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Electrophysiological Evidence of Monosynaptic Excitatory Transmission Between Granule Cells After Seizure-Induced Mossy Fiber Sprouting

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Scharfman, Helen E., Anne L. Sollas, Russell E. Berger, and Jeffrey H. Goodman. Electrophysiological evidence of monosynaptic excitatory transmission between granule cells after seizure-induced mossy fiber sprouting. *J Neurophysiol* 90: 2536–2547, 2003; 10.1152/jn.00251.2003. Mossy fiber sprouting is a form of synaptic reorganization in the dentate gyrus that occurs in human temporal lobe epilepsy and animal models of epilepsy. The axons of dentate gyrus granule cells, called mossy fibers, develop collaterals that grow into an abnormal location, the inner third of the dentate gyrus molecular layer. Electron microscopy has shown that sprouted fibers from synapses on both spines and dendritic shafts in the inner molecular layer, which are likely to represent the dendrites of granule cells and inhibitory neurons. One of the controversies about this phenomenon is whether mossy fiber sprouting contributes to seizures by forming novel recurrent excitatory circuits among granule cells. To date, there is a great deal of indirect evidence that suggests this is the case, but there are also counterarguments. The purpose of this study was to determine whether functional monosynaptic connections exist between granule cells after mossy fiber sprouting. Using simultaneous recordings from granule cells, we obtained direct evidence that granule cells in epileptic rats have monosynaptic excitatory connections with other granule cells. Such connections were not obtained when age-matched, saline control rats were examined. The results suggest that indeed mossy fiber sprouting provides a substrate for monosynaptic recurrent excitation among granule cells in the dentate gyrus. Interestingly, the characteristics of the excitatory connections that were found indicate that the pathway is only weakly excitatory. These characteristics may contribute to the empirical observation that the sprouted dentate gyrus does not normally generate epileptiform discharges.

INTRODUCTION

Mossy fiber sprouting is one of the most extensively studied forms of synaptic plasticity, perhaps because it involves such extensive structural changes within the dentate gyrus, and it occurs not only in experimental animals but also in humans. The phenomenon refers to changes in the terminal projections of axons of dentate gyrus granule cells, which are known as the "mossy fibers." After a variety of stimuli, mossy fibers develop collaterals that terminate in an abnormal location, the inner molecular layer of the dentate gyrus. This layer contains the proximal dendrites of granule cells and also contains the processes of various nongranule cells. Indeed, it has been shown that sprouted fibers project to the region where both the dendrites of granule cells and inhibitory neurons are located, the inner molecular layer (Buckmaster and Dudek 1999; Buckmaster et al. 2002; Cavazos et al. 2003; Franck et al. 1995; Isokawa et al. 1993; Kotti and Riekkinen 1997; Lynch and Sutula 2000; Okazaki et al. 1995; Represa et al. 1993; Ribak and Peterson 1991; Sutula et al. 1998; Wenzel et al. 2000; Zhang and Houser 1999). A subset of these studies have shown with electron microscopy that sprouted mossy fibers make synapses in the inner molecular layer (Buckmaster et al. 2002; Cavazos et al. 2003; Franck et al. 1995; Okazaki et al. 1995; Represa et al. 1993; Ribak and Peterson 1991; Wenzel et al. 2000; Zhang and Houser 1999).

Mossy fiber sprouting can occur after various experimental manipulations and is robust after severe seizures. Thus mossy fiber sprouting has been demonstrated in the kainic acid and pilocarpine models of epilepsy (Nadler 1981; Represa et al. 1990; Turski 1989), after kindling (Elmer et al. 1996; Garcia-Cairasco et al. 1996; Represa et al. 1989, 1993; Sutula et al. 1988), electroconvulsive shock (Gombos et al. 1999; Vaidya et al. 1999), tetanus toxin (Anderson et al. 1999), alumina gel (Ribak et al. 1998), pentylenetetrazol (Golarai et al. 1992), in mutants with spontaneous seizures (Amano et al. 1999; Qiao and Noebels 1993), and temporal lobe epilepsy (especially nontumor associated cases; Babb et al. 1991; Cavazos et al. 1991; Houser et al. 1990; Sutula et al. 1989). Mossy fiber sprouting has also been reported following lesions or deafferentation of the hippocampus, trauma, stroke, ischemia, and feline immunodeficiency virus (Arvidsson 2001; Frotscher and Zimmer 1983; Golarai et al. 2001; Gould and Tanapat 1997; Hannesson et al. 1997; Laurberg and Zimmer 1981; Liu et al. 1998; Mitchell et al. 1999; Mohapel et al. 1997; Onodera et al. 1990; Santhakumar et al. 2001; Shetty and Turner 1999; West and Dewey 1984; Zimmer 1973). Although it has not been definitively proven, mossy fiber sprouting has often been considered to contribute to hippocampal hyperexcitability, particularly in temporal lobe epilepsy.

Electrophysiological studies have provided evidence that the sprouted fibers excite granule cells. Some of the first indications came from hippocampal slices from kainic acid–treated rats, which showed that stimulation in the hilus, which was thought to contain primarily granule cell axons, could produce orthodromic population spikes from the granule cell layer

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(Tauck and Nadler 1985). Since that time, a variety of methods and preparations have been used to show the excitatory effects of sprouted mossy fibers on granule cells, although this usually requires disinhibition or elevation of extracellular potassium (Cronin et al. 1992; Franck et al. 1995; Hardison et al. 2000; Lynch and Sutula 2000; Lynch et al. 2000; Molnar and Nadler 1999; Okazaki and Nadler 2001; Patrylo and Dudek 1998; Patrylo et al. 1999; Wuarin and Dudek 1996, 2001).

Others have argued that the sprouted fibers may actually enhance inhibition. This is based primarily on the strong paired-pulse inhibition observed in epileptic rats or mice with sprouting (Buckmaster and Dudek 1997b; Colling et al. 1997; Sloviter 1992) and recordings from human epileptic hippocampus (Wilson et al. 1998). There is also a high threshold for maximal dentate activation in rats with sprouting (Buckmaster and Dudek 1997a). In addition, anatomical data suggest that sprouted fibers innervate inhibitory neurons (Kotti and Riekkinen 1997; Ribak and Peterson 1991), although the proportion appears low (Buckmaster et al. 2002; Cavazos et al. 2003). The fact that these "interneurons" have highly divergent axons makes the net effect difficult to predict.

Indeed, one of the complexities of predicting the influence of sprouted fibers is the fact that the dentate gyrus network, even in normal rats, is complex. Thus stimulation at many sites in the dentate gyrus is likely to recruit both granule cells and other cell types, either antidromically or orthodromically. After seizures, there is variable hilar cell loss (Buckmaster and Jongen-Relo 1999; Cavazos et al. 1994; Covolan and Mello 2000; Mouritzen-Dam 1982), addition of new granule cells (Covolan et al. 2000; Gray and Sundstrom 1998; Parent et al. 1997; Sankar et al. 2000; Scharfman et al. 2000; Scott et al. 1998), increased expression of GABA in granule cells (Cao et al. 1996; Gutierrez 2000; Schwarzer and Sperk 1995; Sloviter et al. 1996), and changes in afferent input that occur as a consequence of damage in the entorhinal cortex and other areas of the brain. One could argue that these changes make the network even more complicated. Moreover, sprouting of cholinergic fibers and GABAergic neurons occur after seizures, in addition to mossy fiber sprouting (Davenport et al. 1990; Holtzmann and Lowenstein 1995; Mathern et al. 1997).

Therefore a direct approach was taken to assess the functional effect of mossy fiber sprouting. Randomly selected pairs of granule cells in hippocampal slices of epileptic rats and controls were recorded simultaneously. Based on past experience, which demonstrated that even robust pathways required a large sample size to find monosynaptically connected pairs of cells (Scharfman 1994b, 1995b; Scharfman et al. 1990), it was anticipated that a large sample of paired neurons would be required. So that numerous granule cells could be sampled as quickly as possible, sharp electrodes were used in hippocampal slices.

METHODS

Animal care and use met the guidelines set by the National Institutes of Health and the New York State Department of Health. All chemicals were purchased from Sigma (St. Louis, MO) unless otherwise noted.

Pilocarpine treatment

Adult male Sprague-Dawley rats (180–240 g) were obtained from Taconic (Germantown, NY), injected with atropine methylbromide (1 mg/kg sc), and 30 min later, injected with pilocarpine hydrochloride (380 mg/kg ip) as previously described (Scharfman et al. 2000). Diazepam (5 mg/kg ip, Wyeth, Philadelphia, PA) was injected after 1 h of status. Animals that had status epilepticus had repetitive behavioral seizures over the subsequent months and therefore were considered "epileptic." Saline controls rats were the same age and were treated identically, but saline was administered rather than pilocarpine.

Intracellular recordings in hippocampal slices

SLICE PREPARATION. Hippocampal slices (400 μm thick) were prepared from ether-anesthetized rats after decapitation. After one hemisphere of the brain was immersed in ice-cold buffer ["sucrose-buffer," containing (in mM) 126.0 sucrose, 5.0 KCl, 2.0 $CaCl_2$, 2.0 $MgSO_4$, 26.0 $NaHCO_3$, 1.25 NaH_2PO_4, and 10.0 D-glucose), it was sliced in the horizontal plane using a Vibroslice (Stoelting Instruments, Wood Dale, IL). Slices were immediately placed on a nylon net in a recording chamber (Fine Science Tools), which was modified to increase humidity in the area containing slices and to increase the fluid level so that slices were submerged except for the upper surface. They were warmed to 30–31°C, and humidified with 95% O_2 -5% CO_2. Thirty minutes after slices were placed in the chamber, sucrose buffer was switched to one containing NaCl, substituted equimolar for sucrose ("NaCl buffer"). Recordings began 30 min thereafter until approximately 7 h after the dissection. Flow rate was approximately 1 ml/min.

RECORDING AND STIMULATION. Recordings were made with intracellular glass electrodes (0.75 mm ID, 1.0 mm OD, World Precision Instruments, Sarasota, FL) that were pulled horizontally (Model P97, Sutter Instruments, Novato, CA) and filled with 4% neurobiotin (Vector Labs, Burlingame, CA) in 1 M potassium acetate, so that resistance was 60–140 MOhm. Intracellular data were collected using an intracellular amplifier with a bridge circuit (Axoclamp 2B, Axon Instruments, Foster City, CA), and the bridge was balanced whenever current was passed. Data were collected using a digital oscilloscope (Nicolet Instruments, Madison, WI) and also digitized and saved on tape (Neurocorder DR-484, Cygnus Technology, Delaware Water Gap, PA). Off-line analysis was conducted using Nicolet software and Origin 6.1 (OriginLab, Northampton, MA).

Cells that were impaled were first screened to ensure that they were healthy [stable resting potential, over −65 mV for granule cells, overshooting action potentials (APs)]. The outer molecular layer was stimulated by placing a monopolar, Teflon-coated stainless steel wire (75 μm OD) on the border of the outer molecular layer and the fissure. Stimuli were square pulses (10–200 μA, 10–20 μs) triggered at 0.02–0.05 Hz (Pulsemaster, World Precision Instruments) using a stimulus isolator (Isoflex, A.M.P.I. Products, Jerusalem, Israel).

Data analysis

INTRINSIC PROPERTIES. Analysis of intrinsic properties were made as previously described (Scharfman 1995a; Scharfman et al. 2000). Resting potential was defined as the difference between the potential while intracellular and that recorded after withdrawing the microelectrode from the cell. Input resistance was defined by the steepest slope of the *I-V* curve based on steady-state responses to a family of current pulses (0.05–1.0 nA, 150 ms).

AP characteristics were based on a single AP at threshold, evoked by current injected intracellularly ($\leq$0.6 nA, 150-ms pulse) at resting potential. AP amplitude was measured from resting potential to peak. Total AP duration was measured from the start of the rising phase of the AP until the point during the repolarization phase when the AP had repolarized. Half-width was the width of the AP at half-amplitude (amplitude measured from the start of the rising phase to the peak).

UNITARY EPSPS. Unitary EPSP amplitudes were measured at resting potential for the postsynaptic cell (between −67 and −78mV) and were measured from the baseline just before the presynaptic AP to the peak of the EPSP. Time to peak was measured in two ways: from the peak of the presynaptic AP to the peak of the EPSP, or from the end of the capacitative artifact of the presynaptic AP to the peak of the postsynaptic EPSP

(Table 1). Rise times were measured from the point on the EPSP that was 10% of its amplitude to the point that reached 90% or the amplitude. Half-duration was defined as the time from the peak of the presynaptic AP until the point on the EPSP decay that was equal to one-half its peak amplitude. These measurements were made using 7–13 EPSPs occurring in response to directly evoked single APs triggered in the presynaptic cell at 1 Hz by fixed amplitude current pulses. Only PSPs >0.5 mV were included, because smaller events were difficult to distinguish from the noise level of our recordings.

STATISTICS. Statistics were determined using PSI-plot (Version 5.0, Poly Software International, Salt Lake City, UT). Statistical significance was set at $P < 0.05$.

Anatomy

INTRACELLULAR LABELING AND PROCESSING. Neurobiotin (Vector Labs) was injected from the recording electrode in the presynaptic cell using repetitive depolarizing current pulses (+0.3–0.5 nA, 20 ms, 30 Hz, 10–20 min) after electrophysiological data were collected. Immediately after the experiment, slices were immersed in fixative (4% paraformaldehyde, pH 7.4) and refrigerated. Slices were immersed in agar the next day and refrigerated in 2% paraformaldehyde overnight. Agar was peeled away, and slices were sectioned (50 μm thick) using a vibratome (Ted Pella, Redding, CA). Following incubation overnight in 0.5% Triton-X 100, sections were washed in Tris buffer (3 times for 5 min), incubated in 0.3% H_2O_2 in 10% methanol for 30 min, washed, incubated in ABC (ABC standard kit, Vector Labs) in 0.1% Triton-X 100 in Tris, washed in Tris, incubated in diaminobenzidine (Polysciences, Warrington, PA; 50 mg/100 ml Tris) and 0.1% $NiNH_3SO_4$ until the cell could be fully visualized (10–30 min), washed in Tris, dehydrated in a series of graded alcohols (10 min each: 70, 90, 95, 100, and 100%), cleared in xylene, and coverslipped in Permount (Fisher Scientific, Pittsburgh, PA). Slides were examined using an Olympus BX-51 microscope and a digital camera (Model S60671, Optronics, Goleta, CA) with accompanying software (Stereo Investigator, Microbrightfield, Colchester, VT and AdobePhotoshop 5.0). Drawings were made from tracings of the printouts of digital images.

IMMUNOCYTOCHEMISTRY. The hemisphere contralateral to the one used for slicing was placed in ice-cold sucrose-artificial cerebrospinal fluid (ACSF) immediately after the brain was dissected, and immersion-fixed (4% paraformaldehyde, pH 7.4) immediately after slices from the contralateral hemisphere were placed in the recording chamber. The hemisphere, immersed in fixative, was placed on a rotator at room temperature for ≥6 h and refrigerated in fixative for ≥3 days. The tissue was sectioned (50 μm) using a vibratome (Ted Pella) and subsequently processed immunocytochemically using an antibody to Neuropeptide Y (polyclonal; 1:30,000; Peninsula Labs, Belmont, CA) to demonstrate mossy fiber sprouting. Neuropeptide Y is a robust marker of mossy fibers after seizures (Sperk et al. 1996; Vezzani et al. 1996), and previous studies showed that it consistently labeled sprouted mossy fibers in the inner molecular layer to the same extent as the other commonly used stains for mossy fibers, such as Timm stain (Scharfman et al. 1999, 2000). Detailed immunocytochemical methods have been described previously (Scharfman et al. 2000).

RESULTS

This study was based on 903 simultaneous recordings from two granule cells in rats that had pilocarpine-induced status epilepticus followed by recurrent spontaneous seizures. In saline-treated controls, 285 pairs were recorded. For a given granule cell, potential connectivity was tested with ≤45 other granule cell in the same slice.

Slices were made between 4.5 and 8.75 mo after status epilepticus. Only slices from the ventral hippocampus were tested because this area had more robust mossy fiber sprouting relative to dorsal hippocampus after pilocarpine-induced status (Scharfman et al. 2002) as was previously reported for kainic acid–treated rats (Buckmaster and Dudek 1997b). Granule cells were recorded in close proximity to each other (within 250 μm) and were located in the suprapyramidal blade (the blade closest to CA1, also referred to as the dorsal or lateral blade) and crest regions of the dentate gyrus.

TABLE 1. *Parameters of unitary EPSPs*

Pair No.	Amplitude [Base to Peak] (mV)	Time to Peak [Onset to Peak*] (ms)	Time to Peak [AP to Peak†] (ms)	10–90% Rise Time (ms)	Half Duration (ms)	Failure Rate (%)
1 (n = 9)						
Mean	1.74	3.72	5.89	2.90	17.07	59.1
SE	0.52	1.51	1.50	1.13	21.87	
2 (n = 10)						
Mean	2.20	3.68	5.53	2.53	14.90	68.8
SE	1.11	1.18	1.59	0.74	9.69	
3 (n = 10)						
Mean	1.90	4.10	5.59	2.77	12.91	52.6
SE	0.40	1.80	0.89	0.79	16.09	
4 (n = 7)						
Mean	1.73	5.58	6.43	3.39	14.22	78.0
SE	0.80	2.41	2.69	1.22	15.94	
5 (n = 11)						
Mean	1.76	4.35	5.86	2.31	19.00	61.5
SE	0.65	1.37	1.94	0.39	14.20	
6 (n = 8)						
Mean	2.17	3.22	5.04	2.07	11.33	71.4
SE	0.40	1.56	2.00	0.79	10.75	

Values are listed as mean ± SE and n = sample size. Characteristics of unitary excitatory postsynaptic potentials (EPSPs) from 6 pairs of monosynaptic connections between granule cells of rats with mossy fiber sprouting. Means are from 7 to 13 consecutive EPSPs that were evoked by presynaptic action potentials triggered at 1 Hz. Presynaptic action potentials were evoked by fixed amplitude current pulses at 1 Hz. Failures are excluded. Time to peak was measured from the end of the capacitative artifact of the presynaptic action potential to the peak of the unitary event (*) or from the peak of the presynaptic action potential to the peak of the unitary EPSP (†). For definitions of amplitude, rise-time and half-duration, see METHODS.

Characteristics of the unitary PSP

In all paired recordings, bidirectional tests were conducted, i.e., tests to examine whether one granule cell was presynaptic to the second or the second was presynaptic to the first. This was tested by first injecting current into one cell to elicit an AP and examining the second cell for any membrane potential changes that occurred immediately after the first cell's AP. Each potential postsynaptic cell was examined at a range of holding potentials (approximately −50 to −90 mV) as APs were repeatedly elicited in the potential presynaptic cell. In addition, the reverse was tested, i.e., current injection was used to evoke an AP in the second cell to test whether it produced an EPSP in the first cell. There was never any evidence of electrical coupling, i.e., membrane potential changes in one cell during an AP of the other cell. There also was no evidence for changes in membrane potential of one cell when subthreshold currents were injected in the other cell.

In 6 of the 903 (0.66%) paired recordings from epileptic rats, there was evidence of monosynaptic transmission from one granule cell to the other. Thus a depolarization occurred in one granule cell immediately after an AP that was triggered in the other granule cell by current injection (Fig. 1*A*). Thus the presynaptic AP immediately preceded the onset of the depolarization in the postsynaptic cell. This depolarization was considered excitatory because it could trigger APs (Fig. 1*B*), although APs could only be triggered if the postsynaptic cell was depolarized close to its threshold (Fig. 1*B*). It was also considered to be an EPSP because it was depolarizing at membrane potentials that were depolarized to the reversal potential of $GABA_A$ receptor-mediated inhibitory PSPs (IPSPs; approximately −70 mV).

In saline control rats (2.5–5 mo after saline injection), 285 pairs of granule cells were recorded simultaneously, and there was no evidence of monosynaptic connections in these cells. The frequency of monosynaptic connections was significantly different in epileptic animals (6/903, or approximately 1 connection per 150 pairs tested) versus saline controls (0/285; $\chi^2 = 4.84$, $P < 0.05$).

The average peak amplitudes of the unitary EPSPs were calculated from 7 to 13 consecutive EPSPs evoked by presynaptic APs triggered at 1 Hz. Any postsynaptic response that was <0.5 mV was excluded because it could not be discriminated with confidence from noise. Averaging all events was not a useful approach because of the high "failure" rate (see below). The data for each pair are shown in Table 1; mean amplitude for the six pairs was 1.9 ± 0.9 mV. The mean time to peak of all pairs was 4.1 ± 0.3 ms, calculated from the end of the capacitative artifact of the presynaptic AP to the peak of the unitary PSP. Calculated from the peak of the presynaptic AP to the peak of the unitary PSP, mean time to peak was 5.7 ± 0.1 ms. The mean 10–90% rise time was 2.6 ± 0.2 ms. The mean half-duration was 14.9 ± 1.1 ms. Amplitude histograms are shown in Fig. 2.

Unitary events did not always increase in amplitude with hyperpolarization. Only one of the pairs that were tested demonstrated an increase in EPSP amplitude with hyperpolarization. Importantly, small EPSPs (i.e., 2–3 mV in peak amplitude, evoked by weak molecular layer stimulation) did not increase with hyperpolarization either. These data may reflect the decrease in granule cell input resistance with hyperpolarization from approximately −45 to −85 mV (Scharfman 1994a; Thomson et al. 1998).

In all of the neurons that appeared to be monosynaptically connected, there were presynaptic APs that were not necessarily followed by a postsynaptic depolarization. These events could be failures of synaptic transmission or represent unitary events that were below the limits of detection (i.e., <0.5 mV). The average rate of such events was 65.2 ± 3.8% ($n = 6$; Table 1).

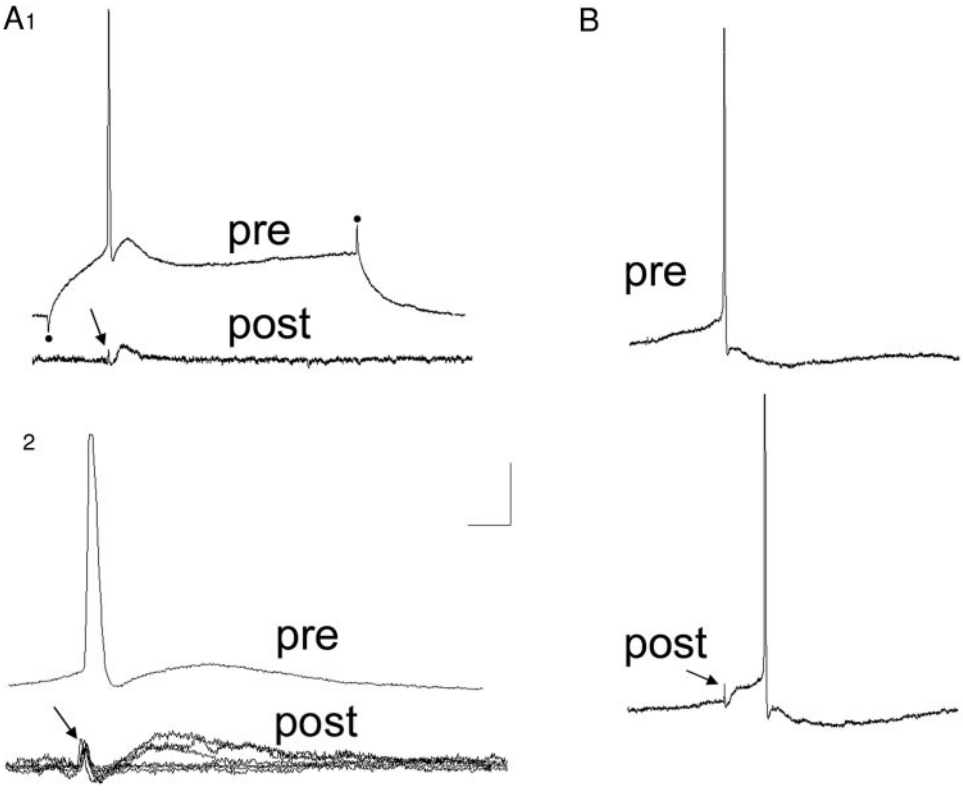

FIG. 1. Monosynaptic connections between granule cells in slices from rats with mossy fiber sprouting. *A*: recordings from a pair of simultaneously recorded granule cells are shown. *1*: presynaptic neuron (*top*). *Bottom*: postsynaptic neuron. Intracellular current (a 150-ms rectangular current pulse; start and end of the pulse are marked by the dots) was used to trigger an action potential (AP) in the presynaptic cell. Immediately thereafter, a small depolarization occurred in the postsynaptic cell. An arrow marks the capacitative artifact of the presynaptic cell's AP. Calibration: presynaptic cell, 20 mV, 30 ms; postsynaptic cell, 4 mV, 30 ms. *2*: recordings from the same pair of neurons with higher gain. Several postsynaptic responses are overlapped to show the variability in the response to the presynaptic AP. Calibration: presynaptic cell, 20 mV, 4 ms; postsynaptic cell, 3 mV, 4 ms. *B*: in a different pair of granule cells, tonic intracellular current was used to depolarize both the putative presynaptic (*top*) and postsynaptic (*bottom*) cells. A spontaneous AP in the presynaptic cell triggered an AP in the second cell. Membrane potentials: *top*, −55 mV; *bottom*, −54 mV. Calibration: 15 mV, 25 ms.

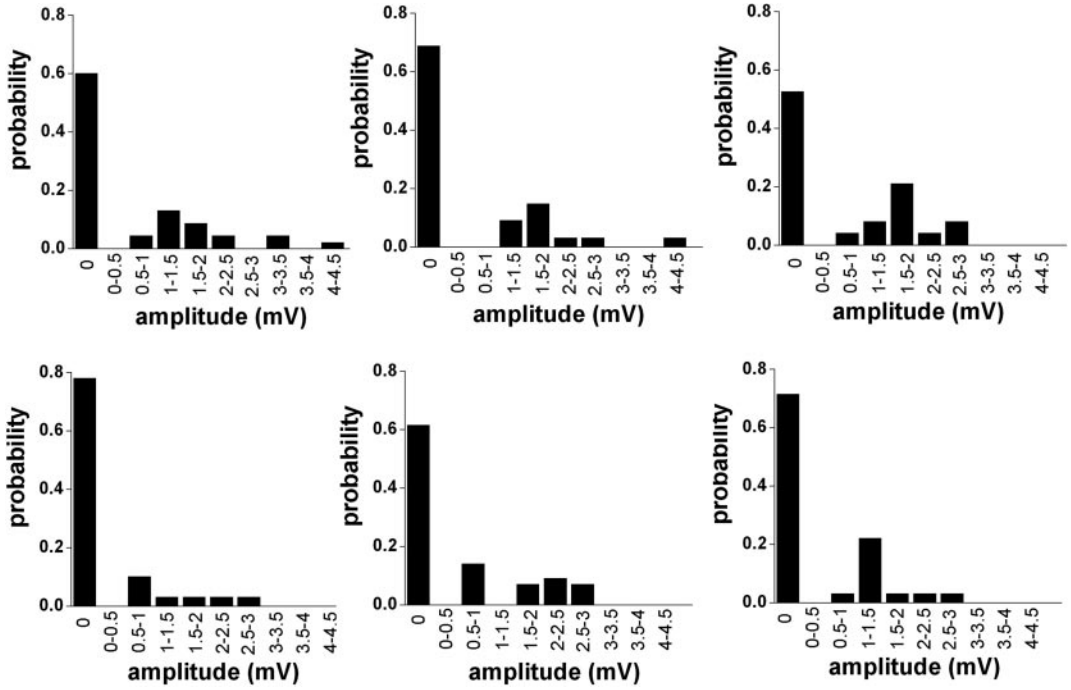

FIG. 2. Characteristics of unitary postsynaptic potentials (PSPs) evoked in pairs of monosynaptically connected granule cells. Amplitude histograms are shown for 6 pairs of granule cells that were monosynaptically connected. Unitary excitatory PSPs (EPSPs) were evoked by a single presynaptic AP triggered at 1 Hz using intracellular current pulses. Amplitudes were binned as 0 amplitude (undetectable responses of the postsynaptic cell) and in 0.5-mV increments, excluding 0–0.5 mV. The latter was excluded from analysis because events that were <0.5 mV in amplitude could not be discriminated with confidence from baseline noise.

Frequency depression

Following more than one presynaptic AP, there was evidence of frequency depression of unitary EPSPs. Frequency depression was defined as a decrease in the amplitude of the second EPSP when two presynaptic APs occurred in close succession. Presynaptic APs were triggered using interspike intervals between 5 and 25 ms (Fig. 3). The amplitude of the second EPSP was 8.8 ± 0.62% of the first (mean of 3 or more trials per pair, $n = 4$ pairs). This large decline (by >90%) was in part due to a high incidence of failures of synaptic transmission by the second AP. Thus the mean (8.8%) reflects the average of failures (defined here as events that were 0 mV in amplitude) and those events that reached amplitudes that were detectable. Higher numbers of APs were not tested systematically because of strong spike frequency adaptation of granule cells, which is a characteristic that distinguishes this cell type (Mott et al. 1997; Scharfman 1995a; Staley et al. 1992; Wang et al. 2000; Williamson et al. 1993).

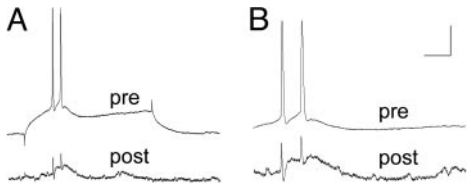

FIG. 3. Frequency depression of unitary EPSPs. *A*: recordings from a pair of granule cells. Presumed presynaptic cell is shown at the *top* and the postsynaptic cell at the *bottom*. A pulse of intracellular current was injected into the presynaptic cell so that more than 1 AP occurred. Calibration: presynaptic cell, 20 mV, 25 ms; postsynaptic cell, 4 mV, 25 ms. *B*: recordings in *A* are shown at higher gain. Calibration: presynaptic cell, 20 mV, 4 ms; postsynaptic cell, 2.5 mV, 4 ms.

Polysynaptic connections

All pairs were tested for electrical, polysynaptic, and monosynaptic connections. In other words, any membrane potential change that occurred in one cell was examined further, whether it started during, immediately after, or several milliseconds after an AP in the other cell. In five simultaneous recordings from granule cells that were not monosynaptically connected, there was evidence of polysynaptic connections. Four recordings were from epileptic tissue (4/903, 0.44%) and one was from a saline control rat (1/285; 0.32%). These frequencies were not statistically different (χ^2 test, $P > 0.05$). These events appeared to be inhibitory because they were hyperpolarizing when the membrane potential of the postsynaptic cell was set to a depolarized level (e.g., depolarized to −70 mV). An example is shown in Fig. 4.

These putative IPSPs appeared to be disynaptic because there was a delay between the presynaptic AP and the onset of the postsynaptic potential (Fig. 4). These recordings may represent the recurrent feedback pathway (granule cell-GABA neuron-granule cell). Notably, the frequency of detectable postsynaptic responses after presynaptic APs (51.2 ± 7.65%) was no greater than that determined for the putative monosynaptic EPSPs (χ^2 test, $P > 0.05$). This is consistent with previous descriptions of the granule cell-GABA neuron synapse, which is highly reliable (Geiger et al. 1997), and contrasts with the low reliability of granule cell-granule cell connections described above.

Verification of mossy fiber sprouting

In all animals, mossy fiber sprouting was demonstrated in the ventral hippocampus of the hemisphere contralateral to the one used for slices. Figure 5, *A* and *B* shows an example of a hori-

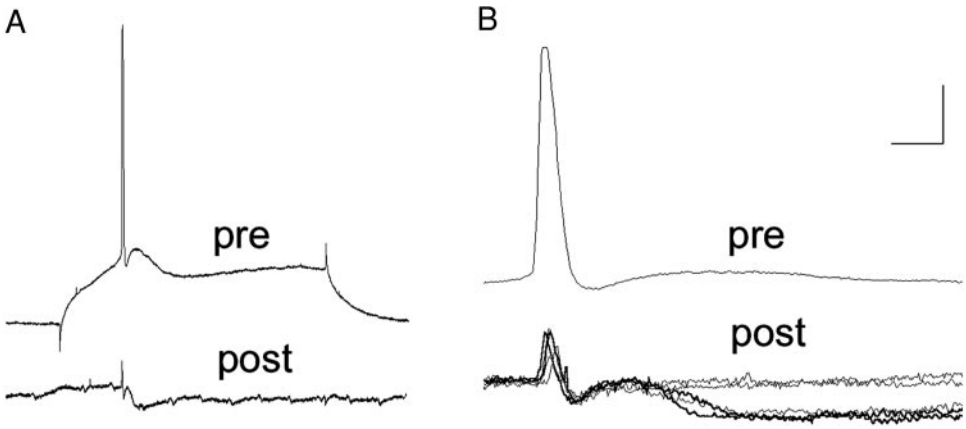

FIG. 4. Presumed disynaptic inhibition of granule cells. *A*: simultaneous recording from 2 granule cells is shown. A current pulse injected intracellularly into the presumed presynaptic cell evoked a hyperpolarization in the postsynaptic neuron. Calibration: presynaptic cell, 20 mV, 25 ms; postsynaptic cell, 4 mV, 25 ms. *B*: recordings from the same neurons as shown in *A* are illustrated at higher gain. Several responses of the postsynaptic neuron to a single presynaptic AP are superimposed. Calibration: postsynaptic cell, 20 mV, 2 ms; presynaptic cell, 2 mV, 2 ms.

zontal section from ventral hippocampus that was stained with antisera to neuropeptide Y (NPY) to demonstrate mossy fiber sprouting. NPY was used as a marker of granule cell axons in epileptic rats (Scharfman et al. 2000, 2002; Sperk et al. 1996).

Identification of presynaptic and postsynaptic cells

In the area of the slice where impalements were made (the granule cell layer), both granule cells and nongranule cells are present. Therefore it was important to verify that the neurons which were identified as pre- or postsynaptic cells in our recordings met criteria used to identify granule cells. Electrophysiological and anatomical methods were used for this purpose.

Electrophysiology showed that intrinsic properties were similar to those previously reported for granule cells (Table 2) (Scharfman et al. 2000; Staley et al. 1992; Wang et al. 2000). Thus resting potentials were hyperpolarized relative to other cell types. APs of granule cells were broad, and granule cell APs were followed by triphasic afterhyperpolarizations (AHP; Fig. 1), distinct from the AHPs of the GABAergic neurons and mossy cells (Scharfman 1995a,b; Scharfman et al. 2000). In

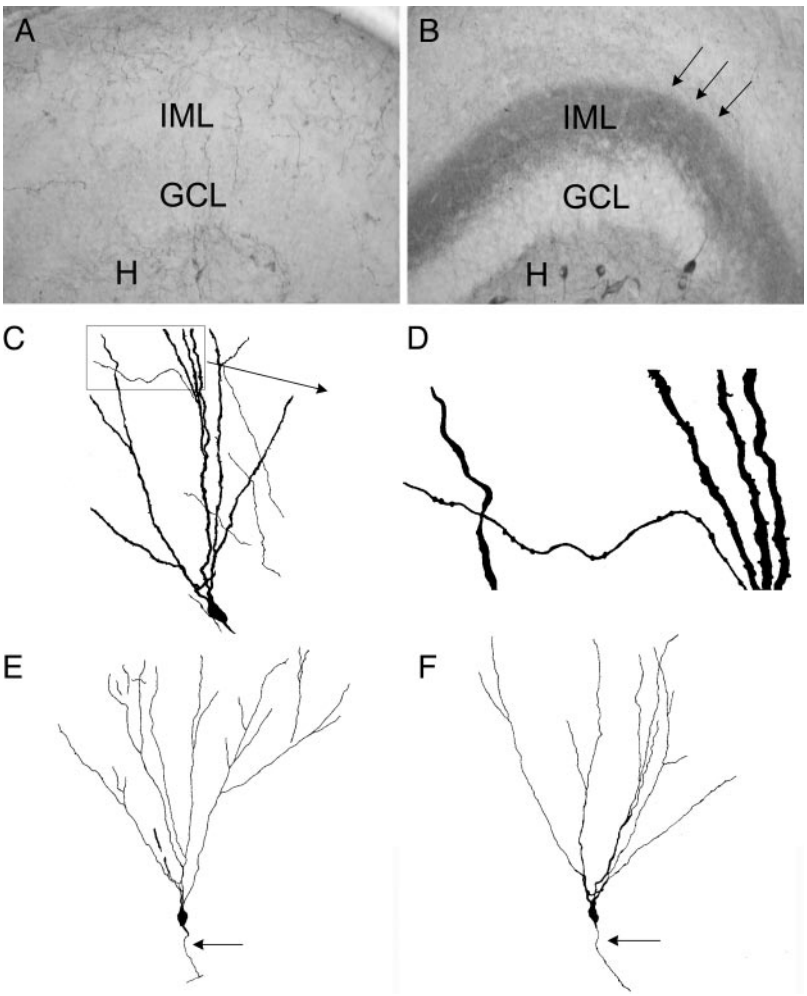

FIG. 5. Anatomical examination of mossy fiber sprouting and morphology of recorded granule cells. *A*: section through the crest of dentate gyrus of a saline-treated control rat is shown, stained using an antibody to neuropeptide Y. Dorsal (e.g., area CA1) is to the right, and lateral (e.g., area CA3) is down. IML, inner molecular layer; GCL, granule cell layer; H, hilus. Calibration: 100 μm. *B*: section through the crest of the dentate gyrus at the same septotemporal level as *A*, but in a rat that had pilocarpine-induced status epilepticus and recurrent seizures. The sections in *A* and *B* were processed concurrently. Arrows point to an immunoreactive band in the IML that is not present in *A*. Arrowhead points to an immunoreactive cell on the border of the hilus and granule cell layer. Calibration: same as *A*. *C*: drawing of a neurobiotin-labeled granule cell that was recorded simultaneous to another granule cell that appeared to be connected monosynaptically. The soma, dendrites, and proximal axon are shown, as well as some of the collaterals of the axon that were present in the molecular layer. Calibration (in *A*): 20 μm. *D*: higher magnification of the boxed portion of *C* illustrates an axon collateral with numerous varicosities. *E* and *F*: drawings of other neurobiotin-labeled neurons in slices that were recorded simultaneously and demonstrated monosynaptic connectivity. Arrows point to the axons. Calibration (in *A*): 40 μm.

TABLE 2. *Membrane properties of pre- and postsynaptic granule cells*

	Resting Potential (mV)	Input Resistance (Mohms)	Time Constant (ms)	Action Potential		
				Amplitude (mV)	Duration (ms)	Half-width (ms)
Presynaptic						
Mean	72.8	47.5	7.83	101.2	1.98	0.183
SE	2.0	2.1	0.70	1.4	0.05	0.015
n	6	6	6	6	6	6
Postsynaptic						
Mean	71.3	53.3	8.50	99.2	1.94	0.190
SE	1.8	3.1	0.76	2.4	0.67	0.094
n	6	6	6	6	6	6

Values are listed as mean ± SE and n = sample size. Membrane properties of the granule cells that were monosynaptically connected. Characteristics of action potentials that were measured were the amplitude, duration and half-width. For measurements of membrane properties, see METHODS.

addition, granule cells were distinguished by their strong spike frequency adaptation (Mott et al. 1997; Scharfman 1992; Staley et al. 1992; Wang et al. 2000; Williamson et al. 1993).

Morphology also confirmed that the recorded cells were granule cells (Fig. 5, *C–D*). In 5 of the 12 cells that were synaptically connected, neurobiotin labeling showed that these neurons met established criteria for granule cells: *1*) round or oval cell body, *2*) spiny, apical dendritic tree extending into the molecular layer, and *3*) axon entering the hilus and collateralizing in that region. Figure 5*C* illustrates one of the granule cells that evoked a monosynaptic depolarization in a simultaneously recorded granule cell located approximately 50 μm away. Portions of the axon from the cell in Fig. 5*C* were evident in and around the inner molecular layer near the cell body and were studded with varicosities (Fig. 5*D*). Morphological examination of filled axons revealed collaterals in the molecular layer in two of four putative presynaptic cells. In the other cells, the axon was not labeled beyond the proximal segment (Fig. 5, *E* and *F*). Numerous varicosities were present along axon collaterals, and no "giant" mossy fiber boutons were observed. This is consistent with previous reports that sprouted mossy fibers with giant terminals in the molecular layer are not commonly observed in epileptic rodents (Buckmaster and Dudek 1999; Cavazos et al. 2003; Okazaki et al. 1995; Sutula et al. 1998), although they have been identified in some cases (Represa et al. 1993) and in human epileptic tissue (Isokawa et al. 1993; Zhang and Houser 1999).

DISCUSSION

Summary

The results provide evidence for monosynaptic connections between granule cells in pilocarpine-treated rats with mossy fiber sprouting. Thus all-or-none depolarizations were recorded in granule cells after an AP was evoked by intracellular current injection in a simultaneously recorded granule cell. The latency was consistent with a monosynaptic pathway.

Factors contributing to the low frequency of detected monosynaptic connections

One could interpret the low frequency of detected connections relative to the number of sampled neurons as an indication that the pathway that was studied is weak. However, there are several methodological issues that indicate such a conclusion is premature at the present time. For example, monosynaptic connections in the dentate gyrus, such as the mossy fiber synapse onto mossy cells, appear difficult to detect with paired recordings (Scharfman et al. 1990), yet this is considered a robust pathway. Therefore the low frequency of detected connections could be related to the method used rather than a paucity of actual connections in situ.

Characteristics of the unitary EPSP

Several characteristics of the unitary EPSP indicate that it is relatively weak in its ability to depolarize granule cells. First, a presynaptic AP often failed to produce a detectable postsynaptic depolarization. This may reflect failure of synaptic transmission, perhaps because of a low safety factor or branch point failure along mossy fiber axons. Indeed, mossy fibers of untreated (Acsady et al. 1998; Claiborne et al. 1986) and epileptic rats (Buckmaster and Dudek 1999; Okazaki et al. 1995; Represa et al. 1993; Sutula et al. 1998) branch extensively, and because these fibers are thin and unmyelinated, branch point failure may be common. In a study using a different approach, one that used laser photostimulation to examine unitary-like events produced by sprouted mossy fibers, failure rate was also extremely high (approximately 70%) (Molnar and Nadler 1999). The results of Molnar and Nadler (1999) support the results presented here, that this pathway has a high failure rate. Whether this indicates that the pathway is weak is hard to judge because high failure rates have also been reported for other unitary events in the CNS (Allen and Stevens 1994; Deuchars et al. 1994; Thomson et al. 1993). Allen and Stevens (1994) proposed that a high failure rate may actually be more characteristic of synapses in the CNS than a low failure rate.

It is important to note that failure rate could have been overestimated. This is due to the fact that extremely small events were poorly discriminated from noise. Another factor that could contribute to an overestimation of failures is that EPSPs could have been shunted by GABAergic inhibition, which is normally strong in granule cells (Otis et al. 1991). GABAergic shunting has been observed in other studies of monosynaptically connected neurons in the dentate gyrus, for example, at the hilar synapse of CA3 pyramidal cell axons on mossy cells (Scharfman 1994b). One could argue that the tissue used was already disinhibited, given the reduced numbers of inhibitory neurons after pilocarpine-induced status (Obenaus et al. 1993). But several types of GABAergic neu-

rons survive (Houser and Esclapez 1996), particularly in the animals that have an anticonvulsant administered 1 h after status begins (Scharfman et al. 2000), as was the case in this study. Furthermore, it has been shown that GABAergic neurons can sprout after seizures (Davenport et al. 1990; Mathern et al. 1997), and their level of GABA also may increase (Esclapez and Houser 1999). As mentioned above, dentate gyrus inhibition in many studies actually seems increased, not decreased, after status epilepticus.

Indeed, there are many studies that have used disinhibition to reveal a powerful underlying recurrent excitatory circuit in epileptic rats or human epileptic tissue (Cronin et al. 1992; Franck et al. 1995; Lynch and Sutula 2000; Lynch et al. 2000; Patrylo and Dudek 1998; Patrylo et al. 1999; Wuarin and Dudek 1996, 2001). However, unitary-like events evoked by laser photostimulation of sprouted mossy fibers showed a failure rate similar to the one we report here, despite the fact that the tissue was disinhibited (Molnar and Nadler 1999), and ours was not. This comparison suggests that disinhibition may actually not have influenced the failure rate that we observed.

Another indication that the granule cell-granule cell synapse was relatively ineffective was the observation that increased frequency of presynaptic discharge led to frequency depression, not facilitation. This would make it unlikely that this synapse would contribute to the evolution of epileptiform activity in hippocampus after high-frequency input from, for example, the entorhinal cortex. However, other frequencies besides those tested in this study might have led to facilitation if they had been tested. Indeed, if lower frequencies than 1 Hz had been used to examine effects of single APs, failure rate and PSP amplitude may have changed dramatically. This may be relevant to the transition to seizures in epileptic rats, in which inhibition may be strong normally, but fragile nevertheless. Under these conditions, the underlying excitatory circuits may contribute to seizures when inhibition deteriorates (Buhl et al. 1996; Wu and Leung 2001).

On the basis of unitary amplitude alone, one could argue that the unitary EPSP would not necessarily be weaker than other unitary EPSPs that have been previously described. This is because the mean amplitude was within the range of other unitary EPSPs in hippocampus and cortex (Debanne et al. 1995; Feldmeyer et al. 2002; Larkman et al. 1997a,b; Markram et al. 1997; Miles 1990; Miles and Wong 1986, 1987; Thomson and Bannister 1998; Thomson and Deuchars 1997; Thomson et al. 1995). It was actually greater than some reports of the unitary amplitude of EPSPs produced at the CA3 pyramidal synapse onto CA1 pyramidal cells (Sayer et al. 1989, 1990).

Another reason why unitary EPSPs may have been underestimated is that our recordings were made at temperatures that were lower than the physiological range, and it has been shown that lowering temperature can decrease unitary events, slow their kinetics, increase failure rate, and affect temporal summation of EPSPs (Hardingham and Larkman 1998; Jack et al. 1994; Pyott and Rosenmund 2002; Trevelyan and Jack 2002).

Sampling bias is important to consider, because it is possible that other connections among granule cells would have demonstrated other characteristics. We suspect sampling bias because axons that were labeled only had small boutons in the inner molecular layer, yet we know that "giant" mossy fiber boutons exist in the normal rat (Blackstad and Kjaerheim 1961; Chicurel and Harris 1989; Hamlyn 1961). Giant boutons have been found along sprouted axons in nonepileptic (Frotscher and Zimmer 1983) and epileptic rats (Represa et al. 1993), but they can be rare (Buckmaster and Dudek 1999; Cavazos et al. 2003; Okazaki et al. 1995; Sutula et al. 1998). Synapses made by giant boutons might have stronger excitatory effects than those synapses with small terminals because they appear to do so in normal tissue (Henze et al. 1997, 2000, 2002; Jonas et al. 1993; Scharfman et al. 1990). Furthermore, the ventral (inferior) blade was not sampled in this study, and recent data suggest stronger mossy fiber sprouting and stronger excitatory effects in that blade (Scharfman et al. 2002), although it has also been shown that the supragranular blade may contain more dense synaptic innervation by sprouted mossy fibers (Sutula et al. 1998). In summary, a higher frequency of connected cells and unitary events with other characteristics may have been obtained if other sites in the dentate gyrus had been sampled.

Variations in the effects of the mossy fiber synapse

Taken together with other studies of mossy fiber synapses, it appears that mossy fibers can have variable effects on their targets. This variability is suggested by comparing the results of this study to those using normal, young rats to examine mossy fiber input to GABAergic neurons (Geiger et al. 1997), hilar GABAergic neurons (Scharfman et al. 1990), hilar mossy cells (Scharfman et al. 1990), or unitary EPSP/Cs of CA3 pyramidal cells using the technique of "minimal" stimulation (Henze et al. 1997, 2000, 2002; Jonas et al. 1993; Walker et al. 2001; Williams and Johnston 1991). The synapses on hilar cells and pyramidal cells in normal rats appear relatively robust compared with the sprouted fiber synapse reported here, because unitary size was larger, failure rate was lower, and frequency facilitation was often quite strong.

One factor that may have diminished the effects of mossy fiber transmission in the current study, relative to studies described above in normal rats, is the fact that there are mechanisms to limit EPSPs in the dentate gyrus that might be greater in epileptic animals. One example is presynaptic inhibition, which can be mediated by $GABA_B$ receptors (Mott and Lewis 1994), metabotropic receptors (Aronica et al. 1997; Hardison et al. 2000; Manzoni et al. 1995; Okazaki and Nadler 2001), NPY (Klapstein and Colmers 1993), opiates (Bausch et al. 1998), or somatostatin (Tallent and Siggins 1999). Some of these might be more effective in the epileptic brain because of increased peptide levels and receptors (e.g., NPY; Sperk et al. 1996; Vezzani et al. 1996). It is also conceivable, although not yet proven, that concurrent release of glutamate and GABA from granule cells decreases EPSPs. This possibility is suggested by the evidence that the level of GABA expressed by granule cells increases after seizures (Cao et al. 1996; Gutierrez 2000; Schwarzer and Sperk 1995; Sloviter et al. 1996).

Comparison to conditions without sprouting

Although no evidence for monosynaptic connections were found in tissue without sprouting, it is not possible to exclude the possibility that such connections occur in normal animals. Thus there are suggestions in the literature that some mossy fibers normally innervate granule cells (Molnar and Nadler 1999; Okazaki et al. 1999). We cannot exclude the possibility that the monosynaptic connections we detected in epileptic

tissue would be present in control tissue if we had conducted more tests. However, the frequency of detected connections was approximately 1 per 150 pairs sampled in epileptic tissue, and at this frequency, one would expect that at least 1 pair would have been detected in the 285 pairs sampled in saline controls. Statistical analysis supported this argument.

Implications for understanding the net effect of mossy fiber sprouting

These data provide an explanation at the level of single synapses for several observations in the literature about mossy fiber sprouting in epileptic rats. Thus substantial anatomic and physiologic evidence exists that supports the hypothesis that the sprouted fibers allow recurrent excitation of granule cells (Buckmaster and Dudek 1999; Buckmaster et al. 2002; Dudek et al. 1994; Masukawa et al. 1992; Mathern et al. 1993; Pollard et al. 1995; Represa et al. 1990). However, the underlying recurrent excitatory circuits appear to require disinhibition or other manipulations to be detected (Cronin et al. 1992; Franck et al. 1995; Hardison et al. 2000; Lynch and Sutula 2000; Lynch et al. 2000; Molnar and Nadler 1999; Okazaki and Nadler 2001; Okazaki et al. 1999; Patrylo and Dudek, 1998; Patrylo et al. 1999; Wu and Leung 2001; Wuarin and Dudek 1996, 2001).

Our data indeed show that there are recurrent excitatory synapses among granule cells in epileptic rats, but several aspects of this monosynaptic pathway indicate that its functional effect may not be robust in and of itself. This is consistent with the observation that the epileptic rat with mossy fiber sprouting does not necessarily have frequent spontaneous seizures, and when probed in vivo, appears to have increased granule cell inhibition rather than increased excitation (Buckmaster and Dudek 1997a,b; Colling et al. 1997; Sloviter 1992).

Therefore one would predict that the net effect of mossy fiber sprouting will only become clear when we understand the nature and the number of recurrent synapses, not just among granule cells, but also synaptic reorganization among other dentate neurons. Furthermore, it will be necessary to understand the impact of other changes in the epileptic brain, including alterations in axonal structure (Pierce and Milner 2001) and neurotransmitters/neuromodulators (Elmer et al. 1996; de Lanerolle et al. 1998; Gall 1993; Mathern et al. 1997, 1998).

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DISCLOSURES

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