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Decreased Hippocampal Inhibition and a Selective Loss of Interneurons in Experimental Epilepsy

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The occurrence of seizure activity in human temporal lobe epilepsy or status epilepticus is often associated with a characteristic pattern of cell loss in the hippocampus. An experimental model that replicates this pattern of damage in normal animals by electrical stimulation of the afferent pathway to the hippocampus was developed to study changes in structure and function that occur as a result of repetitive seizures. Hippocampal granule cell seizure activity caused a persistent loss of recurrent inhibition and irreversibly damaged adjacent interneurons. Immunocytochemical staining revealed unexpectedly that γ-aminobutyric acid (GABA)–containing neurons, thought to mediate inhibition in this region and predicted to be damaged by seizures, had survived. In contrast, there was a nearly complete loss of adjacent somatostatin-containing interneurons and mossy cells that may normally activate inhibitory neurons. These results suggest that the seizure-induced loss of a basket cell–activating system, rather than a loss of inhibitory basket cells themselves, may cause disinhibition and thereby play a role in the pathophysiology and pathology of the epileptic state.

IN HUMAN EPILEPSY, AN EPILEPTIC "focus," made up of an aggregate of abnormally discharging cells, develops as a result of physical injury or an unknown intrinsic mechanism ("idiopathic" epilepsy). Excitatory neural activity originating in the epileptic focus spreads to other brain regions, causing the behavioral and motor manifestations of the disorder. Seizure activity in the hippocampus, which occurs in temporal lobe epilepsy, the most common form of epilepsy, or status epilepticus, a condition of continuous seizure discharge, is often associated with a characteristic pattern of hippocampal damage. Irreversible cell loss is common in the hilus of the hippocampal area dentata and in the CA1 and CA3 pyramidal cell layers. The dentate granule and CA2 pyramidal cells are relatively resistant (*1*).

We developed an experimental epilepsy model (*2*) to study the functional and structural changes that occur as a result of seizure activity and may modify the course of the epileptic state or cause behavioral deficits. In this model, the pattern of hippocampal damage seen in the human epileptic brain (*1*) is induced in normal animals by electrical stimulation of the main excitatory pathway to the hippocampus (*2*). This experimental approach was designed to circumvent some of the interpretive problems inherent in previous studies (*3*) in which convulsant, and often directly neurotoxic, compounds are used to initiate seizures by usually unknown mechanisms. It is difficult to determine the role of seizure activity itself in seizure-associated brain damage when convulsant drugs are used since damage that might be caused by seizure activity cannot be readily distinguished from damage caused by direct neurotoxic actions of the drug. In addition, convulsants cause widespread seizure activity, motor convulsions, and widespread damage that precludes a comparison between damaged and undamaged sides of the same brain.

The model developed in this laboratory involves the induction of focal seizure activity with electrical stimulation of a single excitatory pathway. The mechanism by which seizure activity is induced is known, the seizures are evoked from an identified cell population directly innervated by the stimulated pathway and the seizure duration is controlled precisely. The resulting damage is restricted primarily to the stimulated hippocampus, thus permitting a comparison of structure and function in the stimulated and unstimulated hippocampi of the same brain. Since motor convulsions do not occur, the changes induced by seizures are not the result of metabolic effects associated with convulsions or a cessation of breathing. Furthermore, hippocampal granule cell seizure activity is recorded throughout the stimulation period, indicating that there is no lack of oxygenation in the hippocampus during the period when structure and function change. As a result, studies in this and other laboratories have demonstrated that it is the increased activity in excitatory hippocampal pathways that irreversibly damages cells (*2, 3*), probably by the release of excitatory amino acids in neurotoxic concentrations (*4*).

In recent studies of this model, we noted that the damage to dentate interneurons caused by granule cell seizure activity was associated with an acute decrease in granule cell recurrent inhibition (*2*). Inhibition is thought to be mediated by local interneurons that utilize the inhibitory amino acid γ-aminobutyric acid (GABA) as a neurotransmitter (*5*). It has been hypothesized that the loss of GABA-containing cells and the inhibition they produce shifts the excitatory-inhibitory balance, leading to seizure activity (*6*). Accordingly, we tentatively predicted

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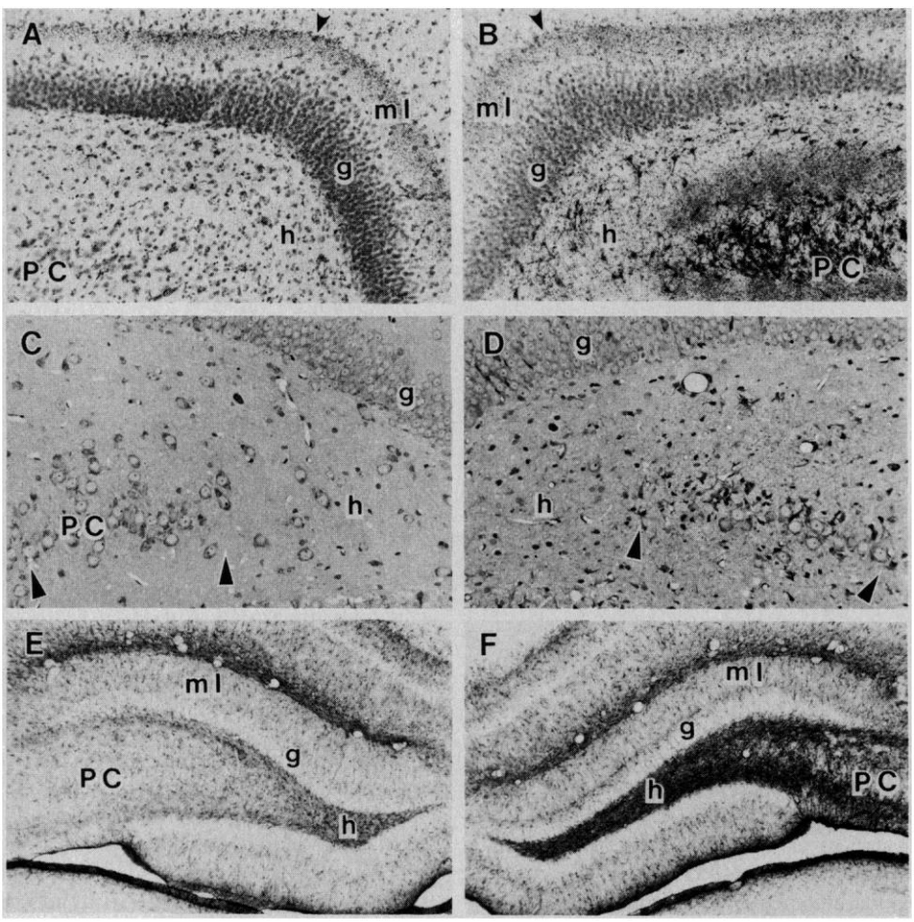

Fig. 1. Effects of seizure activity on hippocampal neurons and glia. (**A**) Unstimulated and (**B**) stimulated area dentata of the same rat 3 days after 2 hours of continuous 20-Hz stimulation. Sections (50 μm thick) stained by the silver stain method (*8*) show degenerating cells (B)in the hilus (h) and pyramidal cell layer (PC). The inner molecular layers (ml) of both hippocampi (A and B) contain stained elements (arrowheads) indicative of degenerating synaptic terminals of damaged hilar cells in (B). (**C** and **D**) Two weeks after 24-hour intermittent stimulation, hilar cell (h) and CA3 pyramidal cell loss (region between arrowheads) is evident. Glycol methacrylate sections (4 μm thick) were stained with methylene blue–basic fuchsin. (**E** and **F**) Immunocytochemical staining of glial fibrillary acidic protein (GFAP) 2 weeks after 24-hour stimulation shows that the hilus (h) in (F) is reduced in width and that increased GFAP staining, possibly indicative of reactive gliosis, has resulted. Sections in (E) and (F) are from the brain shown in Fig. 2. Fixation in (A) and (B): perfusion with 4% paraformaldehyde in 0.1*M* tris buffer, *p*H 7.4. Postfixed overnight at room temperature in perfusate. Fixation in (C) to (F): perfusion with cold 2% paraformaldehyde in 0.1*M* sodium acetate buffer, *p*H 6.5, for 3 minutes (no saline prewash) followed by 30 minutes with cold 2% paraformaldehyde and 0.01% glutaraldehyde in 0.1*M* sodium borate buffer, *p*H 8.5. Postfixed overnight in cold 2% paraformaldehyde, *p*H 8.5. Magnifications: ×30 in (A) and (B); ×116 in (C) and (D); ×75 in (E) and (F).

that the observed decrease in granule cell inhibition may be long-lasting and the result of seizure-induced damage to GABA-containing inhibitory interneurons (*2*). The experiments described in this report were designed to determine whether repetitive seizures cause persistent changes in inhibition and to identify the damaged interneurons, whose loss might contribute to functional abnormalities associated with the epileptic state.

Extracellular field potentials were evoked and recorded in vivo in urethane-anesthetized male Sprague-Dawley rats by stimulating the main excitatory afferent to the hippocampus, the perforant path (*2*). Recurrent inhibition was evaluated by the twin-pulse technique (*2, 5*) before and after seizure activity was induced. Epileptiform activity was induced in the hippocampal granule cells by stimulating the perforant path at 2 Hz (twin pulses 40 msec apart) for 24 hours combined with intermittent 10-second trains of single stimuli at 20 Hz every minute throughout the 24-hour period. Other animals were stimulated continuously at 20 Hz (single pulses) for shorter periods (0.5 to 2.0 hours) to determine the minimum period of continuous discharge that can be tolerated before irreversible damage results. Both procedures involved a stimulus voltage of 20 V and a duration of 0.1 msec to avoid hydrolysis and damage at the electrode tip. Identical recording and stimulating electrodes were placed on the unstimulated side to allow comparison of structure and function in the unstimulated and stimulated hippocampi of each animal. Control animals were treated identically except that no stimuli were delivered between tests of inhibition. Detailed methods have been published previously (*2*). Three or 14 days after stimulation, animals were anesthetized with urethane (1.25 g/kg subcutaneously), evaluated for the relative state of recurrent inhibition, and killed by intracardiac perfusion-fixation. The animals' brains were removed and processed for degeneration and for immunocytochemical and Nissl staining (*7, 8*).

Three days after 24-hour intermittent stimulation, recurrent inhibition was decreased or absent (*9*). Silver staining indicative of neuronal degeneration (*8*) revealed damaged cell bodies of CA3 and CA1 pyramidal neurons and dentate hilar cells in the stimulated hippocampus only (Fig. 1, A and B). Silver-stained particles indicative of degenerating synaptic terminals were visible in the hippocampal regions innervated by the damaged cells. In the area dentata, stained particles were present in both the outer and

Table 1. Survival of immunoreactive hippocampal dentate neurons 2 weeks after 24-hour intermittent stimulation of the perforant path. Alternate 50 μm-thick Vibratome sections of each brain formed four groups (one for each antiserum) with 5 to 15 sections per group. The total number of immunoreactive dentate neurons were counted in each antiserum group by an individual unaware of the treatment, and the mean number of cells per section was used as a single number for each antiserum group. The means (± SEM) in the table derive from four stimulated animals.

	Number of immunoreactive neurons per section			
	Somatostatin	GABA	CCK	VIP
Unstimulated side	54.1 ± 2.0	65.9 ± 9.0	16.1 ± 1.7	5.1 ± 0.9
Stimulated side	11.0 ± 1.7*	57.0 ± 2.4†	16.5 ± 1.2†	4.5 ± 0.7†
Percent of control	20	86	102	88

*$P < 0.001$ (df = 3), Student's *t* test. †Not significantly different, $P > 0.05$.

inner molecular layers, which are innervated by the somatostatin-containing hilar cells and the hilar mossy cells, respectively (*7, 10, 11*). Continuous stimulation for 2 hours produced damage similar to that caused by intermittent stimulation but relatively more damage to the hilar cells that innervate the inner molecular layer (Fig. 1, A and B). Briefer continuous stimulation (45 to 60 minutes) irreversibly damaged many hilar cells but few CA3 pyramidal cells. Surprisingly, the inhibitory, pyramidal-shaped basket cells that lie within the granule cell layer (*7, 12*) and that were predicted to be damaged by seizure activity (*2*), were unstained 3 days after either stimulation procedure in hundreds of silver-stained sections evaluated (Fig. 1B).

Two weeks after intermittent stimulation, inhibition was still absent (*9*). Nissl staining revealed that most hilar cells in the stimulated hippocampus had been phagocytized by this time (Fig. 1, C and D), and reactive gliosis was evident (Fig. 1, E and F). Since inhibition was absent even though there was no apparent damage to inhibitory basket cells, it was necessary to identify the damaged cells and determine whether GABA-containing inhibitory neurons had, in fact, survived.

Two populations of hippocampal dentate interneurons can be discriminated in the rat on the basis of the neuroactive substances they contain. One consists of GABA-containing interneurons (*7, 12, 13*), different subsets of which contain cholecystokinin (CCK) or vasoactive intestinal polypeptide (VIP) or both (*13*). The other is a population of GABA-negative, but somatostatin (SS)-positive neurons whose cell bodies lie in the dentate hilus adjacent to the GABA-positive interneurons (*7, 14*) and apparently form part of the ipsilateral associational-commissural (IAC) projections from the hilus to the dentate molecular layer (*10, 11*). A third group of dentate interneurons, which does not exhibit GABA- or peptide-like immunoreactivity (*11*), lies within the hilus among the SS-containing interneurons. These cells, called mossy cells, project mainly to the inner dentate molecular layer (*11*). Unlike the GABA-mediated basket cell system, the IAC projections originating in the hilus are probably excitatory in part (*11*) and may increase granule cell inhibition by directly exciting the adjacent GABA-containing inhibitory interneurons (*15*).

Immunocytochemical staining of alternate sections of each intermittently stimulated brain (n = 14) for GABA-, CCK-, VIP- and SS-like immunoreactivity 2 weeks after stimulation revealed a selective loss of SS-immunoreactive somata throughout the dorsal hippocampal area dentata on the stimulated side of the brain (Fig. 2, A and B). Cell counts of stimulated versus unstimulated hippocampi of each brain (Table 1) showed an approximate 80% loss (range, 70% to > 90%) of SS-positive dentate hilar interneurons throughout the dorsal hippocampus. Nissl staining of adjacent sections of each brain (Fig. 1, C and D) confirmed that the loss of immunocytochemical staining was due to an actual loss of hilar cells in the stimulated hippocampi and not simply to decreased antibody binding. There were no significant differences in the numbers of interneurons with GABA-, CCK- or VIP-like immunoreactivity between the stimulated and unstimulated hippocampi of each brain (Table 1) as shown in Fig. 2, C to H. The stimulated area dentata exhibited increased staining of CCK-positive fibers in the inner molecular layer (Fig. 2, E and F),

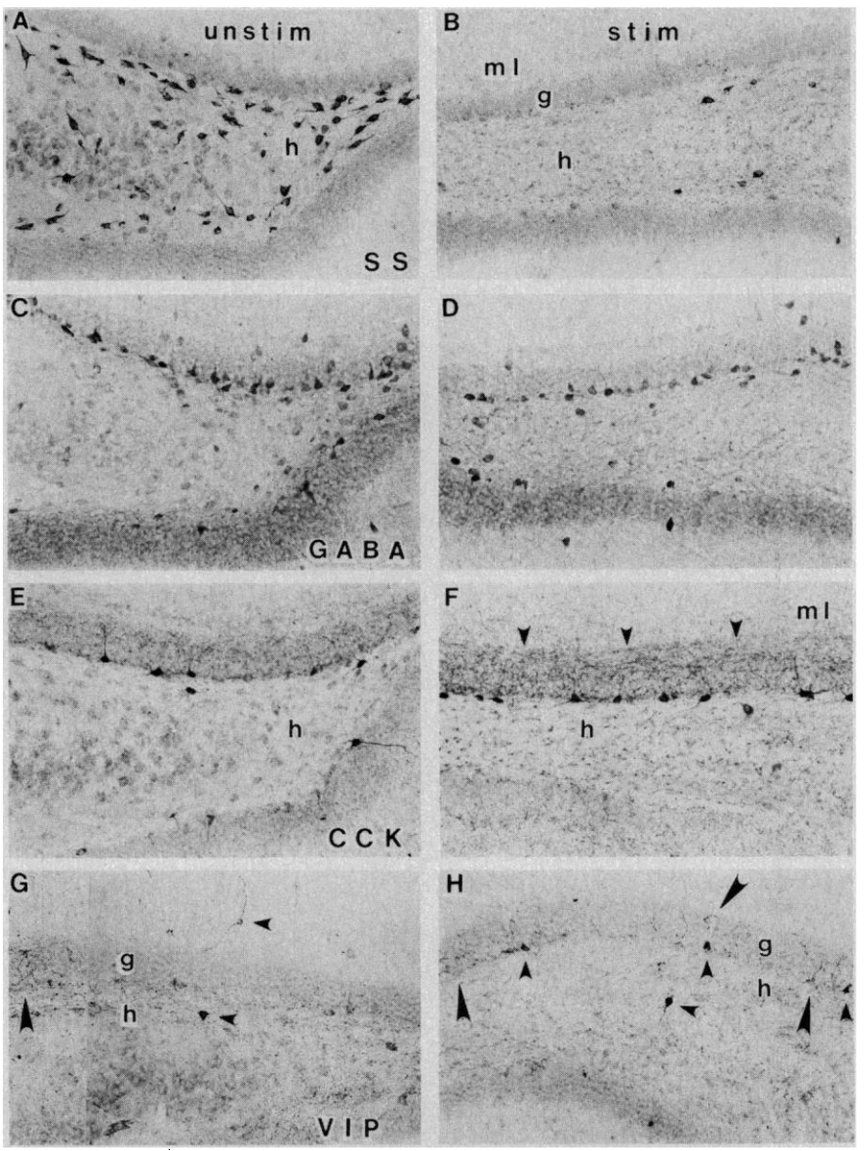

Fig. 2. Effect of seizure activity on the survival of hippocampal dentate interneurons in adjacent sections of the same brain. (**A** and **B**) Somatostatin-like immunoreactivity (SS-LI) in unstimulated (A) and stimulated (B) area dentata 2 weeks after 24-hour intermittent electrical stimulation of the perforant path. SS-LI neurons are lost in the hilus (h) of the stimulated side. This representative degree of cell loss was evident throughout the dorsal hippocampus. (**C** and **D**) GABA-like immunoreactivity. GABA-containing dentate interneurons survived despite the near total loss of SS-LI somata in immediately adjacent sections. (**E** and **F**) Cholecystokinin (CCK)-like immunoreactivity. The subset of GABA-containing cells that contain CCK (*3*) have survived (F). (**G** and **H**) Vasoactive intestinal polypeptide (VIP)–like immunoreactivity. Although there are relatively few VIP-immunoreactive cells, the presence of VIP-positive cells (small arrowheads) and fibers (large arrowheads) in the granule cell layer (g) and hilus (h) are evident in both stimulated and unstimulated hippocampi. Sections were stained immunocytochemically (*18*) with the protein A–avidin–biotin–horseradish peroxidase–diaminobenzidine method described elsewhere (*7*). Fixation as stated in Fig. 1 for (C) to (F). Magnification, ×75.

possibly a response by basket cells to the loss of the terminals of the IAC pathway.

Control animals, subjected to all procedures except that no stimuli were delivered between tests of inhibition, exhibited no obvious decrease in inhibition, no damaged somata after degeneration staining, and no obvious loss of immunocytochemically stained neurons. Some, but not all, control animals exhibited terminal degeneration in the outer molecular layers of both hippocampi, presumably due to damage by the stimulating electrode to perforant path fibers that innervate these layers in both hippocampi.

These results demonstrate, first, that the GABA-containing basket cells predicted to be most sensitive to the effects of seizure activity (*2*) are, in fact, relatively impervious to the excitatory input they receive from the granule cells. Second, they demonstrate that the immediately adjacent population of SS-containing interneurons and mossy cells, which also receive excitatory granule cell activity (*11*), are remarkably sensitive to the seizure activity they receive (*16*). In fact, dentate hilar cells are more sensitive than any other hippocampal cell type, since 1 hour of stimulation causes hilar neuron damage without damaging the next most sensitive cells, the CA3 pyramidal neurons of the endfolium. The reason that immediately adjacent populations of interneurons, both of which receive input from dentate granule cells, exhibit such a striking difference in susceptibility to seizure-induced damage is unknown.

Since dentate hilar interneurons form the IAC projections (*10*) that apparently activate GABA-containing basket cells of the dentate region (*15*), the seizure-induced loss of hilar interneurons, including either or both the SS-containing hilar cells and the immunocytochemically unstained mossy cells (*16*), may explain the seizure-associated loss of inhibition. However, the survival of dentate GABA-containing cells, as demonstrated by immunocytochemical staining, does not preclude the possibility that these inhibitory cells are functionally impaired. Therefore, the present results do not establish a causal relation between the loss of inhibition in granule cells and the selective loss of hilar interneurons. Clearly, it will be necessary to elucidate the normal function of each interneuron population, determine whether hilar interneurons are an obligatory element in the process of inhibition, and test the hypothesis that granule cell seizure activity leaves the GABA-mediated inhibitory system intact but relatively deafferented and unexcited by the loss of cells that normally activate it.

Third, these findings suggest that selective damage to hilar SS-containing cells and mossy cells may occur in human epileptics as a consequence of intermittent seizures characteristic of temporal lobe epilepsy, or even after a single episode of status epilepticus (*1*). A loss of these cells might lead to decreased inhibition, more seizures, and more damage. Although the consequences of hippocampal damage in humans are incompletely understood, they may be related to behavioral and memory impairments that have been associated with epilepsy (*17*). Therefore, these experimental results provide the rationale for investigating the survival of specific interneuron populations in human hippocampi removed during surgical resection for intractable epilepsy and correlating the type of cell loss with the clinical condition.

Finally, although these findings are not necessarily contradictory to the view that epilepsy is the result of a primary loss of GABA-synthesizing neurons (*6*), they demonstrate that a seizure-associated decrease in inhibition can occur without the loss of GABA-containing inhibitory neurons. If a basket cell–activating system is irreversibly damaged by seizure activity in some human epileptics, a resulting loss of hippocampal inhibition could lead to the development of a hippocampal focus or an increase in the spread of seizure activity that causes the clinical manifestations of the condition. Consequently, the possible efficacy of somatostatin or its analogs in preventing seizure activity and its consequences warrants investigation.

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