Evidence of Functional Mossy Fiber Sprouting in Hippocampal Formation of Kainic Acid-Treated Rats[1]

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Abstract

In the rat hippocampal formation, degeneration of CA4-derived afferent fibers provokes the growth of mossy fiber collaterals into the fascia dentata. These aberrant fibers subsequently form granule cell-granule cell synapses. The hippocampal slice preparation was employed to determine whether these recurrent connections are electrophysiologically functional. Hippocampal slices were prepared 12 to 21 days after the bilateral destruction of CA4 neurons with either intracerebroventricular or intravenous kainic acid (KA). In slices from control rats, antidromic stimulation of the mossy fibers elicited a single population spike in the granular layer of the fascia dentata. In contrast, when slices from some KA-treated rats were similarly tested, antidromic stimulation elicited multiple population spikes. This effect was not reproduced by blocking inhibitory transmission with bicuculline methiodide. Slices from other KA-treated rats fired a single population spike, but an antidromic conditioning volley increased the amplitude of a subsequent antidromic population spike by 5 to 15%. In slices from control rats, on the other hand, an antidromic conditioning volley always either decreased or failed to alter the amplitude of an antidromic test response. Superfusion with Ca^{2+}-free medium containing 3.8 mM Mg^{2+} reversibly abolished all effects of KA administration. Abnormal responses to antidromic stimulation correlated with the loss of CA4 neurons and the growth of supragranular mossy fiber collaterals in the same animals.

These results suggest that supragranular mossy fiber collateral sprouts form a functional recurrent excitatory circuit. These aberrant connections may further compromise hippocampal function already disrupted by neuronal degeneration, such as by facilitating seizure activity.

Administration of the potent convulsant kainic acid (KA) reproduces in rats lesions remarkably similar to Ammon's horn sclerosis (AHS) in humans (for review, see Nadler, 1981). AHS, a well recognized pathological finding in the brains of temporal lobe epileptics, involves extensive degeneration of hippocampal pyramidal cells, with sparing of granule cells (Corsellis and Meldrum, 1976). The effect of clinical or experimentally induced AHS on hippocampus-dependent behaviors and on epileptogenesis depends not only on the loss of hippocampal neurons and their connections, but also on any synaptic rearrangements that result from this lesion. Such changes in connectivity have been demonstrated after a variety of lesions to the hippocampal area of experimental animals (reviewed by Cotman and Nadler, 1978), and they are likely to occur also in the brains of temporal lobe epileptics (De Lorenzo and Glaser, 1981; De Lorenzo et al., 1982).

Clinical and KA-induced experimental AHS involve the degeneration of hippocampal CA4 neurons (that is, the morphologically diverse neurons that lie in the dentate hilus between the dendrites of area CA3c pyramidal cells and the hilar border of the granular layer) as well as pyramidal cells. CA4 neurons give rise to the associational and commissural pathways that terminate on the proximal third of granule cell dendrites in the ipsilateral and contralateral fascia dentata, respectively (Gottlieb and Cowan, 1973; Swanson et al., 1978; Berger et al., 1981; Laurberg and Sørensen, 1981). Degenerating associational-commissural afferent fibers are replaced, in part, by nascent collaterals of the hippocampal mossy fibers (axons of the dentate granule cells) (Nadler et al., 1980). Mossy fiber collateral sprouts grow across the granular layer of the fascia dentata and form a plexus in the denervated lamina. Such mossy fiber growth can be provoked not only by KA lesions, but also by other lesions of the associational-commissural projection (Laurberg and Zimmer, 1981). At least some of these aberrant fibers make synaptic connections with dendrites of granule cells (Frotscher and Zimmer, 1983). Thus, destruction of CA4-derived projections creates aberrant granule cell-granule cell connections in the rat fascia dentata.

If these recurrent mossy fiber synapses were electrophysiologically functional, they would endow the granule cells with excitatory input that is not normally present. A single stimulus might then elicit repetitive discharge. We have explored this possibility with use of the hippocampal slice preparation. Preliminary reports of this work have been presented (Tauck and Nadler, 1982; Nadler et al., 1983).

Materials and Methods

KA lesions. Male Sprague-Dawley rats (45 to 60 days of age; Harlan Sprague-Dawley, Madison, WI) were injected with KA either intracerebroventricularly (ICV) or intravenously. For ICV administration, rats were anesthetized with pentobarbital (55 mg/kg, i.p.), and 2.34 to 3.75 nmol of KA in a 1-μl volume of artificial cerebrospinal fluid (Elliott, 1969) were injected during a 30-min period into each lateral ventricle. For intravenous administration, unanesthetized rats were injected in a lateral tail vein with 11 mg/kg of KA dissolved in isotonic saline. Intravenous injections usually destroyed a greater percentage of CA4 neurons that did ICV injections, and they more consistently provoked the growth of supragranular mossy fiber collaterals. Otherwise, results did not differ with the route of administration. Control animals were injected with the appropriate vehicle alone or were not injected. Data from both types of control were indistinguishable.

Preparation of hippocampal slices. Hippocampal slices were prepared 12

to 21 days after administration of KA or vehicle. This interval was chosen because the growth of mossy fiber collaterals is already maximal in 12 days (Laurberg and Zimmer, 1981). We observed no obvious difference in electrophysiological or morphological data between slices from rats that survived 12 or 21 days after KA administration.

Alternate transverse slices of 500 μm thickness cut from the middle third of the hippocampal formation were used for electrophysiological studies. The remaining slices from the same hippocampi were saved for histological analysis. For electrophysiological studies, hippocampal slices were suspended upon a nylon net in recording chambers of the type described by White et al. (1978). Elliott's (1969) artificial cerebrospinal fluid (composition: 122 mM NaCl, 25 mM NaHCO$_3$, 3.1 mM KCl, 1.3 mM CaCl$_2$, 1.2 mM MgSO$_4$, 0.4 mM KH$_2$PO$_4$, and 10 mM D-glucose (pH 7.4), gassed continuously with water-saturated 95% O$_2$:5% CO$_2$) was recirculated through each superfusion chamber for a 1.5- to 2-hr period before experimentation commenced. During this time the fluid level was maintained just below the top surface of the slice. The temperature of the superfusion medium was 31 ± 1°C. Slices from KA-treated and control rats were studied either separately or concurrently; differences between the two groups were the same in either case. Blind electrophysiological analyses were not attempted, since cell death and gliosis were visually obvious in hippocampal slices from KA-treated rats.

Stimulation and recording. Cathodal constant current pulses of 50- to 100-μsec duration were applied through the inner wire of a concentric bipolar electrode (inner Pt/Ir wire, 25 μm in diameter; outer stainless steel cylinder, 100 μm in diameter). Glass micropipettes filled with either 3 M NaCl or 2 M NaCl, 1 M sodium citrate (2 to 15 megohms impedance) were used to record extracellular field potentials. These signals were filtered above 1 kHz, amplified, displayed on an oscilloscope, and recorded on magnetic tape. Signals recorded on tape were digitized, averaged, and drawn with an X-Y plotter.

Antidromic population spikes were recorded from the granular layer of the suprapyramidal blade midway between the end and apex of the granule cell arch. The stimulating electrode was placed in the mossy fiber bundle where it exits the hilus. In slices from control rats, antidromic spikes were insensitive to superfusion with Ca^{2+}-free medium and were abolished by selectively cutting the mossy fibers. Therefore, our antidromic volleys probably lacked any significant orthodromic component and did not fire the granule cells by spread of current to the cell bodies.

Mossy fibers were stimulated with paired pulses delivered at an interpair interval of 30 sec or more. The amplitude (in millivolts) of antidromic granule cell population spikes displayed on the oscilloscope was measured from base line to peak negativity. Because the stimulus artifact sometimes obscured the onset of the antidromic spike, the base line was taken as the point just before onset of the second stimulus of the pair. Slices were retained for study only if maximal stimuli evoked at least an 8-mV antidromic population spike. The stimulus intensity was adjusted to evoke an initial submaximal antidromic population spike of 3 to 5 mV. Then stimulus pairs were delivered with various interstimulus intervals (ISIs). The mossy fibers were stimulated 5 to 10 times with each ISI, and amplitudes of the population spike(s) were averaged. The mean amplitude of the response to the second stimulus in each pair was compared to the mean amplitude of the response evoked by the first stimulus. In slices from some KA-treated rats, additional (presumably orthodromic) population spikes followed the antidromic spike. To measure the amplitude of these additional spikes, we determined the difference between apparent onset of the spike and its peak negativity and between its apparent offset and peak negativity. Spike amplitude was calculated by averaging these two values. Population spike amplitude serves as a measure of the number of granule cells brought to threshold by the stimulus, provided that the amplitude of individual action potentials remains constant (Andersen et al. 1971).

Application of experimental media. These studies were conducted with slices submerged in the superfusion medium. Media equilibrated with 95% O$_2$:5% CO$_2$ were superfused at a rate of 2 ml/min from pressurized flasks. Ca^{2+}-free medium was prepared by replacing CaCl$_2$ with twice the concentration of MgCl$_2$ (final Mg^{2+} concentration of 3.8 mM). Electrophysiological responses were first obtained during superfusion with normal medium. Slices were then superfused with bicuculline methiodide (BMI)-containing or Ca^{2+}-free medium for 10 to 15 min before any additional data were collected. By this time, stimulation of perforant path fibers in Ca^{2+}-free medium no longer evoked a field potential, and in BMI-containing medium it evoked multiple population spikes (Ault and Nadler, 1983).

Histology. Alternate slices from the middle third of the hippocampal formation were reserved for heavy metal staining. These slices were maintained in buffered Na$_2$S solution (Danscher, 1981) for about 1 hr and were then fixed in 10% neutral buffered formalin for about 2 hr. Each slice was then embedded flat in Tissue Tek II O.C.T. compound (Miles Laboratories, Naperville, IL) and cut into frozen sections. Sections were thaw-mounted onto coated sides and stained for the presence of heavy metal cations by Timm's sulfide silver method as modified by Danscher (1981). Additional slices were transferred to neutral buffered formalin directly after cutting, maintained in this solution for 3 hr, and then embedded and cut into frozen sections. Slide-mounted sections were stained with cresyl violet.

After all experiments had been completed, the histological sections were evaluated without knowledge of the electrophysiological data. Sections stained with cresyl violet were used to estimate the extent to which CA4 neurons had been destroyed. Those cut through the temporal third of the hippocampal formation were chosen for analysis, because the cell bodies
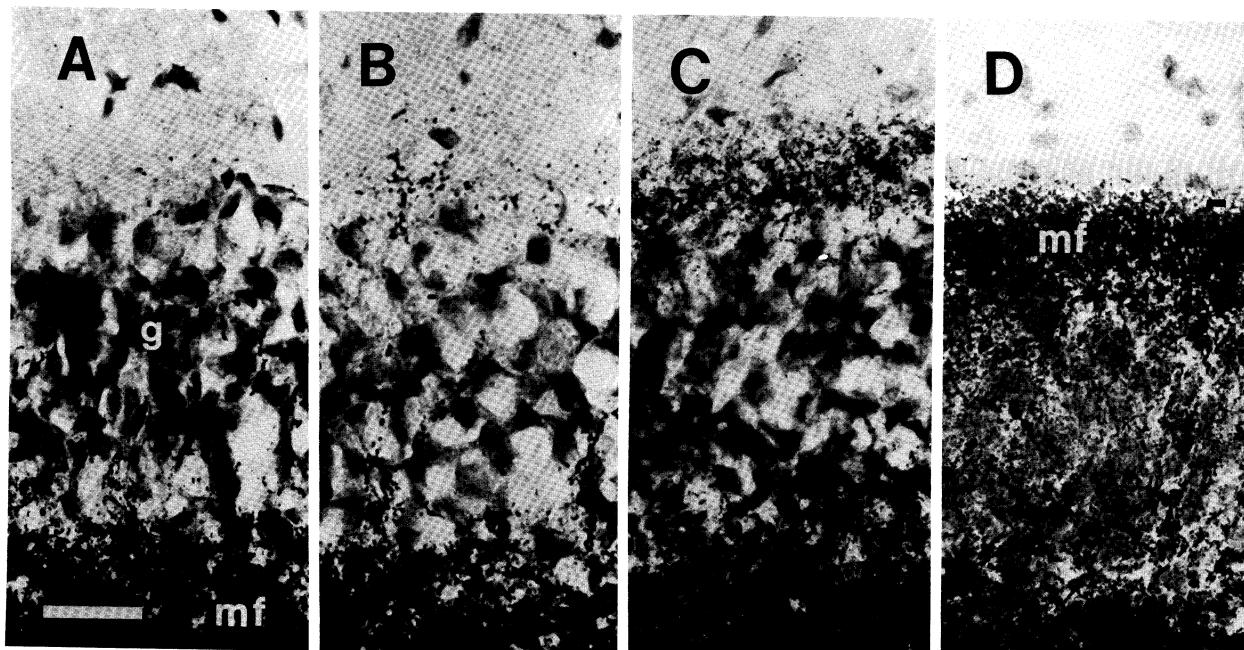
Figure 1. Effect of KA administration on the pattern of Timm's sulfide silver staining in the rat fascia dentata. Sections shown were counterstained with cresyl violet. *A*, Section from control rat. Timm's score of 0. *g*, granular layer; *mf*, mossy fibers. *B* to *D*, Sections from rats treated with KA. *B*, Timm's score of 1; *C*, Timm's score of 2; *D*, Timm's score of 3. Note the dense supragranular band of mossy fiber-like staining in *D*. Calibration: 0.03 mm.
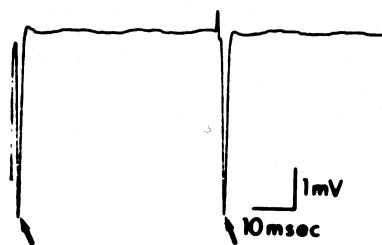
Control   KA-Treated   KA-Treated
                                 Potentiation   Multiple Firing
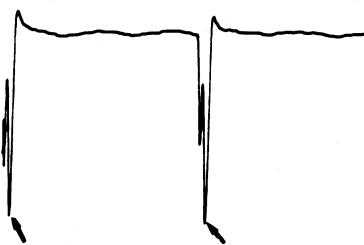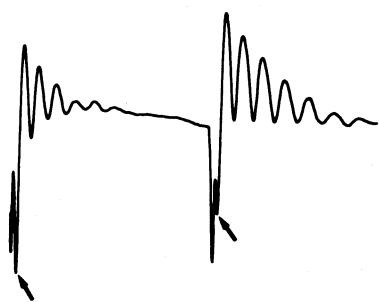

Figure 2. Effects of KA administration on the response of dentate granule cells to antidromic stimulation of the mossy fibers. A pair of identical submaximal electrical pulses were delivered with an ISI of 50 msec. *Arrows* here and in Figure 3 indicate the antidromic population spike. The X-Y plotter broadened the stimulus artifact associated with the second pulse of the pair.

from which associational-commissural fibers originate are mainly located there (Swanson et al. 1978). CA4 cells were counted in three sections from each animal with the aid of a camera lucida. Only cell fragments larger than 10 μm in diameter were included in the count, thus excluding glia and displaced granule cells (Gaarskjaer and Laurberg, 1983). Values from each section were averaged.

Sections stained for the presence of heavy metal cations were used to estimate the extent of recurrent mossy fiber growth. Mossy fiber boutons were recognized by their characteristic black staining, indicative of their unusually high content of heavy metal cation, mainly Zn^{2+} (Haug, 1973). Sections from each animal were assigned a score from 0 to 3 related to the quantity of supragranular mossy fiber-like staining (Fig. 1). Sections with no or only occasional supragranular mossy fiber-like staining were scored 0. Sections with scattered mossy fiber-like staining above all parts of the granular layer were given a score of 1. Sections that exhibited either patches of heavy mossy fiber-like staining interspersed with regions of sparser staining or a continuous band of staining intermediate in intensity between sections scored 1 and 3 were given a score of 2. Finally, sections with a dense, continuous band of supragranular mossy fiber-like staining were assigned a score of 3.

Results

Electrophysiology

Slices from control rats. Antidromic activation of the granule cells elicited a single negative-going population spike in the granular layer (Fig. 2). When paired antidromic stimuli were delivered with ISIs of 15 to 120 msec, the response to the second stimulus of the pair was always smaller than or equal to that evoked by the first. Paired-pulse depression was greatest (about 10%) with an ISI of less than 30 msec. Superfusion with neither Ca^{2+}-free medium nor 100 μM BMI significantly altered these results (Tauck, 1983). Accordingly, we conclude that paired-pulse depression of the antidromic population spike probably did not depend on recurrent GABAergic inhibition. As expected, however, 100 μM BMI markedly depressed recurrent inhibition as assayed with antidromic-orthodromic stimulus pairs.

Slices from KA-treated rats. Slices from animals treated with KA could be divided into three groups, based on their response to antidromic stimulation of the mossy fibers. Slices from about half of the KA-treated rats were electrophysiologically indistinguishable from those of controls. Slices from six other KA-treated rats fired multiple population spikes in response to a single antidromic stimulus (Figs. 2 and 3). These negative-going spikes were separated from each other by 4 to 6 msec and were superimposed on a positive wave whose amplitude varied directly with the total number of spikes. Such a response was never observed in slices from control rats. Superfusion with Ca^{2+}-free medium reversibly abolished the second and succeeding population spikes of the multispike complex, as
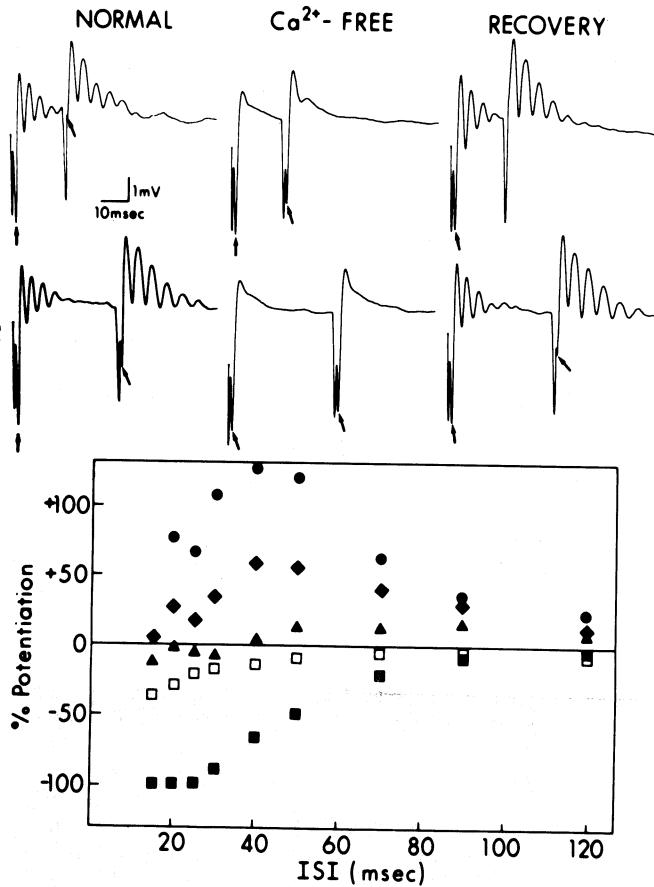

Figure 3. Multiple population spikes in response to paired submaximal antidromic stimuli in a slice from a KA-treated rat. Results are taken from an experiment in which the antidromic population spike was followed by several additional spikes. *Top, 1:* 20 msec ISI; *2:* 40 msec ISI. Note that with an ISI of 20 msec there was little evidence of an antidromic population spike in response to the second stimulus of the pair in normal medium. *Bottom,* Potentiation or depression of different components of the multispike complex by a conditioning volley as a function of ISI. ■, antidromic population spike; ▲, first additional spike; ◆, second additional spike; ●, third additional spike; □, antidromic population spike in Ca^{2+}-free medium. Responses in Ca^{2+}-free medium were indistinguishable from those obtained in slices from control rats bathed in either medium.
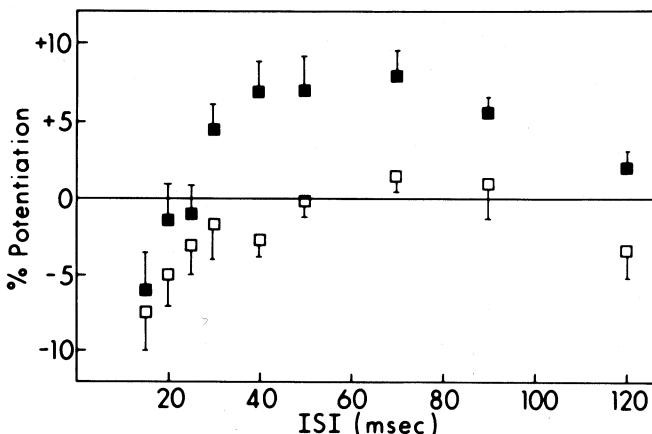
Figure 4. Responses of dentate granule cells to paired submaximal antidromic stimuli in slices from three KA-treated rats and the effect of Ca^{2+}-free medium. These slices fired a single antidromic population spike. ■, mean values in normal medium (±SEM); □, mean values in Ca^{2+}-free medium. Two-way analysis of variance gave $p < 0.01$ for effect of ISI and $p < 0.05$ for effect of Ca^{2+}. Responses in Ca^{2+}-free medium were indistinguishable from those obtained in slices from control rats bathed in either medium.

well as the underlying positive wave, but did not affect the initial (antidromic) spike (Fig. 3). The second and succeeding spikes exhibited paired-pulse potentiation, as did the underlying positive wave. Later spikes potentiated more than earlier spikes. A conditioning stimulus usually also increased the total number of spikes in the complex. Paired-pulse potentiation of the initial (antidromic) population spike was observed in these slices only when the antidromic spike was followed by just one additional population spike. When the first antidromic stimulus of the pair elicited several additional spikes, however, the amplitude of the second antidromic spike was less than that of the first, particularly with short ISIs (Fig. 3). Both effects of a conditioning stimulus on the antidromic population spike amplitude were Ca^{2+} dependent.

We attempted to reproduce the repetitive firing observed in slices from KA-treated rats by allowing control slices to deteriorate with time and by superfusing freshly prepared control slices with 100 μM BMI. Multiple firing could be provoked by driving the mossy fibers antidromically 11.5 hr after placing the slice in the chamber, just before all electrically evoked activity ceased. However, the second and succeeding population spikes were of low and variable amplitude and did not exhibit paired-pulse potentiation. Antidromic stimulation in the presence of BMI failed to elicit more than one population spike, however great the stimulus intensity. Thus, multiple firing of granule cell populations did not arise from deterioration of these neurons or simply from inactivation of GABAergic inhibition.

Slices from 10 additional KA-treated rats, like those from controls, generated only one antidromic population spike per stimulus, and paired-pulse depression was obtained with ISIs less than 30 msec (Figs. 2 and 4). However, when pairs of antidromic stimuli with ISIs of 30 to 90 msec were presented, the response to the second stimulus of the pair exceeded the response to the first by a maximum of 5 to 15% ($p < 0.01$ for effect of KA treatment compared to controls, $p < 0.01$ for interaction between KA treatment and ISI, two-way analysis of variance). Superfusion of the slice with Ca^{2+}-free medium reversibly eliminated paired-pulse potentiation of the antidromic spike (Fig. 4).

We frequently checked the response to antidromic stimulus pairs of other slices from the same animals with use of just one or two ISIs. All slices from the same rat gave the same result (none potentiated, all potentiated, or all generated multispike complexes).

Histology

Extent of KA lesions. In a separate series of animals, we confirmed that neither intravenous nor ICV KA destroyed dentate granule cells.
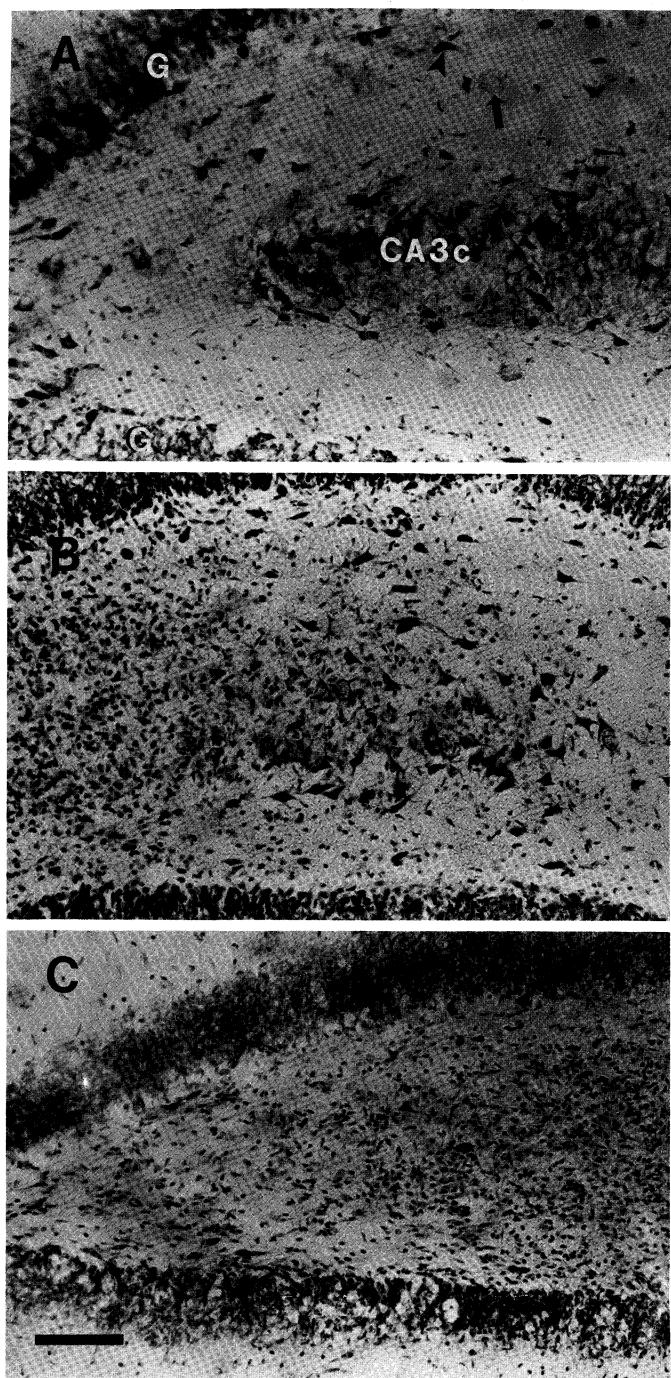
Figure 5. Loss of neurons from the dentate hilus 12 to 21 days after intravenous administration of KA (11 mg/kg). A, Section from an animal injected with saline. The pyramidal cell layer of area CA3c and the granular layers (G) are labeled. Area CA4 is the region between the granule and pyramidal cell layers. This region contains sparsely distributed normal-appearing (arrow) and dark (arrowhead) neurons. Dark cell transformation was considered artifactual and such neurons were counted as intact. B, Section from KA-treated rat that exhibited paired-pulse potentiation of the antidromic granule cell population spike, but fired no additional spikes. Neurons have been lost from the CA3-CA4 area, where there is substantial gliosis. C, Section from KA-treated rat that fired a multispike complex in response to antidromic stimulation of the mossy fibers. Neurons are virtually absent from areas CA4 and CA3c. These regions are gliotic and the dentate hilus has shrunken. Cresyl violet stain. Calibration: 0.1 mm.

Figure 6. Number of CA4 neurons in sections cut from temporal hippocampal slices 12 to 21 days after KA administration. Data from KA-treated rats are related to their electrophysiological response to paired antidromic stimulation of the mossy fibers. Number of animals: A, 19; B, 16; C, 10; D, 5. Group B differed from controls at $p < 0.05$, and groups C and D differed from controls at $p < 0.01$ (one-way analysis of variance).

Figure 7. Timm's scores of sections cut from hippocampal slices 12 to 21 days after KA administration. See "Materials and Methods" and Figure 1 for scoring criteria. Data from KA-treated rats are related to their electrophysiological response to paired antidromic stimulation of the mossy fibers. Number of animals: A, 22; B, 13; C, 10; D, 6. Groups B, C, and D differed from controls at $p < 0.01$ (Fisher's exact test).

Discussion

Our results demonstrate that intravenous or ICV administration of KA can alter the response of dentate granule cells to stimulation of their axons. Normally, the granule cells fire a single antidromic population spike whose amplitude is not increased by an antidromic conditioning stimulus. Two changes were recorded in the present study. First, granule cells in slices from some KA-treated rats fired multiple population spikes in response to a single antidromic stimulus. Second, in slices from many KA-treated rats, a conditioning antidromic stimulus potentiated the response to an identical test stimulus. All effects of KA administration were Ca^{2+} dependent.

The most persuasive evidence that recurrent mossy fiber collaterals mediated these abnormal granule cell responses is the excellent correlation between electrophysiological and histological data. In general, the greater the loss of CA4 neurons, the more dense was the growth of supragranular mossy fiber collaterals and the more abnormal was the response to antidromic mossy fiber stimulation. Only slices from animals with clear evidence of recurrent mossy fiber collateral growth were capable of firing multiple population spikes and slices from five other such animals exhibited paired-pulse potentiation of single antidromic spikes. In no case did a slice that lacked supragranular mossy fiber collaterals exhibit these responses. The lack of abnormal electrophysiological response in slices from three rats which nevertheless had developed extensive recurrent collaterals may perhaps be explained by excessive damage to the relatively fragile giant mossy fiber boutons in cutting those particular slices. In addition, the action of nascent mossy fiber synapses may sometimes have been masked by other, as yet unknown, consequences of KA administration.

Other lines of evidence also tend to support synaptic mediation of these KA-induced electrophysiological changes. Both paired-pulse potentiation and repetitive firing were reversibly abolished by superfusion with Ca^{2+}-free medium. Although dependence of an evoked response on extracellular Ca^{2+} does not in itself prove the involvement of a synaptic mechanism, it remains nonetheless one of the essential criteria. In addition, antidromically evoked multispike complexes evidenced substantial paired-pulse potentiation. This form of

Of the neurons that project to the fascia dentata, only those in area CA4 degenerated in any significant number.

Our cell counts clearly demonstrated that KA administration reduced the number of CA4 neurons in a manner that correlated with the electrophysiological data (Figs. 5 and 6). Sections from animals that fired multiple population spikes in response to single antidromic stimuli contained fewer CA4 neurons than any of the sections from control animals. Sections from the other two groups of KA-treated rats contained more CA4 neurons, but still significantly fewer than controls.

Growth of supragranular mossy fiber collaterals. Sparsely distributed supragranular mossy fiber collaterals were often, but not always, observed at the location chosen for electrophysiological recordings in sections cut from control slices (Figs. 1A and 7). In contrast, a moderate-to-dense band of these fibers was always present in sections from animals that fired multiple population spikes in response to a single antidromic stimulus (Figs. 1, C and D, and 7). Individual mossy fibers could frequently be followed from their origin beneath the granular layer through this layer to the supragranular zone. Similarly extensive mossy fiber growth was evident in half the cases that exhibited paired-pulse potentiation but fired just a single population spike. Of 13 KA-treated rats that responded to antidromic stimulation of the mossy fibers indistinguishably from controls, only 3 showed unequivocal evidence of mossy fiber collateral growth.

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