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Highly Specific Neuron Loss Preserves Lateral Inhibitory Circuits in the Dentate Gyrus of Kainate-Induced Epileptic Rats

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Patients with temporal lobe epilepsy display neuron loss in the hilus of the dentate gyrus. This has been proposed to be epileptogenic by a variety of different mechanisms. The present study examines the specificity and extent of neuron loss in the dentate gyrus of kainate-treated rats, a model of temporal lobe epilepsy. Kainate-treated rats lose an average of 52% of their GAD-negative hilar neurons (putative mossy cells) and 13% of their GAD-positive cells (GABAergic interneurons) in the dentate gyrus. Interneuron loss is remarkably specific; 83% of the missing GAD-positive neurons are somatostatin-immunoreactive. Of the total neuron loss in the hilus, 97% is attributed to two cell types—mossy cells and somatostatinergic interneurons. The retrograde tracer wheat germ agglutinin (WGA)-apoHRP-gold was used to identify neurons with appropriate axon projections for generating lateral inhibition. Previously, it was shown that lateral inhibition between regions separated by 1 mm persists in the dentate gyrus of kainate-treated rats with hilar neuron loss. Retrogradely labeled GABAergic interneurons are found consistently in sections extending 1 mm septotemporally from the tracer injection site in control and kainate-treated rats. Retrogradely labeled putative mossy cells are found up to 4 mm from the injection site, but kainate-treated rats have fewer than controls, and in several kainate-treated rats virtually all of these cells are missing. These findings support hypotheses of temporal lobe epileptogenesis that involve mossy cell and somatostatinergic neuron loss and suggest that lateral inhibition in the dentate gyrus does not require mossy cells but, instead, may be generated directly by GABAergic interneurons.

Key words: hippocampus; dentate gyrus; GABA; GAD; somatostatin; interneurons; inhibition; epilepsy

Neuron loss in the hilus of the dentate gyrus is a key neuropathological feature of temporal lobe epilepsy (Falconer et al., 1964; Margerison and Corsellis, 1966; Mouritzen Dam, 1980; Babb et al., 1984), the most common type of epilepsy in adults (Engel et al., 1997). Hilar neuron loss has been proposed to be epileptogenic by several different mechanisms. The loss of excitatory neurons in the hilus might trigger or permit granule cell axon reorganization (Tauck and Nadler, 1985). Granule cell axon reorganization could result in positive feedback in the dentate gyrus (Wuarin and Dudek, 1996) and contribute to the collapse of inhibition during periods of intense activity (Buhl et al., 1996). The loss of inhibitory interneurons in the hilus could reduce inhibitory control and contribute to seizure genesis (de Lanerolle et al., 1989).

Previous studies have reported that, in patients and models of temporal lobe epilepsy, γ-aminobutyric acidergic (GABAergic) interneurons in the dentate gyrus are spared, despite a significant loss of glutamatergic neurons (Sloviter, 1987; Babb et al., 1989; Davenport et al., 1990). However, Houser and colleagues used *in situ* hybridization for glutamic acid decarboxylase (GAD)-mRNA, a sensitive method for detecting GABAergic neurons, and found a significant loss of GAD-positive neurons in the hilus of pilocarpine-treated rats, a model of temporal lobe epilepsy (Obenaus et al., 1993; Houser and Esclapez, 1996). There are many different types of GABAergic interneurons (Freund and Buzsáki, 1996). Different interneuron classes are likely to have specialized functional roles (Buhl et al., 1994; Miles et al., 1996) and distinct consequences if they were lost. Therefore, it is important to identify which, and to what extent, different classes of interneurons are missing in temporal lobe epilepsy. The present study uses *in situ* hybridization, immunocytochemistry, and the optical fractionator method to estimate the extent and specificity of neuron loss in the kainate-treated rat model of temporal lobe epilepsy.

Mossy cells, which are glutamatergic (Soriano and Frotscher, 1994; Wenzel et al., 1997), are the predominant neuron type of the hilus (Amaral, 1978; Liu et al., 1996). The loss of excitatory mossy cells and subsequent, and seemingly paradoxical, hyperexcitability were reconciled by the dormant basket cell hypothesis of temporal lobe epileptogenesis (Sloviter, 1987, 1994). The hypothesis proposed that mossy cells synaptically activate basket cells in lateral regions of the dentate gyrus that, in turn, inhibit granule cells. Therefore, the loss of mossy cells would disrupt lateral inhibition and thereby cause seizures. To test this hypothesis, we previously measured lateral inhibition in the dentate gyrus and found that it persists in kainate-induced epileptic rats, despite significant hilar neuron loss (Buckmaster and Dudek, 1997a). This finding suggests that lateral inhibition is not generated indirectly by vulnerable mossy cells but instead by direct axon projections of surviving inhibitory interneurons. The present study uses retrograde neuronal labeling to identify cells with appropriate axon projections for generating lateral inhibition.

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The combination of retrograde neuronal labeling with *in situ* hybridization for GAD-mRNA was used to determine whether persistent, laterally projecting neurons are GABAergic interneurons or mossy cells.

MATERIALS AND METHODS

Animals and retrograde tracer injections. All experimental procedures were performed according to protocols approved by an Institutional Animal Care and Use Committee. Male Sprague Dawley rats (Charles River, Wilmington, MA), weighing 200 gm, were treated with kainic acid (Sigma, St. Louis, MO) dissolved in 0.9% NaCl and administered (5 mg/kg, i.p.) at 1 hr intervals until the rat experienced at least 4 hr of recurrent motor seizures. The average cumulative dose was 53 mg/kg. Age-matched control rats received a similar volume of vehicle. The rats were used in a retrograde tracer experiment 5–12 months after kainate treatment.

Wheat germ agglutinin (WGA)-apoHRP-gold was synthesized according to the protocol of Basbaum and Menetrey (1987). Briefly, 50 ml of 0.01% gold chloride (Sigma) in distilled water was brought to a boil. Then 2 ml of 1% sodium citrate aqueous solution [Electron Microscopy Sciences (EMS), Fort Washington, PA] and 100 μl of 1% tannic acid aqueous solution (Sigma) were added to the boiling gold chloride solution. After cooling, the solution was brought to pH 8.2–8.4 with 0.2 M potassium carbonate (Sigma). Finally, 500 μg of lectin from *Triticum vulgaris* (wheat germ) conjugated to inactivated peroxidase (Sigma, L0390) was added to 33 ml of the gold chloride solution. After a vigorous stirring for 5 min, 330 μl of 10% polyethylene glycol aqueous solution (Sigma) was added to the protein–gold solution. The solution was centrifuged at 18,000 rpm for 4 hr at 4°C, and the soft pellet containing the concentrated WGA-apoHRP-gold complex was stored at 4°C until used.

Rats were anesthetized (50 mg/kg pentobarbital, i.p.) and placed in a stereotaxic apparatus. A volume-calibrated glass micropipette containing the WGA-apoHRP-gold solution, with a tip outer diameter of 62–84 μm, was directed toward the dentate gyrus at the following stereotaxic coordinates: 4.1 mm posterior to bregma, 1.8 mm lateral to the midline, and 3.2 mm below the dura. Tracer was expelled by air pressure pulses as described by Amaral and Price (1983). Briefly, 10–30 msec duration pulses at 20–40 psi were applied every 5–10 sec for 5–30 min to expel 100 nl of tracer. After a 5–20 min wait, the micropipette was withdrawn slowly, and the wound was closed; the rats recovered from anesthesia and were returned to their home cage.

Fixation and tissue processing. Fourteen days after retrograde tracer injection the rats were anesthetized deeply (100 mg/kg pentobarbital, i.p.) and perfused with 0.9% NaCl, followed by 4% paraformaldehyde and 0.5% glutaraldehyde (EMS) in 0.1 M phosphate buffer (PB; pH 7.4) at 4°C. Each rat's head was packed with ice during perfusion of the fixative. The brain was post-fixed overnight in the same fixative at 4°C; then it was transferred to 30% sucrose in 0.1 M PB at 4°C until equilibration. The hippocampus ipsilateral to the tracer injection site was isolated, straightened, frozen, and sectioned perpendicular to the septotemporal axis to produce transverse sections with a sliding freezing microtome set at 30 μm. Ten series of sections were collected in cryoprotectant solution consisting of 30% ethylene glycol and 25% glycerol in 50 mM PB, which was treated with 0.05% diethylpyrocarbonate (DEPC) to inactivate RNase activity. One series of sections was processed for staining with thionin, and the rest were stored at −70°C until they could be processed with tissue from other rats.

One series of sections was single-labeled for retrogradely transported WGA-apoHRP-gold, and another series first was reacted to visualize the retrograde tracer and then was double-labeled for GAD67-mRNA. Whenever possible, solutions were treated with 0.05% DEPC and autoclaved before use. The WGA-apoHRP-gold was visualized by means of a silver intensification reaction (IntenSE M Silver Intensification Kit, Amersham, Arlington Heights, IL) as recommended by the manufacturer, with minor modifications (Jongen-Rêlo and Amaral, 1998, 1999). Free-floating sections were rinsed in 0.1 M PB, followed by rinses in 10 mM PBS, pH 7.4, and 0.2 M citrate buffer, pH 7.4. Then the sections were reacted with the silver reaction mixture from the intensification kit for 20 min. The reaction was stopped by a rinsing in 0.2 M citrate buffer and 0.1 M PB.

To visualize GABAergic neurons, we used *in situ* hybridization for the mRNA encoding GAD, because Houser and colleagues demonstrated that GABAergic neurons in the dentate gyrus are labeled more reliably with *in situ* hybridization for GAD-mRNA than with immunocytochemical methods for GABAergic cell bodies, especially in the hilus (Obenaus et al., 1993; Houser and Esclapez, 1994; Esclapez and Houser, 1995). *In situ* hybridization was performed as described previously (Houser and Esclapez, 1994; Jongen-Rêlo and Amaral, 1998, 1999; Jongen-Rêlo et al., 1999). Briefly, rat GAD67 c-RNA probes were obtained by *in vitro* transcription of previously described GAD-cDNA (kindly provided by Dr. A. Tobin, UCLA). RNA probes were produced by transcription of GAD67-DNA, using a nonradioactive RNA labeling kit (Boehringer Mannheim, Indianapolis, IN). Sections were washed in 10 mM PBS and incubated sequentially in 0.01% Triton X-100 in PBS, 0.2 μg/ml proteinase K in 50 mM Tris, pH 7.4, 5 mM EDTA, and 2 mg/ml glycine (all from Sigma) in PBS. Sections were prehybridized for 1 hr in a solution containing 50% formamide (Fluka, Ronkonkoma, NY), 750 mM NaCl (Sigma), 25 mM EDTA, 25 mM piperazine-*N,N′*-bis 2-ethanesulfonic acid (Sigma), 0.2% sodium dodecyl sulfate, 0.02% Ficoll (BDH Chemicals, Carle Place, NY), 0.02% polyvinylpyrrolidone (BDH Chemicals), 0.02% bovine serum albumin (BDH Chemicals), 250 μg/ml poly A (Sigma), and 250 μg/ml salmon sperm DNA (Boehringer Mannheim). Sections were hybridized for 16–19 hr in a humid chamber at 50°C in a solution consisting of the prehybridization solution with the addition of the digoxygenin-labeled RNA probe at a concentration of 2–4 μl/ml, with 100 mM dithiothreitol (Boehringer Mannheim), 4% dextran sulfate (Sigma), and 250 μg/ml tRNA (Boehringer Mannheim). After hybridization the sections were subjected to RNase treatment and stringency washes as described previously (Jongen-Rêlo and Amaral, 1998). Sections were processed for immunodetection of the digoxygenin label with reagents of the nonradioactive nucleic acid detection kit (Boehringer Mannheim). Then the sections were mounted on gelatin-coated slides and coverslipped with Crystalmount (Biomedia, Foster City, CA) and Permount.

Two subtypes of GAD—GAD65 and GAD67—have been found to be sensitive markers for labeling GABAergic cells in the brain (Houser and Esclapez, 1994; Pitkänen and Amaral, 1994; Fukuda et al., 1997; Jongen-Rêlo and Amaral, 1998; Jongen-Rêlo et al., 1999). Houser and Esclapez (1994) found that, in rats, neurons in the hilus of the dentate gyrus are labeled more strongly for GAD65-mRNA than for GAD67-mRNA, but after prolonged incubation in the color substrate the two populations of labeled neurons were stained similarly for both GAD-mRNAs. Therefore, the genes encoding for the two subtypes of GAD are likely to be present in all GABAergic neurons. In our hands, pilot experiments revealed stronger labeling with GAD67-mRNA than GAD65-mRNA, and we therefore chose GAD67-mRNA as the marker for GABAergic cells.

Immunocytochemical labeling of somatostatinergic neurons was performed as described previously (Buckmaster et al., 1994; Buckmaster and Dudek, 1997a). Briefly, sections were rinsed in 0.1 M PB and treated with 1% $NaBH_4$ in 0.1 M PB for 30 min to reduce nonspecific staining (Kosaka et al., 1986). After more rinses in 0.1 M PB the sections were treated with 1% H_2O_2 for 1 hr to suppress endogenous peroxidase activity, and then they were rinsed in 0.1 M PB and 0.1 M TRIS-buffered saline (TBS; pH 7.4) before treatment with a blocking solution consisting of 3% goat serum, 2% bovine serum albumin (BSA), and 0.3% Triton X-100 in 0.05 M TBS for 1 hr. Sections were rinsed in 0.1 M TBS and then incubated for 36 hr at 4°C in anti-somatostatin serum (1:2500; Peninsula Laboratories, Belmont, CA; IHC 8001) diluted in 1% goat serum, 0.2% BSA, and 0.3% Triton X-100 in 0.05 M TBS. After being rinsed in 0.1 M TBS, the sections were incubated for 2 hr in biotinylated goat anti-rabbit serum (1:500; Vector Laboratories, Burlingame, CA) in secondary diluent consisting of 2% BSA and 0.3% Triton X-100 in 0.05 M TBS. After more rinses in 0.1 M TBS the sections were incubated for 2 hr in avidin–biotin–horseradish peroxidase complex (1:500; Vector Laboratories) in secondary diluent. After rinses in 0.1 M TBS and 0.1 M TB, pH 7.6, the sections were placed for 10 min in chromogen solution consisting of 0.02% diaminobenzidine, 0.04% NH_4Cl, and 0.015% glucose oxidase in 0.1 M TB and then were transferred to fresh chromogen solution with 0.1% β-D-glucose for 16 min. The reaction was stopped in rinses of 0.1 M TB, and the sections were mounted and dried on gelatin-coated slides. To enhance staining, we defatted the sections in 50% chloroform and 50% ethanol, rehydrated them in a series of ethanols, placed them in 0.005% OsO_4 (EMS) for 10 min, rinsed them in water, and placed them in 0.05% thiocarbohydrazide (TCH; EMS) for 5 min. After a rinsing in water, the OsO_4 and TCH steps were repeated. Then the sections were dehydrated in a series of ethanols and xylenes and were coverslipped with DPX.

Data analysis. Data analysis was performed by an investigator who was blind to the experimental subjects' treatment. The volume of the retrograde tracer injection site was estimated by using the Cavalieri method

(Gundersen and Jensen, 1987). A 1/10 series of sections was processed by using only the silver intensification method to reveal WGA-apoHRP-gold. A microscope (Eclipse, Nikon, Melville, NY) equipped with a motorized stage (Ludl Electronic Products, Hawthorne, NY) and camera (Dage MTI, Michigan City, IN) and Neurolucida software (MicroBrightField, Colchester, VT) were used to outline and measure injection site areas. The same intensities of dark-field illumination and camera adjustment settings were used on all sections.

Another 1/10 series of sections was double-labeled for WGA-apoHRP-gold and GAD67-mRNA. A microscope (Axioskop, Zeiss, Oberkochen, Germany) equipped with a 100× objective, high-resolution motorized stage (Ludl), and Lucivid (MicroBrightField) and Stereo Investigator software (MicroBrightField) were used to count retrogradely labeled GAD-positive neuron profiles (GABAergic interneurons) and retrogradely labeled GAD-negative neuron profiles in the hilus (putative mossy cells) in sections not included in the tracer injection site. The hilus was defined by its border with the granule cell layer and by straight lines drawn from the ends of the granule cell layer to the proximal end of the CA3 pyramidal cell layer.

The optical fractionator method was used to estimate neuron numbers (West et al., 1991; Buckmaster and Dudek, 1997b). Starting from a random position near the septal pole of the hippocampus, several 1/20 series of sections were collected and processed for thionin staining, *in situ* hybridization for GAD67-mRNA, or somatostatin-immunoreactivity. The average number of sections analyzed per hippocampus was 13. Total section thickness was used for dissector height, and only labeled somata that were not cut at the upper surface of the section were counted. This modification of the optical fractionator method facilitates analysis of tissue sectioned thinly to enhance staining; however, it increases the probability of underestimating cell numbers. There would be no effect on the relative values of control versus kainate-treated rats, because both groups were analyzed identically. To estimate the number of thionin-stained hilar neurons per dentate gyrus, we sampled an average of 16% of the hilar area randomly and systematically (counting frame, 40 × 40 μm; counting grid, 100 × 100 μm); an average of 289 cells was counted per hippocampus. To estimate the number of GAD-positive neurons per dentate gyrus, we sampled an average of 16% of the area of the entire dentate gyrus randomly and systematically (counting frame, 40 × 40 μm; counting grid, 100 × 100 μm); an average of 270 cells, identified by their position with respect to strata (i.e., hilus, granule cell layer, or molecular layer), was counted per hippocampus. To estimate the extent of neuron loss in kainate-treated rats, we used the following formula: 100% − (average number of neurons in kainate-treated rats ÷ average number of neurons in control rats). To ensure that comparable septotemporal levels were used to compare the number of neurons per section, we recorded the section position as a percentage of the distance from the septal pole to the temporal pole, and the data were binned, averaged, and plotted.

RESULTS

Animals

Twenty-one rats were used in this study. Although the rats were not observed systematically for seizure activity, spontaneous motor seizures were observed in 8 of the 10 kainate-treated rats and in 0 of the 11 controls. The kainate treatment protocol used in this study produces chronic, spontaneous motor seizures in at least 95% of the subjects (Buckmaster and Dudek, 1997b; Hellier et al., 1998).

Neuron loss

It has been proposed that mossy cells are the major vulnerable cell type in the hilus (for review, see Buckmaster and Schwartzkroin, 1994; Sloviter, 1994). However, identifying and quantifying them has been hampered by the heterogeneity of neuron types in this region and the lack of a convenient marker that is specific for mossy cells. Mossy cells are glutamatergic (Soriano and Frotscher, 1994; Wenzel et al., 1997) and possibly the only non-GABAergic neuron class in the hilus. Therefore, their number can be estimated by counting all thionin-stained hilar neurons and subtracting the GAD-positive hilar neurons (Fig. 1). In the present study the control rats have ~30,000 GAD-negative hilar neurons (putative mossy cells) per dentate gyrus, which account for 64% of the total hilar population (range, 53–79%; Table 1). GAD-negative hilar neurons are distributed all along the septotemporal axis of the hippocampus, with fewer neurons in sections near the septal pole and more neurons in sections approaching the temporal pole of the hippocampus (Fig. 2). Kainate-treated rats have significantly fewer GAD-negative hilar neurons than controls (Table 1). The extent of mossy cell loss ranges from 0 to 94%, and the average is 52%. The most extensive loss occurs near the temporal pole of the hippocampus, but other levels are affected also (Fig. 2).

The loss of GABAergic neurons could reduce the level of inhibition in the dentate gyrus and contribute to seizure genesis. Control rats ($n = 9$ with a complete series of sections for stereological analysis) have 35,901 ± 1660 (mean ± SEM; range, 28,233–42,220) GAD67-mRNA-positive neurons per dentate gyrus with 27% in the molecular layer, 26% in the granule cell layer, and 47% in the hilus (Fig. 3*A*). GAD-positive neurons are most numerous in sections near the temporal pole of the hippocampus (Fig. 3*B*). Kainate-treated rats ($n = 8$) have 31,251 ± 1397 (range, 25,176–35,427) GAD67-mRNA-positive neurons per dentate gyrus with 31% in the molecular layer, 28% in the granule cell layer, and 41% in the hilus (Fig. 3*A*). Thus, kainate-treated rats lose an average of 13% of their GAD-positive neurons per dentate gyrus. In kainate-treated rats, as compared with controls, there are significantly fewer GAD-positive neurons in the hilus ($p < 0.03$, *t* test) but no significant difference in the granule cell layer or molecular layer (Figs. 1*A,B*, 3*A*). The septotemporal distribution of GAD-positive neurons reveals that the loss is most severe near the temporal pole of the hippocampus (Fig. 3*B*).

Somatostatin-immunoreactive interneurons in the hilus are GABAergic (Somogyi et al., 1984; Kosaka et al., 1988; Esclapez and Houser, 1995). It is unclear to what extent somatostatin-immunoreactive interneurons, versus other GABAergic interneurons, account for GAD-positive cell loss. In the present study, tissue was available from a subset of control ($n = 6$) and kainate-treated ($n = 8$) rats for somatostatin-immunocytochemistry. Control rats have 9140 ± 595 (mean ± SEM, range = 8400–12,100) somatostatin-immunoreactive interneurons, representing 26% of the total population of GAD-positive neurons in the entire dentate gyrus. In the dentate gyrus the somata of virtually all somatostatin-immunoreactive neurons are located in the hilus (Sloviter and Nilaver, 1987; Kosaka et al., 1988; Buckmaster et al., 1994). Dividing the number of somatostatin-immunoreactive interneurons by the number of GAD-positive neurons in the hilus reveals that 54% of the GAD-positive hilar neurons are somatostatin-immunoreactive. This finding confirms a previous report that somatostatin-immunoreactive interneurons are the most abundant interneuron type in the hilus of the dentate gyrus of control rats, representing ~50% of all GABAergic hilar neurons (Houser and Esclapez, 1996). Compared with controls, kainate-treated rats lose 37% of their somatostatin-immunoreactive interneurons (mean ± SEM; 5740 ± 501; range, 3480–7660; $p < 0.001$, *t* test), with most of the neuron loss occurring near the temporal pole (Fig. 4), as reported previously (Buckmaster and Dudek, 1997b). Thus, there is an average of 3400 fewer somatostatin-immunoreactive interneurons in the hilus of kainate-treated versus control animals. In these same groups of rats, controls have an average of 35,363 GAD67-mRNA-positive neurons per dentate gyrus, whereas kainate-treated rats have 4112 fewer. Therefore, 83% of the GAD-positive neuron loss is attributed to the loss of somatostatin-

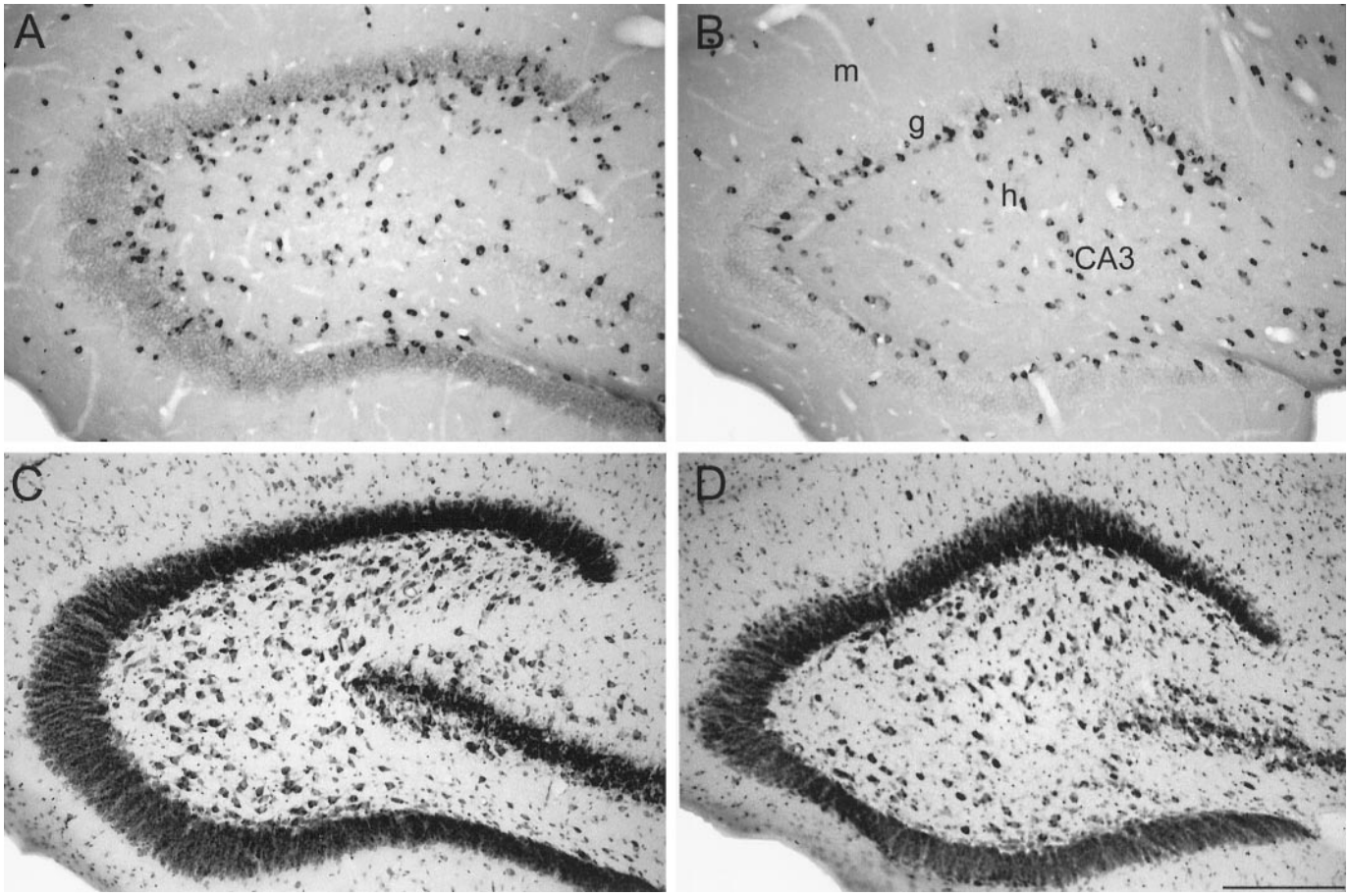

Figure 1. Nonisotopic *in situ* hybridization for GAD67-mRNA (*A, B*) and thionin staining (*C, D*) in the dentate gyrus of a control rat (*A, C*) and a kainate-treated rat (*B, D*). *m,* Molecular layer; *g,* granule cell layer; *h,* hilus; CA3, proximal end of CA3 pyramidal cell layer. Scale bar, 250 μm.

immunoreactive interneurons. There is a significant correlation between the number of somatostatin-immunoreactive neurons and the number of GAD67-mRNA-positive hilar neurons per dentate gyrus (Fig. 5; $r^2 = 0.42$; $F < 0.01$, ANOVA).

Retrograde tracer experiments

Retrograde tracer injections were made to identify neurons with appropriate axon projections for generating lateral inhibition along the septotemporal axis of the dentate gyrus. The average septotemporal length of the straightened hippocampus is 7.8 mm. The mean septotemporal span of the tracer injection site is 1.1 mm or 14% of the total septotemporal length. The mean volumes of the injection sites are not significantly different between the control (0.30 ± 0.06 mm^3; mean ± SEM) and the kainate-treated group (0.23 ± 0.03 mm^3; $p > 0.5$, *t* test). Tracer injection sites are located near the septal end of the hippocampus and span all strata of the dentate gyrus (Figs. 6, 7). In all cases, retrogradely labeled cells in the dentate gyrus are found in sections extending septally and temporally from the injection site. Most of the retrogradely labeled cells are hilar neurons (Fig. 7), but, in sections close to the injection site, retrogradely labeled somata are found in the granule cell layer and molecular layer as well as in the hilus (Fig. 8). Beyond the sections that contain the injection site, very few, if any, granule cells appear to be retrogradely labeled, even in the kainate-treated group in which granule cells had undergone axon reorganization. The number of retrogradely labeled neurons

Table 1. Number of neurons in the hilus of control and kainate (KA)-treated rats

	Control	KA-treated
n (rats)	7	8
Thionin-stained		
Mean	46,734	27,321*
SEM	4241	3653
Range	34,965–68,782	15,875–49,336
CE[a]	0.13	0.07
CV[b]	0.24	0.38
GAD67-mRNA-positive		
Mean	16,801	12,950**
SEM	1383	818
Range	11,443–21,757	8,484–16,492
CE	0.08	0.07
CV	0.22	0.18
GAD67-mRNA-negative[c]		
Mean	29,933	14,371***
SEM	4466	3253
Range	19,116–54,305	1906–32,844

*$p = 0.04$, **$p < 0.03$, ***$p < 0.02$, *t* tests.

[a]Estimated intra-animal coefficient of error.

[b]Observed inter-animal coefficient of variation.

[c]Calculated by subtracting the number of GAD-positive hilar neurons from the number of thionin-stained hilar neurons.

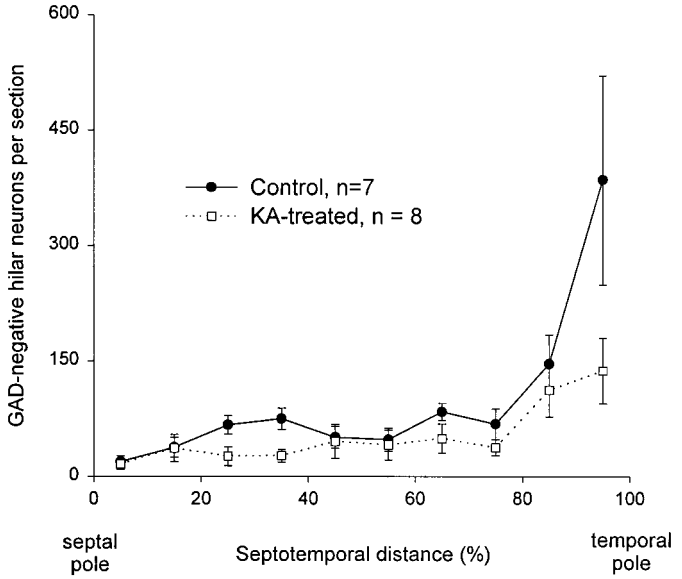

Figure 2. Septotemporal distribution of GAD67-mRNA-negative hilar neurons (putative mossy cells) in control and kainate-treated (*KA-treated*) rats. Error bars indicate SEM.

counted was correlated significantly with the number of thionin-stained hilar neurons per dentate gyrus ($r^2 = 0.63$; $F < 0.01$, ANOVA), but not with the volume of the injection site ($r^2 = 0.06$; $F > 0.70$, ANOVA). Given the loss of hilar neurons in kainate-treated rats, it is not surprising that more retrogradely labeled neuron profiles were counted in control (759 ± 67; mean ± SEM) versus kainate-treated rats (332 ± 94; $p < 0.001$, t test).

With bright-field illumination, retrogradely labeled cells are clearly evident by black particles accumulated in their somata. The particles can be seen easily through the pink reaction product of GAD-positive neurons; therefore, it is possible to identify retrogradely labeled GAD-positive and GAD-negative neurons (Fig. 8). Lateral inhibition between regions of the dentate gyrus separated by 1 mm septotemporally has been demonstrated previously in control rats (Sloviter and Brisman, 1995), and it persists in kainate-induced epileptic rats, despite significant hilar neuron loss (Buckmaster and Dudek, 1997a). To determine whether GABAergic interneurons and/or glutamatergic mossy cells are present and have appropriate axon projections for generating lateral inhibition in kainate-treated rats after hilar neuron loss, we plotted the number of retrogradely labeled GAD-positive neurons in the dentate gyrus and the number of retrogradely labeled GAD-negative hilar neurons with respect to septotemporal distance from the tracer injection site (Fig. 9). Retrogradely labeled GAD-positive neurons are most abundant adjacent to the injection site, and they are found consistently at least 1 mm away. The number and distribution of retrogradely labeled GAD-positive neurons is similar in control and kainate-treated rats (Fig. 9*A*). More numerous retrogradely labeled GAD-negative hilar neurons are found extending up to 4 mm from the injection site toward the temporal pole in controls and in some kainate-treated rats (Fig. 9*B*). The kainate-treated group has fewer retrogradely labeled GAD-negative hilar neurons per section when compared with the control group. Despite similar treatment with kainic acid, individual rats display different degrees of hilar neuron loss (Buckmaster and Dudek, 1997b). Results of the three kainate-

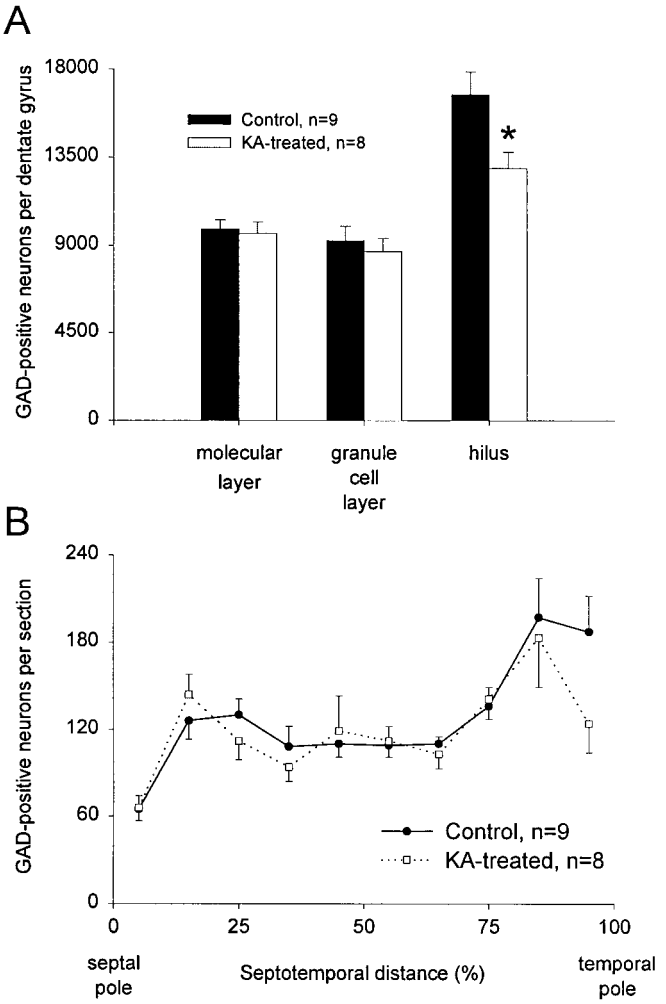

Figure 3. Compared with controls, kainate-treated rats have fewer GAD67-mRNA-positive cells in the hilus (*A*) and at the temporal pole of the hippocampus (*B*). Error bars indicate SEM; $^*p < 0.03$, t test.

treated rats with the fewest retrogradely labeled GAD-negative hilar neurons have been segregated and plotted. In those cases, virtually all of the retrogradely labeled putative mossy cells are missing (Fig. 9*B*). Despite the absence of retrogradely labeled mossy cells, these three kainate-treated rats have the number and distribution of retrogradely labeled GAD-positive neurons similar to other kainate-treated and control rats (Fig. 9*A*). An example of a kainate-treated rat in which retrogradely labeled neurons are preserved in sections within ± 1.2 mm of the injection site, but are lost in sections beyond, is shown in Figure 7*B*.

DISCUSSION

The principal findings of this study include the following. (1) In the dentate gyrus of kainate-treated rats an average of 52% of putative mossy cells and 13% of GABAergic interneurons are lost. The temporal part of the hippocampus is affected most severely. (2) In kainate-treated rats, GABAergic interneuron loss is specific to the hilus, and the loss of somatostatinergic interneurons accounts for 83% of the GABAergic interneuron loss. (3) In the dentate gyrus of control rats, mossy cells and some GABAergic interneurons have axon projections that extend at least 1 mm along the septotemporal axis of the hippocampus. (4) Compared

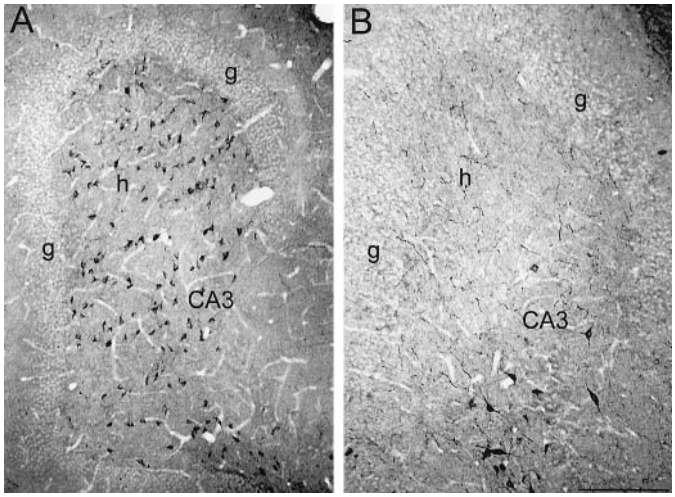

Figure 4. Somatostatin-immunoreactivity in the dentate gyrus of the temporal hippocampus in a control rat (*A*) and a kainate-treated rat (*B*). Note the lack of somatostatin-immunoreactive interneurons in the hilus (*h*) of the kainate-treated rat, despite darkly labeled neurons in the CA3 field. *g*, Granule cell layer; *CA3*, proximal end of the CA3 pyramidal cell layer. Scale bar, 250 μm.

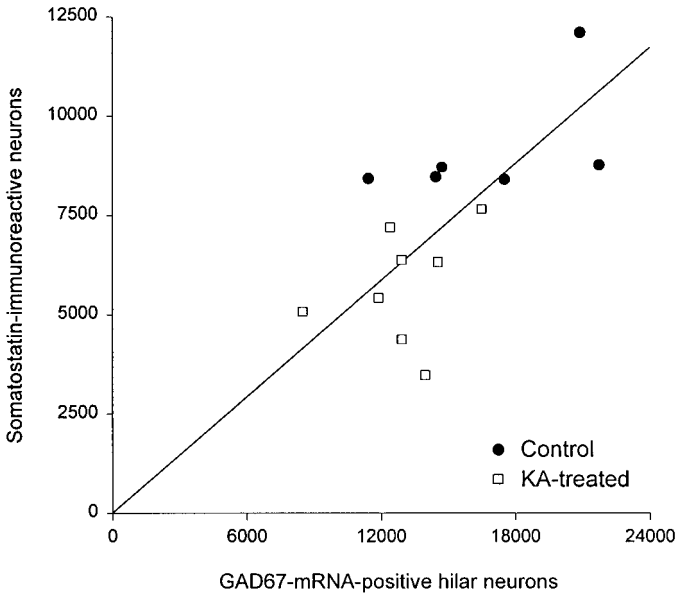

Figure 5. There is a significant correlation ($r^2 = 0.42$; $F < 0.01$, ANOVA) between the number of somatostatin-immunoreactive interneurons and the number of GAD67-mRNA-positive hilar neurons per dentate gyrus in control and kainate-treated (*KA-treated*) rats.

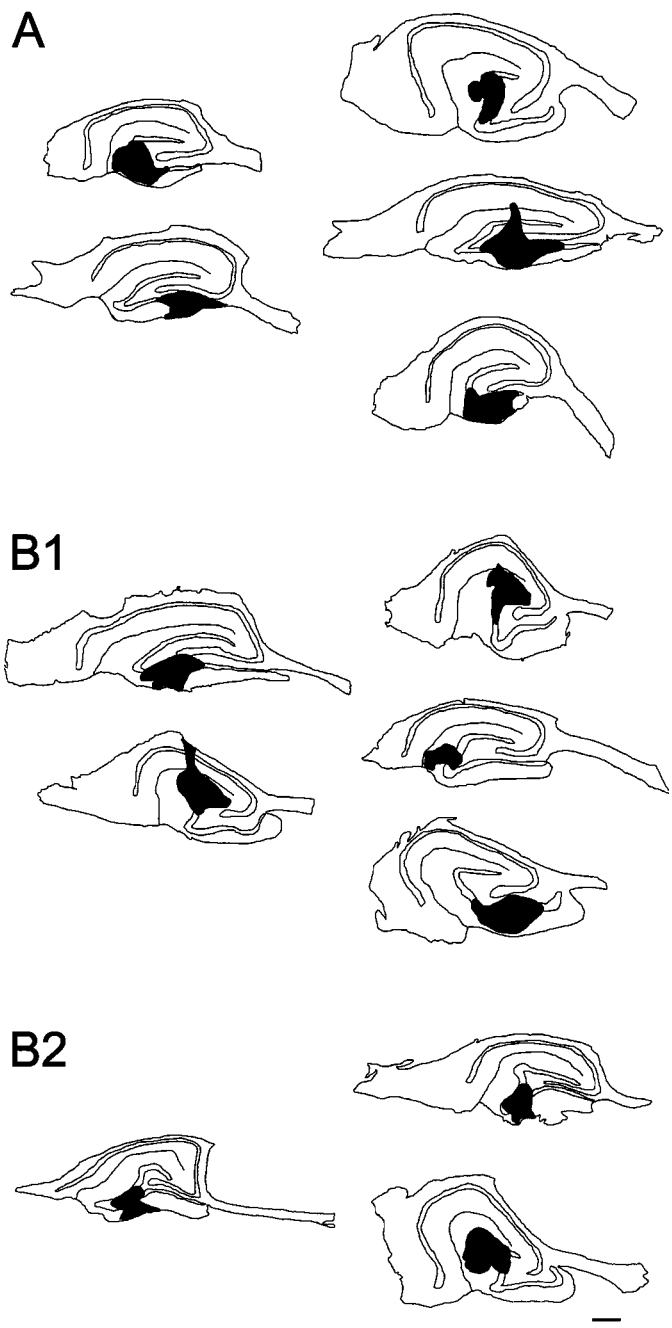

Figure 6. WGA-apoHRP-gold injection sites in control (*A*) and kainate-treated rats (*B*). Injection sites are indicated by the *black areas. B1,* Sections from the kainate-treated rats with the most retrogradely labeled GAD67-mRNA-negative hilar neurons. *B2*, Sections from the kainate-treated rats with the fewest retrogradely labeled GAD67-mRNA-negative hilar neurons (see Fig. 9). The *top section* of the *left column* of *A* and *B2* are from the animals for which the sections are shown in Figure 7, *A* and *B*, respectively. Scale bar, 500 μm.

with controls, kainate-treated rats have fewer mossy cells with long axon projections into a retrograde tracer injection site in the dentate gyrus. In some kainate-treated rats virtually all of the retrogradely labeled mossy cells are missing; however, GABAergic interneurons with long axon projections into a retrograde tracer injection site survive.

Highly specific neuron loss in the dentate gyrus of epileptic rats

Mossy cells are glutamatergic neurons in the hilus (Soriano and Frotscher, 1994; Wenzel et al., 1997). They have been visualized with Golgi staining or intracellular labeling and have been identified on the basis of dendritic and axonal characteristics (Amaral, 1978; Ribak et al., 1985; Frotscher et al., 1991; Buckmaster et al., 1993). Quantifying mossy cell numbers has been hindered by a lack of sensitive and specific markers for this cell type in rats. Recently, mossy cells in rats have been found to be immunoreactive for calcitonin gene-related peptide, but that staining re-

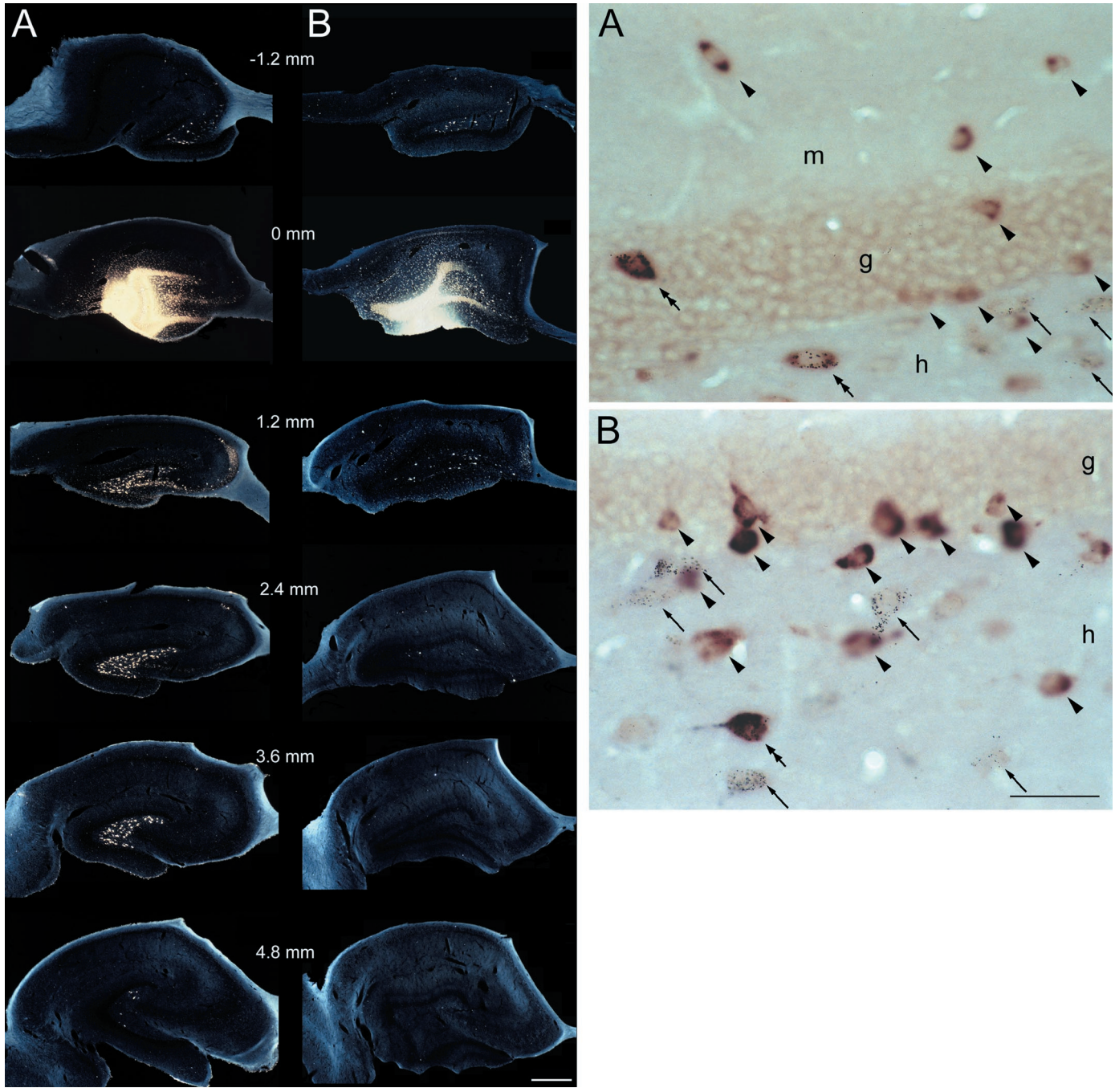

Figure 7. Dark-field photomicrographs of silver-intensified WGA-apoHRP-gold-labeled neurons in the dentate gyrus of a control rat (*A*) and a kainate-treated rat (*B*). Distance from the injection site (*0 mm*) is indicated, with negative numbers toward the septal pole and positive numbers toward the temporal pole. The control rat displays numerous retrogradely labeled hilar neurons in sections at least 3.6 mm from the injection site. The kainate-treated rat displays fewer labeled neurons and only in sections near the injection site. Scale bar, 500 μm.— *Figure 8.* Neurons in the dentate gyrus of control (*A*) and kainate-treated rats (*B*) are single- and double-labeled for WGA-apoHRP-gold and GAD67-mRNA. Sections are 1.2 mm from the center of the injection site toward the temporal pole. *Arrowheads* indicate GAD-positive neurons that were not retrogradely labeled. *Arrows* indicate retrogradely labeled GAD-negative cells in the hilus (putative mossy cells). *Double arrows* indicate retrogradely labeled GAD-positive cells. *m,* Molecular layer; *g,* granule cell layer; *h,* hilus. Scale bar, 50 μm.

quires colchicine injections, and mossy cells in the septal part of the hippocampus do not express this marker (Freund et al., 1997). In the present study the mossy cells were identified as GAD67-mRNA-negative hilar neurons. This approach overestimates the number of mossy cells to the extent that there are other types of GAD-negative hilar neurons. The GABAergic nature of spiny calretinin-immunoreactive interneurons in the hilus has been controversial (Miettinen et al., 1992; Soriano and Frotscher, 1993; Freund and Buzsáki, 1996), but recently they have been shown to be somatostatinergic (Catania et al., 1998) and, therefore, GABAergic (Somogyi et al., 1984; Kosaka et al., 1988; Esclapez and Houser, 1995). Cholinergic neurons in the dentate gyrus may

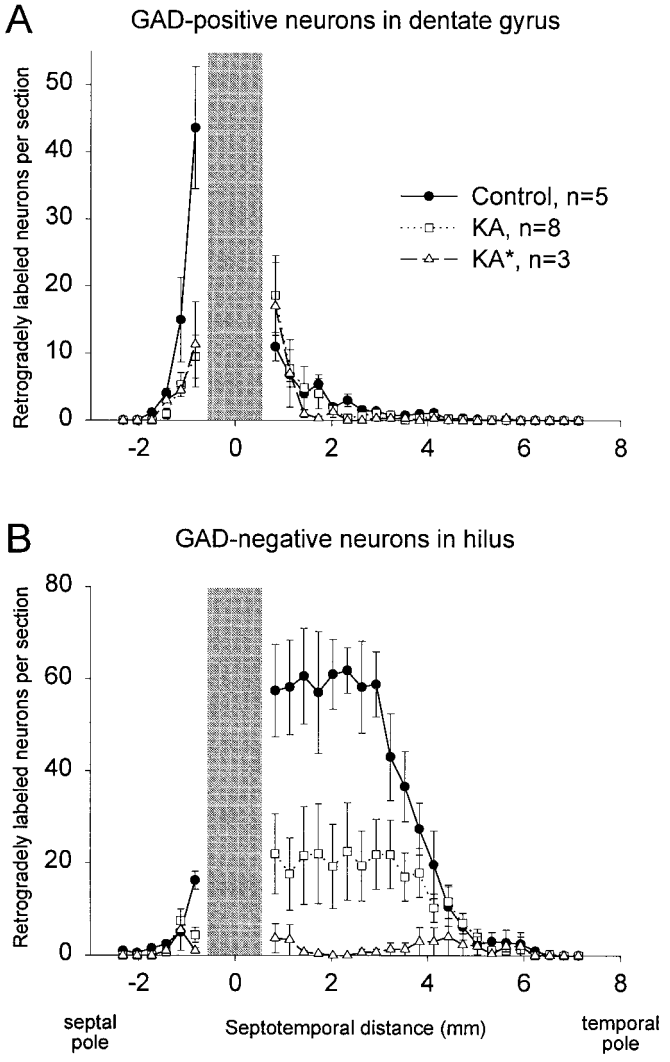

Figure 9. Distribution of retrogradely labeled neurons along the septotemporal axis of the hippocampus. *A,* Number of retrogradely labeled GAD67-mRNA-positive cells per section in all strata of the dentate gyrus. *B,* Number of retrogradely labeled GAD67-mRNA-negative hilar neurons (putative mossy cells) per section in control and kainate-treated rats. The injection site, which spans an average of 1.1 mm septotemporally, is centered at 0 septotemporal distance and is indicated by the *gray region* in the graph. Kainate-treated rats are represented as the entire group (*KA*) and as a subset of the three animals with the fewest retrogradely labeled GAD-negative hilar neurons (*KA**). The control and kainate-treated groups have similar numbers and distributions of retrogradely labeled GAD-positive neurons, which are most abundant in the sections adjacent to the injection site and extend at least 1 mm from the injection site (*A*). Control rats have numerous GAD-negative hilar neurons in sections extending 4 mm from the injection site (*B*). KA-treated rats have fewer putative mossy cells than controls, and three KA-treated rats (*KA**, *triangles*, and *dashed lines*) have almost no retrogradely labeled GAD-negative hilar neurons.

be GAD-negative, but they are extremely rare (Frotscher and Leranth, 1985). We believe that estimating the number of GAD-negative hilar neurons is the best method currently available for estimating the number of mossy cells.

In tissue from patients and models of temporal lobe epilepsy, there is significant hilar neuron loss (Dam, 1980; Babb et al., 1984; Cavazos and Sutula, 1990; Buckmaster and Dudek, 1997b) and degeneration of axon collaterals in the inner molecular layer, where mossy cell axons concentrate (Nadler et al., 1978; Sloviter, 1987; Obenaus et al., 1993). It has been assumed that the loss of mossy cells contributes substantially to hilar neuron loss in epilepsy. The results of the present study verify this view. To the best of our knowledge, this is the first report of mossy cell numbers in control and epileptic animals. Control rats have ~30,000 mossy cells per dentate gyrus, and kainate-treated rats lose an average of 52%, which accounts for 80% of the total neuron loss in the hilus. Mossy cells project axon collaterals septotemporally (Amaral and Witter, 1989) and synapse with the proximal dendrites of granule cells in the ipsilateral hippocampus (Buckmaster et al., 1996). Therefore, their loss is likely to reduce the spread of excitability along the septotemporal axis of the dentate gyrus. Mossy cells also project axon collaterals commissurally to the inner molecular layer of the contralateral dentate gyrus (Berger et al., 1980), where they might synapse with granule cells and/or interneurons (Seress and Ribak, 1984). Therefore, their loss could have mixed effects on the spread of excitability to the contralateral dentate gyrus. Mossy cell death might play an indirect role in synaptic reorganization of granule cell axons by vacating postsynaptic targets and triggering, or permitting, the formation of excitatory recurrent collaterals (Nadler et al., 1980; Cavazos and Sutula, 1990).

Somatostatin is a marker for a major class of GABAergic interneurons in the hilus (Somogyi et al., 1984; Kosaka et al., 1988; Esclapez and Houser, 1995). The loss of hilar somatostatinergic interneurons is the best documented and most consistent interneuron deficit in tissue from patients with temporal lobe epilepsy (de Lanerolle et al., 1989; Robbins et al., 1991). Mathern et al. (1995) report that in temporal lobe epilepsy patients the number of hilar somatostatinergic interneurons is reduced to <20% of control values. Many rat models of temporal lobe epilepsy display significantly fewer hilar somatostatinergic interneurons as compared with controls (Johansen et al., 1987; Sloviter, 1987; Freund et al., 1991; Lowenstein et al., 1992; Sperk et al., 1992; Magloczky and Freund, 1993; Mitchell et al., 1995; Schwarzer et al., 1995; Houser and Esclapez, 1996; Buckmaster and Dudek, 1997b). However, it has not been known to what extent somatostatinergic interneurons, versus other GABAergic interneuron classes, account for the loss of GAD-positive neurons in epileptic tissue. The present study reveals that the loss of somatostatinergic interneurons in kainate-treated rats is remarkably specific, accounting for 83% of the total loss of GAD-positive neurons in the dentate gyrus. The loss of somatostatinergic interneurons is correlated with the loss of GAD-positive hilar neurons and, as previously reported (Buckmaster and Dudek, 1997b), with the loss of Nissl-stained hilar neurons. These findings suggest that the loss of somatostatinergic interneurons is attributable to cell death and not just the loss of immunoreactivity.

The loss of hilar somatostatinergic interneurons might compromise inhibitory mechanisms in the dentate gyrus. Their axon projections extend to the middle-outer molecular layer of the dentate gyrus (Bakst et al., 1986), where they form symmetric synaptic contacts with granule cell dendrites (Leranth et al., 1990). Therefore, they might inhibit perforant path input to granule cells. Somatostatin has been reported to have anticonvulsant effects (Vezzani et al., 1991; Monno et al., 1993; Tallent and Siggins, 1999) (but see Havlicek and Friesen, 1979; Higuchi et al., 1983). Many somatostatinergic neurons in the hilus coexpress neuropeptide Y (Köhler et al., 1987), which has antiepileptic effects (Baraban et al., 1997).

In kainate-treated rats, mossy cells and somatostatin-

immunoreactive interneurons account for 97% of the hilar neuron loss—a remarkable degree of specificity. The sparing of GABAergic interneurons, other than somatostatin-immunoreactive interneurons, may explain why lateral inhibition persists in the dentate gyrus of kainate-induced epileptic rats (Buckmaster and Dudek, 1997a).

Possible circuits underlying lateral inhibition in the dentate gyrus

Lateral inhibition is a fundamental mechanism of neural processing (Shepherd and Koch, 1998), and it occurs along the septotemporal axis of the rat dentate gyrus (Sloviter and Brisman, 1995; Buckmaster and Dudek, 1997a). Mossy cells have been proposed to drive lateral inhibition in the dentate gyrus by projecting axon collaterals along the septotemporal axis of the hippocampus to excite distant GABAergic basket cells that in turn inhibit granule cells (Sloviter, 1994). This has been an important concept, because it provided a potential mechanistic link between the loss of mossy cells, which occurs after many different epileptogenic treatments or injuries (for review, see Buckmaster and Schwartzkroin, 1994; Sloviter, 1994), and the development of hyperexcitability. The present study identifies retrogradely labeled mossy cells (GAD-negative hilar neurons) and shows that these cells are virtually absent in some kainate-induced epileptic rats. Therefore, it is unlikely that mossy cell death contributes to temporal lobe epileptogenesis by disrupting lateral inhibition in the dentate gyrus. However, mossy cells might contribute in a minor way to lateral inhibition, because their local axon collaterals in the hilus form synapses with GABAergic interneurons (Buckmaster et al., 1993, 1996; Scharfman, 1995; Wenzel et al., 1997) and the interneurons could extend long axon projections to lateral regions of the dentate gyrus. However, this is unlikely to be a major mechanism, because lateral inhibition persists despite mossy cell loss.

Alternatively, it has been proposed that GABAergic interneurons directly generate lateral inhibition by projecting axon collaterals along the septotemporal axis to synapse with and inhibit distant granule cells (Buckmaster and Dudek, 1997a). The results of the present study support this view. Kainate treatment caused extensive loss of hilar neurons (up to 67%), but retrogradely labeled GABAergic interneurons survived with axon projections extending at least 1 mm along the septotemporal axis. Previous studies have shown that some individual GABAergic interneurons in the dentate gyrus extend axon collaterals far along the septotemporal axis of the hippocampus (Struble et al., 1978; Buckmaster and Schwartzkroin, 1995; Sik et al., 1997). Future studies may identify which classes of GABAergic interneurons contribute to the circuits that generate lateral inhibition in the dentate gyrus.

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