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Stereological analysis of GluR2-immunoreactive hilar neurons in the pilocarpine model of temporal lobe epilepsy:

correlation of cell loss with mossy fiber sprouting

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Abstract

Mossy fiber sprouting and the genesis of ectopic granule cells contribute to reverberating excitation in the dentate gyrus of epileptic brain. This study determined whether the extent of sprouting after status epilepticus in rats correlates with the seizure-induced degeneration of GluR2-immunoreactive (GluR2+) hilar neurons (presumptive mossy cells) and also quantitated granule cell-like GluR2-immunoreactive hilar neurons. Stereological cell counting indicated that GluR2+ neurons account for 57% of the total hilar neuron population. Prolonged pilocarpine-induced status epilepticus killed 95% of these cells. A smaller percentage of GluR2+ neurons (74%) was killed when status epilepticus was interrupted after 1–3.5 h with a single injection of phenobarbital, and the number of residual GluR2+ neurons varied among animals by a factor of 6.2. GluR2+ neurons were not necessarily more vulnerable than other hilar neurons. In rats administered phenobarbital, the extent of recurrent mossy fiber growth varied inversely and linearly with the number of GluR2+ hilar neurons that remained intact ($P = 0.0001$). Thus the loss of each GluR2+ neuron was associated with roughly the same amount of sprouting. These findings support the hypothesis that mossy fiber sprouting is driven largely by the degeneration of and/or loss of innervation from mossy cells. Granule cell-like GluR2-immunoreactive neurons were rarely encountered in the hilus of control rats, but increased 6- to 140-fold after status epilepticus. Their number did not correlate with the extent of hilar cell death or mossy fiber sprouting in the same animal. The morphology, number, and distribution of these neurons suggested that they were hilar ectopic granule cells.

Keywords

Hippocampus; dentate gyrus; epilepsy; mossy cell; axonal reorganization; granule cell; mossy fiber

Introduction

A unique feature of temporal lobe epilepsy and animal models of this disorder is the anatomical reorganization of the dentate gyrus (Nadler, 2003). Many hilar neurons degenerate and are replaced, in part, by ectopic granule cells (Parent et al., 1997; Scharfman et al., 2000; McCloskey et al., 2006). In addition, some granule cells in epileptic rat brain are found to have a basal dendrite, a process found less frequently in normal brain (Spigelman et al., 1998;

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Buckmaster and Dudek, 1999; Ribak et al., 2000). Finally, normal granule cells, granule cells with a basal dendrite, and hilar ectopic granule cells become synaptically connected by recurrent mossy fibers, forming a reverberating network unique to epileptic brain. This pathway can reduce the threshold for granule cell synchronization (Tauck and Nadler, 1985; Cronin et al., 1992; Masukawa et al., 1992; Patrylo and Dudek, 1998; Hardison et al., 2000; Okazaki and Nadler, 2001; Gabriel et al., 2004) and may therefore facilitate participation of these cells in seizures. The new circuitry may also increase inhibitory drive to granule cells, however. Recurrent mossy fibers make some contacts with inhibitory interneurons (Buckmaster et al., 2002; Sloviter et al., 2006), and reverberating excitation among granule cells may allow these neurons to be driven repeatedly by a mossy fiber volley rather than just once.

Recurrent mossy fiber growth was first described in the “dentate island” preparation, in which the granule cell body layer and molecular layer of the dentate gyrus are isolated from the underlying hilus (Zimmer, 1974). Both in this preparation and in the kainic acid model of temporal lobe epilepsy (Nadler et al., 1980a), mossy fibers were shown to invade the inner part of the dentate molecular layer, which had been denervated by degeneration of the associational-commissural fibers. The associational-commissural projection arises from mossy cells of the dentate hilus (Ribak et al., 1985), which are considered highly vulnerable to seizure-induced damage (Sloviter, 1987; Buckmaster and Jongen-Rêlo, 1999; Blümcke et al., 2000; Sloviter et al., 2003). It was therefore proposed that mossy fibers formed synapses with the proximal portion of the granule cell dendrite specifically in response to interruption of the associational-commissural fibers or degeneration of mossy cells. Several studies found a statistically significant correlation between the loss of total hilar neurons and the extent of mossy fiber sprouting (Tauck and Nadler, 1985; Babb et al., 1991; Masukawa et al., 1995; Buckmaster and Dudek, 1997; Nissinen et al., 2001). However, direct evidence for the involvement of mossy cell death is lacking and some have questioned whether mossy cell death and mossy fiber sprouting are, in fact, related (Ratzliff et al., 2002). Indeed some sparse recurrent mossy fiber growth can occur in response to stimuli that produce no detectable cell death (Bender et al., 2003). Furthermore, some inhibitory hilar interneurons are also killed by seizures, and their death might theoretically provoke mossy fiber growth. We therefore sought to determine whether the extent of recurrent mossy fiber growth correlates in a statistically significant way with the loss of mossy cells in rats subjected to pilocarpine-induced status epilepticus.

This study required the identification and quantitation of mossy cells. Because the excitatory mossy cells are intermixed with inhibitory hilar interneurons, a specific marker was required. The best available at present is GluR2 immunoreactivity (Leranth et al., 1996; Sloviter et al., 2001, 2003). Practically all telencephalic glutamate neurons express the GluR2 subunit of the AMPA receptor. The AMPA receptor mediates fast excitatory transmission at the vast majority of glutamate synapses. The presence of a GluR2 subunit in the AMPA receptor complex prevents flux of divalent cations through the channel and thus protects the neuron from excitotoxic death due to Ca^{2+} and/or Zn^{2+} overload (Weiss and Sensi, 2000). Some inhibitory neurons also express GluR2, but usually to a much lesser degree than glutamate neurons. Sloviter et al. (2001) reported that <1% of the GluR2-immunoreactive (GluR2+) neurons in the dentate hilus were also immunoreactive when subjected to a sensitive “mirror” method for visualizing GABA. Moreover, when the number of GluR2/3-immunoreactive hilar neurons and hilar neurons that expressed GAD67 mRNA (presumptive inhibitory interneurons) were determined in the same rats, the total accounted for 98.4% of all hilar neurons (Bender et al., 2003). This result suggests little overlap between the two neuronal populations. Therefore GluR2 immunoreactivity served as a useful, albeit imperfect, marker of hilar mossy cells in our study.

In all control rats, we observed a small population of GluR2-immunoreactive hilar neurons that appeared morphologically distinct from the presumptive mossy cells. The size, shape, and

immunoreactivity of these cells closely resembled those of dentate granule cells in the cell body layer. Because their number increased dramatically after status epilepticus, we quantitated them separately from the presumptive mossy cells.

Materials and methods

Pilocarpine-induced status epilepticus

Male Sprague-Dawley rats (175-200 g; Zivic Laboratories, Pittsburgh, PA) were injected with pilocarpine hydrochloride (335-365 mg/kg, i.p.) 30 min after pretreatment with (-)-scopolamine methyl bromide and terbutaline hemisulfate (2 mg/kg, i.p., each). Most rats treated in this manner developed status epilepticus, defined as a continuous limbic motor seizure of stage 2 or higher (Racine, 1972). In most instances, status epilepticus was terminated 1, 2, or 3.5 h after onset with a single injection of sodium phenobarbital (50 mg/kg, i.p.). The behavioral seizure did not subside immediately; compulsive head nodding (stage 2) persisted for between 45 min and 2 h. In other instances, status epilepticus was allowed to self-terminate after 6-8 h.

Animals were treated in accordance with guidelines of the National Institutes of Health and all protocols were approved in advance by the Duke University Institutional Animal Care and Use Committee.

Perfusion/fixation, preparation of hippocampal sections, and staining

Animals were studied 10-25 wk after pilocarpine administration. The controls were rats of the same age that had not been treated with pilocarpine. Rats were perfused transcardially with 500 ml of 0.1% (w/v) Na₂S in 0.15 M sodium phosphate buffer, pH 7.4, followed by 500 ml of 4% (w/v) paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4. Subsequent steps followed the protocol described by Buckmaster and Dudek (1997). The brain was immersed in fixative for 4 h at 4°C, the hippocampi were dissected and the isolated hippocampi were immersed in 30% (w/v) sucrose, 0.1 M sodium phosphate buffer (PB), pH 7.4, until they equilibrated. Hippocampi were straightened as much as possible without damaging them and frozen onto a microtome chuck with powdered solid CO₂. Only one hippocampus from each rat was used in these studies. It was considered acceptable to analyze just one hippocampus, because mossy fiber sprouting is bilaterally symmetrical after pilocarpine-induced status epilepticus (Okazaki et al., 1995). Serial transverse sections of 30-μm thickness were cut in a cryostat and transferred to PB. Starting from the rostral end of the hippocampus, a set of 6 consecutive sections was saved and the next 9 were discarded. This procedure was repeated until all the tissue had been cut. On average, each hippocampus yielded 18 sets of serial sections. The first section of each set was used for GluR2 immunocytochemistry, the second was stained with cresyl violet, and the third was used for Timm histochemistry. The remaining sections were retained in PB at 4°C in case a histological procedure had to be repeated.

For GluR2 immunocytochemistry, sections were washed twice for 10 min each with PB and then incubated for 50 min in phosphate-buffered 1% (v/v) H₂O₂. After incubation for 10 min in PB, the sections were exposed sequentially to 0.1% (v/v) Triton X-100 in PB (PB-T; 2 × 10 min), 10% (v/v) normal goat serum in PB-T (90 min), PB-T (3 × 10 min), rabbit anti-GluR2 antiserum (Chemicon AB1768-25UG, Temecula, CA; 1:200 dilution with 2% (v/v) normal goat serum/PB-T) (17.5 h at 4°C then 20 min at room temperature), PB-T (3 × 10 min), biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA; 1:200 dilution with 2% (v/v) normal goat serum/PB-T) (1 h), PB (3 × 10 min), avidin-biotin-horseradish peroxidase (Vectastain Elite ABC kit; Vector Laboratories) (1 h), PB (3 × 10 min) and 0.06% (w/v) 3,3'-diaminobenzidine/0.01% (v/v) H₂O₂ (~2 min).

The primary Chemicon GluR2 antibody was directed against a 16 amino acid peptide corresponding to amino acids 827-842 located 20 residues from the C-terminus of GluR2. The antibody was raised in rabbits and purified as described by Petralia et al. (1997). Its specificity was characterized thoroughly by those investigators. In addition, immunoreactivity was absent in mice with a targeted disruption of the gene that encodes GluR2 (Sans et al., 2003).

Mossy fiber terminals were visualized with use of the Timm stain for heavy metals, as described by Danscher (1981).

Stereological analysis

Quantitative analyses were performed without knowledge of any other data on that animal. The number of Nissl-stained and GluR2+ neurons in the dentate hilus was computed by the optical fractionator method (Gundersen et al., 1988; West et al., 1991) with use of Stereo Investigator (MicroBrightField, Colchester, VT). Total section thickness (~12 µm) was determined by the software; the 2 µm closest to the cut surfaces of the section were excluded from the analysis. Only “caps” were counted. For GluR2+ neurons, a cap was defined as an immunoreactive cell body whose top surface came into focus while raising the microscope stage. The entire hilus was used as the counting frame. The hilus was defined as the area bounded by the lower edge of the granule cell body layer and straight lines drawn from the ends of the granule cell arch to the end of the pyramidal cell body layer in area CA3c. Neurons within one cell diameter of the granule cell body layer were excluded from the counts.

The total number of hilar GluR2+ neurons per hippocampus (N) was computed from the formula:

$$N = \Sigma Q^- \cdot t/h \cdot 1/asf \cdot 1/ssf$$

where ΣQ^- is the total number of neurons counted, t is the section thickness (= 12 µm), h is the dissector height (= 8 µm), asf is the area sampling fraction (= 1) and ssf is the fraction of sections sampled (= 1/15). In the hilus of control rats, 926 ± 44 GluR2+ caps were actually counted. Immunoreactive neurons having the same diameter (8-12 µm) as dentate granule cells were counted separately from the larger (30-40 µm-diameter) GluR2+ neurons.

For sections stained with cresyl violet, a cap was defined as a neuronal nucleus whose top surface came into focus while raising the microscope stage. Neurons within one cell diameter of the granule cell body layer and cells having the same diameter as dentate granule cells or smaller were excluded from the analysis. The latter criterion excluded glia and ectopic granule cells. Caps within the counting frame and those transected by the top and/or right edge of the frame were counted, whereas those transected by the bottom and/or left edge of the frame were not. Counting frames (68 × 68 µm; scan grid, 100 × 100 µm) were systematically and randomly distributed throughout the hilus. About 46% of the total hilar cross-sectional area was sampled. The total number of hilar neurons was computed with the same formula used to compute GluR2 + hilar neurons, except that asf was 0.4625. In the hilus of control rats, 716 ± 38 caps were actually counted.

The number of stained neurons was computed for each one-sixth of the dentate hilus from the caudal to the rostral pole of the hippocampus (levels 1-6). Cell counts from the sections cut at each level were averaged to obtain a single value for the number of stained neurons per section. The number of stained neurons in the caudal part of the hilus was calculated as the sum of neurons computed for levels 1-3 and the number in the rostral part of the hilus was the sum computed for levels 4-6. Neuron loss in pilocarpine-treated rats was determined by dividing the mean number of neurons in pilocarpine-treated rats by the mean number in control rats, expressing the result as a percentage, and subtracting it from 100%. The standard error of the mean was calculated as described by McLean and Welch (1971).

Each rat was assigned a mean Timm score according to criteria similar to those described by Okazaki et al. (1995). Scores were assigned as follows: 0, no or only occasional supragranular mossy fiber-like Timm stain; 1, scattered mossy fiber-like stain above all parts of the granule cell body layer or denser stain confined to the extreme ends of the blade with only occasional Timm granules in between; 2, dense mossy fiber-like stain above one-third or more of the granule cell body layer with the remainder more sparsely stained or a continuous band of stain intermediate in intensity between sections scored 1 and 3; 3, a narrow, dense and essentially continuous band of supragranular mossy fiber-like stain above the entire granule cell body layer; 4, a denser and broader band of supragranular mossy fiber-like stain, occupying at least a third of the molecular layer at some location. A score was assigned to the suprapyramidal and infrapyramidal blades of the dentate gyrus on each section (mean of 18 sections per rat). These values (mean of 36 per rat) were averaged to obtain a single score for each animal.

All quantitative data are expressed as means $\pm$ S.E.M. unless stated otherwise.

Results

Stereological precision

With regard to our sampling procedures for quantitation of total hilar neurons, the coefficient of error (CE), which measures intra-animal variation due to the sampling procedure, was consistently 0.04 for control rats. The coefficient of variation (CV), which measures interanimal variation due to biological factors was 0.095. CE^2/CV^2 was 0.18, which indicates a low methodological bias in the precision of estimated neuron number (Gundersen et al., 1988; West et al., 1991). For rats that had experienced pilocarpine-induced status epilepticus, $CE = 0.06$ - 0.09 , $CV = 0.19$, and $CE^2/CV^2 = 0.16$ (for $CE = 0.075$). These values also indicate a low methodological bias.

Control rats

The number of hilar neurons per hippocampus, as estimated by quantitative stereology of cresyl violet-stained sections (Fig. 1A-B), ranged from 33,006 to 42,100 in five rats aged 4-5 months ($37,580 \pm 1,594$; Fig. 5). The number of hilar neurons declined progressively when sections were analyzed from the caudal to the rostral dentate gyrus. Hilar neurons were about four times as numerous near the caudal pole (level 1) as they were near the rostral pole (level 6; Figs. 1A-B,6).

All hippocampal granule and pyramidal cells exhibited GluR2 immunoreactivity (Fig. 2). In addition, two morphologically-distinct GluR2-immunoreactive neuronal populations were observed in the dentate hilus (Fig. 4). The much larger population (referred to as GluR2+ neurons) exhibited morphologic features characteristic of mossy cells: namely, an ovoid soma comparable in diameter to that of CA3 pyramidal cells (30-40 μ m) with three or four primary dendrites emerging from it (Amaral, 1978; Ribak et al., 1985). The nucleus was surrounded by abundant intensely GluR2-immunoreactive cytoplasm. The hilus contained a total of $21,542 \pm 849$ neurons of this type (range of 18,450-23,625; Fig. 5). They accounted for $57 \pm 2\%$ of the total hilar neuron population. GluR2+ neurons were considerably more numerous at caudal levels (Figs. 2A-B,6). They accounted for about 68% of total hilar neurons in sections near the caudal pole of the dentate gyrus (level 1), but for only about 31% in sections near the rostral pole (level 6). A small population of GluR2-immunoreactive hilar neurons exhibited morphologic features of dentate granule cells, including a small (8-12 μ m diameter) round-to-oval soma and a thin rim of immunoreactive cytoplasm surrounding the nucleus. These cells are referred to as granule cell-like GluR2-immunoreactive neurons. Neurons of this type were rarely encountered in sections from control rats. Only 90 ± 19 were observed per hippocampus. They were excluded from counts of both total hilar neurons and GluR2+ hilar neurons.

The Timm staining pattern of the dentate molecular layer was as described in previous studies (Okazaki et al., 1995,1999). In the rostral dentate gyrus, only scattered mossy fiber-like Timm granules were present in the supragranular zone (inner third; Fig. 3A). This pattern corresponded to a Timm score of 0. In sections cut from the caudal dentate gyrus, clusters of mossy fiber-like Timm staining were present in the supragranular zone, especially in the infrapyramidal blade (Fig. 3B). This pattern corresponded to a Timm score of 1. At least some of these silver granules are associated with sparsely distributed recurrent mossy fiber boutons that make synaptic contact with dentate granule cells (Molnár and Nadler, 1999; Okazaki et al., 1999).

Rats administered phenobarbital 1-3.5 h after the onset of status epilepticus

Over the range of 1-3.5 h, the exact time at which phenobarbital was administered affected neither the mean loss of total hilar neurons (Fig. 5), the mean loss of GluR2+ hilar neurons (Fig. 5), nor the extent of supragranular mossy fiber growth (Fig. 7). The range of effects obtained is illustrated in panels C-F of figures 1-3. In this regard, one must keep in mind that phenobarbital did not terminate the seizures immediately. Although forelimb clonus, rearing and falling (limbic motor seizure stages 3-5) disappeared within minutes, head nodding (stage 2) persisted for 0.75-2 h. Thus individual differences in the duration of status epilepticus did not correspond exactly to differences between the times that phenobarbital was administered. Because the exact timing of phenobarbital administration did not affect any of the outcome measures in this study, further analysis considered all 21 rats administered phenobarbital between 1 and 3.5 h after the onset of status epilepticus as a single experimental group.

In this group of rats, the total number of hilar neurons was reduced by $75 \pm 1\%$ (range: 66-81%) and the number of GluR2+ hilar neurons by $74 \pm 3\%$ (range: 48-92%). Thus hilar neurons that expressed an immunocytochemically detectable amount of GluR2 were, on average, about as vulnerable to a few hours of pilocarpine-induced status epilepticus as other hilar neurons. Cell counts performed at different locations in the dentate gyrus revealed a rostrocaudal difference in the vulnerability of both total hilar neurons and GluR2+ hilar neurons (Figs. 1,2,6). Although a greater absolute number of neurons was killed at caudal levels of the dentate hilus (levels 1-3), the percentage killed was greater in the rostral dentate hilus (levels 4-6) (total neurons: rostral, $82 \pm 2\%$ and caudal, $68 \pm 3\%$; GluR2+ neurons: rostral, $83 \pm 2\%$ and caudal, $66 \pm 4\%$). Importantly, the number of residual GluR2+ hilar neurons varied 6.2-fold among animals administered phenobarbital (range: 1,800-11,205). In contrast, the total number of residual hilar neurons varied only 1.8-fold among these animals (range: 7,163-12,943). There was not a statistically significant correlation between the number of residual GluR2+ hilar neurons and the total number of residual hilar neurons in the same animal (Fig. 8 top; $P = 0.194$, Pearson product moment correlation).

The 6.2-fold variation in the number of residual GluR2+ hilar neurons provided a sufficient spread of values for comparison with recurrent mossy fiber sprouting. The extent of sprouting varied inversely with the number of GluR2+ hilar neurons that remained intact ($P = 0.0001$, Pearson product moment correlation; Fig. 8 middle). The relationship between mean Timm score and the number of immunostained hilar neurons was not significantly different from linear ($r^2 = 0.55$, $P = 0.68$, runs test for non-linearity). There was no correlation between recurrent mossy fiber growth at any particular rostrocaudal location and the loss of GluR2+ neurons at that same location. The Timm score was either greatest near the caudal and rostral poles of the dentate gyrus ($n = 6$), greatest near the caudal pole ($n = 8$) or the same throughout the rostrocaudal extent of the dentate gyrus ($n = 7$). Regardless, the loss of GluR2+ and total hilar neurons as a percentage of control was always greatest in the rostral dentate gyrus. The mean Timm score did not correlate with the loss of total hilar neurons (Fig. 8 bottom; $P = 0.3019$, Pearson product moment correlation). Our results did not permit an accurate

assessment of this relationship, however, due to the small interanimal variation in the total number of residual hilar neurons.

The number of granule cell-like GluR2-immunoreactive hilar neurons was much greater than in control rats (Figs. 2G-H,9). The hilus contained 4868 ± 768 neurons of this type. Their number varied by about 20-fold from one animal to another. A similar variability was noted in different sections from the same rat. When the number of granule cell-like GluR2-immunoreactive neurons was added to the number of larger hilar neurons, the former accounted for ~34% of the total. The rostrocaudal distribution of these neurons varied considerably from one animal to another, but they were usually most numerous at intermediate-to-midcaudal levels (Fig. 9). There was no statistically significant relationship between the number of these cells and the time of phenobarbital administration. Their number also did not correlate with the loss of total hilar neurons, the loss of GluR2+ hilar neurons, or the extent of mossy fiber sprouting in the same animal ($P >>0.1$, Pearson product moment correlation).

Rats subjected to 6-8 h of status epilepticus without phenobarbital

Rats that were not administered phenobarbital to interrupt status epilepticus suffered devastating hilar lesions (Figs. 1G-H,5). The total number of hilar neurons per hippocampus fell by $88 \pm 1\%$ in six rats. The rostrocaudal gradient of cell loss noted after shorter periods of status epilepticus was abolished and perhaps reversed (Fig. 6). Thus cell loss was relatively greater at caudal levels both in absolute terms and to a lesser degree as a percentage of control. In the caudal half of the dentate hilus, the total number of hilar neurons was reduced by $89 \pm 1\%$ compared with $81 \pm 2\%$ in the rostral half. Compared to rats administered phenobarbital 1-3.5 h after the onset of status epilepticus, an average of 5010 additional hilar neurons were lost when status epilepticus was allowed to self-terminate. Virtually all of the additional loss occurred in the caudal part of the hilus.

GluR2+ hilar neurons were disproportionately vulnerable to prolonging status epilepticus. Hardly any of these cells remained at any rostrocaudal level when phenobarbital was not administered (Figs. 2G-H,5). Their number fell by $95 \pm 1\%$ to 1017 ± 242 . Nearly all (~93%) of the additional hilar neurons killed as a result of allowing status epilepticus to self-terminate were GluR2+ neurons and nearly all of them were located in the caudal part of the hilus.

When results from rats subjected to prolonged status epilepticus were added to the results from rats whose status epilepticus had been interrupted with phenobarbital, the correlation between mossy fiber sprouting and the number of residual GluR2+ hilar neurons became even stronger ($P < 0.000001$, Pearson product moment correlation).

The number of granule cell-like GluR2-immunoreactive hilar neurons (7290 ± 882) was higher on average than in rats that experienced a shorter period of status epilepticus, but the difference was not statistically significant ($P = 0.125$, Student's *t*-test; Fig. 9). When the number of these cells was added to the number of larger hilar neurons that remained after prolonged status epilepticus, granule cell-like GluR2-immunoreactive neurons accounted for ~62% of the total. They thus became the dominant hilar neuron population under these conditions.

All rats that had experienced prolonged pilocarpine-induced status epilepticus exhibited robust supragranular mossy fiber growth (Timm score of 3 or 4) in both suprapyramidal and infrapyramidal blades of nearly every section (Figs. 3G-H,7). Sprouting that corresponded to a Timm score of 4 was observed in sections from the caudal one-fourth of the dentate gyrus in 4 out of 6 rats. Mossy fiber-like Timm stain filled as much as half the molecular layer in these sections. In 3 instances, sprouting of this degree was also present in the rostral one-sixth of the dentate gyrus. Lesser mossy fiber growth was observed in sections cut from intermediate levels; Timm scores of 3 were assigned consistently.

Discussion

Temporal lobe epilepsy is associated with a number of neuropathological changes in the dentate gyrus, including loss of total hilar neurons (Babb et al., 1991; Masukawa et al., 1995), loss of hilar mossy cells (Blümcke et al., 2000), the appearance of granule cell-like neurons in the hilus (Houser, 1992; Parent et al., 2006), and sprouting of mossy fibers into the dentate molecular layer (Represa et al., 1989; Sutula et al., 1989; Babb et al., 1991). The pilocarpine model of temporal lobe epilepsy replicates all these features. This study was undertaken to assess the relationships among these events. Our results suggest that mossy fiber sprouting is related specifically to the loss of hilar mossy cells and that the increased number of granule cell-like hilar neurons is unrelated quantitatively to any of these other changes.

Comparison of hilar neuron numbers in control rats to previous reports

The total number of hilar neurons per hippocampus computed in the present study ($37,580 \pm 1,594$) compares closely to the number reported by Buckmaster and Dudek (1997) ($41,093 \pm 1,284$), who used essentially the same optical dissector approach, and by Miki et al. (2005) ($35,200 \pm 1,600$), who used a physical dissector approach. The similarity of our estimate to previously reported values and the low error coefficients suggest high precision in the stereological estimates of neuronal number.

Our estimate of GluR2+ neurons, presumptive mossy cells, also agrees well with previous work. Bender et al. (2003) determined that GluR2/3-immunoreactive neurons accounted for 37.5% of all neurons in the rostral portion of the hilus. Similarly, our counts in the rostral hilus (levels 5 and 6) revealed that 38% of hilar neurons were GluR2+. Buckmaster and Jongen-Rêlo (1999) estimated the number of mossy cells in the rat by subtracting the number of hilar neurons that expressed detectable GAD67 mRNA from the total hilar neuron population. This calculation assumed that all hilar neurons are either GABA interneurons, and thus express GAD67 mRNA, or mossy cells. Their value of ~64% mossy cells was just slightly above our estimate of $57 \pm 2\%$ based on GluR2 immunocytochemistry. Our estimate could theoretically be complicated by three sources of error. First, although mossy cells appeared to be intensely immunoreactive, we cannot exclude the possibility that some of them expressed an immunocytochemically undetectable amount of GluR2 protein. If some mossy cells were missed for this reason, then we would have underestimated their number somewhat. Second, some GluR2+ hilar neurons may have been inhibitory interneurons rather than mossy cells. Previous studies suggest that this error should have been small, especially since the rabbit antibody developed by Petralia et al. (1997) was used (Sloviter et al., 2001; Bender et al., 2003). Third, it is possible that only the top of the cell penetrated the counting frame in some instances, causing the cell to be misclassified as a granule cell-like GluR2-immunoreactive hilar neuron. This source of error must also have been small, because few granule cell-like GluR2-immunoreactive hilar neurons were encountered in control rats.

GluR2+ neurons were much more numerous in the caudal than in the rostral part of the hilus. This distribution closely resembles that of hilar neurons that lack expression of GAD67 mRNA (Buckmaster and Jongen-Rêlo, 1999), suggesting again that both approaches quantitated the same neuronal population, i.e., mossy cells.

Because dentate granule cells express GluR2 immunoreactivity and a small number of these neurons is present in the hilus of normal rats (Marti-Subirana et al., 1986; Scharfman et al., 2003), it seems likely that the granule cell-like GluR2-immunoreactive hilar neurons were, in fact, granule cells. Recently, McCloskey et al. (2006) reported stereological data that indicated about an order of magnitude higher number of hilar ectopic granule cells (HEGCs) in normal rats than the number of granule cell-like GluR2-immunoreactive hilar neurons we found. These authors quantitated HEGCs based on their expression of the nuclear protein PROX-1. The

discrepancy between our values might be explained by non-specificity of PROX-1. All dentate granule cells appear to express this protein and most other hippocampal neuron populations clearly do not. It is possible, however, that a small population of hilar interneurons also expresses PROX-1 or perhaps a different nuclear protein that cross-reacts with the PROX-1 antibody. Alternatively, many HEGCs, at least in control rats, may not express immunocytochemically detectable GluR2. If that is the case, they would differ dramatically in this respect from normally-located granule cells.

Changes in the hilar neuron population produced by pilocarpine-induced status epilepticus

Pilocarpine-induced status epilepticus produced remarkably extensive and consistent hilar lesions. Individual rats subjected to the same protocol varied little with respect to the percentage of total hilar neurons destroyed. Nearly all hilar neurons (88%) were killed when status epilepticus was allowed to self-terminate after 6-8 h. Even when phenobarbital was administered 1-3.5 h after the onset of status epilepticus, 75% of hilar neurons were killed. These results suggest that few hilar neurons are truly seizure-resistant. Sloviter et al. (2003) reached a similar conclusion based on observation of hippocampal sections prepared from rats that had experienced prolonged status epilepticus after perforant path stimulation or administration of kainic acid. Although the actual number of hilar neurons lost was greatest near the caudal end of the hilus, the percentage loss was greater in the rostral half, at least when status epilepticus was interrupted with phenobarbital. This result contrasts with observations in kainic acid-treated rats (Buckmaster and Jongen-Rêlo, 1999) and in humans with medically-intractable temporal lobe epilepsy (Babb et al. 1984; Masukawa et al., 1996). In these studies, the percentage loss was greatest at the caudal pole. We observed greater hilar neuron loss in the caudal dentate gyrus only when status epilepticus was allowed to self-terminate and even then the difference was not large. Thus pilocarpine-treated rats appear to differ in this respect.

Pilocarpine-induced status epilepticus also markedly reduced the population of GluR2+ hilar neurons. Theoretically, a loss of GluR2-immunoreactive neurons after status epilepticus could be explained either by neuronal death or down regulation of GluR2 expression. Several observations favor mossy cell degeneration as the probable explanation. First, counts of total hilar neurons demonstrated a substantial seizure-induced loss, especially when seizures were allowed to continue without anticonvulsant. The number of GluR2+ hilar neurons in normal rats greatly exceeded the total number of hilar neurons that survived prolonged status epilepticus. Thus the loss of hilar neurons must have included mossy cells. Second, silver impregnation demonstrated in pilocarpine-treated rats both the degeneration of hilar neurons and a terminal-like pattern of degeneration in the inner portion of the dentate molecular layer that corresponded to the termination of associational-commissural fibers (Okazaki et al., 1999). Similar patterns of degeneration have been reported in other models of temporal lobe epilepsy (Nadler et al., 1980a,b; Sloviter, 1983; Sloviter et al., 1996, 2003). Electron microscopy confirmed the degeneration of associational-commissural synaptic terminals (Nadler et al., 1980c; Sloviter et al., 2003). Finally, it is doubtful that mossy cells could survive after a marked down regulation of GluR2. An abrupt and selective loss of GluR2 mRNA and protein occurs in vulnerable neurons after transient cerebral ischemia, seizures, and other insults (Pellegrini-Giampietro et al., 1997). This loss is observed shortly before the neurons become irreversibly damaged. The consequent expression of AMPA receptor channels that lack the GluR2 subunit is thought to trigger cell death, because such channels are highly permeable to the toxic ions Ca^{2+} (Hollmann et al., 1991) and Zn^{2+} (Yin and Weiss, 1995). Thus down regulation of GluR2 expression to the extent that it becomes undetectable by immunocytochemistry would probably kill mossy cells.

The death of mossy cells contributes substantially to hilar damage in both humans with epilepsy (Blümcke et al., 2000) and in kainic acid-treated rats (Buckmaster and Jongen-Rêlo, 1999). In

kainic acid-treated rats, mossy cells appeared more vulnerable to seizures than the inhibitory interneurons of the hilus. We found this to be true also in pilocarpine-treated rats, provided that status epilepticus was allowed to self-terminate. However, mossy cells appeared no more vulnerable on average than other hilar neurons in animals subjected to a shorter period of status epilepticus. In kainic acid-treated rats, degeneration of inhibitory hilar interneurons was largely confined to the subpopulation that co-expresses somatostatin with GABA and accounts for about half the total (Buckmaster and Jongen-Rêlo, 1999). In the present study, degeneration of inhibitory hilar neurons could be approximated by comparing the effects of status epilepticus on the total and GluR2+ hilar neuron populations. These comparisons indicate that pilocarpine-induced status epilepticus killed a greater percentage of inhibitory hilar interneurons than kainic acid-induced status epilepticus, and more interneuronal types than just somatostatin-immunoreactive cells must have been involved. The extent of hilar lesions produced by kainic acid-induced seizures depends on the extent to which dentate granule cells are activated during the seizure (Sloviter et al., 2003). The difference between kainic acid-induced and pilocarpine-induced seizures might thus be related to a more consistent activation of dentate granule cells by pilocarpine-induced seizures.

The apparent loss of inhibitory hilar interneurons in the present study also appears substantially greater than that reported in pilocarpine-treated rats by Kobayashi and Buckmaster (2003), even in rats administered anticonvulsant. Status epilepticus was terminated with anticonvulsant at similar times in both studies, but we used just a single injection of phenobarbital, whereas Kobayashi and Buckmaster (2003) used repeated injections of diazepam. This seemingly minor difference in the protocol may explain the rather different hilar lesions. Kobayashi and Buckmaster (2003) also reported that the percentage loss of inhibitory interneurons was greater at more caudal levels of the dentate gyrus, whereas the loss in the present study was greater at rostral levels if anticonvulsant was used. These different outcomes emphasize that variations in animal treatment can be highly significant, even within the same epilepsy model.

Mossy cells did not respond uniformly to status epilepticus. One group of mossy cells, consisting of nearly all those in the rostral dentate gyrus and most of those in the caudal dentate gyrus, degenerated even when phenobarbital was administered one hour after the onset of status epilepticus. The remaining mossy cells, located almost exclusively in the caudal dentate gyrus, were killed only by prolonged status epilepticus (6-8 h). This result is consistent with the observation of Scharfman et al. (2001) that many mossy cells survived when pilocarpine-induced status epilepticus was terminated with diazepam after 1 h. The difference in seizure susceptibility of mossy cells located at different rostrocaudal levels of the dentate gyrus correlates well with previous evidence of mossy cell heterogeneity (Freund et al., 1997; Fujise et al., 1998; Fujise and Kosaka, 1999; Jinno et al., 2003).

Pilocarpine-induced status epilepticus markedly increased the number of granule cell-like GluR2-immunoreactive hilar neurons, by two orders of magnitude in some instances. Their number did not correlate with any other measure examined in this study, including the duration of status epilepticus, the total hilar neuron loss, the loss of GluR2+ hilar neurons, and the extent of mossy fiber sprouting. This effect of status epilepticus therefore appears largely independent of neuronal degeneration and axon sprouting. In addition, the rostrocaudal distribution of these neurons varied considerably. Our results resemble those reported for PROX-1-immunoreactive hilar neurons in pilocarpine-treated rats (McCloskey et al., 2006). The number of PROX-1-immunoreactive hilar neurons increased by an order of magnitude after status epilepticus, the increase varied substantially from one rat to another, and no consistent rostrocaudal gradient was evident. Based on the morphology and immunoreactivity of granule cell-like GluR2-immunoreactive hilar neurons and the corresponding changes in the number of PROX-1-immunoreactive hilar neurons, we suggest that the increase in granule cell-like GluR2-immunoreactive hilar neurons after status epilepticus reflects the generation and aberrant

migration of new granule cells. We cannot, however, exclude the possibility that status epilepticus induces some of the smaller inhibitory interneurons of the hilus to express GluR2, as suggested by other investigators (Sloviter et al., 2003). If this indeed occurs and the expression of GluR2 is sufficient to render these neurons detectable by immunocytochemistry, then our estimates of HEGC number may be somewhat higher than the true values.

Correlation between mossy fiber sprouting and loss of GluR2+ hilar neurons

A major finding of this study was that the extent of supragranular mossy fiber growth varied inversely with the number of mossy cells that remained intact. The relationship was not significantly different from linear. Thus the loss of each mossy cell was associated with roughly the same amount of mossy fiber growth. In view of this result, the lack of correlation between mossy fiber sprouting and total hilar neuron loss implies that sprouting is unrelated to the loss of GluR2-immunonegative hilar neurons (presumptive inhibitory interneurons). It should be borne in mind that, although supragranular mossy fiber growth correlated strongly with mossy cell loss, such evidence is insufficient by itself to prove a causal relationship. Conceivably, another factor that covaries with mossy cell death might contribute to mossy fiber sprouting. A definitive test would require assessment of sprouting after the selective and relatively complete removal of mossy cells. No method for making the required mossy cell lesion exists at present. Our findings therefore provide the best support currently obtainable for the hypothesis that seizure-induced supragranular mossy fiber sprouting is driven largely by the degeneration of mossy cells and/or their associational-commissural projection.

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Fig. 1.

Loss of cresyl violet-stained hilar neurons 10-25 wk after pilocarpine-induced status epilepticus. Additional results from these animals are presented in the corresponding panels of figures 2 and 3. A,C,E,G: Rostral dentate gyrus; B,D,F,H: Caudal dentate gyrus. A,B: Control rat. H, hilus; GCL, granule cell body layer. C,D: Rat administered phenobarbital 2 h after the onset of status epilepticus. Note the loss of rostral hilar neurons with less noticeable change in the caudal dentate hilus. These sections illustrate the least hilar neuron damage observed in animals whose status epilepticus was interrupted with phenobarbital. E,F: Another rat administered phenobarbital 2 h after the onset of status epilepticus. Note the substantial loss of both rostral and caudal hilar neurons. These sections illustrate the greatest hilar neuron

damage observed in animals whose status epilepticus was interrupted with phenobarbital. G,H: Rat whose status epilepticus was allowed to self-terminate after 6-8 h. Note the nearly complete loss of normal-sized hilar neurons. Many neurons of a size comparable to dentate granule cells are present. Numerous small neurons of this type were present in the hilus of all rats that had developed status epilepticus, but were much less numerous in the hilus of control rats. Scale bar = 200 μm .

Fig. 2.

Loss of GluR2+ hilar neurons 10-25 wk after pilocarpine-induced status epilepticus. Additional results from these animals are presented in the corresponding panels of figures 1 and 3.

A,C,E,G: Rostral dentate gyrus; B,D,F,H: Caudal dentate gyrus. A,B: Control rat. Note the greater number and density of GluR2+ hilar neurons in the caudal dentate gyrus. The size of these large multipolar neurons is comparable to that of CA3 pyramidal cells in the same section and their cytoplasm is intensely immunoreactive. H, hilus; GCL, granule cell body layer. C,D: Rat administered phenobarbital 2 h after the onset of status epilepticus. Few large GluR2+ neurons remain in the rostral dentate hilus. Arrows indicate an intact neuron of this type. In contrast, many GluR2+ neurons are still present in the caudal dentate hilus. E,F: Another rat

administered phenobarbital 2 h after the onset of status epilepticus. Practically no large GluR2⁺ neurons remain in the rostral dentate hilus, but some granule cell-like GluR2-immunoreactive neurons are evident. A few large GluR2⁺ neurons can be seen in the caudal dentate hilus beneath the granule cell body layer of the suprapyramidal blade (arrow), but the great majority of GluR2-immunoreactive hilar neurons resemble granule cells. G,H: Rat whose status epilepticus was allowed to self-terminate after 6-8 h. Many granule cell-like GluR2-immunoreactive neurons are visible in the dentate hilus, but few GluR2⁺ hilar neurons (arrow) remain. Scale bar = 200 μ m.

Fig. 3.

Supragranular Timm stain 10-25 wk after pilocarpine-induced status epilepticus. Additional results from these animals are presented in the corresponding panels of figures 1 and 2. Micrographs illustrate changes in the suprapyramidal blade of the dentate gyrus. A,C,E,G: Rostral dentate gyrus; B,D,F,H: Caudal dentate gyrus. The arrows in panels C-H point to supragranular Timm stain indicative of recurrent mossy fiber sprouting. A,B: Control rat. Mossy fiber boutons in stratum lucidum of area CA3 and the dentate hilus stain darkly, indicating a high content of heavy metal. Note the near absence of such staining above the granule cell body layer (G). C,D: Rat administered phenobarbital 2 h after the onset of status epilepticus. In this animal, supragranular mossy fiber-like Timm stain appeared mainly at the

ends (panel C) and apex (panel D) of the granule cell arch. A Timm score of 2 was assigned to the infrapyramidal and suprapyramidal blades in both the rostral and caudal dentate gyrus. This result indicates a modest degree of mossy fiber sprouting. E,F: Another rat administered phenobarbital 2 h after the onset of status epilepticus. A dense, but narrow, band of supragranular mossy fiber-like Timm stain is present in the suprapyramidal blade of both the rostral and caudal dentate gyrus. Similar staining was observed in the infrapyramidal blade. Thus robust mossy fiber sprouting was present in all parts of the dentate gyrus. A Timm score of 3 was assigned to both blades of the rostral and caudal dentate gyrus. G,H: Rat whose status epilepticus was allowed to self-terminate after 6-8 h. A narrow dense band of mossy fiber-like Timm stain is present in the suprapyramidal blade of the rostral dentate gyrus, but this band is even denser and much thicker in the caudal dentate gyrus (arrows). A Timm score of 3 was assigned to both blades of the rostral dentate gyrus and a score of 4 to both blades of the caudal dentate gyrus. Scale bar = 200 μ m.

Fig. 4. GluR2-immunoreactive neurons in the dentate hilus. G, granule cell body layer. A: Control rat. The GluR2+ hilar neurons exhibit morphologic features characteristic of mossy cells, including intense immunoreactivity, multiple processes and cell body size much larger than that of granule cells. White arrows indicate some examples. B: Rat whose status epilepticus was allowed to self-terminate after 6-8 h. None of the immunoreactive hilar neurons in this view exhibits mossy cell-like morphology. Instead, their size, shape and immunoreactivity correspond to those of granule cells in the cell body layer. Black arrows indicate some examples. Scale bar = 100 μ m.

Fig. 5.

Loss of hilar neurons as a function of the time at which phenobarbital (PB) was administered after the onset of pilocarpine-induced status epilepticus. Values are the mean number of neurons in the hilus of the entire dentate gyrus $\pm$ S.E.M. N = 5 control (Con) rats, 7 rats administered phenobarbital 1 h after the onset of status epilepticus, 8 rats administered phenobarbital 2 h after onset, 6 rats administered phenobarbital 3.5 h after onset, and 6 rats whose status epilepticus was allowed to self-terminate after 6-8 h (no PB). Both the total number of hilar neurons and the number of GluR2+ hilar neurons were reduced markedly whether or not status epilepticus was interrupted with phenobarbital. In animals administered phenobarbital, status epilepticus reduced both populations to a similar degree and results did

not differ significantly with the time of administration. When status epilepticus was prolonged by omitting phenobarbital administration, the additional neuron loss came disproportionately from the GluR2+ population.

Fig. 6.

Loss of hilar neurons from different locations in the dentate gyrus 10-25 wk after pilocarpine-induced status epilepticus. The dentate gyrus was divided into 6 levels from the caudal to the rostral end, and the number of hilar neurons in each section was averaged to yield a single value for each level. Results are expressed as the mean number of neurons per section $\pm$ S.E.M. for 6 control rats (Con), 21 rats administered phenobarbital (PB) 1-3.5 h after the onset of status epilepticus, and 6 rats whose status epilepticus was allowed to self-terminate after 6-8 h. Where S.E.M. is not shown, it was smaller than the symbol. In control rats, both hilar neurons as a whole and GluR2+ neurons specifically were most numerous near the caudal end of the dentate gyrus and least numerous near the rostral end. Prolonged status epilepticus reduced both

populations to basal numbers that did not differ along the caudorostral axis. In rats administered phenobarbital to interrupt status epilepticus, many neurons in the caudal part of the hilus survived, whereas those in the rostral part did not.

Fig. 7.

Relationship between the extent of recurrent mossy fiber sprouting and the time at which phenobarbital (PB) was administered. The mean Timm score, calculated as described in Materials and methods, was used as a measure of mossy fiber sprouting. Each filled circle represents the result obtained from one rat and the horizontal lines indicate the mean value for that group. Consistently robust sprouting was obtained from rats whose status epilepticus was allowed to self-terminate after 6-8 h (no PB). The extent of sprouting varied considerably in rats administered phenobarbital to terminate status epilepticus, and there was no consistent difference among rats treated with phenobarbital at 1, 2, or 3.5 h after onset. Mean Timm scores of control rats are always greater than 0 and less than 1.

Fig. 8.

The extent of recurrent mossy fiber sprouting, as measured by the mean Timm score, was inversely related to the number of GluR2+ hilar neurons that survived pilocarpine-induced status epilepticus ($P = 0.0001$, Pearson product moment correlation). This relationship did not differ significantly from linearity ($P = 0.68$, runs test). In contrast, the mean Timm score did not correlate significantly with the total number of hilar neurons that survived ($P = 0.3019$, Pearson product moment correlation), nor did the loss of GluR2+ hilar neurons correlate significantly with the loss of total hilar neurons ($P = 0.194$, Pearson product moment correlation). Only data from rats administered phenobarbital between 1 and 3.5 h after the onset of status epilepticus were used in this analysis.

Fig. 9.

Top, relationship between the number of granule cell (GC)-like GluR2-immunoreactive hilar neurons and the time at which phenobarbital (PB) was administered. Each filled circle represents the result obtained from one rat and the horizontal lines indicate the mean value for that group. Con, control. The number of granule cell-like GluR2-immunoreactive hilar neurons varied among animals by more than an order of magnitude. Despite a trend toward a higher mean cell number when status epilepticus was allowed to self-terminate after 6-8 h (No PB), the difference from animals that had experienced a shorter period of status epilepticus was not statistically significant ($P = 0.125$, Student's t -test). Bottom, caudorostral distribution of

granule cell-like GluR2-immunoreactive hilar neurons. Data from all rats that had developed status epilepticus are included. See figure 6 for other details.