Mossy Fiber–Granule Cell Synapses in the Normal and Epileptic Rat Dentate Gyrus Studied With Minimal Laser Photostimulation

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Molnár, Péter and J. Victor Nadler. Mossy fiber–granule cell synapses in the normal and epileptic rat dentate gyrus studied with minimal laser photostimulation. J. Neurophysiol. 82: 1883–1894, 1999. Dentate granule cells become synaptically interconnected in the hippocampus of persons with temporal lobe epilepsy, forming a recurrent mossy fiber pathway. This pathway may contribute to the development and propagation of seizures. The physiology of mossy fiber–granule cell synapses is difficult to characterize unambiguously, because electrical stimulation may activate other pathways and because there is a low probability of granule cell interconnection. These problems were addressed by the use of scanning laser photostimulation in slices of the caudal hippocampal formation. Glutamate was released from a caged precursor with highly focused ultraviolet light to evoke action potentials in a small population of granule cells. Excitatory synaptic currents were recorded in the presence of bicuculline. Minimal laser photostimulation evoked an apparently unitary excitatory postsynaptic current (EPSC) in 61% of granule cells from rats that had experienced pilocarpine-induced status epilepticus followed by recurrent mossy fiber growth. An EPSC was also evoked in 13–16% of granule cells from control groups. EPSCs from status epilepticus and control groups had similar peak amplitudes (~30 pA), 20–80% rise times (~1.2 ms), decay time constants (~10 ms), and half-widths (~8 ms). The mean failure rate was high (~70%) in both groups, and in both groups activation of N-methyl-D-aspartate receptors contributed a small component to the EPSC. The strong similarity between responses from the status epilepticus and control groups suggests that they resulted from activation of a similar synaptic population. No EPSC was recorded when the laser beam was focused in the dentate hilus, suggesting that indirect activation of hilar mossy cells contributed little, if at all, to these results. Recurrent mossy fiber growth increases the density of mossy fiber–granule cell synapses in the caudal dentate gyrus by perhaps sixfold, but the new synapses appear to operate very similarly to preexisting mossy fiber–granule cell synapses.

INTRODUCTION

A common feature of temporal lobe epilepsy (Babb et al. 1991; Franck et al. 1995; Houser et al. 1990; Represa et al. 1989; Sutula et al. 1989) and of animal models of epilepsy (Buckmaster and Dudek 1997b; Mello et al. 1993; Nadler et al. 1980; Stanfield 1989; Sutula et al. 1988) is the development of a dense recurrent mossy fiber pathway in the hippocampal formation. Recurrent mossy fiber growth creates monosynaptic excitatory connections among dentate granule cells (Okazaki et al. 1995, 1999; Wuarin and Dudek 1996). There is, at present, no anatomic evidence for such connections in the normal brain. In nonepileptic animals, dentate granule cells have been shown to resist the propagation of seizures through the limbic circuit (Lothman et al. 1992). Although granule cell discharge can be synchronized by nonsynaptic mechanisms (Swetzer et al. 1992), this occurs only during very strong afferent bombardment (Lothman et al. 1992). The recurrent mossy fiber pathway, if sufficiently powerful, could reduce the threshold for granule cell synchronization, thus enhancing the participation of these cells in seizures. Considerable evidence appears to support this view (Buckmaster and Dudek 1997a; Cronin et al. 1992; Masukawa et al. 1992; Patrylo and Dudek 1998; Tauck and Nadler 1985), although some investigators have offered alternative interpretations (Kotti et al. 1997; Longo and Mello 1997; Sloviter 1992).

As one approach toward understanding the role of the recurrent mossy fiber pathway in limbic seizures, we sought to characterize the electrophysiological properties of mossy fiber–granule cell synapses. In a previous study, we evoked excitatory postsynaptic currents (EPSCs) by antidromic stimulation of the mossy fibers in hippocampal slices from rats with recurrent mossy fiber growth (Okazaki et al. 1999). These responses appeared to be largely or entirely monosynaptic, as indicated by our ability to record an N-methyl-D-aspartate (NMDA) receptor–mediated EPSC in the presence of an α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)/kainate receptor antagonist. In addition, we recorded antidromically evoked EPSCs in some granule cells from control rats. These responses also appeared to be largely monosynaptic, based again on the observation of an NMDA receptor–mediated EPSC. The use of antidromic electrical stimulation raises at least the theoretical concern that excitatory pathways other than the mossy fibers might have contributed to the synaptic response. To study mossy fiber–granule cell synapses in a way that is not compromised by simultaneous activation of any other pathway, the stimulus must be restricted to presynaptic granule cells. Our electron microscopic (Okazaki et al. 1995) and electrophysiological (Okazaki et al. 1999) data suggested a low probability of connection between pairs of granule cells, even after robust recurrent mossy fiber growth. Furthermore, published anatomic studies provide no assistance in localizing coupled cell pairs. Individual granule cells project not only to their close neighbors, but also to granule cells some distance away (Buckmaster and Dudek 1999; Okazaki et al. 1995; Sutula et al. 1998). These considerations suggested that simultaneous recording from granule cell pairs would have a low probability of success. Accordingly, we studied the properties of mossy fiber–granule cell synapses with the use of scanning laser photostimulation (Katz...
and Dalva 1994). By using highly focused ultraviolet (UV) light to release glutamate from an inactive (“caged”) precursor, extracellular glutamate reached a concentration high enough to cause action potential firing by only a small number of granule cells, and a large number of photostimulation sites could be explored in a short period of time.

**M E T H O D S**

**Pilocarpine-induced status epilepticus**

Recurrent mossy fiber growth was provoked by inducing status epilepticus with pilocarpine. Male Sprague-Dawley rats (175–225 g; Zivic-Miller Laboratories, Allison Park, PA) received a single injection of pilocarpine (330–360 mg/kg ip) preceded 30 min earlier with scopolamine methyl bromide and terbutaline hemisulfate (2 mg/kg ip, each) to block peripheral side effects and maintain respiration. Status epilepticus was defined as a continuous limbic motor seizure of stage 2 or higher (Racine 1972). Status epilepticus was terminated after 3–5 h with a single injection of phenobarbital sodium (50 mg/kg ip). Some pilocarpine-treated rats did not develop status epilepticus. These animals were used as drug-treated controls. Age-matched rats were used as untreated controls.

**Preparation and incubation of hippocampal slices**

Animals were studied 10–30 wk after the administration of pilocarpine. The rat was decapitated under ether anesthesia, the brain was removed, and 400-μm-thick transverse slices were cut from the caudal third of the hippocampal formation with a vibratome. Slices used for electrophysiological recording corresponded to horizontal plates 98–100 of Paxinos and Watson (1986). Slices reserved for Timm histochemistry were taken from a level of the hippocampal formation immediately rostral to this, corresponding to plates 101–103. For electrophysiological studies, the slices were transferred to a beaker of artificial cerebrospinal fluid (ACSF, which contained (in mM) 122 NaCl, 25 NaHCO₃, 3.1 KCl, 1.8 CaCl₂, 1.2 MgSO₄, 0.4 KH₂PO₄, and 10 D-glucose, pH 7.4] and oxygenated at room temperature for at least 1.5 h with 95% O₂-5% CO₂. Some pilocarpine-treated rats did not develop status epilepticus. These animals were used as drug-treated controls. Age-matched rats were used as untreated controls.

**Whole cell patch-clamp recording**

A slice was transferred to a glass-bottom Plexiglas submersion-type recording chamber mounted on the stage of a Nikon Optiphot-2 upright microscope (Nikon, Melville, NY) connected to a Noran Odyssey confocal imaging system (Noran Instruments, Middleton, WI). The chamber was filled with ACSF that was recirculated at a rate of 4 ml/min at room temperature (22–24°C). The total volume of superfusion medium was 10 ml. Patch electrodes were pulled from borosilicate glass (1.5 mm OD, 1.1 mm ID, Sutter Instruments, Novato, CA) and had a tip resistance of 5–7 MΩ. The tip of the electrode was filled by vacuum with a solution that contained (in mM) 140 cesium gluconate, 15 HEPES, 3.1 MgCl₂, 1 CaCl₂, 11 EGTA, pH 7.2 and 276 mosm. The electrode was then backfilled with internal solution that contained (in mM) 120 cesium gluconate, 10 HEPES, 2 MgATP, 1 EGTA, 5 creatine phosphate, 20 units/ml creatine phosphokinase, 10 QX-314 (N-(ethylidloicaine) chloride, and 0.1 fluorescein-dextran (10,000 MW), pH 7.2 and 276 mosm. Creatine phosphate, creatine phosphokinase, and ATP, constituting an ATP-regenerating system, were included to minimize the rundown of NMDA receptor-mediated currents (Rosenmund and Westbrook 1993). In current-clamp experiments, potassium gluconate replaced cesium gluconate, and QX-314 was omitted. The superfusion medium contained 30 μM bicuculline methiodide to block GABA₆ receptors mediated currents. Activation of postsynaptic GABA₆ receptors was prevented by the use of a cesium-based internal solution that included QX-314, but not GTP.

Recordings were made from granule cells located in the infrapyramidal blade of the dentate gyrus near the apex of the granule cell arch, because Timm histochemistry indicated that recurrent mossy fiber growth is densest there (Okazaki et al. 1995). Gigaohm seals were formed by the “blind” approach (Blanton et al. 1989) on granule cell bodies located at least 30 μm on the upper surface of the slice. Whole cell access was obtained in current-clamp mode; only cells with Vₘ more than –70 mV on break-in (after correction for a 10-mV liquid junction potential) were accepted for study. Resting membrane potentials for granule cells from the three treatment groups were as follows: status epilepticus, –78 ± 4 mV (mean ± SD, n = 79); treated controls, –77 ± 3 mV (n = 71); untreated controls, –79 ± 2 mV (n = 47). Input resistances for these cells after intracellular dialysis with the cesium gluconate–based internal solution were as follows: status epilepticus, 440 ± 150 MΩ; treated controls, 620 ± 160 MΩ; untreated controls, 660 ± 150 MΩ. In five granule cells from the treated control group, input resistance after intracellular dialysis with the potassium gluconate–based internal solution was 180 ± 30 MΩ. Granule cell identity was confirmed by visualizing intracellular fluorescein (excitation: 488 nm, 515 nm barrier filter) and observation of strong spike-frequency adaptation during a suprathreshold depolarization.

Recordings were made with an Axon Instruments (Foster City, CA) Axopatch ID patch-clamp amplifier beginning ~20 min after achieving whole cell access. Series resistances ranged from 6 to 22 MΩ and were compensated ~50%. Signals were filtered at 2 kHz, digitized at 10 kHz, and stored to disk with use of a TL 1–125 digitizing board and PClamp6 (Axon Instruments, Foster City, CA).

**Scanning laser photostimulation**

An 80-nW Coherent Enterprise 653 argon ion UV laser (Coherent Laser Group, Santa Clara, CA) was used for photostimulation. The laser was coupled to the epifluorescence input of the microscope by a fiber optic cable, and the output passed through an Olympus (Melville, NY) water-immersion, UV-corrected ×40 objective (NA, 0.7; working distance, 3.2 mm). The effective diameter of the laser beam within the plane of focus, uncorrected for light scattering within the slice, was 5.3 μm (Fig. 1). Shutter opening was controlled by PClamp6.

To locate a presynaptic granule cell, 200 μM γ-(CNB-caged) L-glutamate (Molecular Probes, Eugene, OR) was added to the superfusion medium. The laser beam was initially focused at a site in the granule cell body layer ~75 μm from the recorded cell on the side opposite the apex of the granule cell arch. Then five pulses of UV light (4 ms, 50 mW) were applied at 10-s intervals. If none of the stimuli evoked an EPSC, the laser beam was moved 25 μm farther from the recorded cell (by moving the microscope stage), and another five pulses were applied. Stimuli continued to be applied at 25-μm intervals. If no EPSC had been recorded when the laser beam reached the end of the granule cell body layer, uncaging sites were surveyed in a similar manner on the opposite side of the recorded cell. The search ended when laser photostimulation evoked an EPSC in the recorded cell. A test was considered unsuccessful if this procedure failed to evoke an EPSC at any of 20–80 uncaging sites in that slice. We avoided exposing the region within 75 μm of the recorded cell to UV irradiation to minimize contamination of the recording by direct glutamate current and to avoid UV-induced cytotoxicity. Exposure of granule cells to the laser beam in the absence of caged glutamate evoked neither an inward current nor cell firing.

When a presynaptic granule cell was located, the laser power was reduced to the minimum required to evoke a visually identifiable EPSC. The stimulus frequency was reduced to 1/15 s. In most experiments, EPSCs were recorded at alternating holding potentials of –80 and –20 mV. In some early experiments, recordings were initially
angular current pulses of 100 \( \text{F} \)quinoxaline (NBQX) was added to the superfusion medium. Rect-

Effect of \( \gamma-(CNB-caged) L\)-glutamate on NMDA receptors

A bipolar stimulating electrode (25 \( \mu \)m diam insulated nichrome wire, tip separation \( \approx 0.3 \) mm) was inserted into the perforant path where it crosses the subiculum. To block the AMPA-kainate component of the EPSC, 5 \( \mu \)M 2,3-dihydroxy-6-nitro-7-sulfamyl-benzo-(F)quinoxaline (NBQX) was added to the superfusion medium. Rectangular current pulses of 100 \( \mu \)s duration were applied with a Grass (W. Warwick, RI) stimulator and stimulus isolator every 30 s. Stimulus strength was adjusted to evoke a 150- to 200-pA inward synaptic current recorded at a holding potential of \( -20 \) mV. The concentration of caged glutamate was the same as that used for scanning laser photostimulation.

Visualilation of synaptic connections

In some experiments, DiI was applied to the uncaging spot after the electrophysiological recordings had been completed. DiI was dissolved in hot cod liver oil (Hosokawa et al. 1995) at a concentration of 1% (wt/vol) and pressure ejected through a glass micropipette (10 \( \mu \)m tip opening) with a Picospritzer (General Valve, Fairfield, NJ). The diameter of the region labeled by DiI was \( \approx 50 \) \( \mu \)m. In these experiments, the recorded cell was filled with 0.6% (wt/vol) Lucifer yellow instead of fluorescein-dextran. The slice was then fixed in 4% (wt/vol) paraformaldehyde, 0.1 M sodium phosphate buffer, pH 7.4, at 4°C for 7 days. It was then embedded in an albumin-gelatin mixture (Okazaki et al. 1995), and sections of 50-\( \mu \)m thickness were cut with a vibratome. Fluorescent dyes were visualized under the confocal microscope by excitation at 488 nm and use of a 510-nm barrier filter. Images were captured and stored with use of Image-1 software (Universal Imaging, West Chester, PA). The sections were also viewed under an epifluorescence microscope equipped with DiI and Lucifer yellow cubes.

Timm histochemistry

Slices remained in the \( \text{Na}_2\text{S} \) solution for 90 min and were then stored in phosphate-buffered 10% Formalin at 4°C for 1–2 days. They were then embedded in albumin-gelatin, and 30-\( \mu \)m-thick sections were prepared with a vibratome. Slide-mounted sections were stained for the presence of heavy metals as described by Danscher (1981) and lightly counterstained with cresyl violet.

Data analysis

Waveforms were analyzed off-line with functions incorporated in PClamp6. Traces without an observable stimulus-evoked inward current were averaged, and the averaged trace was electronically subtracted from traces that showed a stimulus-evoked current. Half-width and decay time constant (\( \gamma \)) were obtained with built-in PClamp routines, whereas EPSC amplitude, latency to onset, and 20–80% rise time were measured manually with cursors. These parameters were determined from each recording, and the results were averaged to yield single values for each synaptic connection. Spontaneous synaptic events were not usually observed during the 100-ms period after laser photostimulation, but occasionally a spontaneous event overlapped a portion of the evoked response. In those instances, measurements were restricted to the parameters that were uncontaminated. Multiple responses were defined as two or more inward currents time locked to the stimulus whose onset latency distributions did not overlap.

Materials

\( \gamma-(CNB-caged) L\)-glutamate, fluorescein dextran, DMNB-caged fluorescein-dextran (10,000 MW), Lucifer yellow, and DiI were purchased from Tocris Cookson (Bristol, UK), bicuculline methiodide from Research Biochemicals (Natick, MA), and cesium hydroxide (99.9%; 50 wt%) from Aldrich (Milwaukee, WI). \( \text{Na}_2\text{S} \) was solved in hot cod liver oil (Hosokawa et al. 1995) at a concentration of 1% (wt/vol) and pressure ejected through a glass micropipette (10 \( \mu \)m tip opening) with a Picospritzer (General Valve, Fairfield, NJ). The diameter of the region labeled by DiI was \( \approx 50 \) \( \mu \)m. In these experiments, the recorded cell was filled with 0.6% (wt/vol) Lucifer yellow instead of fluorescein-dextran. The slice was then fixed in 4% (wt/vol) paraformaldehyde, 0.1 M sodium phosphate buffer, pH 7.4, at 4°C for 7 days. It was then embedded in an albumin-gelatin mixture (Okazaki et al. 1995), and sections of 50-\( \mu \)m thickness were cut with a vibratome. Fluorescent dyes were visualized under the confocal microscope by excitation at 488 nm and use of a 510-nm barrier filter. Images were captured and stored with use of Image-1 software (Universal Imaging, West Chester, PA). The sections were also viewed under an epifluorescence microscope equipped with DiI and Lucifer yellow cubes.

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Materials

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RESULTS

Timm histochemistry

In slices from both pilocarpine-treated rats that did not develop status epilepticus (treated controls) and age-matched,
untreated rats (untreated controls), scattered clusters of mossy fiber–like Timm staining were present in the supragranular zone of the dentate molecular layer near the recording site (Fig. 2). Pilocarpine-induced status epilepticus caused the appearance of dense Timm staining at this site in every animal. Previous studies demonstrated that dense Timm staining of the supragranular zone signifies the robust growth of recurrent mossy fibers, at least some of which make synaptic contact with granule cells (Frotscher and Zimmer 1983; Okazaki et al. 1995; Sutula et al. 1989).

**Granule cell action potentials evoked by laser photostimulation**

Preliminary studies determined the conditions necessary for uncaged glutamate to depolarize a relatively small number of granule cells to threshold. In 15 experiments, the laser power was adjusted to 50 mW, and the duration of shutter opening was varied. The membrane potential of the recorded cell was clamped at $-80$ mV, and flashes of UV light were focused on the soma. A 2-ms light flash, the shortest duration tested, evoked an inward current of $\sim 100$-pA peak amplitude. A maximal current was produced with a pulse duration of 16 ms.

The granule cell’s response to a 4-ms, 50-mW exposure to UV light, which had produced a large, but submaximal, glutamate current, was examined in current-clamp mode. The focus of laser stimulation was moved to different locations, and the response to 10 light flashes presented at 15-s intervals was recorded at each location. When glutamate was uncaged by focusing the laser beam on the center of the soma, the cell fired one to three action potentials (Fig. 3). At this site, the laser power could be reduced to as low as 10–15 mW without loss of the response (although the number of action potentials per stimulus was reduced to one). The minimum peak glutamate current or injected electrical current required to depolarize a granule cell to threshold was $\sim 150$ pA. When the laser beam was focused on the perimeter of the soma, on the apical dendrite, within 12 $\mu$m from the center of the soma in the $xy$ plane or within 50 $\mu$m from the center of the soma in the $z$-axis, a 4-ms, 50-mW stimulus evoked a single action potential. Every light flash evoked an action potential during trial periods that lasted as long as 25 min. At greater distances from the recorded cell, laser photostimulation evoked an action potential, if at all, only when the laser power or duration of shutter opening was substantially increased. In each of seven experiments, there was an abrupt transition between a location at which photostimulation always evoked an action potential in the recorded cell and a location at which no action potentials could be evoked. We never found a site at which the light flash evoked an action potential in some trials, but not in others. Such variable responses, if they occurred at all, must have been rare, because the maximal width of the transition zone between sites at which laser photostimulation with 4-ms, 50-mW pulses always evoked an action potential and sites at which it never evoked an action potential was $\leq 2$ $\mu$m (the limit of resolution of our stage drive; shaded area between $2$ and $2$ in Fig. 3A). This result indicates that, in subsequent experiments, the presynaptic granule cell fired an action potential in response to every photostimulus.

The region of the cell body layer in which granule cells fired an action potential would be expected to have had an hourglass shape centered on the focal plane (Pettit et al. 1997). To approximate the number of granule cells brought to threshold by the 4-ms, 50-mW flash of UV light used for scanning, one must estimate the volume of this oddly shaped region. However, it is sufficient for the present purpose to compute maximal and minimal values based on the volume of simpler shapes. Considering the region of action potential firing as the frusta of a double cone provides an overestimate of the volume, whereas considering the region as a cylinder provides an underestimate. Assuming that the laser beam filled the objective, the double frusta model yields an estimate of $68,161$ $\mu$m$^3$ [$V = \frac{2}{3} \cdot (\pi r_1^2 + \pi r_2^2 + \sqrt{\pi r_1^2 \cdot \pi r_2^2}) \cdot h$, where $r_1$ (radius of activated region within the focal plane $= 12$ $\mu$m) and $r_2$ (radius of activated region 50 $\mu$m above or below the focal plane $= 17.3$ $\mu$m) are the radii of the bases of each frustum and $h$ (greatest distance above or below the focal plane at which...
granule cells were activated = 50 μm) is the height of each frustum. The cylinder model yields an estimate of 45,239 μm³ [V = π · r² · h, where r (12 μm) is the radius of the cylinder and h (100 μm) is the total height of the cylinder]. Granule cell density in aldehyde-fixed rat brain was determined to be 0.0008 cell/μm³ (Boss et al. 1985). Applying a correction of 30% for shrinkage during fixation (Mott et al. 1997) yields a granule cell density of 0.00065 cell/μm³ in a hippocampal slice. From these values, we estimate that each exposure to UV light brought between 25 and 38 granule cells to threshold. It should be noted that in many experiments the minimal conditions for evoking an EPSC in the recorded cell required less total UV light exposure per stimulus than that provided by the standard pulse used for scanning. Thus the stimulation parameters were reduced, sometimes by half or more. In these instances, correspondingly fewer granule cells were activated.

**EPSCs evoked by uncaging glutamate in the granule cell body layer**

Minimal photostimulation in the granule cell body layer most frequently evoked an EPSC in granule cells from rats that had developed status epilepticus, with subsequent recurrent mossy fiber growth. We were able to locate a presynaptic granule cell in 61% of these experiments (Table 1). On average, seven uncaging sites were tested before a connection was found (range: 1–20). Thus an average of between 175 and 266 granule cells had to be activated before a synaptic response was evoked in the recorded cell.

**TABLE 1. Properties of the mossy fiber–granule cell EPSC evoked by minimal photostimulation**

<table>
<thead>
<tr>
<th>Condition</th>
<th>All EPSCs</th>
<th>First EPSC only</th>
<th>Status epileptic</th>
<th>Untreated control</th>
<th>Status epileptic</th>
<th>Untreated control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( I ), pA</td>
<td>( \tau ), ms</td>
<td>( \tau ), ms</td>
<td>( \tau ), ms</td>
<td>( \tau ), ms</td>
<td>( \tau ), ms</td>
</tr>
<tr>
<td>Granule Cells</td>
<td>With EPSC/Total</td>
<td>Rise Time, ms</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All EPSCs</td>
<td>( -30.9 \pm 12.5 )</td>
<td>( 1.2 \pm 0.6 )</td>
<td>( 10.9 \pm 5.7 )</td>
<td>( 8.6 \pm 4.9 )</td>
<td>( 69 \pm 13 )</td>
<td></td>
</tr>
<tr>
<td>Status epileptic</td>
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<td>( 1.2 \pm 0.6 )</td>
<td>( 10.9 \pm 5.7 )</td>
<td>( 8.6 \pm 4.9 )</td>
<td>( 69 \pm 13 )</td>
<td></td>
</tr>
<tr>
<td>Treated control</td>
<td>( -25.9 \pm 9.9 )</td>
<td>( 1.3 \pm 0.5 )</td>
<td>( 10.8 \pm 2.8 )</td>
<td>( 8.0 \pm 2.4 )</td>
<td>( 71 \pm 12 )</td>
<td></td>
</tr>
<tr>
<td>Untreated control</td>
<td>( -33.7 \pm 12.8 )</td>
<td>( 0.9 \pm 0.3 )</td>
<td>( 7.5 \pm 2.4 )</td>
<td>( 7.0 \pm 1.4 )</td>
<td>( 73 \pm 16 )</td>
<td></td>
</tr>
<tr>
<td>First EPSC only</td>
<td>( 48/79 ) (61)</td>
<td>( 1.3 \pm 0.5 )</td>
<td>( 11.3 \pm 5.7 )</td>
<td>( 9.1 \pm 4.8 )</td>
<td>( 69 \pm 13 )</td>
<td></td>
</tr>
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<td>Status epileptic</td>
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<td>( 1.3 \pm 0.5 )</td>
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<td>( 9.1 \pm 4.8 )</td>
<td>( 69 \pm 13 )</td>
<td></td>
</tr>
<tr>
<td>Treated control</td>
<td>( 11/71 ) (16)</td>
<td>( 1.2 \pm 0.4 )</td>
<td>( 10.4 \pm 3.1 )</td>
<td>( 7.3 \pm 2.5 )</td>
<td>( 67 \pm 11 )</td>
<td></td>
</tr>
<tr>
<td>Untreated control</td>
<td>( 6/47 ) (13)</td>
<td>( 0.9 \pm 0.3 )</td>
<td>( 7.5 \pm 2.4 )</td>
<td>( 7.0 \pm 1.4 )</td>
<td>( 73 \pm 16 )</td>
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</tr>
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</table>

Values are mean ± SD. They were obtained at a holding potential of −80 mV and a stimulation rate of 0.067 Hz. Numbers in parentheses are percentages. Rise time was measured between 20 and 80% of the peak amplitude. Decay of the response was best fit by a monoexponential function. Attempts to fit the data to the sum of 2 exponential functions usually produced negative values for one of the components. All EPSCs: calculations based on the assumption that multiple responses always resulted from activation of more than one presynaptic cell. First EPSC only: calculations based on the assumption that multiple responses always resulted from repetitive activity of a single presynaptic cell. Only one granule cell was studied in each slice. The slices were prepared from 52 rats in the status epilepticus group, 33 rats in the treated control group, and 21 rats in the untreated control group. * Proportion of successful experiments was significantly greater than for the control groups (\( P < 0.001 \), \( \chi^2 \) test).
recorded. With use of the same procedure, synaptic connections were also located in 16% of the experiments with pilocarpine-treated control rats and in 13% of the experiments with age-matched, untreated rats. On average, 11 uncaging sites were tested in slices from treated controls and 9 in slices from untreated controls before a synaptic connection was found (range: 3–22). The latency from the beginning of the light flash to the onset of the EPSC averaged 30 ms (range: 9–94 ms). There was no significant difference in onset latency among the treatment groups. In studies of this type, onset latency is determined predominantly by the highly variable and indeterminable interval between the photostimulus and cell firing (Katz and Dalva 1994). Latency values cannot be related to synaptic delay or axonal conduction.

Synaptic currents evoked by minimal photostimulation exhibited properties consistent with their identification as unitary EPSCs. They had a smooth rising phase (Fig. 4A), appeared at a constant latency (±5 ms) with respect to the onset of the light flash and exhibited all-or-none behavior when the laser power was varied (Fig. 5). In a few instances, the rising phase of the synaptic current exhibited a slightly jagged or inflected appearance (e.g., Fig. 4B). This irregularity may have resulted from a slightly asynchronous release of transmitter from different sites in the same bouton, as described for some unitary mossy fiber EPSCs recorded in CA3 pyramidal cells (Jonas et al. 1993). Responses were abolished by addition of 2 mM Cd²⁺ to the superfusion medium.

Multiple responses were commonly observed with the present experimental approach. These consisted of two to four EPSCs that appeared at distinctly different times after the photostimulus (Fig. 4B). Of the 48 granule cells from the status epilepticus group in which EPSCs were recorded, multiple responses were recorded in 18. Multiple responses were also recorded in 7 of the 11 successful experiments from the treated control group. No multiple responses were recorded in granule cells from the untreated control group. Reducing either the laser power or duration of the light flash usually did not reduce the number of temporally distinct responses. In some experiments, the individual EPSCs exhibited very similar peak amplitudes and response kinetics. They may therefore have arisen from repetitive firing of a single presynaptic cell. In other experiments, the individual EPSCs exhibited distinctly different peak amplitudes and/or response kinetics, suggesting that they arose from the glutamate-evoked firing of more than one presynaptic cell. Because we could not distinguish between these two mechanisms with certainty, quantitative data were compiled in two ways: by assuming that each temporally distinct EPSC arose from the activation of a different presynaptic cell and by assuming that multiple responses always arose from repetitive activity in a single presynaptic cell. However, the two calculations produced nearly indistinguishable results.

Properties of the EPSC evoked by minimal photostimulation did not vary significantly among the treatment groups. The EPSC had a mean peak amplitude of ~30 pA, 20–80% rise time of ~1.2 ms, decay time constant (τ) of ~10 ms and half-width of ~8 ms (Table 1). Peak amplitudes varied over a

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![Graph](image-url)

**FIG. 5.** EPSC evoked by minimal photostimulation exhibited all-or-none behavior in response to a change in stimulus strength. EPSCs were recorded at a holding potential of ~80 mV. The shutter was opened for 4 ms each time. For each laser power, 15–30 light flashes were delivered at 15-s intervals. Values are means ± SD for all EPSCs evoked. The plot shows results from a representative experiment. Similar results were obtained in 4 additional experiments.
5.5-fold range (12.8–70.5 pA). Response kinetics for EPSCs in granule cells from untreated controls were generally slightly faster than for EPSCs in the other treatment groups, but the differences were not statistically significant.

The mean failure rate was ~70%. That is, only ~3 of 10 photostimuli evoked an EPSC in the recorded cell (Fig. 4A). Few, if any, failures could have resulted from our inability to distinguish a synaptic response from noise. The smallest EPSC recorded in this study had a peak amplitude of 12 pA, about 3 times the peak-to-peak noise in our recordings.

In some granule cells, laser photostimulation evoked a large, complex inward current (Fig. 4C). The peak amplitude of these responses ranged from 100 to 500 pA. They appeared at varying latencies and were evoked in some trials and not others. In some experiments, they were evoked by the same stimuli that also evoked one or more minimal EPSCs in the recorded cell. However, 70% of the large, complex inward currents occurred in the absence of a minimal EPSC. These responses were most commonly observed in granule cells from the status epilepticus group (16/79, 20%), but also appeared in 7 of 71 granule cells (10%) from the treated control group and in 1 of 47 granule cells (2%) from the untreated control group. They closely resembled delayed inward currents evoked by antidromic stimulation of the mossy fiber pathway in the presence of a GABA<sub>A</sub> receptor antagonist (Okazaki et al. 1999). The antidromically evoked delayed inward currents were abolished by AMPA/kainate receptor antagonists. Thus they were considered to be polysynaptic EPSCs that resulted from reverberating excitation among granule cells. Unfortunately, we could not use AMPA/kainate receptor antagonists to test whether the large, complex inward currents observed in the present study were also polysynaptic EPSCs, because AMPA receptors must be activated in order for uncaged glutamate to fire granule cells.

**NMDA receptors contribute to the EPSC**

To determine whether the EPSC evoked by minimal photostimulation has an NMDA receptor–mediated component, we compared evoked responses recorded at alternating holding potentials of −80 and −20 mV (Fig. 6). A late component of the EPSC with slow decay kinetics appeared at a holding potential of −20 mV, but was not apparent at a holding potential of −80 mV (Fig. 6, A and B). The NMDA receptor antagonist D-AP5 (50 μM) was then added to the superfusion medium. Addition of D-AP5 usually abolished the evoked response, but the response could always be recovered simply by increasing the duration of shutter opening. This action of D-AP5 presumably resulted from block of NMDA receptors whose activation by uncaged glutamate contributed to depolarization of the presynaptic cell. After the EPSC was recovered, it could be seen that D-AP5 barely affected the response recorded at −80 mV, but it abolished the late, slow component of the response recorded at −20 mV (Fig. 6C). Electronic subtraction of traces recorded at −20 mV before and after superfusion with D-AP5 revealed a small NMDA component (Fig. 6D). Similar results were obtained in nine granule cells from the status epilepticus group and six granule cells from the treated control group. An NMDA/AMPA ratio was computed by dividing the peak amplitude of the NMDA receptor–mediated EPSC recorded at −20 mV by the peak amplitude of the AMPA/kainate receptor–mediated EPSC recorded at −80 mV. There was no significant between-group difference. Mean values (±SD) were as follows: status epilepticus group, 0.19 ± 0.06; treated control group, 0.16 ± 0.05.

**γ-(CNB-caged) L-glutamate did not desensitize NMDA receptors**

High concentrations of γ-(CNB-caged) L-glutamate have been found to activate NMDA receptors (Kandler et al. 1998). One possible reason for the relatively small amplitude of the NMDA receptor–mediated EPSC was that caged glutamate desensitized NMDA receptors. To evaluate this possibility, we studied the effect of caged glutamate on the pharmacologically isolated NMDA receptor–mediated EPSC evoked by perforant path stimulation. In five experiments, addition of 200 μM caged glutamate to the superfusion medium altered neither the peak amplitude of the response nor the clamp current. This result indicates that the compound did not activate or desensitize NMDA receptors under the present experimental conditions.

**Laser photostimulation in the dentate hilus did not evoke an EPSC**

The only other known excitatory projection to granule cells that could have been activated by laser photostimulation is the dentate associational pathway. This pathway originates from mossy cells within the dentate hilus (Buckmaster et al. 1992; Ribak et al. 1985). We attempted to activate associational connections by laser photostimulation directed at mossy cells. We were mainly concerned with activation of associational synapses in slices from control rats, because most mossy cells are believed to die during status epilepticus (Scharfman and Schwarzkoelin 1990; Sloviter 1987). Therefore age-matched,
untreated control rats were used in this study. The laser beam was focused within the region of the dentate hilus in which presynaptic mossy cells were most likely to be found.

We surveyed a total of 601 uncaging sites in 22 slices from 15 rats. Only one granule cell was studied in each slice. The laser parameters (4 ms, 50 mW) were the same as those used to locate presynaptic granule cells. In none of these experiments did laser photostimulation evoke an EPSC.

Visualizing potential mossy fiber–granule cell connections

To visualize presynaptic mossy fibers, DiI was pressure ejected at the site of glutamate uncaging. In 12 experiments where minimal photostimulation evoked an EPSC (10 from the status epilepticus group and 1 each from the treated control and untreated control groups), a single fluorescent axon was observed to traverse the molecular layer approximately perpendicular to the dendrites of the recorded granule cell (Fig. 7). In three additional experiments on slices from rats that had developed status epilepticus, DiI ejection labeled two axons with a similar trajectory that coursed parallel to each other at different depths within the molecular layer. The labeled axons were studded with brightly fluorescent varicosities, the largest of which were ~2 μm diam. Both confocal and fluorescence microscopy showed that one of these varicosities was in close apposition to a dendritic branch of the recorded cell. These contact points were located as close as 22 μm and as distant as 130 μm from the center of the soma. Two-thirds of them were located between 50 and 85 μm away. The size of the labeled boutons and the location of the contact sites matches the description of recurrent mossy fibers provided by retrograde labeling with biocytin (Okazaki et al. 1995). There was no correlation between the number of temporally distinct EPSCs evoked by photostimulation and the number of axons labeled in the same experiment. Only a single EPSC was evoked in each of the three experiments in which two putatively presynaptic axons were labeled and only a single axon was labeled in two experiments in which multiple responses were observed.

In six slices (4 from the status epilepticus group and 2 from the treated control group), DiI was pressure ejected at a site where photostimulation failed to evoke an EPSC. In none of these experiments was a fluorescent axon observed to cross the dendritic tree of the recorded cell.

DISCUSSION

Our results reinforce and extend the existing electrophysiological evidence for synaptic connections between dentate granule cells (Okazaki et al. 1999; Wuarin and Dudek 1996). They indicate that some mossy fiber–granule cell synapses are normally present in the rat dentate gyrus, that pilocarpine-induced status epilepticus substantially increases the number of these synapses, that EPSCs evoked by activating preexisting and newly formed synapses have very similar properties, and that both AMPA/kainate and NMDA receptors mediate transmission at these sites.

We evaluated the possibility that some of the EPSCs evoked by minimal photostimulation arose from activation of an associational (granule cell–mossy cell–granule cell) circuitry within the slice. Hilar mossy cells have been shown to make monosynaptic excitatory synapses on granule cells (Scharfman 1995). They are highly excitable, because their normal resting membrane potential is only ~7–8 mV above their action potential threshold (compared with ~30 mV for granule cells; Staley et al. 1992), inhibitory postsynaptic potentials (IPSPs) are small and their input resistance is relatively high (Scharfman and Schwartzkroin 1988). Thus mossy cells are readily activated by excitatory input from mossy fibers (Scharfman et al. 1990). In the present study, the glutamate-evoked firing of as many as 25–38 granule cells per UV light flash could potentially have activated mossy cells. However, we did not activate a single mossy cell–granule cell connection by laser photostimulation of the dentate hilus in 601 attempts: the probability of evoking an EPSC by focusing the laser beam in the granule cell body layer with use of the same power and shutter open time was much higher. Similarly, Wuarin and Dudek (1996) observed no change in the spontaneous excitatory postsynaptic potential (EPSP) frequency when they applied glutamate to the dentate hilus of hippocampal slices from kainate-treated rats. In contrast, spontaneous EPSP frequency was markedly increased by similar glutamate applications to the granule cell body layer. In addition, the properties of minimal EPSCs from the status epilepticus and control groups were indistinguishable; the major difference was that these responses were much more frequently evoked in granule cells from the status epilepticus group. This result was opposite to what would be expected if minimal photostimulation had evoked associational synaptic responses, because mossy cells are more numerous in slices from control rats. We therefore conclude that minimal photostimulation in the granule cell body layer rarely, if ever, activated associational synapses on granule cells. Perhaps essentially all associational circuitry was cut during preparation of our slices. More likely, mossy cells, being extremely vulnerable to insults that raise intracellular calcium (Scharfman and Schwartzkroin 1989), simply fail to survive slice preparation and incubation in older rats.
Properties of the mossy fiber–granule cell EPSC evoked by minimal photostimulation

The mean peak amplitude (≈30 pA) of the EPSC evoked by minimal photostimulation fell between reported values obtained under similar recording conditions for mossy fiber synapses on CA3 pyramidal cells (≈70 pA) (Jonas et al. 1993) and for hippocampal synapses made by small terminals with one or at most two release sites (=15 pA) (Allen and Stevens 1994; Kneisler and Dingledine 1995; Raastad et al. 1992). This result appears intuitively reasonable for recurrent mossy fiber synapses, assuming that each fiber usually makes only a single contact with each granule cell. All other factors being equal, peak amplitude would be expected to vary with the number of release sites per bouton. This appears to be the case for mossy fiber synapses on CA3 pyramidal cells: the quantal content of different EPSCs (Jonas et al. 1993) varies over the same range as the number of synaptic contacts per bouton (Chicurel and Harris 1992). Mossy fiber boutons that contact dentate granule cells usually have multiple release sites, but are, on average, only about one-third to one-half the size of mossy fiber boutons that contact CA3 pyramidal cells (Okazaki et al. 1995). The largest of these boutons in the inner third of the dentate molecular layer is ≈2 μm diam as determined by retrograde labeling with biocytin (Okazaki et al. 1995) and by DiI labeling (present study). However, many recurrent mossy fiber boutons are <2 μm and may contact only a single spine. Thus the relatively large size of and the 5.5-fold variability in the minimal EPSC amplitude appear anatomically reasonable.

Response kinetics were relatively slow compared with values reported for mossy fiber synapses on other postsynaptic cells. DiI labeling suggests that this difference probably cannot be explained by greater attenuation and filtering in the dendritic tree. Although we cannot be certain that the labeled contact site represented the synapse that was studied electrophysiologically, the distribution of potential contact sites provides information on the range of synaptic placements relative to the recording site at the soma. The synaptic currents we studied were probably generated at roughly the same distance from the somatic voltage clamp as in studies of CA3 pyramidal cells (Jonas et al. 1993), and errors due to imperfect space clamp are likely to have been small (Carnevale et al. 1997; Spruston et al. 1993). Our use of higher resistance patch electrodes, which we found necessary in slices from older rats, may have exaggerated the effect of any series resistance errors. However, the slower response kinetics may also reflect differences among postsynaptic cells in their receptor mechanisms and in the extent to which postsynaptic receptors are saturated with released glutamate. In addition, much younger rats were used to study mossy fiber synapses on other cell types; response kinetics of mossy fiber EPSCs may change with age. Finally, response kinetics of the mossy fiber–granule cell EPSC may be influenced to some degree by the nonuniform electrototoxic structures of granule cells (Carnevale et al. 1997).

The consistently high failure rate (~70%) observed in this study did not result from variable activation of the presynaptic granule cell by uncaged glutamate. Rather, transmission at mossy fiber–granule cell synapses may be relatively unreliable. A similarly high failure rate was reported for Schaffer collateral-commissural synapses (Allen and Stevens 1994). The percentage of failures at mossy fiber–CA3 pyramidal cell synapses depends on the stimulation rate, the greatest incidence of failures occurring during stimulation at ≈0.5 and >2 Hz (Jonas et al. 1993). During stimulation at 1 Hz, the failure rate was ~11%. An average failure rate of 33% was reported for the mossy fiber–dentine basket cell synapse during stimulation at 0.2–0.33 Hz (Kneisler and Dingledine 1995). Thus the lower failure rate observed in previous studies of mossy fiber synapses compared with the present study may reflect the use of higher stimulus frequencies. Mossy fiber synapses on CA3 pyramidal cells exhibit dramatic frequency facilitation when activated at rates of 0.1–1 Hz (Salin et al. 1996). A stimulus frequency of 1/15 s, as employed in the present study, may be a worst case for transmission failure. The high failure rate at Schaffer collateral-commissural synapses results mainly from the probabilistic nature of transmitter release at sites of low release probability (Allen and Stevens 1994). For the mossy fiber pathway, the possibility of conduction failure should also be considered, due to the impedance mismatch between the thin axon and large bouton (Lüscher and Shiner 1990). It should be noted, however, that some of the minimal EPSCs we recorded, especially in the status epilepticus group, may not have been monosynaptic. They could have been the end product of reverberating excitation among granule cells; indeed we found evidence of such polysynaptic activity in some experiments. We cannot rule out the possibility that the higher failure rate of polysynaptic transmission influenced our results.

NMDA receptors contribute to the mossy fiber–granule cell EPSC, as they do to mossy fiber EPSCs recorded in CA3 pyramidal cells (Weisskopf and Nicoll 1995) and dentate basket cells (Kneisler and Dingledine 1995). We found no difference between status epilepticus and control groups in the relative size of the NMDA component; the peak amplitude of the NMDA component recorded at −20 mV was a small fraction (~20%) of the AMPA/kainate component recorded at −80 mV in each case. In contrast, a preceding status epilepticus usually increased the NMDA/AMPA ratio to a value of 1.4–2.2 when mossy fibers were activated by antidromic electrical stimulation (Okazaki et al. 1999). In light of the present results, this effect cannot easily be explained by a change in the NMDA receptor itself. One possibility is that the size of the NMDA component depends, in part, on spillover of glutamate from mossy fiber synapses on nearby granule cells. Due to the much higher affinity of NMDA receptors for glutamate compared with AMPA/kainate receptors, glutamate diffusing from the synaptic cleft predominantly activates NMDA receptors (Kullmann and Asztely 1998). The region of the dentate molecular layer into which recurrent mossy fibers grow normally has a high density of NMDA receptors (Monaghan and Cotman 1985). The effect of spillover is limited by active transport and is thus particularly prominent at a less than physiological recording temperature (Asztely et al. 1997), such as that used in both our previous (Okazaki et al. 1999) and present studies. The spillover effect is also limited by the number of activated glutamate synapses in close proximity to the synapses under study. With respect to the recurrent mossy fiber pathway, antidromic electrical stimulation activates many more synapses than minimal photostimulation, and seizure-induced mossy fiber growth increases the likelihood that antidromic stimulation will release glutamate from a large number of nearby terminals. According to this view, the enhanced contribution of NMDA receptors to the mossy fiber–granule cell EPSC evoked by antidromic stimulation is simply the inevitable consequence of increasing the synaptic density of the...
Mossy fiber growth increases the number of recurrent synapses on dentate granule cells

Our results reinforce the suggestion from our previous work (Okazaki et al. 1999) that mossy fibers make recurrent synapses with granule cells in the normal brain. Although there is no published anatomic evidence for the existence of these synapses, mossy fiber–like Timm stain is present in the supragranular zone at some locations, especially near the caudal end of the dentate gyrus (Gaarskjaer 1978; Haug 1974). It has remained unclear to what extent these presumptive recurrent mossy fibers innervate granule cells, as opposed to interneurons. Wuarin and Dudek (1996) reported that application of glutamate to the granule cell body layer failed to evoke an excitatory response in granule cells from normal rats. However, the likelihood of activating connections between granule cells probably depends on the site in the dentate gyrus at which recordings are made. In slices immediately rostral to those we used for electrophysiological recording, scattered clusters of Timm granules were present in the inner third of the molecular layer near the recording site. This is the same location in which Dil-labeled axons were found. Our results suggest that these Timm granules do, in fact, represent mossy fiber boutons and that at least some of these boutons engage in synaptic contact with granule cells. Recurrent mossy fiber synapses may synchronize granule cell discharge in regions of the dentate gyrus where their numbers are significant.

Laser photostimulation much more readily evoked an EPSC in slices from rats that had developed status epilepticus, but the EPSC was virtually identical to control with respect to its mean peak amplitude, duration, kinetics, onset latency, failure rate, and contribution from NMDA receptors. Thus the new mossy fiber synapses appear to operate very similarly to preexisting synapses, but there are many more of them. We were able to evoke an EPSC in about four times as many slices from the status epilepticus group and, in successful experiments, a connection was found after ~30% fewer uncaging trials. Therefore reactive growth after pilocarpine-induced status epilepticus may increase the density of mossy fiber–granule cell synapses in the caudal dentate gyrus about sixfold.

It should be noted that our results provide only a rough estimate of granule cell interconnectivity. The finding that 175–266 granule cells had to be activated in slices from the status epilepticus group before a synaptic connection was located does not necessarily imply a 0.5% probability of interconnection in vivo. Some recurrent circuitry may have been cut when the slices were prepared. Furthermore, this study was designed to identify and characterize mossy fiber–granule cell synapses, not to estimate their absolute numbers; the search for a synaptic connection ended whenever a connection was found. Additional studies specifically directed at the quantitation of granule cell interconnectivity are needed.

Functional implications of the recurrent mossy fiber projection

All rats that develop status epilepticus after administration of pilocarpine invariably exhibit chronic limbic motor seizures after a latent period of 1–3 wk (Mello et al. 1993; Lemos and Cavaleiro 1996). Synaptic connections among pyramidal cells serve as the anatomic substrate for epileptiform burst firing in area CA3 (Miles et al. 1984). Recurrent mossy fiber growth creates circuitry in the dentate gyrus similar to that normally present in the CA3 area. Given that dentate granule cells are difficult to recruit into epileptiform discharge and thus present a barrier to seizure propagation (Lothman et al. 1992), the new mossy fiber–granule cell connections may contribute to seizures in pilocarpine-treated animals. The recurrent mossy fiber circuit may also be contributory in persons with epilepsy whose hippocampi have undergone this same synaptic rearrangement.

The circumstances under which mossy fiber–granule cell synapses contribute to epileptogenesis remain to be defined. Our results suggest that the synchronous activation of about five such synapses may be sufficient to bring a granule cell to threshold under our experimental conditions (~150 pA inward current required / ~30 pA unitary synaptic current). However, transmission at these synapses appears to be relatively unreliable, granule cells normally fire action potentials in vivo at low rates (Jung and McNaughton 1993), and these cells are subject to strong GABA inhibition (Freund and Buzsáki 1996; Otis et al. 1991). These considerations suggest that the synchronous activation of recurrent mossy fibers might fail to bring more than a small percentage of granule cells to threshold. Conversely, other factors might enhance the ability of recurrent mossy fibers to promote seizures. Both the higher temperature and the background depolarization due to perforant path input present in vivo will increase granule cell excitability. Furthermore, if mossy fiber–granule cell synapses exhibit marked activity-dependent facilitation similar to that of mossy fiber synapses on CA3 pyramidal cells (Salin et al. 1996), then the recurrent mossy fiber pathway might preferentially support synchronous granule cell discharge when it is driven at particular frequencies or in particular firing patterns that maximize facilitation. Activation of the recurrent pathway at frequencies near 5 Hz may also enhance its synchronizing capability by depressing GABA inhibition (Mott et al. 1993). Finally, granule cells may interact through electrotonic, as well as synaptic, coupling (MacVicar and Dudek 1982).

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REFERENCES


