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Hippocampal injury, atrophy, synaptic reorganization, and epileptogenesis after perforant pathway stimulation-induced status epilepticus in the mouse

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Abstract

Prolonged dentate granule cell discharges produce hippocampal injury and chronic epilepsy in rats. In preparing to study this epileptogenic process in genetically altered mice, we determined whether the background strain used to generate most genetically altered mice, the C57BL/6 mouse, is vulnerable to stimulation-induced seizure-induced injury. This was necessary because C57BL/6 mice are reportedly resistant to the neurotoxic effects of kainate-induced seizures, which we hypothesized to be related to strain differences in kainate's effects, rather than genetic differences in intrinsic neuronal vulnerability. Bilateral perforant pathway stimulation-induced granule cell discharge for four hours under urethane anesthesia produced degeneration of GluR2-positive hilar mossy cells and peptide-containing interneurons in both FVB/N (kainate-vulnerable) and C57BL/6 (kainate-resistant) mice, indicating no strain differences in neuronal vulnerability to seizure activity. Granule cell discharge for two hours in C57BL/6 mice destroyed most GluR2-positive dentate hilar mossy cells, but not peptide-containing hilar interneurons, indicating that mossy cells are the neurons most vulnerable to this insult. Stimulation for 24 hours caused extensive hippocampal neuron loss and injury to the septum and entorhinal cortex, but no other detectable damage. Mice stimulated for 24 hours developed hippocampal sclerosis, granule cell mossy fiber sprouting, and chronic epilepsy, but not the granule cell layer hypertrophy (granule cell dispersion) produced by intrahippocampal kainate. These results demonstrate that perforant pathway stimulation in mice reliably reproduces the defining features of human mesial temporal lobe epilepsy with hippocampal sclerosis. Experimental studies in transgenic or knockout mice are feasible if electrical stimulation is used to produce controlled epileptogenic insults.

Keywords

hippocampus; epileptogenesis; rat; mouse; dentate gyrus

INTRODUCTION

Prolonged hippocampal granule cell discharges in rats evoked by perforant pathway stimulation cause a selective loss of vulnerable hippocampal neurons, and damage to synaptically connected extra- hippocampal neurons (Sloviter and Damiano, 1981; McIntyre

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et al., 1982; Sloviter, 1983). Cell death may be directly epileptogenic (Sloviter, 1987; 1991; 1994; Bumanglag and Sloviter, 2008), or it may serve as a triggering stimulus that initiates secondary epileptogenic mechanisms (Tauck and Nadler, 1985; Parent et al., 1997; Chang and Lowenstein, 2003). The cellular processes mediating excitation-induced neuron death, neuroprotection, and injury-associated epileptogenesis are incompletely understood (Henshall and Simon, 2005), but studies in mice have begun to elucidate these processes (Schauwecker et al., 2000; Schauwecker, 2002; 2006; Hatazaki et al., 2007). Prolonged perforant pathway stimulation in rats is a useful animal model of seizure-induced brain damage and epileptogenesis because it reliably reproduces the hippocampal pathology and hippocampal epileptogenesis that characterizes the human neurological condition, and avoids the use of chemoconvulsant drugs and the direct damage caused by intrahippocampal stimulation or injection (Sloviter et al., 2007; Bumanglag and Sloviter, 2008).

Before studying epileptogenesis in transgenic or gene knockout mice, we sought to determine whether the electrical stimulation paradigm that causes hippocampal sclerosis and epileptogenesis in rats produces similar pathology and epilepsy in normal mice. One possible obstacle to studying genetically altered mice relates to the finding that the hippocampus of the C57BL/6 background strain used for generating many genetically altered mice (Brinster et al., 1985; Mátyás et al., 2004), is resistant to the excitotoxic effects of kainate-induced seizure activity (Schauwecker and Steward, 1997; Schauwecker, 2002; 2006; McCord et al., 2008). To differentiate between strain differences in the actions of kainate, and possible strain differences in the intrinsic vulnerability of hippocampal neurons to the same excitotoxic insult, we assessed the hippocampal response to a minimally injurious duration of afferent excitation in the mouse strains reported to be vulnerable (FVB/N) or resistant (C57BL/6) to kainate-induced seizure activity (Schauwecker and Steward, 1997). We then addressed the main goal of this study, which was to determine whether prolonged perforant pathway stimulation reliably produces hippocampal sclerosis, synaptic reorganization, and a permanent epileptic state in C57BL/6 mice, as it does in rats. It has also been reported in mice that intrahippocampal injection of kainate produces massive hypertrophy of the granule cell layer (Suzuki et al., 1995; Bouillier et al., 1999; Riban et al., 2002), which apparently results from a loss of reelin expression that normally maintains the laminar structure of the granule cell layer (Heinrich et al., 2006). Therefore, we determined whether granule cell dispersion is an obligatory consequence of seizure-induced hippocampal damage and epileptogenesis in mice, or if it is a unique result of intrahippocampal kainate injection possibly unrelated to, or not required by, the epileptogenic process.

METHODS

Animal treatment

Male C57BL/6 and FVB/N mice (6-12 weeks; 20-25g; Harlan; Indianapolis, IN) were treated in accordance with the guidelines of the National Institutes of Health for the humane treatment of animals, and the methods used were approved by the University of Arizona Institutional Animal Care and Use Committee. Eighty mice were used in this study, including twelve FVB/N mice (9 stimulated and 3 sham controls), and sixty-eight C57BL/6 mice. The sixty-eight C57BL/6 mice included forty-four mice stimulated for two hours (n=6), four hours (n=9), or 24 hours (n=29), five mice that received an intrahippocampal injection of kainate, two mice injected with 0.9% sodium chloride as vehicle controls, fourteen mice used as sham stimulated controls, and three naive mice used to assess normal anatomy.

Electrophysiological and video monitoring methods

Mice were anesthetized with urethane (1.9 g/kg sc; 12.5 mg urethane/ml 0.9% saline; Sigma Chemical, St. Louis, MO), and their temperature was maintained at 37°C with a thermostatically-controlled blanket (Harvard Instruments, Holliston, MA). Bipolar stimulating electrodes were placed in the angular bundles of the perforant pathways, and one recording electrode was placed in the left dentate granule cell layer, as previously described (Kienzler et al., 2006). The optimal locations of the stimulating electrodes were determined by maximizing the evoked responses to perforant pathway stimulation (Andersen et al., 1966). Electrical stimulation consisted of 15-20 V paired-pulse stimuli delivered at 2 Hz, with a 40 msec interpulse interval, plus 10 sec long, 20 Hz stimulus trains of single, 15-20 V stimuli delivered through the same electrodes once per minute for two, four, or 24 hours. Biphasic stimuli (0.1 msec duration) were generated by a Grass S88 stimulator and SIU5 stimulus isolation unit (Grass Instruments, West Warwick, RI). Evoked and spontaneous granule cell layer activities were amplified and recorded digitally at a 10 kHz sampling rate (AD Instruments, Mountain View, CA). Continuous (24/7) video-monitoring began two months after stimulation in some animals utilizing a Panasonic model 9622 color CCD day/night infrared camera and surveillance software (Ben Software, London, UK). Video files were played back and observed at high speed (6-16X) to detect behavioral seizures. Spontaneous behavioral seizures were scored according to the Racine scale (Racine, 1972). Only behavioral seizures clearly identifiable by video analysis on review at normal playback speed as Stage 3-5 motor seizures were judged to be epileptic seizures.

Intrahippocampal kainate injection

Five C57BL/6 mice under chloral hydrate anesthesia (0.7 g/kg sc; Sigma Chemical) were injected with kainic acid (0.2 µg/ 50 nl saline; Ocean Produce International, Shelburne, Nova Scotia, Canada) into the left dentate gyrus to produce mice with granule cell layer hypertrophy (Suzuki et al., 1995; Bouilleret et al., 1999; Riban et al., 2002). The injection location (the inner blade of the granule cell layer) was identified using a kainate-filled recording electrode attached to a 1 µl Hamilton microsyringe by optimizing the characteristic granule cell responses to perforant pathway stimulation, as previously described (Zappone and Sloviter, 2004). Two mice were injected with 50 nl of 0.9% sodium chloride as vehicle controls.

Perfusion-fixation and tissue treatment

Mice were anesthetized with urethane and perfused through the aorta by gravity-feed with saline for two min followed by 4% paraformaldehyde in 0.1M phosphate buffer, pH 7.4, for 10 min. Other mice, intended for Timm staining (Sloviter, 1982), were perfused with 0.1% sodium sulphide in 0.1M phosphate buffer, pH 7.4, for one min prior to the saline and paraformaldehyde solutions described above. After storage of the intact mice overnight at 4°C, brains were removed from the skull and 40 µm-thick sections were cut in 0.1M Tris (hydroxymethylaminomethane) buffer, pH 7.6, using a Vibratome. In most cases, brains were first cut in the horizontal plane from the base of the brain, and then the remaining block was cut coronally to obtain dorsal hippocampal sections. All histological and immunocytochemical procedures were as previously described (Sloviter et al, 2006). Adjacent sections were mounted on subbed slides for subsequent Nissl (1% cresyl violet) and Fluoro-Jade B staining (FJB; Schmued and Hopkins, 2000). Other sections remained in Tris buffer for subsequent immunocytochemistry.

Immunocytochemistry protocol

Sections were mounted on Superfrost Plus slides, air dried, and placed in 0.1 M 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 1:2,000; AB1768; lot No. 22070361; Chemicon, Temecula, CA); 2) mouse anti-NeuN (diluted 1:10,000; MAB377; lot No. 22021251; Chemicon); 3) rabbit anti-somatostatin-28 (diluted 1:100,000; IH8004; lot No. 971457; Peninsula, Torrance, CA); 4) rabbit anti-Neuropeptide Y (diluted 1:100,000; IH7172; lot No. 991025; Peninsula). 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 minutes minimum).

Slides were incubated in biotinylated secondary antibody solution (1:2,000 dilution of goat anti-mouse or goat anti-rabbit; Vector, Burlingame, CA) in Tris-BSA-Triton X buffer for two hours, washed in the same buffer, and then incubated for two hours in avidin-biotin-HRP complex (Vector Elite kit diluted 1:1,000 in Tris-BSA-Triton X buffer). Slides were then washed in Tris (3×5 min minimum) and incubated in a hydrogen peroxide-generating diaminobenzidine (DAB) solution (100 ml 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 buffer, dehydrated in graded ethanols and xylene, and coverslipped with Permount.

Immunocytochemical controls included omission of the primary antisera, which eliminated all specific staining and revealed no non-specific staining due to the subsequent steps. In addition, incubation of the primary antisera solutions with their respective antigens abolished all staining, indicating that none of the polyclonal antibodies present resulted in any detectable non-specific staining at the high antisera dilutions used in these studies.

Antisera specificity

Rabbit anti-glutamate receptor subunit 2 (diluted 1:2,000; AB1768; lot No. 22070361; Chemicon, Temecula, CA) 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 (Petrulia et al., 1997). The antiserum generated against this sequence, which shares 6 amino acids with the GluR3 subunit, was purified by removing antibodies that recognized the corresponding unconjugated and conjugated GluR3-containing peptides, producing an antiserum that was specific for the GluR2 subunit, and did not recognize any other glutamate receptor subunits with the use of either Western blots or immunocytochemistry (Petrulia et al., 1997).

Mouse anti-NeuN (diluted 1:10,000; MAB377; lot No. 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 (Mullen et al., 1992). NeuN was used solely as a marker of hippocampal neurons. Although NeuN is constitutively expressed in all hippocampal neurons, to our knowledge, it is not expressed by all neurons of all species (Sarnat et al., 1998; Weyer and Schilling, 2003; Kumar and Buckmaster, 2007), and its expression can become undetectable after brain insults that do not result in neuronal death (McPhail et al., 2004; Unal-Cevik et al., 2004; Collombet et al., 2006). Therefore, Nissl staining was used to verify that loss of NeuN expression was due to cell death.

Rabbit anti-somatostatin-28 (diluted 1:100,000; IH8004; lot No. 971457; Peninsula, Torrance, CA). This polyclonal antiserum was raised against the S28 form of somatostatin (H-Ser-Ala-Asn-Ser-Asn-Pro-Ala-Met-Ala-Pro-Arg-Glu-Arg-Lys-Ala-Gly-Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Cys-OH disulfide bond). In cross-reactivity studies involving adsorption of the diluted primary antiserum with peptide solutions at a

concentration of 10 ug/ml, the rabbit anti-somatostatin immunostaining of hilar interneurons was abolished by adsorption with somatostatin 28 (Peninsula Labs), but not by identical adsorption with Neuropeptide Y (human/rat; Peninsula Labs), somatostatin 14 (Peninsula Labs), Substance P (Sigma Chemical), or cholecystokinin fragment 26-33 amide (CCK8), non-sulfated (Sigma Chemical). In addition, hippocampal somatostatin-like immunoreactivity obtained with this rabbit antiserum was qualitatively identical to that produced by two monoclonal antisera (Soma 8 and Soma 10; kindly provided by A.J. Malcolm of the Medical Research Council Regulatory Peptide Group, University of British Columbia) that recognize two different somatostatin epitopes, and have been fully characterized previously (Buchan et al., 1985). In addition, the distribution of somatostatin-like immunoreactivity using this rabbit antiserum was similar to the distribution of mRNA detected by in situ hybridization (Naus and Bloom, 1988; Naus et al., 1988; Priestley et al., 1991).

Rabbit anti- Neuropeptide Y (diluted 1:100,000; IH7172; lot No. 991025; Peninsula). This polyclonal antiserum was raised against the following 36 amino acid sequence: H-Tyr-Pro-Ser-Lys-Pro-Asp-Asn-Pro-Gly-Glu- Asp-Ala-Pro-Ala-Glu-Asp-Met-Ala- Arg-Tyr-Tyr-Ser-Ala-Leu-Arg-His-Tyr-Ile-Asn-Leu-Ile-Thr-Arg-Gln- Arg-Tyr-NH₂. The cross-reactivities with NPY (human, rat), NPY (porcine), and NPY (13-36, 16-36, 18-36, 20-36, porcine) were 100%, and the cross-reactivities with pancreatic polypeptide (human) and NPY 22-36 (porcine) were 1.6% and 1.5%. There was no cross-reactivity with NPY 26-36, Peptide YY, VIP (human, porcine, rat), or somatostatin 28. In cross-reactivity studies involving adsorption of the diluted primary antiserum with peptide solutions at a concentration of 10 ug/ml, the rabbit anti-NPY immunostaining of hilar interneurons was abolished by adsorption with Neuropeptide Y (human/rat; Peninsula Labs), but not with somatostatin 28, somatostatin 14, Substance P, or cholecystokinin fragment 26-33 amide (CCK8), non-sulfated. The distribution of NPY-like immunoreactivity was similar to that of the NPY mRNA in situ hybridization (Vezzani et al., 1996). In addition, our use of this antiserum solely as a marker of NPY-positive neurons in the dentate gyrus confirmed that the pattern of staining is identical to that observed in previous studies of NPY immunostaining in the mouse (Mátyás et al., 2004). All patterns of immunoreactivity were qualitatively similar to the characteristic staining patterns shown in previous studies (Bakst et al., 1986; Sloviter and Nilaver, 1987; Sloviter, 1991; Mátyás et al., 2004; Sun et al., 2007).

Light and fluorescent microscopic imaging methods

Brightfield and fluorescent 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 similar conditions of exposure. Fluorescent FluoroJade-B-stained sections were photographed and then converted to grayscale and inverted, making FluoroJade-B-positive cells to appear black on a white background.

Hilar neuron counting and area measurement

To assess the approximate extent of acute injury caused by 4 hr of perforant pathway stimulation, we measured the area of the hilus in each section using the Adobe Photoshop CS3 Extended Measurement program, and counted the number of fluorescent Fluoro-Jade B (FJB)-stained hilar neuron somata in 6 dorsal hippocampal sections (every fifth section) from each identically stimulated FVB/N (n=3) and C57BL6/N (n=3) mouse perfusion-fixed 1 day after the start of 4 hr of bilateral perforant pathway stimulation. Only hilar neuron somata in stimulated animals were counted because stimulated mice exhibited no fluorescent cells outside the hilus of the dorsal dentate gyrus, and sham control animals exhibited no

fluorescent hippocampal neurons. Ventral hippocampal sections contained no FJB-positive cells and were not further evaluated.

To estimate the extent of hilar neuron loss 4 days after 4 hr of perforant pathway stimulation in FVB/N and C57BL6/N mice, we analyzed every fifth section from the dorsal and ventral hippocampus from sham control- and experimental animals that were immunostained for NeuN-, glutamate receptor subunit 2 (GluR2)-, neuropeptide Y (NPY)-, and somatostatin (SS). The same method was used to compare the loss or survival of hilar neuron subpopulations in C57BL6/N mice stimulated for 2 or 4 hr, which addressed the identity of the most vulnerable cell subpopulations. The hilar cell counts of immunopositive neurons included all neuronal somata within the hilus that contained a visible nucleus, were not in contact with the granule cell layers, and were outside the somal and dendritic regions of area CA3c. Hilar neurons were not counted in Nissl-stained sections because neurons could not be reliably differentiated from the many glial cells that proliferate after seizure-induced damage.

To estimate the extent of hippocampal atrophy 6-8 months after 24 hours of stimulation in C57BL6/N mice, we calculated whole hippocampal and subregional volumes. This was done by measuring the areas of the whole hippocampus and of 4 hippocampal subregions (the granule cell and molecular layers of the dentate gyrus together, the hilus of the dentate gyrus, area CA1, and area CA3) in every other section in the entire series of coronal sections that contained any hippocampal tissue. Measurements were made in 3 sham control mice and 4 stimulated mice perfusion-fixed 6-8 months post-stimulation. Areas were measured using the Adobe Photoshop CS3 Extended Measurement feature to calculate the area bounded by an irregular border. Whole hippocampal area included the dentate gyrus and the hippocampus “proper,” excluding the fimbria. Hilar area was measured by outlining the area enclosed between the two blades of the granule cell layer, excluding the granule cell layer itself and the pyramidal cell somata and dendrites of area CA3c. The total hippocampal volume was calculated by multiplying the area of each section by the thickness of two sections (80 μ m), because every other section was measured. The accuracy of area measurement was verified by measuring the area of a 1 mm square on a micrometer, which yielded an area of 1.003 mm² by the Adobe Photoshop CS3 Extended Measure program. Group means were compared using Student’s *t*-test.

We applied Abercrombie’s correction method (Abercrombie, 1946; Saper, 1996) to the cell counts per unit area to reduce the overcounting of cells caused by changes in cell size if a significant difference in cell size exists within one class of cells between control and experimental groups. Therefore, the nuclear diameter of each cell class (hilar mossy cells, NPY- and SS-positive hilar interneurons) was measured using the CS3 Adobe Photoshop measuring tool. The number of counted cells with visible nuclei per unit area in sections 40 μ m thick was divided by the section thickness plus the diameter of the mossy cell nucleus, for example, according to the formula: $N=n*t/t+d$, where *N* is the true value (cells per unit area), “*n*” is the observed cell count per unit area, *t* is the section thickness, and *d* is the diameter of the object counted.

RESULTS

Hippocampal injury after four hours of perforant pathway stimulation in FVB/N and C57BL/6 mice

We first determined a duration of bilateral perforant pathway stimulation that reliably produced minor hippocampal injury in FVB/N mice, which is the strain reported to be vulnerable to the excitotoxic effects of kainic acid-induced seizure activity (Schauwecker and Steward, 1997; Schauwecker, 2002; 2006). This was done to determine whether the

kainate-resistant strain (C57BL/6 mice) exhibited less, the same, or more damage than FVB/N mice subjected to the identical afferent stimulation. Four hours of bilateral perforant pathway stimulation in urethane-anesthetized FVB/N (kainate-vulnerable) mice evoked granule cell population spikes and epileptiform discharges throughout the period of stimulation in all nine mice stimulated. Mice were perfusion-fixed one, four, or eight days after the start of four hours of stimulation ($n=3$ mice per survival time). Multiple survival periods were assessed to minimize the risk of missing the acute neurodegenerative stage when only one time point is analyzed. Sections from these mice were stained with FluoroJade-B (FJB) to detect acute neurodegeneration (Schmued and Hopkins, 2000), for NeuN immunoreactivity, a neuronal marker (Mullen et al., 1992), and were Nissl-stained to confirm that loss of NeuN immunoreactivity reflected neuronal loss. All three FVB/N mice perfusion-fixed one day after four hours of stimulation exhibited FJB-positive hilar neurons bilaterally in the dorsal hippocampus, but no other hippocampal neuron injury was detected (Fig. 1, A2, A3). FVB/N mice perfusion-fixed four or eight days post-stimulation exhibited no acutely degenerating FJB-positive cells, but did exhibit hilar neuron loss in the dorsal hippocampus as evidenced by the loss of NeuN-positive- and Nissl-stained hilar neurons (Fig. 1, C1-C4). Identical stimulation in C57BL/6 mice produced similar qualitative results, with all nine mice exhibiting granule cell population spikes and epileptiform discharges throughout the period of stimulation, as well as FJB-positive hilar neurons bilaterally in the dorsal hippocampus one day post-stimulation (Fig. 1, B). All C57BL/6 mice perfusion-fixed four or eight days post-stimulation exhibited loss of NeuN-positive and Nissl-stained hilar neurons in the dorsal hippocampus similar to that seen in FVB/N mice (Fig. 1, D1-D4).

Quantitative analysis of 6 equally-spaced (every fifth) sections from the dorsal hippocampus of each of the FVB/N and C57BL/6 sham-stimulated control mice, and those perfusion-fixed one day after 4 hr of stimulation, revealed that both groups of mice exhibited a similar extent of acute hilar neuron injury following the same excitatory insult. Sham-stimulated control mice of both strains exhibited no FJB-positive hilar neurons. FVB/N mice stimulated for 4 hours exhibited an average of 38.2 ± 4.3 FJB-positive hilar neuron somata per section (467.6 ± 76.4 cells/mm²), and identically stimulated C57BL/6 mice exhibited an average of 47.2 ± 4.0 FJB-positive hilar neuron somata per section (621.3 ± 71.1 cells/mm²), group means that were not significantly different from each other ($P>0.05$). Thus, both FVB/N and C57BL/6 strains exhibited qualitatively and quantitatively indistinguishable hilar neuron injury one day after four hours of granule cell epileptiform discharges evoked by perforant pathway stimulation, indicating no detectable strain difference in intrinsic neuronal vulnerability to seizure-induced injury. Counting NeuN-positive hilar neurons from control mice and mice perfusion-fixed four days post-stimulation revealed that both strains normally contained a similar number of NeuN-positive hilar neurons per dorsal hippocampal section, and that both strains suffered a significant and similar extent of loss of NeuN-positive hilar neurons (~73%) following four hours of perforant pathway stimulation (Fig. 2).

Which hilar neurons are most sensitive to excitotoxic injury?

The unexpected observation that only four hours of stimulation-induced granule cell discharges in C57BL/6 mice resulted in extensive hilar neuron loss enabled us to identify the most vulnerable hilar neuron subpopulation. This was done simply by decreasing the duration of afferent stimulation to two hours. Whereas four hours of bilateral perforant pathway stimulation (Fig. 3, B) decreased the number of both GluR2-positive hilar neurons (putative excitatory hilar mossy cells; Léránth et al., 1996) and NPY- and somatostatin-positive hilar interneurons, two hours of identical stimulation preferentially affected the hilar GluR2-positive hilar neurons, and spared the peptide-containing inhibitory interneurons (Fig. 3, C). The quantitative results four days post-stimulation were consistent with the qualitative observations. Whereas four hours of stimulation reduced the numbers of GluR2-

positive neurons and NPY- and SS-positive interneurons in the dorsal hippocampus by 84%, 45%, and 45%, respectively, two hours of identical stimulation preferentially decreased the number of GluR2-positive hilar neurons by ~70%, but had no significant effect on the numbers of NPY- and SS-positive interneurons in co-processed sections (Fig. 4).

Hippocampal injury after 24 hours stimulation in C57BL/6 mice

In this experiment, and the one following, we determined whether 24 hours of bilateral perforant pathway stimulation reproduced the defining features of mesial temporal lobe epilepsy with hippocampal sclerosis, i.e. selective injury to vulnerable hippocampal neurons, limited extra-hippocampal pathology, mossy fiber sprouting, hippocampal atrophy, and spontaneous epileptic seizures (Sloviter et al., 2007).

Acute neuropathology—C57BL/6 mice stimulated bilaterally for 24 hours were perfusion-fixed one and three days after the end of stimulation to assess acute neuropathology. FJB-positive neurons were limited to the hippocampus, septum, and entorhinal cortex (Fig. 5) in all mice assessed at these short survival periods. Within the hippocampus, FJB-positive neurons included dentate hilar neurons, CA1 and CA3 pyramidal cells, as well as a small number of granule cells. FJB staining of sections along the longitudinal axis of the hippocampus revealed a consistent pattern of neuronal pathology throughout the dorsal hippocampus, extending into the ventral hippocampus (Fig. 6).

Histology following long-term survival—Mice that survived for 6-8 months after 24 hours perforant pathway stimulation were used to assess chronic neuronal loss, mossy fiber sprouting (Nadler et al., 1980), and hippocampal shrinkage. Figure 7 shows NeuN-immunostained coronal sections from 3 sham control and 3 stimulated C57BL/6 mice. Stimulated mice exhibited highly reproducible and significant bilateral hippocampal atrophy compared to sham controls (Fig. 7), and this atrophy was caused by the extensive loss of hilar neurons and CA1-CA3 pyramidal cells (their acute injury is shown in Figures 5 and 6). Quantitative measurements of the areas of the hippocampus (including the dentate gyrus and the hippocampus proper), and of 4 hippocampal subregions confirmed the qualitative findings. Stimulated mice exhibited a 43% loss of total hippocampal volume (Fig. 8). The dorsal half of the hippocampus decreased in volume by 65%, and the ventral half of the hippocampus was 32% smaller in volume than in the sham controls (Fig. 8). Similar volume decreases were observed in all hippocampal subregions analyzed, with the hilus reduced in volume to 40% of control, and the granule cell and molecular layers reduced to 48% of control. The volume of area CA3 was reduced to 18% of control, and area CA1 volume was reduced to 34% of control (Fig. 8). Measurement of nuclear size in hilar neurons and CA1 and CA3 pyramidal cells revealed no significant difference between experimental and control groups, consistent with the atrophy being the result of neuron loss rather than cell shrinkage.

Timm staining in mice that had survived for eight months after 24 hours of bilateral perforant pathway stimulation revealed the presence of mossy fiber sprouting throughout the dorsal hippocampus in all four randomly selected mice processed for Timm staining (Fig. 9,F), an expected consequence of hilar mossy cell loss (Jiao and Nadler, 2007). However, none of the 15 mice that survived for eight months post-stimulation exhibited the massive granule cell layer hypertrophy observed in mice following intrahippocampal kainate injection (Fig. 9,B). This previously described granule cell layer hypertrophy (Suzuki et al., 1995; Bouillier et al., 1999; Riban et al., 2002) is apparently the result of a kainate-induced suppression of reelin expression (Heinrich et al., 2006), an effect apparently not caused by perforant pathway stimulation-induced hippocampal seizure activity.

Spontaneous epilepsy after 24 hours perforant pathway stimulation

We used continuous (24/7) video monitoring to determine whether C57BL/6 mice exhibited spontaneous Stage 3-5 behavioral seizures (Racine, 1972) following bilateral perforant pathway stimulation. Individual mice were monitored by a single camera so that subtle behaviors could be detected. The observation in rats that identical electrical stimulation revealed a five week latent period before spontaneous Stage 3-5 seizures were first detected (Bumanglag and Sloviter, 2008) precluded continuous observation starting immediately after stimulation because of the time needed to watch the videos of multiple animals for months at a time if seizure latency and frequency were to be accurately determined. Therefore, we viewed two weeks of continuous video recordings from four randomly selected mice that were recorded between six and twenty weeks post-stimulation. This was done to determine whether 24 hours of perforant pathway stimulation produced a chronic epileptic state in C57BL/6 mice. All four mice exhibited at least one spontaneous Stage 3-5 behavioral seizure (forepaw clonus and rearing lasting approximately 20-60 sec) during the two week observation period, and one of the four mice exhibited five Stage 3-5 seizures on one day, and a sixth seizure eight days later.

DISCUSSION

The main original findings of this study are as follows. First, both FVB/N and C57BL/6 mice were found to be highly and similarly vulnerable to hippocampal seizure activity evoked by afferent excitation, and to be more vulnerable than rats to the identical stimulation paradigm. Second, the threshold stimulation duration for producing hippocampal damage (two hours) selectively affected GluR2-positive hilar mossy cells (Léránth et al., 1996), but spared vulnerable hilar peptide-containing inhibitory interneurons (Sloviter, 1987), demonstrating that hilar mossy cells are the hippocampal neuron population most vulnerable to this insult in mice. Third, the granule cell layer hypertrophy (granule cell dispersion) caused by intrahippocampal kainate injection (Suzuki et al., 1995; Bouillier et al., 1999; Riban et al., 2002; Heinrich et al., 2006) was not observed in any mice following stimulation-induced hippocampal damage, suggesting that massive granule cell layer hypertrophy is an effect of intrahippocampal kainate injection that may be unrelated to epileptogenesis. Fourth, mice that exhibited hippocampal neuron loss, synaptic reorganization, hippocampal atrophy, and limited extrahippocampal damage also became chronically epileptic. Thus, this animal model appears to exhibit the defining characteristics of human mesial temporal lobe epilepsy with hippocampal sclerosis (Margerison and Corsellis, 1966; French et al., 1993; Engel, 1996).

Lack of genetic strain differences in neuronal vulnerability to hippocampal seizure activity

Mice exhibit strain-dependent differences in the extent of hippocampal damage following systemic kainate administration, and these strain differences have been interpreted as indicating genetically-determined differences in intrinsic neuronal vulnerability to insult (Schauwecker and Steward, 1997; Schauwecker, 2002; 2006; McCord et al., 2008). Our observation that hippocampal neurons in both “kainate-vulnerable” and “kainate-resistant” strains appeared equally and highly vulnerable to the same stimulation-induced insult suggests that the observed strain differences following kainate may reflect differences in the way different mouse strains respond to kainic acid, rather than reflecting any significant strain differences in intrinsic neuronal vulnerability. The likelihood that C57BL/6 mice exhibit less damage after systemic kainate injection simply because the hippocampal insult is less intense in C57BL/6 mice than in FVB/N mice is supported by the observation that kainate caused prolonged c-fos expression in the hippocampus of FVB/N (“kainate-vulnerable”) mice, but not in the hippocampus of C57BL/6 (“kainate-resistant”) mice (Schauwecker et al., 2000). We have previously reported that kainate-induced status

epilepticus in rats often bypasses the hippocampus, failing to evoke granule cell epileptiform discharges despite generalized seizures and widespread extra-hippocampal brain damage (Sloviter et al., 2003). Therefore, apparent strain differences in the effects of kainate in mice probably reflect greater or lesser degrees of hippocampal recruitment during behavioral status epilepticus, or pharmacokinetic strain differences, rather than genetic differences in neuronal vulnerability.

Extreme vulnerability of excitatory hilar mossy cells

Having previously shown that vulnerable hilar neurons of the urethane-anesthetized rat were not destroyed by four hours of perforant pathway stimulation (Sloviter and Lowenstein, 1992), and were only minimally affected by eight hours of stimulation (Sloviter et al., 2003), we were surprised to find that four hours of identical perforant pathway stimulation in mice produced extensive loss of both hilar mossy cells and hilar peptide-containing interneurons. Decreasing the duration of stimulation enabled us to determine that two hours of granule cell excitation destroyed ~70% of GluR2-positive mossy cells in the dorsal hippocampus, but had no detectable effect on the peptide-containing hilar interneurons also targeted by the mossy fiber pathway. These data in mice indicate that hilar mossy cells are the hippocampal neurons most vulnerable to the excitotoxic effects of mossy fiber pathway excitation. The available data in rats (Lowenstein et al., 1992; Sloviter, 1987; 1991; Sloviter et al., 2003; Zappone and Sloviter, 2004), and now in mice, clearly indicate that dentate hilar mossy cells are among the first neurons to die in response to an excitotoxic insult. The extreme vulnerability of hilar mossy cells in mice and rats is consistent with their extensive loss in human hippocampi from patients suffering from mesial temporal lobe epilepsy with hippocampal sclerosis (Blümcke et al., 1999; 2000). Why mossy cells are the most vulnerable hippocampal cell type is unclear. One possibility is differences in the density of excitatory input from mossy fiber terminals to mossy cells and to intermingled peptide-containing interneurons. A second possibility is a limited ability of mossy cells to maintain low intracellular calcium, which is presumed to play a causal role in excitation-induced neuronal injury (Griffiths et al., 1982; 1984; Scharfman and Schwartzkroin, 1989). However, the data in this study provide no directly relevant information regarding the mechanisms underlying mossy cell vulnerability.

Perforant pathway stimulation as a reliable initiator of hippocampal sclerosis, mossy fiber sprouting, and epileptogenesis in the mouse

The utility of animal models that involve systemically-administered chemoconvulsant drugs is limited by high rates of non-response and lethality, severe and variable brain damage, and the failure to produce hippocampal atrophy or hippocampal-onset epilepsy reliably (Harvey and Sloviter, 2005; Sloviter, 2005; Bumanglag and Sloviter, 2008). Our results in urethane-anesthetized mice indicate that perforant pathway stimulation, which avoids the use of chemoconvulsants entirely, is an effective method for reliably producing the defining features of human mesial temporal lobe epilepsy with hippocampal sclerosis (Margerison and Corsellis, 1966; French et al., 1993; Engel, 1996). The finding that the C57BL/6 background strain used to generate many transgenic and knockout mice is not resistant to seizure-induced hippocampal damage indicates that experimental studies in wild-type and genetically altered mice are feasible if systemic chemoconvulsant use is avoided. The minimal variability with which perforant pathway stimulation produced selective hippocampal damage, mossy fiber sprouting, and limited damage in the septum and entorhinal cortex suggests that this model may be particularly useful for identifying genes that mediate or influence cell death, synaptic reorganization, and epileptogenesis. The clarification in this study of several unresolved issues, including the lack of strain differences in hippocampal neuron vulnerability to seizure activity, and the possible unimportance of granule cell layer hypertrophy to the epileptogenic process, suggests that

stimulation-induced injury is a highly reproducible method for producing epilepsy associated with a relatively small number of structural abnormalities. This feature of stimulation-induced injury should facilitate the effort to identify epileptogenic pathology and likely epileptic structures, and to elucidate epileptogenic mechanisms. The latency between the initial insult and clinical epilepsy, and whether the hippocampus is a source of the spontaneous seizures we observed in these animals, remain to be determined.

Which post-injury abnormalities mediate epileptogenesis?

The ultimate challenge in any experimental epilepsy study is to determine which of the many changes that occur in response to a brain insult are causally related to the subsequent development of epilepsy (Sloviter, 2008). In this study, prolonged granule cell discharges evoked by perforant pathway activation caused extensive hippocampal injury, very limited extra-hippocampal injury restricted to the septum and entorhinal cortex, mossy fiber sprouting, hundreds if not thousands of other effects not assessed, and epilepsy. The minimal variability between stimulated animals, the minimal lethality, and the absence of non-responders suggest that this animal model can be used productively to study the epileptogenic process, and to develop neuroprotective and anti-epileptogenic treatments. The ability to control the intensity and duration of the excitatory insult should facilitate efforts to determine which pathology, if any, causes or triggers epileptogenesis. Future studies that determine which durations of stimulation do, or do not, cause epilepsy, that determine the latency between injury and both subclinical and clinical epilepsy, and that begin to assess the process in genetically altered mice, should provide answers to the central question of which of the many effects of brain injury might be causally related to the still poorly understood process of injury-associated epileptogenesis.

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Figure 1.

Acute neurodegeneration in the FVB/N and C57BL/6 mouse hippocampus one and four days after four hours of bilateral perforant pathway stimulation. (A1 and B1) NeuN-immunoreactivity in the normal FVB/N (A1) and C57BL/6 (B1) hippocampus showing normal dorsal hippocampal anatomy. (A2 and B2) FluoroJade-B (FJB) staining one day after bilateral perforant pathway stimulation for four hours. Note that both mouse strains exhibited numerous FJB-positive neurons, but only in the hilus of the dentate gyrus (h), with no detectable FJB staining detected in any other hippocampal subregion. (A3 and A4) FJB-positive cells in the hilus at higher magnification. (C and D) Hilar neuron loss four days after four hours of perforant pathway stimulation in FVB/N and C57BL/6 mice. (C1) NeuN-immunostaining in the sham control FVB/N dentate gyrus. (C2) NeuN-immunoreactivity showing absence of NeuN-positive hilar neurons (h) after four hours of perforant pathway stimulation. (C3) Nissl-stained section from the sham control FVB/N dentate gyrus. (C4) Nissl-stained section after four hours of perforant pathway stimulation showing that the loss of NeuN-positive hilar neurons reflects neuronal death rather than loss of NeuN immunoreactivity alone. (D1-4) the identical series of photographs in C57BL/6 mice

showing similar hilar neuron loss regardless of genetic strain. Note that green FJB fluorescence was photographed, converted to grayscale, and then inverted to produce the images of black FJB-positive, acutely degenerating neurons on a white background. Calibration bar: 600 um in A1-B2; 330 um in A3-B3; 350 um in C and D.

Figure 2.

Hilar neuron loss four days after four hours of perforant pathway stimulation in FVB/N (kainate-vulnerable) and C57BL/6 (kainate-resistant) mice. The area of the hilus was measured in square millimeters using the Adobe Photoshop CS3 Extended Measurement program that calculates the area bounded by an irregular border. The number of NeuN-positive hilar neuron somata with a visible nucleus was counted within this area and the numbers of neurons were expressed per square millimeter of area. Note that both strains exhibited a similar seizure-induced hilar neuron loss following electrical stimulation-induced seizure activity. Both stimulated groups were significantly different from their respective controls ($P < 0.01$), but not from each other ($P > 0.05$) by Student's *t*-test.

Figure 3.

Hilar neuron loss after two or four hours of bilateral perforant pathway stimulation in C57BL/6 mice. (A1-A5) Sections from a sham control mouse showing the normal patterns of Nissl staining (A1), and immunoreactivities in the C57BL/6 mouse for NeuN (A2), GluR2 subunit (A3), neuropeptide Y (A4), and somatostatin (A5). (B1-B5) Four days after four hours of perforant pathway stimulation, extensive loss of all hilar subtypes is apparent. (C1-C5) Four days after two hours of perforant pathway stimulation, GluR2-positive hilar neurons are extensively affected (C-3), but neuropeptide Y- and somatostatin-positive hilar neurons in adjacent sections survive. Calibration bar: 210 μ m in all panels.

	NeuN		GluR2		NPY		SS	
	cells/ mm ²	% of control	cells/ mm ²	% of control	cells/ mm ²	% of control	cells/ mm ²	% of control
control	404.9 ± 15.6	100.0 ± 3.9	347.4 ± 7.0	100.0 ± 2.0	195.8 ± 3.3	100.0 ± 1.7	123.1 ± 5.7	100.0 ± 4.6
4h stim	118.2 ± 19.4	29.2 ± 4.8	54.6 ± 28.8	15.7 ± 8.3	107.5 ± 8.9	55.2 ± 6.7	68.0 ± 8.3	55.2 ± 6.7
2h stim	187.4 ± 16.2	46.3 ± 4.0	103.0 ± 12.3	29.7 ± 3.5	197.5 ± 6.2	100.9 ± 3.2	120.9 ± 4.0	98.2 ± 3.3

Figure 4.

Selective hilar neuron loss after two or four hours of perforant pathway stimulation. Note that four hours of stimulation-induced granule cell discharges decreased the numbers of all three hilar neuron subtypes (glutamate receptor subtype 2 (GluR2)-, Neuropeptide Y (NPY)-, and somatostatin (SS)-positive hilar interneurons, whereas two hours of stimulation selectively decreased the number of GluR2 subunit-positive hilar neurons. All four-hour means were significantly different from their respective controls ($P < 0.01$), as were the NeuN and GluR2 means in the two-hour groups. The NPY and SS groups in the two-hour group were not significantly different from control ($P > 0.05$) by Student's *t*-test. The area of the hilus was measured in square millimeters using the Adobe Photoshop CS3 Extended Measurement program that calculates the area bounded by an irregular border. The number of immunoreactive hilar neuron somata was counted within this area and these numbers were expressed per square millimeter of area. Note that only cells with a visible nucleus were counted. Therefore, the means indicate the approximate extent of cell loss in experimental animals, but not the total number of neurons present.

Figure 5.

FluoroJade-B staining one day after 24 hours of bilateral perforant pathway stimulation in the urethane-anesthetized C57BL/6 mouse. (A) Bilateral afferent stimulation resulted in extensive acute degeneration of hilar neurons, and CA1 and CA3 pyramidal cells. (B) A coronal section of the whole mouse brain illustrates the selectivity of the damage evoked by afferent stimulation. Despite confirmed hippocampal granule cell discharging throughout the 24 hours stimulation period, acutely FJB-positive neurons were restricted to the hippocampus (A,B), septum (C), and the entorhinal cortex, in which the FJB-positive cells were only detectable in horizontal sections (D). Note that green FJB fluorescence was photographed, converted to grayscale, and then inverted. Calibration bar: 450 μ m in A; 1 mm in B; 400 μ m in C and D.

Figure 6.

FluoroJade-B staining throughout the longitudinal axis of the hippocampus one day after 24 hours of bilateral perforant pathway stimulation in the urethane-anesthetized C57BL/6 mouse. Note that acute degeneration of dentate hilar neurons and hippocampal pyramidal cells was consistently present in coronal sections at all levels throughout the dorsal hippocampus (A-C), and decreased in extent in the ventral hippocampus (D) with most FJB-positive cells in the hilus. The presence of acutely degenerating neurons in the entorhinal cortex of horizontal sections (D) was a highly localized (arrows) and consistent feature following 24 hours of afferent stimulation. Asterisks in (D) mark the stimulating electrode tracks. Note that green FJB fluorescence was converted to grayscale and inverted. Calibration bar: 400 μ m in A-C; 300 μ m in D.

Figure 7.

Hippocampal atrophy in dorsal and ventral hippocampus eight months after 24 hours of bilateral perforant pathway stimulation in the urethane-anesthetized C57BL/6 mouse. NeuN immunostaining in all sections. (A1-A3) Normal NeuN immunostaining in three different sham-stimulated mice that survived for eight months following sham treatment. (B1-B3) Identical NeuN immunostaining in co-processed sections from three stimulated mice. Note the loss of dentate hilar neurons and hippocampal pyramidal cells, and the resulting hippocampal atrophy. This pattern of hippocampal neuron loss was present throughout the dorsal hippocampus in all stimulated animals. (A4 and A5) Ventral hippocampal sections showing less extensive hippocampal neuron loss and hippocampal atrophy in the ventral hippocampus (these sections are from the mouse shown in panels A2 and B2). Calibration bar: 500 μ m.

	sham control		24h stimulation	
	10 ⁻² mm ³	% of control	10 ⁻² mm ³	% of control
total hippocampus	1010.0 ± 13.8	100.0 ± 1.4	573.4 ± 18.7	56.8 ± 1.9
dorsal hippocampus	344.5 ± 14.8	100.0 ± 4.3	121.0 ± 8.9	35.1 ± 2.6
ventral hippocampus	668.7 ± 5.5	100.0 ± 0.8	452.4 ± 27.7	67.7 ± 4.1
hilus	10.9 ± 0.6	100.0 ± 5.3	4.4 ± 0.4	40.5 ± 3.5
DG	104.3 ± 3.5	100.0 ± 3.4	54.3 ± 5.6	52.1 ± 5.3
CA3	107.6 ± 3.0	100.0 ± 2.8	18.2 ± 2.5	17.9 ± 2.3
CA1	155.7 ± 2.8	100.0 ± 1.8	53.6 ± 3.9	34.4 ± 2.5

Figure 8.

Hippocampal volume changes in dorsal and ventral hippocampus six months after 24 hours of bilateral perforant pathway stimulation in the urethane-anesthetized C57BL/6 mouse. The areas of every other section throughout the entire rostro-caudal extent of the hippocampus were measured using the Adobe Photoshop CS3 Extended Measurement program that calculates the area bounded by an irregular border. Five areas in coronal sections 4 stimulated- and 3 sham-stimulated mice were measured, including the entire hippocampus, the dentate granule cell and molecular layers (DG), the hilus of the dentate gyrus, and areas CA1 and CA3. Note that hippocampal shrinkage was greater in the dorsal than the ventral hippocampus. Asterisks denote the experimental group means that were significantly different from their respective controls (** P<0.01; *P<0.05, by Student's t-test).

Figure 9.

Absence of granule cell layer hypertrophy and presence of mossy fiber sprouting after 24 hours of bilateral perforant pathway stimulation in the C57BL/6 mouse. (A) Nissl-stained section from a control animal two months after intrahippocampal saline injection. (B) Nissl-stained section from an experimental animal two months after intrahippocampal kainic acid (0.2 ug). Note granule cell layer hypertrophy. (C and D) NeuN-immunostaining in the dentate gyrus from a sham-stimulated control mouse (C) and a stimulated mouse (D), showing that despite confirmed granule cell discharging and extensive hilar neuron loss (h), the granule cell hypertrophy caused by kainic acid (B) was not reproduced by perforant pathway stimulation. (E and F) Timm staining in the dentate gyrus from the same sham control (E) and stimulated (F) mice, showing the presence of granule cell mossy fiber sprouting above the granule cell layer (arrows) eight months post-stimulation. Calibration bar: 600 um in A and B; 180 um in C-F.