline-treated controls were allowed to survive for 10 weeks post-SE, during which all 4 kainate-treated rats became chronically epileptic. Only rats exhibiting early granule cell hyperexcitability were used for this ultrastructural analysis because acute granule cell hyperexcitability and extensive hilar neuron loss are closely correlated (Sloviter, 1991; Sloviter et al., 2003), and the dense mossy fiber sprouting that follows extensive hilar neuron loss (Sloviter, 1992) was a necessary condition of the experimental design.

We took two ultrastructural approaches to determine whether mossy fiber sprouting in the inner molecular layer of the dentate gyrus involved synaptic connections between aberrant granule cell axons and inhibitory interneurons. First, we examined ultrathin sections of the dentate gyrus from control and kainate-treated animals, and searched for interneuron somata in the granule cell layer that were sectioned in a plane that contained both the soma and a traceable apical dendrite. In this way, we could identify granule cell layer interneuron somata and then examine the synaptic input to their dendrites in the inner molecular layer. Granule cell layer interneuron somata were differentiated from surrounding granule cells on the basis of their location at the base of, or within the granule cell layer, and their characteristic morphological features (Ribak and Anderson, 1980). Second, we examined sections subjected to post-embedding GABA immunocytochemistry to identify GABA-immunoreactive postsynaptic targets of abnormally large presynaptic terminals in the neuropil of the inner molecular layer.

Conventional electron microscopy—In ultrathin sections of control tissue, 8 interneurons in sections from 2 saline controls (5 cells in one animal and 3 cells in the other rat) exhibiting the morphological features of dentate basket cells (Ribak and Anderson, 1980) were identified and their dendrites were followed into the inner molecular layer. These neurons of control animals received presynaptic input from small boutons on both their somata and dendrites, as illustrated in Figure 4, A. A similar qualitative analysis of the granule cell layers in sections from chronically epileptic, kainate-treated rats also found interneurons that appeared similar to those in control tissue with respect to their innervation by relatively small boutons. However, other granule cell layer interneurons in the same 4 kainate-treated rats, which exhibited the morphological features of dentate basket cells, possessed a density and pattern of presynaptic input that was not observed in control tissue. In 5 cases in which granule cell layer interneurons were sectioned in a plane that permitted the proximal dendrite to be followed into the inner molecular layer (1 interneuron in each of 3 kainate-treated rats and 2 interneurons in the 4th animal), the somata and proximal dendrites of these interneurons were densely covered with large presynaptic terminals that exhibited the morphological features of mossy fiber terminals (Figs. 4,5). A qualitative analysis of synaptic structure in the neuropil of the mossy fiber termination zone in the inner molecular layer also revealed obvious differences between sections from control and kainate-treated rats. Whereas control animals consistently exhibited relatively small presynaptic terminals terminating on dendritic spines of presumed granule cells (Fig. 6, A), synaptic terminals in the same dendritic region of kainate-treated rats were much larger (Fig. 6, B, C), and exhibited the morphological features of large mossy fiber terminals, i.e. large terminals packed with synaptic vesicles, and containing numerous mitochondria and dense-core vesicles (Amaral and Dent, 1981; Claiborne et al., 1986).

Immunocytochemical electron microscopy—To complement the qualitative survey of interneuron innervation described above, we examined sections of the same tissue blocks after post-embedding immunostaining for GABA. First, we looked at low magnification for

large neuronal somata located at the base of the granule cell layer. At higher magnification, we verified that these cell bodies were GABA-immunoreactive and showed characteristics of interneuron cell bodies such as infolded nuclei, large amounts of perinuclear rough endoplasmic reticulum, and intranuclear inclusions (Ribak and Anderson, 1980). We then traced, in serial sections, the ascending dendrites of these neurons to the inner molecular layer and studied their input synapses. In control tissue, GABA-positive dendrites of granule cell layer somata were contacted by GABA-negative- and GABA-positive boutons of approximately similar size (Fig. 7, A1 and A2). The GABA-negative boutons established asymmetric synaptic contacts, whereas the GABA-positive boutons formed symmetric synapses (Fig. 7, A2). Thin sections from the kainate-treated rat that exhibited the best ultrastructural preservation (shown in Figs. 4 and 5) were GABA-immunostained in parallel with sections from a matched control animal. This analysis revealed similar types of GABA-positive and GABA-negative input synapses in both animals. However, in addition to small presynaptic boutons, we observed abnormally large boutons in the inner molecular layer of the kainate-treated rat that were densely filled with clear synaptic vesicles intermingled with a few dense-core vesicles (Fig. 7, B1 and B2). These large boutons, which often emerged from thin, unmyelinated pre-terminal axons (white arrow in Fig. 7, B1), were consistently GABA-negative, and established asymmetric synaptic contacts with the GABA-positive dendrites (Fig. 7, B1 and B2). These qualitative observations were corroborated by measurements of the mean area of all boutons in the inner molecular layer, which was nearly twice as large in the kainate-treated rat, as in control sections (control: $0.237 \pm 0.004 \text{ :m}^2$; KA-treated: $0.420 \pm 0.008 \text{ :m}^2$; $n=2,100$ boutons counted in 6 sections (350 boutons per section) from the control and 2,100 boutons counted in 6 sections of the kainate-treated animal). For boutons that were specifically in synaptic contact with GABA-positive dendrites, the mean bouton area was similarly nearly twice that of the control value (control: $0.357 \pm 0.022 \text{ :m}^2$; KA-treated: $0.695 \pm 0.040 \text{ :m}^2$, $n=2,100$ boutons from each of the control and kainate-treated animals). A similar difference was observed when the mean perimeter lengths of boutons contacting GABA-positive dendrites were compared (control: $2.638 \pm 0.075 \text{ :m}$; KA-treated: $3.690 \pm 0.123 \text{ :m}$; $n=2,100$ boutons from each of the control and kainate-treated animals). The increase in perimeter reflected, in part, a more convoluted structure of the large boutons that we observed qualitatively in sections from the kainate-treated animal. No similar increase in the size of GABAergic terminals contacting interneuron dendrites in the inner molecular layer was apparent, suggesting a relatively selective change in the size of GABA-negative presynaptic terminals in the inner molecular layer 10 weeks post-SE.

DISCUSSION

The stated purposes of this study were to determine whether dentate granule cells become gradually more excitable as mossy fiber sprouting progresses, and whether aberrant granule cell axons target inhibitory interneurons, as well as granule cells. The primary findings of this study are that: 1) dentate granule cells in awake rats exhibited maximal hyperexcitability (multiple population spikes, decreased paired-pulse suppression, and a lowered seizure discharge threshold) immediately after kainate-induced SE, coincident with the initial neuron loss, and prior to mossy fiber sprouting; 2) initial granule cell hyperexcitability gradually converted to a hyperinhibited state characterized by abnormally increased and prolonged paired-pulse suppression and an elevated seizure discharge threshold, both of which were reversed by the focal application of the GABA_A receptor antagonist bicuculline within the granule cell layer, and; 3) identified inhibitory interneurons of the granule cell layer, and GABA-immunopositive dendrites of the inner molecular layer of chronically hyperinhibited epileptic rats, were prominent targets of large mossy fiber terminals.

Gradual reversal of acute post-SE granule cell hyperexcitability

The repeated assessment of granule cell excitability in the same awake, freely moving animals revealed that maximal granule cell hyperexcitability developed coincidently with neuronal injury, prior to mossy fiber sprouting. Our observation that granule cells became immediately hyperexcitable to orthodromic afferent stimulation, and that this hyperexcitability persisted throughout the first weeks after SE, is consistent with the original description by Tauck and Nadler (1985) of granule cell hyperexcitability in response to antidromic stimulation in hippocampal slices during the 12-21 day period post-SE. Since granule cells are maximally hyperexcitable *in vivo* immediately after SE, and become progressively less excitable as mossy fiber sprouting develops, the early hyperexcitability first reported by Tauck and Nadler (1985), and attributed to mossy fiber sprouting, cannot be caused by synaptic reorganization, which follows, rather than precedes, the hyperexcitability. This conclusion is supported by the original observation of Tauck and Nadler (1985) that granule cell hyperexcitability was not greater at 21 days post-SE than at 12 days post-SE, a time when mossy fiber sprouting is actively increasing in density, and would be predicted to have resulted in increased excitability.

Inhibitory interneurons as targets of mossy fiber sprouting

Our anatomical analyses confirmed that aberrant Timm-positive mossy fibers target dentate inhibitory interneurons, as well as granule cells, as previously reported (Sloviter, 1992; Kotti et al., 1997; Wenzel et al., 2000; Buckmaster et al., 2002; Cavazos et al., 2003), and that aberrant mossy fibers densely innervate inhibitory interneurons exhibiting the morphological characteristics of dentate basket cells. This conclusion is based on the following observations: 1) Timm-positive axonal varicosities were observed to surround parvalbumin-positive interneuron somata and dendrites in the inner molecular layer, which normally lacks this aberrant axonal projection; 2) aberrant Timm-positive fibers often left the main axon plexus in the inner molecular layer to climb the dendrite of a parvalbumin-positive interneuron, suggesting that inhibitory neuron elements just beyond the inner molecular layer may exert an attractive influence on aberrant granule cell axon collaterals not shared by granule cells; 3) electron microscopy revealed that some dentate inhibitory interneurons were virtually encrusted by abnormally large presynaptic terminals forming asymmetrical synapses and exhibiting the morphological features of large mossy fiber terminals; 4) post-embedding GABA immunostaining confirmed that abnormally large GABA-negative terminals of the inner molecular layer formed frequent asymmetrical synaptic contacts with GABA-immunopositive postsynaptic dendrites.

Possible functional implications

Mossy fiber sprouting has been observed in both human (Sutula et al., 1989; Houser et al., 1990; Babb et al., 1991; Zhang and Houser, 1999; de Lanerolle et al., 2003) and experimental tissues (Nadler et al., 1980; Laurberg and Zimmer, 1981; Frotscher and Zimmer, 1983), and its presence has been hypothesized to constitute the formation of epileptogenic recurrent excitatory connections among normally unconnected granule cells (Tauck and Nadler, 1985; Sutula et al., 1988; Represa et al., 1993; Wuarin and Dudek, 1996). The appealing concept of a newly-formed, recurrent excitatory circuit (Tauck and Nadler, 1985) developing within the normally tonically inhibited dentate gyrus (Winson and Abzug, 1978; Misgeld et al., 1986; Lambert and Jones, 1990; Staley and Mody, 1992; Jung and McNaughton, 1993; Chawla et al., 2005), received indirect support from the finding that normal recurrent excitatory connections among CA3 pyramidal cells are tonically suppressed by GABA-mediated inhibition (Miles and Wong, 1987). This finding in a hippocampal region that normally contains recurrent excitatory interconnections implied that if granule cells formed similar recurrent excitatory connections after injury, a decrease in GABA-mediated inhibition hypothesized to occur in epilepsy might occasionally unmask

the interictally suppressed recurrent excitatory circuit lying beneath (Cronin et al., 1992). This hypothesis has had considerable influence on the concept of epileptogenesis despite the observations that: 1) aberrant mossy fibers contact inhibitory interneurons (Sloviter, 1992; Kotti et al., 1997; Wenzel et al., 2000; Buckmaster et al., 2002; Cavazos et al., 2003), as well as granule cells; 2) there is no evidence that granule cells become progressively hyperexcitable *in vivo* as mossy fiber sprouting progresses, and; 3) there is no evidence that granule cells are a source of the spontaneous seizures that define post-SE rats as “epileptic” (Harvey and Sloviter, 2005).

It has nonetheless been proposed that mossy fiber sprouting can be assumed to be predominantly excitatory, and essentially epileptogenic in nature, because individual mossy fibers make more synaptic contacts with granule cells than they do with inhibitory interneurons (Buckmaster et al., 2002). Based on the quantitative finding by these authors that each mossy fiber axon forms relatively few synaptic contacts with inhibitory interneurons, we conclude that the dense asymmetrical synaptic innervation of inhibitory basket cells by abnormally large presynaptic terminals represents a convergent input by many different aberrant mossy fibers onto the relatively small number of interneurons present. Given the extraordinarily divergent output of individual hippocampal inhibitory interneurons, and the ability of small numbers of interneurons to influence a large number of targeted principal cells (Soriano and Frotscher, 1989; Soriano et al., 1990; Cobb et al., 1995; Sik et al., 1995; 1997; Martinez et al., 1996), convergent excitatory input to inhibitory interneurons from a large number of granule cells may result in an amplification of inhibitory output that may underlie the observed granule cell hyperinhibition. Thus, together with the results of our physiological recordings, our anatomical findings do not support the view that mossy fiber sprouting can be assumed to be predominantly excitatory at the network level. To the contrary, mossy fiber sprouting may constitute a predominantly inhibitory feedback circuit.

We suggest that initial granule cell hyperexcitability is the result of the extensive loss of vulnerable hilar neurons (Sloviter, 1987, 1991; Obenaus et al., 1993; Sloviter et al., 2003; Zappone and Sloviter, 2004). The loss of hilar mossy cells, which normally project to the inner molecular layer, presumably triggers mossy fiber sprouting into this denervated subregion, which results in the gradual re-innervation of both inhibitory interneurons and granule cells that were partially deafferented by the initial mossy cell degeneration. Unlike granule cells, dentate inhibitory interneurons normally receive granule cell input (Ribak and Peterson, 1991; Blasco-Ibanez et al., 2000; Seress et al., 2001), and can therefore be assumed to possess all of the components of the intracellular signaling pathways that mediate normal interneuron-granule cell influences. The possibility that granule cells constitutively lack the cellular apparatus to respond fully to abnormal input from other granule cells might underlie the observation that direct interactions among granule cells are relatively weak in the synaptically reorganized dentate gyrus (Scharfman et al., 2003), although this is clearly conjectural.

The finding that granule cells always became less, rather than more, excitable over time is consistent with physiological studies in epileptic patients that indicate chronic hippocampal hyperinhibition (Cherlow et al., 1977; Colder et al., 1996; Wilson et al., 1998). The mechanism by which initial granule cell hyperexcitability is gradually reversed remains unclear, and could conceivably involve a wide variety of mechanisms, including changes in GABA_A receptor dynamics (Coulter, 2000; Cohen et al., 2003; Goodkin et al., 2005). Given that some GABA neurons may act to synchronize principal cell behavior (Isokawa-Akesson et al., 1989), and might conceivably be pro-epileptic in terms of their net effects (Maglóczky and Freund, 2005), it has been hypothesized that an interictal hippocampal hyperinhibitory state might cause spontaneous hippocampal epileptiform discharges (Bragin et al., 2002;

Engel et al., 2003) that then initiate the spontaneous behavioral seizures that develop in these animals (McNamara, 1999; Esclapez and Houser, 1999; Coulter, 2000). However, in a recent study of awake, chronically epileptic, and synaptically reorganized pilocarpine-treated rats, we reported that interictal granule cell hyperinhibition extended to the ictal state (Harvey and Sloviter, 2005). That is, during 235 spontaneous behavioral epileptic seizures, granule cell discharges never preceded the onset of the 235 spontaneous behavioral seizures. We have obtained identical results in kainate-treated rats during 8 spontaneous behavioral seizures observed while recording bilateral granule cell activity in the course of this study (unpublished results). Taken together, these data indicate that granule cell hyperinhibition in post-SE rats has not been shown to collapse or to initiate spontaneous granule cell epileptiform discharges. Thus, the hippocampus cannot be assumed to be a primary epileptic “focus” in rats subjected to prolonged SE initiated by kainate, or by pilocarpine (Harvey and Sloviter, 2005), both of which produce far more severe and multifocal brain damage (Schwob et al., 1980; Turski et al., 1987) than that seen in human temporal lobe epilepsy (Sloviter, 2005).

A recent study by Peng and Houser (2005) reported that epileptic, pilocarpine-treated mice exhibited c-Fos immunostaining in dentate granule cells after spontaneous behavioral seizures, and suggested that granule cells might have been the source of the spontaneous seizures that they observed in their epileptic mice. However, we recently showed in epileptic pilocarpine-treated rats that although granule cell discharges never preceded the onset of behavioral seizures, granule cells became c-Fos-positive when spontaneous seizures recruited granule cells to discharge (Harvey and Sloviter, 2005). Thus, without electrophysiological data to indicate that granule cells discharged prior to the onset of the spontaneous behavioral seizures they observed in mice, the results of Peng and Houser (2005) are consistent with our finding that granule cells can be recruited by seizures that originate elsewhere, and that when they discharge, they become c-Fos-positive (see Figure 12 of Harvey and Sloviter, 2005).

The lack of evidence indicating that the hippocampus of post-SE epileptic rats is itself “epileptic” raises questions about the common assumption that altered hippocampal parameters of structure and function in post-SE rats can be assumed to represent epileptogenic mechanisms. Clearly, *in vitro* analyses of hippocampi removed from post-SE epileptic animals, which exhibit widespread brain damage and seizures of undetermined origin (Bertram, 1997; Bertram et al., 2001; Harvey and Sloviter, 2005), may tend to underemphasize the epileptogenic contribution of the unexamined extrahippocampal structures, and to overemphasize the importance of the hippocampus by assuming, but not demonstrating, that the hippocampi of epileptic animals are “epileptic” (Tauck and Nadler, 1985; Wuarin and Dudek, 1996; Esclapez et al., 1997; Cossart et al., 2001; Bernard et al., 2004).

CONCLUSIONS

Although the cellular mechanisms underlying the initial hyperexcitability and the subsequent hyperinhibition that we observed were not the subject of this study, our *in vivo* recordings made sequentially in the same awake and chronically epileptic rats clearly indicate that granule cells do not become gradually more excitable with time as mossy fiber sprouting progresses. To the contrary, granule cells of epileptic kainate-treated rats consistently become progressively less excitable, as previously reported (Sloviter, 1992; Buckmaster and Dudek, 1997; Wu and Leung, 2001), and with a time course that parallels mossy fiber sprouting, as we recently reported in pilocarpine-treated rats (Harvey and Sloviter, 2005). As a working hypothesis, we suggest that mossy fiber sprouting alters the initial inhibitory interneuron malfunction possibly caused by mossy cell degeneration

(Sloviter, 1991; 1994; Sloviter et al., 2003), by gradually replacing some of the lost mossy cell input to the inner molecular layer. Therefore, rather than playing a primarily excitatory and epileptogenic role, mossy fiber sprouting may reinstate some of the features of normal interneuron-granule cell interactions, thereby restoring the dentate gyrus, to some extent, toward its original state as a powerfully inhibited structure that is relatively resistant to discharging (Winson and Abzug, 1978; Misgeld et al., 1986; Lambert and Jones, 1990; Staley and Mody, 1992; Jung and McNaughton, 1993; Chawla et al., 2005). Although it remains to be demonstrated that mossy fiber sprouting is causally related to the granule cell hyperinhibition that develops *in vivo*, we hypothesize that mossy fiber sprouting may play a clinically important role in retarding seizure spread (keeping subclinical seizures subclinical). Mossy fiber sprouting may also delay the occurrence of each spontaneous clinical seizure by impeding the recruitment of the dentate gyrus, which may play a role as an amplifying “gate” within the temporal lobe (Winson and Abzug, 1977; 1978; Heinemann et al., 1992; Lothman et al., 1992). Regardless, our previous (Harvey and Sloviter, 2005) and present findings in awake, chronically epileptic rats provide direct evidence against the hypothesis that mossy fiber sprouting necessarily constitutes an excitatory epileptogenic circuit in animals that exhibit spontaneous seizures of unknown origin following prolonged status epilepticus and widespread brain damage.

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LITERATURE CITED

- Amaral DG, Dent JA. Development of the mossy fibers of the dentate gyrus: I. A light and electron microscopic study of the mossy fibers and their expansions. *J Comp Neurol.* 1981; 195:51–86. [PubMed: 7204652]
- Andersen P, Holmqvist B, Voorhoeve PE. Entorhinal activation of dentate granule cells. *Acta Physiol Scand.* 1966; 66:448–460. [PubMed: 5927271]
- Babb TL, Kupfer WR, Pretorius JK, Crandall PH, Levesque MF. Synaptic reorganization by mossy fibers in human epileptic fascia dentata. *Neuroscience.* 1991; 42:351–363. [PubMed: 1716744]
- Bernard C, Anderson A, Becker A, Poolos NP, Beck H, Johnston D. Acquired dendritic channelopathy in temporal lobe epilepsy. *Science.* 2004; 305:532–305. [PubMed: 15273397]
- Bertram EH. Functional anatomy of spontaneous seizures in a rat model of limbic epilepsy. *Epilepsia.* 1997; 38:95–105. [PubMed: 9024190]
- Bertram EH, Mangan PS, Zhang D, Scott CA, Williamson JM. The midline thalamus: alterations and a potential role in limbic epilepsy. *Epilepsia.* 2001; 42:967–978. [PubMed: 11554881]
- Blackstad T. Commissural connections of the hippocampal region in the rat, with special reference to their mode of termination. *J Comp Neurol.* 1956; 105:417–537. [PubMed: 13385382]
- Blasco-Ibanez JM, Martinez-Guijarro FJ, Freund TF. Recurrent mossy fibers preferentially innervate parvalbumin-immunoreactive interneurons in the granule cell layer of the rat dentate gyrus. *Neuroreport.* 2000; 11:3219–3225. [PubMed: 11043552]
- Bragin A, Mody I, Wilson CL, Engel J Jr. Local generation of fast ripples in epileptic brain. *J Neurosci.* 2002; 22:2012–2021. [PubMed: 11880532]
- Buckmaster PS, Wenzel HJ, Kunkel DD, Schwartzkroin PA. Axon arbors and synaptic connections of hippocampal mossy cells in the rat *in vivo*. *J Comp Neurol.* 1996; 366:271–292. [PubMed: 8698887]

- Buckmaster PS, Dudek FE. Neuron loss, granule cell axon reorganization, and functional changes in the dentate gyrus of epileptic kainate-treated rats. *J Comp Neurol*. 1997; 385:385–404. [PubMed: 9300766]
- Buckmaster PS, Zhang GF, Yamawaki R. Axon sprouting in a model of temporal lobe epilepsy creates a predominantly excitatory feedback circuit. *J Neurosci*. 2002; 22:6650–6658. [PubMed: 12151544]
- Cavazos JE, Zhang P, Qazi R, Sutula TP. Ultrastructural features of sprouted mossy fiber synapses in kindled and kainic acid-treated rats. *J Comp Neurol*. 2003; 458:272–292. [PubMed: 12619081]
- Chang BS, Lowenstein DH. Mechanisms of disease; epilepsy. *N Engl J Med*. 2003; 349:1257–1266. [PubMed: 14507951]
- Chawla MK, Guzowski JF, Ramirez-Amaya V, Lipa P, Hoffman KL, Marriott LK, Worley PF, McNaughton BL, Barnes CA. Sparse, environmentally selective expression of Arc RNA in the upper blade of the rodent fascia dentata by brief spatial experience. *Hippocampus*. 2005; 15:579–586. [PubMed: 15920719]
- Cherlow DG, Dymond AM, Crandall PH, Walter RD, Serafetinides EA. Evoked response and after-discharge thresholds to electrical stimulation in temporal lobe epileptics. *Arch Neurol*. 1977; 34:527–531. [PubMed: 889493]
- Claiborne BJ, Amaral DG, Cowan WM. A light and electron microscopic analysis of the mossy fibers of the rat dentate gyrus. *J Comp Neurol*. 1986; 246:435–458. [PubMed: 3700723]
- Cobb SR, Buhl EH, Halasy K, Paulsen O, Somogyi P. Synchronization of neuronal activity in hippocampus by individual GABAergic interneurons. *Nature*. 1995; 378:75–78. [PubMed: 7477292]
- Cohen AS, Lin DD, Quirk GL, Coulter DA. Dentate granule cell GABA(A) receptors in epileptic hippocampus: enhanced synaptic efficacy and altered pharmacology. *Eur J Neurosci*. 2003; 17:1607–1616. [PubMed: 12752378]
- Colder BW, Wilson CL, Frysinger RC, Chao LC, Harper RM, Engel J Jr. Neuronal synchrony in relation to burst discharge in epileptic human temporal lobes. *J Neurophysiol*. 1996; 75:2496–2508. [PubMed: 8793759]
- Cossart R, Dinocourt C, Hirsch JC, Merchán-Pérez A, De Felipe J, Ben-Ari Y, Esclapez M, Bernard C. Dendritic but not somatic GABAergic inhibition is decreased in experimental epilepsy. *Nat Neurosci*. 2001; 4:52–62. [PubMed: 11135645]
- Coulter DA. Mossy fiber zinc and temporal lobe epilepsy: pathological association with altered “epileptic” gamma-aminobutyric acid A receptors in dentate granule cells. *Epilepsia*. 2000; 41((Suppl) 6):S96–S99. [PubMed: 10999528]
- Cronin J, Obenaus A, Houser CR, Dudek FE. Electrophysiology of dentate granule cells after kainate-induced synaptic reorganization of the mossy fibers. *Brain Res*. 1992; 573:305–310. [PubMed: 1504768]
- de Lanerolle NC, Kim JH, Williamson A, Spencer SS, Zaveri HP, Eid T, Spencer DD. A retrospective analysis of hippocampal pathology in human temporal lobe epilepsy: evidence for distinctive patient subcategories. *Epilepsia*. 2003; 44:677–687. [PubMed: 12752467]
- Deller T, Nitsch R, Frotscher M. Associational and commissural afferents of parvalbumin-immunoreactive neurons in the rat hippocampus: a combined immunocytochemical and PHA-L study. *J Comp Neurol*. 1994; 350:612–622. [PubMed: 7890833]
- Engel J Jr, Wilson C, Bragin A. Advances in understanding the process of epileptogenesis based on patient material: what can the patient tell us? *Epilepsia*. 2003; 44(Suppl 12):60–71. [PubMed: 14641562]
- Esclapez M, Hirsch JC, Khazipov R, Ben-Ari Y, Bernard C. Operative GABAergic inhibition in hippocampal CA1 pyramidal neurons in experimental epilepsy. *Proc Natl Acad Sci U S A*. 1997; 94:12151–12156. [PubMed: 9342378]
- Esclapez M, Houser CR. Up-regulation of GAD65 and GAD67 in remaining hippocampal GABA neurons in a model of temporal lobe epilepsy. *J Comp Neurol*. 1999; 412:488–505. [PubMed: 10441235]
- Falconer MA, Taylor DC. Surgical treatment of drug-resistant epilepsy due to mesial temporal lobe sclerosis; etiology and significance. *Arch Neurol*. 1968; 19:353–361. [PubMed: 5677186]

- Frotscher M, Zimmer J. Lesion-induced mossy fibers to the molecular layer of the rat fascia dentata: identification of postsynaptic granule cells by the Golgi-EM technique. *J Comp Neurol.* 1983; 215:299–311. [PubMed: 6189867]
- Goodkin HP, Yeh JL, Kapur J. Status epilepticus increases the intracellular accumulation of GABA_A receptors. *J Neurosci.* 2005; 25:5511–5520. [PubMed: 15944379]
- Gorter JA, van Vliet EA, Aronica E, Lopes da Silva FH. Long-lasting increased excitability differs in dentate gyrus vs. CA1 in freely moving chronic epileptic rats after electrically induced status epilepticus. *Hippocampus.* 2002; 12:311–324. [PubMed: 12099483]
- Hardison JL, Okazaki MM, Nadler JV. Modest increase in extracellular potassium unmasks effect of recurrent mossy fiber growth. *J Neurophysiol.* 2000; 84:2380–2389. [PubMed: 11067980]
- Harvey BD, Sloviter RS. Hippocampal granule cell activity and c-Fos expression during spontaneous seizures in awake, chronically epileptic, pilocarpine-treated rats; implications for hippocampal epileptogenesis. *J Comp Neurol.* 2005; 488:441–462.
- Heinemann U, Beck H, Dreier JP, Ficker E, Stabel J, Zhang CL. The dentate gyrus as a regulated gate for the propagation of epileptiform activity. *Epilepsy Res (Suppl).* 1992; 7:273–280. [PubMed: 1334666]
- Houser CR, Miyashiro JE, Swartz BE, Walsh GO, Rich JR, Delgado-Escueta AV. Altered patterns of dynorphin immunoreactivity suggest mossy fiber reorganization in human hippocampal epilepsy. *J Neurosci.* 1990; 10:267–282. [PubMed: 1688934]
- Isokawa-Akesson M, Wilson CL, Babb TL. Prolonged inhibition in synchronously firing human hippocampal neurons. *Epilepsy Res.* 1989; 3:236–247. [PubMed: 2731521]
- Jung MW, McNaughton BL. Spatial selectivity of unit activity in the hippocampal granular layer. *Hippocampus.* 1993; 3:165–182. [PubMed: 8353604]
- Klitgaard H, Matagne A, Vanneste-Goemaere J, Margineanu DG. Pilocarpine-induced epileptogenesis in the rat: impact of initial duration of status epilepticus on electrophysiological and neuropathological alterations. *Epilepsy Res.* 2002; 51:93–107. [PubMed: 12350385]
- Kotti T, Riekkinen PJ Sr, Miettinen R. Characterization of target cells for aberrant mossy fiber collaterals in the dentate gyrus of epileptic rat. *Exp Neurol.* 1997; 146:323–330. [PubMed: 9270041]
- Lambert JDC, Jones RSG. A reevaluation of excitatory amino acid-mediated synaptic transmission in rat dentate gyrus. *J Neurophysiol.* 1990; 64:119–132. [PubMed: 1974917]
- Laurberg S, Zimmer J. Lesion-induced sprouting of hippocampal mossy fiber collaterals to the fascia dentata in developing and adult rats. *J Comp Neurol.* 1981; 200:433–459. [PubMed: 7276246]
- Lothman EW, Stringer JL, Bertram EH. The dentate gyrus as a control point for seizures in the hippocampus and beyond. *Epilepsy Res (Suppl).* 1992; 7:301–313. [PubMed: 1334669]
- Maglóczy Z, Freund TF. Impaired and repaired inhibitory circuits in the epileptic human hippocampus. *Trends Neurosci.* 2005; 28:334–340. [PubMed: 15927690]
- Martinez A, Lubke J, Del Rio JA, Soriano E, Frotscher M. Regional variability and postsynaptic targets of chandelier cells in the hippocampal formation of the rat. *J Comp Neurol.* 1996; 376:28–44. [PubMed: 8946282]
- McNamara JO. Emerging insights into the genesis of epilepsy. *Nature.* 1999; 399(6738 Suppl):A15–22. [PubMed: 10392576]
- Miles R, Wong RK. Inhibitory control of local excitatory circuits in the guinea-pig hippocampus. *J Physiol.* 1987; 388:611–629. [PubMed: 3656200]
- Misgeld U, Deisz RA, Dodt HU, Lux HD. The role of chloride transport in postsynaptic inhibition of hippocampal neurons. *Science.* 1986; 232:1413–1415. [PubMed: 2424084]
- Molnar P, Nadler JV. Mossy fiber-granule cell synapses in the normal and epileptic rat dentate gyrus studied with minimal laser photostimulation. *J Neurophysiol.* 1999; 82:1883–1894. [PubMed: 10515977]
- Nadler JV, Perry BW, Cotman CW. Selective reinnervation of hippocampal area CA1 and the fascia dentata after destruction of CA3-CA4 afferents with kainic acid. *Brain Res.* 1980; 182:1–9. [PubMed: 7350980]

- Obenaus A, Esclapez M, Houser CR. Loss of glutamate decarboxylase mRNA-containing neurons in the rat dentate gyrus following pilocarpine-induced seizures. *J Neurosci.* 1993; 13:4470–4485. [PubMed: 8410199]
- Okazaki MM, Evenson DA, Nadler JV. Hippocampal mossy fiber sprouting and synapse formation after status epilepticus in rats: visualization after retrograde transport of biocytin. *J Comp Neurol.* 1995; 352:515–534. [PubMed: 7721998]
- Parent JM, Lowenstein DH. Mossy fiber reorganization in the epileptic hippocampus. *Curr Opin Neurol.* 1997; 10:103–109. [PubMed: 9146991]
- Patrylo PR, Dudek FE. Physiological unmasking of new glutamatergic pathways in the dentate gyrus of hippocampal slices from kainate-induced epileptic rats. *J Neurophysiol.* 1998; 79:418–429. [PubMed: 9425210]
- Paxinos, G.; Watson, C. The rat brain in stereotaxic coordinates. Academic; San Diego: 1998.
- Peng Z, Houser CR. Temporal patterns of Fos expression in the dentate gyrus after spontaneous seizures in a mouse model of temporal lobe epilepsy. *J Neurosci.* 2005; 25:7210–7220. [PubMed: 16079403]
- Petralia RS, Wang YX, Mayat E, Wenthold RJ. Glutamate receptor subunit 2-selective antibody shows a differential distribution of calcium-impermeable AMPA receptors among populations of neurons. *J Comp Neurol.* 1997; 385:456–476. [PubMed: 9300771]
- Represa A, Jorquera I, Le Gal La Salle G, Ben-Ari Y. Epilepsy induced collateral sprouting of hippocampal mossy fibers: does it induce the development of ectopic synapses with granule cell dendrites? *Hippocampus.* 1993; 3:257–268. [PubMed: 8353609]
- Ribak CE, Anderson L. Ultrastructure of the pyramidal basket cells in the dentate gyrus of the rat. *J Comp Neurol.* 1980; 192:903–916. [PubMed: 7419759]
- Ribak CE, Peterson GM. Intragranular mossy fibers in rats and gerbils form synapses with the somata and proximal dendrites of basket cells in the dentate gyrus. *Hippocampus.* 1991; 1:355–364. [PubMed: 1669315]
- Scharfman HE, Sollas AL, Berger RE, Goodman JH. Electrophysiological evidence of monosynaptic excitatory transmission between granule cells after seizure-induced mossy fiber sprouting. *J Neurophysiol.* 2003; 90:2536–2547. [PubMed: 14534276]
- Schwob JE, Fuller T, Price JL, Olney JW. Widespread patterns of neuronal damage following systemic or intracerebral injections of kainic acid: a histological study. *Neuroscience.* 1980; 5:991–1014. [PubMed: 7402461]
- Seress L, Ribak CE. Direct commissural connections to the basket cells of the hippocampal dentate gyrus: anatomical evidence for feed-forward inhibition. *J Neurocytol.* 1984; 13:215–225. [PubMed: 6726288]
- Seress L, Abraham H, Paleszter M, Gallyas F. Granule cells are the main source of excitatory input to a subpopulation of GABAergic hippocampal neurons as revealed by electron microscopic double staining for zinc histochemistry and parvalbumin immunocytochemistry. *Exp Brain Res.* 2001; 136:456–462. [PubMed: 11291726]
- Sik A, Penttonen M, Buzsaki G. Interneurons in the hippocampal dentate gyrus: an in vivo intracellular study. *Eur J Neurosci.* 1997; 9:573–588. [PubMed: 9104599]
- Sik A, Penttonen M, Ylinen A, Buzsaki G. Hippocampal CA1 interneurons: an in vivo intracellular labeling study. *J Neurosci.* 1995; 15:6651–6665. [PubMed: 7472426]
- Sloviter RS. A simplified Timm stain procedure compatible with formaldehyde fixation and routine paraffin embedding of rat brain. *Brain Res Bull.* 1982; 8:771–774. [PubMed: 6182964]
- Sloviter RS. “Epileptic” brain damage in rats induced by sustained electrical stimulation of the perforant path. I. Acute electrophysiological and light microscopic studies. *Brain Res Bull.* 1983; 10:675–697. [PubMed: 6871737]
- Sloviter RS. Decreased hippocampal inhibition and a selective loss of interneurons in experimental epilepsy. *Science.* 1987; 235:73–76. [PubMed: 2879352]
- Sloviter RS. Permanently altered hippocampal structure, excitability, and inhibition after experimental status epilepticus in the rat: the dormant basket cell hypothesis and its possible relevance to temporal lobe epilepsy. *Hippocampus.* 1991; 1:41–66. [PubMed: 1688284]

- Sloviter RS. Possible functional consequences of synaptic reorganization in the dentate gyrus of kainate-treated rats. *Neurosci Lett*. 1992; 137:91–96. [PubMed: 1625822]
- Sloviter RS. The functional organization of the hippocampal dentate gyrus and its relevance to the pathogenesis of temporal lobe epilepsy. *Ann Neurol*. 1994; 35:640–654. [PubMed: 8210220]
- Sloviter RS. The neurobiology of temporal lobe epilepsy; too much information, not enough knowledge. *CR Biologies*. 2005; 328:143–153.
- Sloviter RS, Brisman JL. Lateral inhibition and granule cell synchrony in the rat hippocampal dentate gyrus. *J Neurosci*. 1995; 15:811–820. [PubMed: 7823182]
- Sloviter RS, Damiano BP. Sustained electrical stimulation of the perforant path duplicates kainate-induced electrophysiological effects and hippocampal damage in rats. *Neurosci Lett*. 1981; 24:279–284. [PubMed: 7279294]
- Sloviter RS, Zappone CA, Harvey BD, Bumanglag AV, Bender RA, Frotscher M. “Dormant basket cell” hypothesis revisited; relative vulnerabilities of dentate gyrus mossy cells and inhibitory interneurons after hippocampal status epilepticus in the rat. *J Comp Neurol*. 2003; 459:44–76. [PubMed: 12629666]
- Somogyi P, Hodgson AJ. Antisera to gamma-aminobutyric acid. III. Demonstration of GABA in Golgi-impregnated neurons and in conventional electron microscopic sections of cat striate cortex. *J Histochem Cytochem*. 1985; 33:249–257. [PubMed: 2579124]
- Soriano E, Frotscher M. A GABAergic axo-axonic cell in the fascia dentate controls the main excitatory hippocampal pathway. *Brain Res*. 1989; 503:170–174. [PubMed: 2611653]
- Soriano E, Nitsch R, Frotscher M. Axo-axonic chandelier cells in the rat fascia dentata: Golgi-electron microscopy and immunocytochemical studies. *J Comp Neurol*. 1990; 293:1–25. [PubMed: 1690225]
- Spencer SS. Neural networks in human epilepsy: evidence of and implications for treatment. *Epilepsia*. 2002; 43:219–227. [PubMed: 11906505]
- Staley KJ, Mody I. Shunting of excitatory input to dentate gyrus granule cells by a depolarizing GABA receptor-mediated postsynaptic conductance. *J Neurophysiol*. 1992; 68:197–212. [PubMed: 1381418]
- Subula T, He XX, Cavazos J, Scott G. Synaptic reorganization in the hippocampus induced by abnormal functional activity. *Science*. 1988; 239:1147–1150. [PubMed: 2449733]
- Subula T, Cascino G, Cavazos J, Parada I, Ramirez L. Mossy fiber synaptic reorganization in the epileptic human temporal lobe. *Ann Neurol*. 1989; 26:321–330. [PubMed: 2508534]
- Tauk DL, Nadler JV. Evidence of functional mossy fiber sprouting in hippocampal formation of kainic acid treated rats. *J Neurosci*. 1985; 5:1016–1022. [PubMed: 3981241]
- Turski L, Cavalheiro EA, Czuczwar SJ, Turski WA, Kleinrok Z. The seizures induced by pilocarpine: behavioral, electroencephalographic and neuropathological studies in rodents. *Pol J Pharmacol Pharm*. 1987; 39:545–555. [PubMed: 3333612]
- Wenzel HJ, Buckmaster PS, Anderson NL, Wenzel ME, Schwartzkroin PA. Ultrastructural localization of neurotransmitter immunoreactivity in mossy cell axons and their synaptic targets in the rat dentate gyrus. *Hippocampus*. 1997; 7:559–570. [PubMed: 9347352]
- Wenzel HJ, Woolley CS, Robbins CA, Schwartzkroin PA. Kainic acid-induced mossy fiber sprouting and synapse formation in the dentate gyrus of rats. *Hippocampus*. 2000; 10:244–260. [PubMed: 10902894]
- Wilson CL, Khan SU, Engel J Jr, Isokawa M, Babb TL, Behnke EJ. Paired pulse suppression and facilitation in human epileptogenic hippocampal formation. *Epilepsy Res*. 1998; 31:211–230. [PubMed: 9722031]
- Winson J, Abzug C. Gating of neuronal transmission in the hippocampus: efficacy of transmission varies with behavioral state. *Science*. 1977; 196:1223–1225. [PubMed: 193192]
- Winson J, Abzug C. Neuronal transmission through hippocampal pathways dependent on behavior. *J Neurophysiol*. 1978; 41:716–732. [PubMed: 207830]
- Wu K, Leung LS. Enhanced but fragile inhibition in the dentate gyrus in vivo in the kainic acid model of temporal lobe epilepsy: a study using current source density analysis. *Neuroscience*. 2001; 104:379–396. [PubMed: 11377842]

- Wuarin JP, Dudek FE. Electrographic seizures and new recurrent excitatory circuits in the dentate gyrus of hippocampal slices from kainate-treated epileptic rats. *J Neurosci.* 1996; 16:4438–4448. [PubMed: 8699254]
- Zappone CA, Sloviter RS. Translaminar disinhibition in the rat hippocampal dentate gyrus after seizure-induced degeneration of vulnerable hilar neurons. *J Neurosci.* 2004; 24:853–864. [PubMed: 14749430]
- Zhang N, Houser CR. Ultrastructural localization of dynorphin in the dentate gyrus in human temporal lobe epilepsy: a study of reorganized mossy fiber synapses. *J Comp Neurol.* 1999; 405:472–490. [PubMed: 10098940]

Figure 1.

Dentate granule cell pathophysiology in awake, freely-moving rats after kainate-induced status epilepticus (SE). A, Granule cell potentials evoked by paired-pulse stimulation of the perforant pathway at 0.1 Hz. A1, in a normal rat prior to kainate injection, afferent stimulation evoked a single granule cell population spike (arrow), which inhibited the response to the second of two identical stimuli (asterisk), illustrating paired-pulse suppression at an interpulse interval of 40 msec. A2, 4 days post-SE in the same animal, granule cell responses to identical afferent stimuli exhibited multiple population spikes and a failure of paired-pulse suppression. A3, hyperexcitable responses were evident throughout the week after kainate-induced SE, and paired-pulse suppression was not restored 7 days

post-SE. A4, 28 days post-SE; note increased paired-pulse suppression of the second population spike (asterisk) compared to the responses to identical afferent stimuli in the same animal at 4 and 7 days post-SE. However, granule cells still responded abnormally to afferent stimulation by generating multiple population spikes in response to the first afferent stimulus. B, gradual restoration of paired-pulse suppression evoked by 0.1 Hz perforant pathway stimulation. Compared to the responses in 4 saline-treated control rats, 5 kainate-treated rats exhibited decreased paired-pulse suppression (measured at a 40 msec interpulse interval) throughout the first week post-SE. Paired pulse suppression recovered gradually over the subsequent 3 weeks. C, Augmented granule cell paired-pulse suppression in chronically epileptic kainate-treated rats >60 days post SE. Percent paired-pulse suppression (ratio of the amplitudes of two evoked population responses) of saline-treated control rats (n=3) and chronically epileptic kainate-treated rats (n=4) at different interpulse intervals (20-100 msec). Note in KA-treated rats, that paired-pulse suppression exists at interpulse intervals at which all control animals exhibited paired-pulse potentiation (asterisks, $p < 0.05$). All rats were implanted with chronic electrodes after the kainate-treated rats were chronically epileptic (>60 days post-kainate or saline treatment). This was done to assess granule cell excitability in the chronic epileptic state without the possibility that the presence of electrodes could have influenced the epileptogenic process.

Figure 2.

Dentate gyrus pathology after kainate-induced status epilepticus (SE). A1, normal immunostaining of the neuronal marker NeuN in a saline-treated control animal, showing the neuronal populations of the normal dentate gyrus, which include granule cells of the granule cell layer (sg, stratum granulosum) and scattered neurons of the hilus (h). A2, normal immunostaining of subunit 2 of the glutamate receptor (GluR2). A3, the Timm-stained dentate gyrus of the same saline-treated control animal (darkfield illumination), showing the normal zinc-positive terminals of the inner molecular layer (arrows), which derive from the mossy cell innervation of this layer (Sloviter, 1982). B, immunostaining and Timm stain 28 days post-SE. B1, B2, note extensive loss of NeuN and GluR2 immunostaining of hilar (h) and CA3 pyramidal cells (CA3) compared to the control in (A). B3, following extensive hilar neuron loss, Timm staining reveals the formation of dense mossy fiber sprouting in the inner molecular layer (arrows), which replaces the lost mossy cell axon plexus to this lamina. C1, 7 days post-SE, detectable, but submaximal, mossy fiber sprouting is evident. C2, In a control animal, Timm-positive elements surround and outline the soma and dendrites of a granule cell layer interneuron (arrow), but there are relatively few Timm-positive elements in the inner molecular layer (iml). C3, in co-processed sections from a kainate-treated rat 7 days post-SE, early mossy fiber sprouting is evident throughout the inner molecular layer, outlining the soma and dendrites (arrows) of an interneuron of the granule cell layer. Scale bar in C3: 100 μ m in A, B, and C1; 25 μ m in C2 and C3.

Figure 3.

Parvalbumin (PV)-positive inhibitory interneurons are targets of aberrant mossy fiber sprouting 17-27 days after SE-induced hilar neuron loss. A1, A2, Low and higher magnification views of a kainate-treated rat 22 days post-SE, showing that early synaptic reorganization in the inner molecular layer (sm; stratum moleculare) targets PV-positive interneurons. Note in (A2) that Timm-positive axonal varicosities leave the inner molecular layer to innervate a PV-positive dendrite (arrows) of an interneuron of the inner molecular layer. B-I, similar scenes in sections from 4 different kainate-treated rats perfusion-fixed 17-27 days post-SE showing that Timm staining during the early post-SE period targets PV-positive interneurons (arrows). Note in panels C, E, G, and I that beyond the limit of the

main Timm-stained plexus in the inner molecular layer, Timm-positive terminals appear to target PV-positive interneurons preferentially, in that most or all of the Timm-stained terminals at the margin of the mossy fiber plexus are apposed to PV-positive dendrites (arrows). A: 22 days post-SE; B: 27 days; C,D,F: 23 days; E,G,I: 17 days; H: 22 days. Scale bar: A1: 25 :m; A2-I: 12.5 :m.

Figure 4.

Ultrastructure of the granule cell layer 10 weeks after saline or kainate (KA)- induced status epilepticus (SE). A, in a saline-treated control, an interneuron of the granule cell layer, exhibiting the characteristics of dentate basket cells (large soma with abundant cytoplasm and an infolded nucleus; Ribak and Anderson, 1980) receives relatively small presynaptic terminals (box magnified in inset) on its ascending dendrite. B, 10 weeks after KA-induced SE, a similar dentate basket cell receives numerous abnormally large presynaptic terminals (shown in inset) exhibiting the morphological characteristics of mossy fiber boutons (cytoplasm with tightly packed vesicles, mitochondria, and dense core vesicles), which form asymmetrical synapses with the dendrite. Scale bar: 5 :m (insets: 1.25 :m).

Figure 5.

Ultrastructure of the granule cell layer 10 weeks after kainate (KA)- induced status epilepticus. A, Higher magnification of the ascending dendrite shown in the previous figure. Note that the dendritic shaft and a rare dendritic spine (sp) receive dense synaptic input from abnormally large presynaptic terminals forming exclusively asymmetrical contacts (arrows). B1, B2, in a serial section of the same interneuron, a different dendritic branch with a dendritic spine (sp) receives abnormally large presynaptic terminals (arrows) over much of the dendritic surface. Scale bar: 1 :m in A; 1.5 :m in B1; 0.75 :m in B2.

Figure 6.

Comparison of the morphological appearances of presynaptic terminals in the dentate inner molecular layer after saline or kainate (KA)- induced status epilepticus (SE). A, in a saline treated control animal, a presynaptic terminal (asterisk) forms asymmetrical synapses on dendritic spines (sp) of presumed granule cell dendrites. B, 10 weeks post-SE, an abnormally large presynaptic terminal forms 3 asymmetrical synapses on dendritic spines (sp) of 3 presumed granule cell dendrites. C, 10 weeks post-SE, a section from a kainate-treated rat shows that the inner molecular layer contains abnormally large presynaptic terminals (asterisks) exhibiting the morphological features of large mossy fiber terminals. Scale bar: 1.5 :m.

Figure 7.

GABA immunocytochemical electron microscopy of the dentate inner molecular layer 10 weeks after saline or kainate (KA)-induced status epilepticus (SE). A1,A2, In control sections, proximal dendrites of GABA-positive interneurons (D) are contacted by small immunonegative boutons (1) establishing asymmetric synaptic contacts, and by GABA-positive terminals (2) forming symmetric synapses. Arrows point to asymmetric synaptic contacts. B1,B2, Ten weeks after kainate injection, numerous large terminals (1), densely filled with clear synaptic vesicles, and containing dense-core vesicles (short arrows), emerge from thin, unmyelinated preterminal axons (white arrow in B1). These mossy fiber boutons

establish asymmetric synaptic contacts (black arrows) with GABA-positive dendritic shafts (D). Scale bar: 0.5 :m.