Synaptic Connections From Multiple Subfields Contribute to Granule Cell Hyperexcitability in Hippocampal Slice Cultures

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Bausch, Suzanne B. and James O. McNamara. Synaptic connections from multiple subfields contribute to granule cell hyperexcitability in hippocampal slice cultures. J Neurophysiol 84: 2918–2932, 2000. Limbic status epilepticus and preparation of hippocampal slice cultures both produce cell loss and denervation. This commonality led us to hypothesize that morphological and physiological alterations in hippocampal slice cultures may be similar to those observed in human limbic epilepsy and animal models. To test this hypothesis, we performed electrophysiological and morphological analyses in long-term (postnatal day 11; 40–60 days in vitro) organotypic hippocampal slice cultures. Electrophysiological analyses of dentate granule cell excitability revealed that granule cells in slice cultures were hyperexcitable compared with acute slices from normal rats. In physiological buffer, spontaneous electrographic granule cell seizures were seen in 22% of cultures; in the presence of a GABA A receptor antagonist, seizures were documented in 75% of cultures. Hilar stimulation evoked postsynaptic potentials (PSPs) and multiple population spikes in the granule cell layer, which were eliminated by glutamate receptor antagonists, demonstrating the requirement for excitatory synaptic transmission. By contrast, under identical recording conditions, acute hippocampal slices isolated from normal rats exhibited a lack of seizures, and hilar stimulation evoked an isolated population spike without PSPs. To examine the possibility that newly formed excitatory synaptic connections to the dentate gyrus contribute to granule cell hyperexcitability in slice cultures, anatomical labeling and electrophysiological recordings following knife cuts were performed. Anatomical labeling of individual dentate granule, CA3 and CA1 pyramidal cells with neurobiotin illustrated the presence of axonal projections that may provide reciprocal excitatory synaptic connections among these regions and contribute to granule cell hyperexcitability. Knife cuts severing connections between CA1 and the dentate gyrus/CA3c region reduced but did not abolish hilar-evoked excitatory PSPs, suggesting the presence of newly formed, functional synaptic connections to the granule cells from CA1 and CA3 as well as from neurons intrinsic to the dentate gyrus. Many of the electrophysiological and morphological abnormalities reported here for long-term hippocampal slice cultures bear striking similarities to both human and in vivo models, making this in vitro model a simple, powerful system to begin to elucidate the molecular and cellular mechanisms underlying synaptic rearrangements and epileptogenesis.

INTRODUCTION

The hippocampus is a brain structure that is important for learning and memory and is a common focus for epileptic seizures. One of the primary afferent pathways into the hippocampus arises from neurons in the entorhinal cortex. Propagation of information from the entorhinal cortex through the hippocampal formation, on a simplistic level, proceeds via a trisynaptic feed-forward excitatory pathway. Information from the entorhinal cortex is transmitted sequentially through a series of excitatory synapses; first to dentate granule cells, from there to CA3 pyramidal cells, and last from CA3 to CA1 pyramidal cells (Amaral and Witter 1989). Thus as the first neurons in this series of excitatory synapses through the hippocampal formation, the dentate granule cells are in a position to limit transmission through the hippocampal network.

The hippocampal formation is an exquisitely seizure-prone structure (Green 1964). Within this structure, the principal cells (i.e., CA1 and CA3 pyramidal cells of the hippocampus and the granule cells of the dentate gyrus) display differing propensities for epileptiform activity and seizures. The CA3 pyramidal cells are most prone to epileptiform activity (Miles and Wong 1986), which is thought to be due in part to recurrent excitatory synapses between CA3 pyramidal cells. In contrast, seizure-like activity is difficult to induce in normal dentate granule cells, which is thought to be due to the intrinsic properties of the granule cells, the lack of recurrent excitatory synapses with their neighboring granule cells and the presence of strong polysynaptic inhibitory synapses onto granule cells. The difficulty in inducing seizures in dentate granule cells, together with work by Lothman and colleagues (Collins et al. 1983), have led to the hypothesis that the granule cells of the dentate gyrus normally serve as a barrier to invasion of epileptiform activity and seizures into the hippocampus. Collins et al. (1983) studied behavioral seizures and 2-deoxy-glucose (2DG) activity during focal application of penicillin to the entorhinal cortex; as long as the 2DG increase was limited to the dentate gyrus itself, little or no behavioral seizure activity occurred. Only after 2DG increases propagated beyond the dentate gyrus did behavioral seizures emerge, leading to the idea that the granule cells served as a barrier to seizure invasion of hippocampal circuitry. Field potential recordings of seizures evoked in entorhinal-hippocampal slices provided direct evidence in support of this idea. Behr et al. (1996) showed that low Mg2+ induced seizures in entorhinal cortex propagated through dentate gyrus to CA3 and CA1 in 4 of 18 slices from...
normal rats; propagation of these entorhinal cortex seizures was blocked by a knife cut between the entorhinal cortex and dentate gyrus (Behr et al. 1998), implying that axons connecting entorhinal cortex with dentate conveyed the seizures to hippocampus. Moreover, limbic epileptogenesis is associated with loss or attenuation of the barrier function (Behr et al. 1998). Taken together, these findings suggest that elimination of the filter function of the granule cells may be a pivotal event in limbic epileptogenesis.

The single most common form of temporal lobe epilepsy in humans is associated with selective neuronal loss termed Ammon’s horn or hippocampal sclerosis (Margerrison and Corsellis 1966) and synaptic reorganization termed mossy fiber sprouting (de Lanerolle et al. 1989; Houser et al. 1990; Sutula et al. 1989). Similarly, mossy fiber sprouting also has been observed in numerous animal models of temporal lobe epilepsy (Mello et al. 1992; Sutula et al. 1988; Tauck and Nadler 1985). Mossy fiber sprouting is the synaptic reorganization of the mossy fiber axons of dentate granule cells into the inner molecular layer of the dentate gyrus (Okazaki et al. 1995; Represa et al. 1993; Sutula et al. 1988); a region almost devoid of mossy fiber collaterals in normal animals (Mello et al. 1992; Ribak and Peterson 1991; Seress 1992; Sutula et al. 1988; Tauck and Nadler 1985). The association between mossy fiber sprouting and an epileptic phenotype has led to the popular hypothesis that one cause of hyperexcitability in the sclerotic, epileptic hippocampus is that the mossy fiber axons of dentate granule cells form synapses with themselves and other granule cells, thus forming a recurrent excitatory network. Indeed, anatomical studies have documented that sprouted mossy fibers do form synapses onto granule cells (Okazaki et al. 1995; Represa et al. 1993; Sutula et al. 1988, 1989; Wenzel et al. 1995), suggestive of recurrent excitatory synapses between granule cells. Thus the idea has emerged that recurrent excitatory synapses between granule cells coincident with mossy fiber sprouting could compromise the ability of the dentate gyrus to act as a barrier to invasion of epileptiform activity into the hippocampus.

Ammon’s horn sclerosis is often preceded by limbic status epilepticus in animals and humans, which causes dramatic cell loss in regions such as entorhinal cortex, septum, and hippocampus as well as denervation of hippocampal structures (Du et al. 1993; Green et al. 1989; Margerrison and Corsellis 1966; Mathern et al. 1996; but see Pennell et al. 1999). Preparation of hippocampal slice cultures also produces dramatic denervation. Although a substantial number of synapses are maintained following slice preparation due to the laminar organization of the hippocampus, profound denervation from extrahippocampal structures occurs. This striking commonality between organotypic hippocampal slice cultures and the sclerotic hippocampus in human temporal lobe epilepsy prompted us to hypothesize that morphological reorganizations and granule cell excitability in hippocampal slice cultures should be similar to those observed in human limbic epilepsy and animal models. To test this hypothesis, we performed electrophysiological analyses of granule cell excitability and morphological analyses of the synaptic organization in long-term organotypic hippocampal slice cultures.

Portions of this manuscript were presented previously in abstract form (Bausch and McNamara 1997; Bausch et al. 1998).

METHODS
Organotypic hippocampal slice cultures
Slice cultures were prepared using the method by Stoppini et al. (1991) as described previously (Routbort et al. 1999). Briefly, postnatal day 11 (P11) Sprague-Dawley rat pups (Zivic-Miller, Zenople, PA) were anesthetized with pentobarbital sodium and decapitated. The brains were removed; hippocampi were dissected aseptically and placed onto an agarose cushion. Hippocampi were then cut into 400-μm transverse sections using a McIlwain tissue chopper and placed into Gey’s balanced salt solution (GBSS, GIBCO BRL) supplemented with 6.5 mg/ml glucose. Sections were separated with a Teflon spatula, and the middle four to six slices of each hippocampus (with the entorhinal cortex removed) were placed onto tissue culture membrane inserts (Millipore) in a tissue culture dish containing medium consisting of 50% minimum essential medium, 25% Hank’s buffered salt solution, 25% heat-inactivated horse serum, 0.5% GlutaMax II, 10 mM HEPES (all from GIBCO BRL), and 6.5 mg/ml glucose (pH 7.2). Medium was changed two to four times per week. Cultures were maintained at 37°C under room air +5% CO2. Physiological recordings and anatomical labeling were performed at 40–60 days in vitro (DIV).

All treatment of animals was according to National Institutes of Health and institutional guidelines.

Acute hippocampal slices
Acute hippocampal slices were isolated from young adult (5–6 wk) male Sprague-Dawley rats (Zivic-Miller, Zenople, PA); the age of the rat approximating the age of slice cultures at time of recording. Rats were anesthetized with halothane and decapitated, and brains were immediately removed and placed in ice-cold buffer. The brain was blocked and attached to a wax block using cyanoacrylate glue, and 400-μm transverse slices were cut using a vibratome. The middle four to six slices of each hippocampus were placed in a submerged holding chamber containing buffer composed of (in mM): 120 NaCl, 3.5 KCl, 1.3 MgSO4, 2.5 CaCl2, 1.24 NaH2PO4, 25.6 NaHCO3, and 10 glucose equilibrated with 95% O2-5% CO2 for at least 1 h before recording.

Electrophysiological recording
For acute hippocampal slices, slices were placed into a recording chamber mounted to a Zeiss Axioskop microscope and held down with a harp made of platinum wire and nylon strings. For hippocampal slice cultures, a portion of the tissue culture insert membrane containing a single slice culture was cut, and the membrane and slice culture were placed into the recording chamber. The membrane was held down with platinum wires. Both acute slices and slice cultures were superfused at room temperature with a recording buffer composed of (in mM): 120 NaCl, 3.5 KCl, 1.3 MgSO4, 2.5 CaCl2, 1.24 NaH2PO4, 25.6 NaHCO3, and 10 glucose, equilibrated with 95% O2-5% CO2. Bicuculline methiodide (BMI, 10 μM; Sigma), d(-)-2-amino-5-phosphonovaleric acid (d-APV, 50 μM; Tocris Cookson), 6-cyano-7-nitrocoumarin (CNQX, 10 μM; Tocris Cookson), and 6-nitro-7-sulfamoylbenzof[1]quinolin-2,3-dione (NBQX, 10 μM; Tocris Cookson) were diluted immediately before use in recording buffer and applied by bath superfusion. Recording pipettes (whole cell, 2–5 MΩ; extracellular, 1–3 MΩ) were pulled on a Flaming-Brown puller and filled with 3 M NaCl for extracellular recordings or with (in mM) 100 K-glucurate, 30 KCl, 10 HEPES, 10 EGTA, 3 MgCl2, and 2 Na2ATP (pH 7.2 with KOH) for whole cell current-clamp recordings. Whole cell recordings were obtained using visual identification and were excluded if series resistance was >10 MΩ. Evoked responses were elicited by stimulation (0.3-ms square pulse, 0.03 Hz, 20–700 μA) of the hilar mossy fiber pathway using a concentric bipolar electrode (MCE-100, Rhodes Medical Supply) and a Grass stimulator.
Current-clamp data were collected within 2–5 min of establishing whole cell configuration. The resting membrane potential (RMP) was read from the amplifier. Input resistance \((R_{in})\) was calculated with pCLAMP software using points from the linear portion of a current-voltage plot of the change in membrane voltage in response to a series of 450-ms, 25- to 50-pA steps. Spike properties were determined by generating a series of 450-ms, 25- to 50-pA steps. Spike threshold was determined as the first current step that elicited an action potential. The number of action potential spikes was counted at 1) threshold for spike generation and 2) following a 200-pA current step.

Dentate granule cell layer field potential recordings were deemed acceptable if hilar stimulation yielded an action potential spike (spike) that immediately followed the stimulus artifact with a response threshold \(\leq 100 \mu A\) (e.g., Fig. 4). The basis for concluding that this waveform was an action potential spike was that the waveform could be abolished with tetrodotoxin (TTX; 1 µM; Calbiochem; data not shown). Given the very short latency and lack of an underlying field excitatory postsynaptic potential (EPSP), this action potential spike was most likely due to the antidromic stimulation and the subsequent synchronous firing of a population of dentate granule cells. The spike immediately following the stimulus artifact in field potential recordings was therefore called an antidromic population spike. Neither the amplitude of the antidromic population spike nor the shape of the waveform was used as criterion for acceptable recordings. Evoked responses were measured at a stimulus intensity sufficient to evoke a maximal response (200–700 µA) in both acute slices and slice cultures. Short-latency EPSP amplitudes (Fig. 8, C–E, right) were measured from baseline to peak positivity (see Fig. 8B, right). Short-latency EPSP durations (Fig. 8, C–E, left) were measured from the point immediately following the antidromic population spike to a point at 50% of the peak amplitude (excluding any spikes) during the decay phase of the EPSP (see Fig. 8B, left). Seizures were defined as a burst of rhythmic activity \(\geq 3\) s in duration that evolved over time and exhibited an abrupt onset and an abrupt termination (see Figs. 1 and 2). Recordings from slice culture experiments investigating seizure activity were analyzed independently by two investigators, and the percentages of cultures showing seizures were averaged; investigators agreed on 15 of 17 traces. Epileptic bursts were defined as bursts of rhythmic spikes or spikes superimposed on positive field potential shifts that were \(\geq 80\) ms in duration, but that did not fit the criteria for seizures (see Fig. 3A, BMI). Spontaneous postsynaptic potential (PSP) data were collected as two to six runs of 30 s duration per run (Fig. 3A). Spontaneous activity in acute slice experiments was monitored on an oscilloscope; no spontaneous activity was noted. PSPs were defined as waveforms that were blocked by antagonists of fast synaptic transmission (BMI + APV + CNQX or NBQX). Data were collected using an Axopatch 1D amplifier (2-kHz analog filter) and pCLAMP or Axotape (Figs. 1 and 2; 3.33- to 10-kHz acquisition rate) software.

Knife cuts between the dentate gyrus and different regions of the hippocampal formation were performed with a scalpel blade under a Reichert Stereo Zoom dissecting microscope 45 min to 1 h prior to electrophysiological experiments as depicted in Fig. 8A. To separate the CA1/CA2 regions of the hippocampus from the dentate gyrus (CA1 cut), two knife cuts were placed in a plane roughly parallel to the hippocampal fissure at the approximate border between stratum radiatum and stratum lacunosum-moleculare; knife cuts extended into stratum radiatum of CA3 and thus severed the Schaeffer collateral projection from CA3 to CA1. To separate the CA3/CA4 region of the hippocampus from the dentate gyrus (CA3 cut) one knife cut was placed at the open end of the dentate hilus in a straight line between the two blades of the granule cell layer. This CA3 cut extended through the CA1/CA2 pyramidal cell layer. CA3 cuts did not isolate the CA3c region from the remainder of the dentate gyrus. Combined cuts of CA1 and CA3 were used to isolate the dentate gyrus/CA3c region from the rest of the hippocampal formation (DG cut). Data from uncut and CA1, CA3, and the combination CA1/CA3 cut (DG...
incubated in ABC elite (Vector) diluted in PBS containing 2% BSA and 0.1% Triton X-100 according to kit instructions overnight at 4°C. Cultures were then rinsed, incubated in 0.05% 3,3’-diaminobenzidine (DAB, Sigma), 0.028% CoCl₂, and 0.020% nickel ammonium sulfate in PBS for 15 min, and treated with 0.05% DAB, 0.028% CoCl₂, 0.02% nickel ammonium sulfate, and 0.00075% H₂O₂ in PBS until staining was evident under an Axiovert 135 microscope at ×100 magnification. Cultures were then rinsed, mounted onto subbed glass slides, dehydrated, cleared in xylens, and coverslipped. The CA3c region was defined as the CA3 pyramidal cell layer located between the blades of the dentate granule cell layer. The CA3a/b region was defined as the CA3 pyramidal cell layer excluding the CA3c region. No attempt was made to differentiate CA1 from CA2. Camera lucida reconstructions were drawn using a Zeiss Axioskop microscope at ×250 magnification.

Statistical analysis

Numbers and error bars represent means ± SE in the stated number of slice cultures except where otherwise stated. All statistical analysis was performed with Sigma Stat software. Data fitting a nonparametric distribution were tested for significance using the Kruskal-Wallis ANOVA by ranks test with Dunn’s post hoc comparison when comparing multiple groups, Mann-Whitney rank sum test when comparing two experimental groups, or a z-test when comparing proportions. Data fitting a normal parametric distribution were tested for significance using a two-way ANOVA with least significance difference (LSD) post hoc comparison when comparing multiple groups or a t-test when comparing two experimental groups. Significance was defined as P < 0.05.

RESULTS

Hyperexcitability in slice cultures

DENTATE GRANULE CELLS IN HIPPOCAMPAL SLICE CULTURES WERE HYPEREXCITABLE. Spontaneous seizures. In the first series of experiments, field potential recordings from the dentate granule cell layer were conducted in an effort to detect the occurrence of spontaneous seizures.

Field potentials were recorded in the dentate granule cell layer for approximately 45 min (48 ± 2 min, mean ± SE) in physiological buffer. Despite the brevity of the recording period, electrographic seizures (Fig. 1) composed of negative spikes superimposed on a relatively flat baseline were observed in 22% of cultures (2 of 9 cultures). The average duration of seizures in these two cultures with seizures was 32 s. The common occurrence of spontaneous seizures detected in physiological buffer in a relatively brief recording period was consistent with a striking increase of excitability of these cultures.

To determine whether the propensity of granule cells to express seizures was under (γ-aminobutyric acid) GABAergic control, field potentials were recorded in the dentate granule cell layer for 46 ± 2 min following application of the GABA₂ receptor antagonist, BMI. In contrast to 22% of slices exhibiting spontaneous seizures in physiological buffer, inclusion of BMI triggered seizures in 75% of cultures (6 of 8 cultures). Unlike seizures observed in physiological buffer, BMI-induced seizures displayed both “tonic” and “clonic” phases (Traynelis and Dingledine 1988) and were observed as spikes superimposed on short-duration positive field potential shifts riding on a long-duration negative shift in baseline potential (Fig. 2). Also, the duration of BMI-induced seizures was 216 ± 106 s
(n = 6); a 675% increase in duration compared with seizures detected in physiological buffer. Interestingly, the percentage of slices showing tonic-clonic seizures (of the type illustrated in Fig. 2) following brief (1.0–2.5 min) or extended (45 min) application of BMI was similar (data not shown) and occurred only during wash-in of the antagonist. Recurrent BMI-induced seizures did not occur in any cultures during the recording period, although spontaneous interictal activity persisted. Spontaneous interictal bursts (Fig. 3A) were evident in 100% of cultures in the presence of BMI but were never observed in the absence of BMI. Spontaneous interictal bursts are likely to be composed of excitatory PSPs (EPSPs) because they were completely abolished following further addition of the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)/kainate (KA) receptor antagonist, NBQX (data not shown). These data show that dentate granule cell hyperexcitability and seizures were under GABAergic control.

Furthermore, although 100% of 3–5 DIV slice cultures (n = 4) displayed spontaneous, recurrent interictal bursts in the presence of BMI, no seizures were observed in these cultures, even at elevated recording temperatures (32–34°C). Thus there appears to be an ongoing process of dentate granule cell epileptogenesis in organotypic hippocampal slice cultures.

**Hilar-evoked population spikes.** The occurrence of spontaneous seizures and interictal bursts detected in dentate field potential recordings led us to assess the excitability of the granule cells themselves; initially the responses of the granule cells to hilar stimulation was investigated.

Based on recordings from acute hippocampal slices isolated from vehicle-treated rats (Cronin et al. 1992; Patrylo and Dudek 1998; Tauck and Nadler 1985; Wuarin and Dudek 1996), a single hilar stimulus was expected to evoke a single population spike in physiological buffer. However, electrical stimulation of the hilus elicited an antidromic population spike (Fig. 4, A–C, arrow) and a short-latency positive field potential shift (Fig. 4A, open arrowhead) recorded in the dentate granule cell layer in 55% of slice cultures (n = 28). These positive field potential shifts were likely to be PSPs because they could be blocked by antagonists of fast synaptic transmission (BMI + D-APV + CNQX or NBQX; see Fig. 5). This short-latency PSP was already evident in two of four slice cultures at 3–5 DIV and 17 of 20 slice cultures by 10–12 DIV (shortest DIV investigated; data not shown), implying that synaptic connections responsible for the PSP were present at this early time point in culture. In the remaining slice cultures, either a second population spike (21% of cultures; Fig. 4B, filled arrowhead) or multiple population spikes (24% of cultures; Fig. 4C, filled arrowhead) were seen immediately following the hilar-evoked population spike.

Eliciting hilar-evoked responses in the presence of BMI resulted in even more pronounced hyperexcitability. Whereas only 24% of cultures exhibited multiple population spikes in response to hilar stimulation in physiological buffer, multiple population spikes were observed in 86% of cultures (n = 21) in the presence of BMI. Inclusion of BMI also increased the mean duration of the short-latency evoked EPSP by eightfold (see Figs. 3B and 5, A and B, BMI; compare Fig. 8, C and D, left, no cut) and caused the appearance of longer latency hilar-evoked PSPs (evoked epileptiform bursting) in 92% of slice cultures (Figs. 3B and 5, A and B, BMI). These longer-latency evoked PSPs are likely to be EPSPs since they can be abolished by antagonists of excitatory glutamatergic transmission (see Fig. 5). These findings demonstrate the powerful control that GABA<sub>A</sub> receptor–mediated inhibition exerts over dentate granule cell hyperexcitability in hippocampal slice cultures.

**GLUTAMATERGIC TRANSMISSION WAS REQUIRED FOR HYPEREXCITABILITY.** Positive PSPs following the hilar-evoked population spike raised the possibility that excitatory glutamatergic synaptic transmission may contribute to granule cell hyperexcitability in slice cultures. Thus the contribution of N-methyl-D-aspartate (NMDA) and AMPA/KA receptor responses to evoked granule cell hyperexcitability in slice cultures was investigated.

**NMDA receptors.** The NMDA receptor antagonist D-APV was used to investigate the contribution of NMDA receptors to granule cell hyperexcitability in slice cultures. As described above, in physiological recording buffer, hilar stimulation elicited a short-latency EPSP immediately following the anti-
FIG. 5. Hilar-evoked PSPs and multiple population spikes require glutamate receptor activation. Extracellular field potentials were evoked using a stimulating electrode placed in the hilar mossy fiber pathway and recorded in the dentate granule cell layer as described in METHODS. A and B: representative traces show that application of BMI caused an increase in the duration of the short-latency EPSP and the appearance of longer-latency EPSPs and population spikes. A: addition of APV to recording buffer containing BMI decreased the duration of the short-latency EPSP, decreased the number of longer-latency EPSPs with superimposed population spikes, and altered the underlying waveform. Further addition of CNQX to recording buffer containing BMI and D-APV abolished both the short-latency and longer-latency EPSPs and population spikes (n = 7 slice cultures). B: addition of NBQX to recording buffer containing BMI did not significantly affect the underlying waveform but did abolish the longer-latency EPSPs with superimposed population spikes in 90% (n = 10 slice cultures) of cultures. Further addition of D-APV to recording buffer containing BMI and NBQX abolished the underlying waveform (n = 8 slice cultures). Stimulus artifacts were removed from all traces. APV, (−)-2-amino-5-phosphonopentanoic acid, 50 µM; BMI, bicuculline methiodide, 10 µM; CNQX, 6-cyano-7-nitroquinoline-2,3-dione, 10 µM; NBQX, 6-nitro-7-sulfamoylbenzof[l]quinoline-2,3-dione, 10 µM. Scale bars in B apply to all traces.

dromic population spike. In the presence of BMI, hilar stimulation yielded a short-latency EPSP and longer-latency epileptiform bursts of EPSPs with superimposed spikes. D-APV, when applied concurrently with BMI, significantly decreased the duration of the short-latency evoked EPSP (Fig. 5A, compare Fig. 8, D and E, left, no cut), altered the underlying waveform (Fig. 5A) and decreased the number of longer-latency hilar-evoked EPSPs with superimposed spikes by 47 ± 10% (Fig. 5A). The short- and longer-latency hilar-evoked EPSPs remaining after NMDA receptor blockade were dependent on AMPA/KA receptor activation as shown by blockade of these components by further addition of the AMPA/kainate receptor antagonist, CNQX (Fig. 5A). D-APV had similar effects on spontaneous interictal bursts but did not significantly affect their frequency (data not shown). Thus NMDA receptor activation contributes to granule cell hyperexcitability in slice cultures.

AMPA/KA receptors. The AMPA/KA receptor antagonist, NBQX, was used to investigate the contribution of AMPA/KA glutamate receptors to granule cell hyperexcitability in slice cultures. NBQX was chosen over other AMPA/KA receptor antagonists because of its high affinity for AMPA/KA receptors and low affinity for the glycine site on the NMDA receptor (Randle et al. 1992). Again, in physiological recording buffer, hilar stimulation elicited an antidromic population spike and short-latency EPSP. In the presence of BMI, hilar stimulation yielded a short-latency EPSP and longer-latency epileptiform bursts of EPSPs with superimposed spikes. NBQX, when applied concurrently with BMI, abolished the hilar-evoked longer-latency EPSPs with superimposed spikes in 90% of cultures (n = 10; Fig. 5B) but had no significant effect on the amplitude or duration of the short-latency hilar-evoked EPSP (Fig. 5B) or on the shape of the underlying waveform (Fig. 5B).

The short-latency evoked EPSP and long-latency negative waveform remaining following AMPA/KA receptor blockade required NMDA receptor activation; both were abolished by further addition of D-APV in all slice cultures (Fig. 5B). NBQX abolished spontaneous EPSPs in all cultures (data not shown). Thus our data clearly demonstrate that glutamatergic transmission mediated through both NMDA and AMPA/KA receptors is required for the expression of the hyperexcitability seen in slice cultures following hilar stimulation.

Two interesting findings emerge from these results. First, activation of NMDA receptors is required for the long-latency hilar-evoked negative waveform. Whether the D-APV–sensitive long-latency negative waveform represents a NMDA receptor–generated conductance or a conductance activated secondarily to NMDA receptor activation is uncertain. Second, whereas NMDA receptor blockade decreased the duration of the short-latency hilar-evoked EPSP, pharmacological blockade of either AMPA/KA nor NMDA receptors alone was sufficient to decrease the amplitude of this waveform (Fig. 5). Thus activation of either AMPA/KA or NMDA receptors is sufficient to account for the short-latency hilar-evoked EPSP. Thus AMPA/KA and NMDA glutamate receptors have distinct, yet related roles in the expression of dentate granule cell hyperexcitability in hippocampal slice cultures.

Possible mechanisms underlying granule cell hyperexcitability

Collectively, our data provide evidence for granule cell hyperexcitability in hippocampal slice cultures in comparison to slices acutely isolated from normal rats. Neither spontaneous seizures nor interictal bursting have been reported in slices acutely isolated from normal rats, even in the presence of BMI (Cronin et al. 1992; Patrylo and Dudek 1998; Wuarin and Dudek 1996). Similarly, hilar-evoked PSPs and multiple population spikes have not been reported in acute slices from normal rats, even following GABA<sub>A</sub> receptor blockade (Cronin et al. 1992; Patrylo and Dudek 1998; Tauck and Nadler 1985; Wuarin and Dudek 1996). Given the dependence of hilar-evoked EPSPs on glutamatergic transmission in slice cultures, we hypothesized that newly formed excitatory glutamatergic synaptic inputs to dentate granule cells may contribute to granule cell hyperexcitability. However, granule excitability also could be influenced by small differences in ion concentration, oxygenation, osmolality, etc., which may be different in our recording conditions compared with conditions used by investigators performing similar experiments in acute slices. Another possibility is that the membrane properties of
granule cells may be different in slice cultures and acute slices. To begin to assess the possible mechanisms contributing to increased granule cell excitability in long-term slice cultures, we investigated each of these possibilities.

**RECORDING CONDITIONS DID NOT PRODUCE GRANULE CELL HYPEREXCITABILITY.** To investigate the possibility that our recording conditions promote granule cell hyperexcitability, granule cell responses were measured in acute hippocampal slices under conditions identical to those used for slice culture recordings. Similar to previous reports (Cronin et al. 1992; Patrylo and Dudek 1998; Tauck and Nadler 1985; Wuarin and Dudek 1996), in both physiological buffer and in the presence of BMI, no seizures or interictal spikes were observed (data not shown), and hilar stimulation elicited a sole antidromic population spike with no PSPs (Fig. 6). Furthermore, glutamate receptor antagonists had no effect on hilar-evoked responses (Fig. 6). Thus our recording conditions were not sufficient to account for granule cell hyperexcitability in slice cultures.

**ALTERED MEMBRANE PROPERTIES CANNOT ACCOUNT FOR GRANULE CELL HYPEREXCITABILITY.** To investigate the possibility that the membrane properties of dentate granule cells may be different in slice cultures and acute slices, whole cell current-clamp recordings were performed. Average RMP of dentate granule cells in hippocampal slice cultures was $-66 \pm 8 \text{ mV}$ ($n = 10$), consistent with the RMP of $-67 \text{ mV}$ measured under similar recording conditions in acutely prepared hippocampal slices (Edwards et al. 1989). Input resistance ($R_{in}$) of granule cells in slice cultures was $135 \pm 11 \text{ M\Omega}$ ($n = 11$), a result not significantly different ($t$-test; $P > 0.05$) from the $186 \pm 23 \text{ M\Omega}$ reported by Staley and colleagues (1992) when a calcium chelator was included in the patch pipette solution (our pipette solution contained the calcium chelator EGTA). Action potential spike threshold for granule cells in slice cultures was $-44 \pm 1.2 \text{ mV}$ ($n = 9$), which is significantly higher than the $-49 \pm 0.3 \text{ mV}$ previously reported for granule cells in acute hippocampal slices (Staley et al. 1992). However, a higher spike threshold should serve to make granule cells in hippocampal slice cultures less excitable than granule cells in acute hippocampal slices. Thus alterations in dentate granule cell membrane properties cannot account for granule cell hyperexcitability in hippocampal slice cultures.

**ANATOMICAL REARRANGEMENTS IN SLICE CULTURES CONTRIBUTE TO GRANULE CELL HYPEREXCITABILITY.** Morphology. To investigate the possibility that newly formed excitatory glutamatergic synaptic inputs to dentate granule cells may contribute to granule cell hyperexcitability, single CA1 pyramidal cells, CA3 pyramidal cells, and dentate granule cells were filled with neurobiotin, and axonal projections of individual neurons were traced.

Axons from most CA1 pyramidal cells (7/9) projected toward the subiculum and/or CA3a/b but remained within the CA1 region (Fig. 7A). However, in 22% of CA1 pyramidal cells (2/9), axon collaterals were detected within the CA3 pyramidical cell layer and the dentate gyrus (Fig. 7B) including the hilus, granule cell, and dentate molecular layers, suggesting that a subpopulation of CA1 pyramidical cells may synapse onto granule cells, CA3 pyramidical cells, and/or hilar neurons. CA1 pyramidical cell dendrites were confined mainly to the CA1 region (Fig. 7A); however, in 22% of CA1 pyramidical cells, dendrites were seen in the dentate molecular layer (not shown).

Thus any neurons that project axons to the molecular layer could synapse onto CA1 pyramidical cell dendrites. These data are suggestive of synapses between CA1 pyramidical cell and neurons in the dentate gyrus.

All ($n = 10$) CA3c pyramidical cells sent axons to str. oriens, pyramidale, and radiatum of CA3a/b and dentate hilus (Fig. 7C). Axon collaterals also were observed in str. radiatum and pyramidale of CA1 (60% of CA3c pyramidical cells; Fig. 7C); collaterals traversed the hippocampal fissure and/or CA3 to reach CA1. In most (90%) CA3c pyramidical cells, axon collaterals were seen in the granule cell layer; in 40%, axon collaterals were detected in the dentate molecular layer (Fig. 7C). These anatomical data suggest that CA3c pyramidical cells could synapse onto most principal cell types in slice cultures. Dentrites of CA3c pyramidical cells were confined to the hilus and subgranular zone (Fig. 7C). Thus neurons that project to the hilus could form synapses with CA3c pyramidical cell dendrites; possible sources include granule cells, hilar neurons, other
CA3 pyramidal cells, and, as shown above, CA1 pyramidal cells. These data suggest direct reciprocal synapses between CA3c pyramidal cells and granule cells and/or a polysynaptic circuit from CA3c pyramidal cells to granule cells via a CA1 pyramidal cell intermediate.

All \((n = 4)\) CA3a/b pyramidal cells projected axons to str. radiatum of CA1 and all layers of CA3a/b/c (Fig. 7D). In 50% of CA3a/b pyramidal cells, axon collaterals were detected within the hilus; in a single CA3 a/b pyramidal cell, collaterals were seen in the granule cell layer (Fig. 7D). These data suggest that, like CA3c pyramidal cells, CA3a/b pyramidal cells could synapse onto most principal cell types in the slice cultures. CA3a/b pyramidal cell dendrites were confined to the CA3 region, a known projection area for granule cells and other CA3 pyramidal cells. These data are suggestive of direct reciprocal synapses between CA3a/b pyramidal cells and granule cells and/or a polysynaptic circuit from CA3a/b pyramidal cells to granule cells via a CA3c or CA1 pyramidal cell intermediate.

Granule cell axons were confined mainly to CA3, the hilus, and str. granulosum and moleculare of the dentate gyrus (Fig. 7, E and F). However, in one granule cell (1/5), an axon collateral was detected within str. lacunosum-moleculare of CA1 (not shown), suggesting the possibility that granule cells could synapse directly onto CA1 pyramidal cells. In all slice cultures, granule cell dendrites were observed to cross the hippocampal fissure and invade str. lacunosum-moleculare (Fig. 7, E and F), a region that is normally devoid of axonal projections from other hippocampal principal cells. However, as shown above, a subset of CA1 pyramidal cells send axon collaterals through str. lacunosum-moleculare, suggesting that CA1 pyramidal cells could synapse onto granule cells in this region under slice culture conditions.

Taken together, anatomical data (present study; Caeser and Aertsen 1991; Frotscher and Gahwiler 1988; but see Zimmer and Gahwiler 1984) suggest that granule cells may form direct (or polysynaptic) reciprocal synaptic connections with neurons in all hippocampal subfields including CA3 a/b, CA3c, and CA1, as well as other with other granule cells and hilar neurons in slice cultures.
Electrophysiology with knife cuts. To examine the possibility that these newly formed synapses were functional and thereby contribute to granule cell hyperexcitability in normal organotypic hippocampal slice cultures, granule cell responses to hilar stimulation were recorded in the absence and presence of knife cuts disconnecting CA1 and/or CA3 regions from the dentate gyrus. Extracellular field potentials were evoked using a stimulating electrode placed in the hilar mossy fiber pathway and recorded in the dentate granule cell layer in (C) physiological buffer, (D) in the presence of BMI or (E) in the presence of BMI and APV as described in Methods. The amplitude (C–E, right) and duration (C–E, left) of the short-latency evoked EPSP were measured following acute knife cuts to separate the dentate granule cell layer from other hippocampal subregions. A: acute knife cuts were performed just prior to recording as described in Methods and were placed between the dentate gyrus and the CA1 (CA1 cut) or CA3 (CA3 cut) regions of the hippocampus. A combination of the CA1 and CA3 cuts (DG cut) was used to completely isolate the dentate gyrus from the rest of the hippocampal formation. B: short-latency EPSP durations were measured from the point immediately following the antidromic population spike to a point at 50% of the peak amplitude (excluding any spikes) during the decay phase of the EPSP. Short-latency EPSP amplitudes were measured from baseline to peak positivity. C: in physiological buffer, the CA1, CA3, and DG cuts all decreased both the duration and the amplitude of the short-latency EPSP. D: in the presence of BMI, the CA1 and DG cuts decreased the duration of the short-latency EPSP, while only the DG cut decreased the amplitude of the short-latency EPSP. E: in the presence of BMI and t-APV, CA1, and DG cuts decreased t-APV effects on short-latency EPSP duration, but did not alter t-APV effects on amplitude. Statistics: numbers and error bars represent the mean ± SE in the stated number of slice cultures. * Significantly different from no cut. ** Significantly different from CA3 cut (ANOVA with LSD post hoc comparison; \( P < 0.05 \)). ACSF, artificial cerebrospinal fluid (physiological buffer); APV, 50 \( \mu \)M. Scale bars in B apply to both traces.
CA1, CA3, and the combination CA1/CA3 (DG) cuts all decreased both the duration and the amplitude of the short-latency EPSP (Fig. 8C). These data suggest that synaptic connections from neurons extrinsic to the dentate gyrus/CA3c region contribute to the short-latency hilar-evoked EPSP recorded in the granule cell layer of hippocampal slice cultures.

GABAergic transmission exerts powerful control over hyperexcitability and excitatory transmission; blockade of GABA_{A} receptors may unmask synaptic connections not detected in physiological buffer. Therefore the effects of acute knife cuts on the short- and longer-latency evoked EPSPs in the presence of the GABA_{A} receptor antagonist, BMI, were investigated to further identify the neurons responsible for generating these events. The CA1 cut decreased the duration but did not significantly affect the amplitude of the short-latency EPSP evoked by hilar stimulation (Fig. 8D). Surprisingly, the CA3 cut had no significant effect on either the duration or the amplitude of the short-latency EPSP (Fig. 8D). The combination CA1/CA3 cut (DG cut) decreased the duration by about 75% and the amplitude by 40% (Fig. 8D).

The effects of knife cuts on BMI-induced longer-latency EPSPs were similar to those observed for the short-latency EPSP. The number of longer-latency evoked EPSPs was partly, but significantly decreased from a median of 5.5 in uncut cultures to 3.5 (−36%) following the CA1 cut and to 3 (−46%) following the combination CA1/CA3 cut (DG cut; Kruskal-Wallis ANOVA by ranks test with Dunn’s post hoc comparison; \( P < 0.05; n = 24 \) slice cultures, no cut; \( n = 26, \) CA1 cut; \( n = 20, \) DG cut). The CA3 cut had no significant effect on the number of longer-latency evoked EPSPs [median of 4 (−27%); \( n = 19 \) slice cultures]. These data again suggest that synaptic connections from neurons extrinsic to the dentate gyrus/CA3c region contribute to hilar-evoked EPSPs recorded in the granule cell layer in hippocampal slice cultures, but that a major source also may originate from neurons intrinsic to the dentate gyrus/CA3c region.

The long duration of the short-latency field EPSP recorded in the presence of BMI suggests summation of multiple delayed synaptic inputs and/or glutamatergic transmission mediated primarily by NMDA receptors. [Blockade of NMDA receptors dramatically decreased the duration of the short-latency EPSP (Fig. 5A); blockade of AMPA/KA receptors had no significant effect on this measure (Fig. 5B).] In an attempt to distinguish between these two possibilities, the contribution of NMDA receptors to the short-latency EPSP was investigated before and after knife cuts. In uncut cultures, the NMDA receptor antagonist, \( \alpha - \text{APV}, \) decreased the duration of the short-latency EPSP by approximately 50% but had little effect on amplitude (12% reduction; Fig. 8E). Knife cuts did not alter the lack of \( \alpha - \text{APV} \) effects on amplitude (Fig. 8E). However, both the CA1 cut and the combination CA1/CA3 (DG) cut decreased the effect of \( \alpha - \text{APV} \) on short-latency EPSP duration by about 40–50%, respectively (Fig. 8E), suggesting that the CA1 cut severed a synaptic circuit with a large NMDA receptor-mediated component. The CA3 cut did not significantly decrease \( \alpha - \text{APV} \) effects on short-latency EPSP duration. Thus the long duration of the short-latency field EPSP appears to be mediated by summation of multiple delayed synaptic inputs from neurons intrinsic to the dentate gyrus and by glutamatergic transmission mediated primarily by NMDA receptors from neurons in CA1.

Taken together, data from physiological experiments using acute knife cuts suggest that synaptic glutamatergic connections arising from neurons in the CA1 and CA3a/b regions of the hippocampus as well as neurons intrinsic to the dentate gyrus/CA3c region contribute to hilar-evoked EPSPs and that CA1 to granule cell synapses exhibit a relatively large NMDA receptor component. These physiological data combined with the anatomical findings provide strong evidence that reciprocal excitatory synapses among dentate granule cells as well as between dentate granule cells and CA1/CA3 pyramidal cells contribute to dentate granule cell hyperexcitability in long-term organotypic hippocampal slice cultures.

DISCUSSION

Field potential recordings of dentate granule cells were performed in organotypic hippocampal slices isolated at P11 and maintained long term (40–60 days) in vitro. The principal findings are the following. Relatively brief recordings conducted in physiological buffer disclosed spontaneous seizures recruiting granule cells in 22% of cultures; recordings conducted in the presence of the GABA_{A} receptor antagonist, BMI, documented seizures in 75% of cultures. Hilar stimulation evoked PSPs and multiple population spikes in both physiological recording buffer and in the presence of BMI. PSPs were eliminated by a combination of NMDA and AMPA/KA receptor antagonists, demonstrating the requirement for excitatory synaptic transmission. These findings stand in sharp contrast to the lack of seizures and to the isolated hilar-evoked population spike without PSPs observed in acute hippocampal slices isolated from normal rats under identical recording conditions. A combination of anatomical and physiological approaches provided strong evidence that newly formed excitatory synaptic connections to the dentate gyrus contribute to granule cell hyperexcitability in slice cultures. Anatomical labeling of individual pyramidal cells in CA3 and CA1 demonstrated axonal projections to the dendritic regions of the dentate granule cells; likewise labeling of individual dentate granule cells disclosed axonal projections to both CA3 and CA1 as well as to dendritic regions of the granule cells themselves. Physiological granule cell responses to hilar stimulation recorded in the absence and presence of knife cuts disconnecting CA1 and/or CA3 regions from the dentate gyrus suggested that these newly formed synapses were functional. Knife cuts severing connections between CA1 and the dentate gyrus greatly reduced measures of synaptic transmission with smaller reductions evident after severing connections between CA3a/b and dentate gyrus. Remarkably, some residual enhanced response of the granule cells to hilar stimulation persisted even after isolation of the dentate gyrus from both CA1 and CA3 a/b. Taken together, these data are consistent with the proposal that reciprocal excitatory synaptic connections among dentate granule cells on the one hand and between dentate granule cells and pyramidal cells in CA3 and CA1 on the other contribute to the striking hyperexcitability of the dentate granule cells in long-term organotypic hippocampal slice cultures. In support of this interpretation, a paper (Gutierrez and Heinemann 1999) appeared during preparation of this manuscript that also provided evidence for functional synaptic pathways between CA1 pyramidal cells and granule cells as well as between granule cells themselves in organotypic hippocampal slice cultures.
Dentate granule cell seizures

The dentate granule cells normally limit invasion of seizure activity into the hippocampus, a function that may be pivotal in preventing epileptic activity in limbic circuitry. Our findings demonstrate that long-term culture of organotypic hippocampal slices reliably produces seizures that recruit dentate granule cells. A diversity of in vitro models of temporal lobe seizures have been described in the past 10–15 yr (Anderson et al. 1986; Avoli et al. 1996; Benedikz et al. 1993; Bragdon et al. 1992; Bruckner et al. 1999; Gutierrez et al. 1999; Jensen and Yaari 1988; Konnerth et al. 1986; McBain et al. 1989; Merlin 1999; Muller 1993; Ogata 1978; Richardson and O’Reilly 1995; Routbort et al. 1999; Schwartzkroin and Prince 1977; Sombati and DeLorenzo 1995; Swann et al. 1993; Tancredi and Avoli 1987; Traynelis and Dingledine 1988). In most instances, seizures are triggered by subjecting a hippocampal slice acutely isolated from a normal animal to some intervention (e.g., ionic manipulation, GABA_A receptor antagonist, etc.). Most often these seizures involve CA3 and/or CA1 pyramidal cells. Only two reports disclose seizures recruiting the seizure-resistant dentate granule cells in acute hippocampal slices; following 4-aminopyridine induced seizures in entorhinal cortex (Avoli et al. 1996) and during low-calcium perfusion combined with exposure to electric fields (Richardson and O’Reilly 1995). Moreover, perfusion of 7–8 DIV hippocampal slice cultures with solutions containing low magnesium evoked seizures involving the dentate granule cells (Gutierrez et al. 1999). By contrast, we report the occurrence of isolated seizures arising spontaneously in brief recordings (45 min) conducted in physiological buffer (e.g., without ionic manipulations or convulsants agents). The occurrence of spontaneous seizures in the present study is all the more surprising because the recordings were performed at 27–29°C, and reduction of temperature from 33 to 28°C has been shown to exert powerful anti-seizure effects in hippocampal slices (Traynelis and Dingledine 1988). The onset of seizures in 75% of cultures within minutes of addition of BMI underscores the powerful effects of GABA_A-mediated inhibition in control of dentate granule cell excitability in these slice cultures. Why addition of BMI to hippocampal slice cultures elicited an isolated seizure rather than recurrent seizures is unclear. However, this finding is consistent with similar data obtained in CA1 following BMI application to hippocampal slice cultures (Scanziani et al. 1994).

Comparison of hippocampal slice cultures with in vivo models and human temporal lobe epilepsy

A critical issue raised by this study is whether abnormalities evident in long-term hippocampal slice cultures are similar to abnormalities in animal models or humans with limbic epilepsy. Indeed, striking parallels emerge.

DENTATE GRANULE CELL SEIZURES AND HYPEREXCITABILITY. However, one notable difference between hippocampal slice cultures and acute slices isolated from animal models or humans with temporal lobe epilepsy is the incidence of seizures involving the granule cells. In our study, relatively brief recordings conducted in physiological buffer disclosed spontaneous seizures including granule cells in 22% of cultures; recordings conducted in the presence of the GABA_A receptor antagonist, BMI, documented seizures in 75% of cultures. These findings contrast sharply with previous reports from numerous laboratories in which investigators recorded extra-cellular granule cell layer field potentials in acute slices isolated from rat or resected human epileptic hippocampus (Cronin et al. 1992; Franck et al. 1995; Isokawa and Fried 1996; Isokawa et al. 1991, 1997; Masukawa et al. 1991; Molnar and Nadler 1999; Okazaki et al. 1999; Patrylo and Dudek 1998; Tauck and Nadler 1985; Urban et al. 1990; Williamson et al. 1995). Surprisingly, none of these investigators reported seizures involving granule cells in either physiological buffer or physiological buffer containing GABA_A receptor antagonists. Several factors could explain this discrepancy including the following: recording duration, maintenance of neuronal network connections, different gap junction coupling, and/or altered ionic homeostasis. The contributions of all factors in a subset of slice culture may be sufficient for seizure initiation; whereas, in acute slices from epileptic hippocampus, these parameters may need to be manipulated. In validation of this idea, Dudek and colleagues (Patrylo and Dudek 1998; Wuarin and Dudek 1996) have reported spontaneous seizures in a subset of acute slices prepared from KA-treated epileptic rats following perfusion with a GABA_A receptor antagonist and elevated extracellular K⁺ (6–9 mM). We are unaware of any similar studies using slices isolated from humans with limbic epilepsy or, indeed, of any reports of spontaneous seizures recruiting granule cells in slices isolated from humans with limbic epilepsy. Such a multifactorial mechanism would likely yield a large degree of variability and heterogeneity in physiological responses and may explain why seizures were observed in only a subset of slice cultures in our study. Once the various mechanisms contributing to dentate granule cell seizures in hippocampal slice cultures are elucidated, this information can guide subsequent investigations into the operative factors contributing to temporal lobe epilepsy in humans and animal models.

Dentate granule cell hyperexcitability in hippocampal slice cultures exhibits many similarities to granule cell hyperexcitability in acute slices isolated from epileptic rat and resected human epileptic hippocampus. Treatment of rats with KA produces limbic status epilepticus that persists for hours; many of these animals develop spontaneous seizures beginning weeks later. Equivalent to findings reported here (Figs. 3 and 4), in physiological buffer, hilar stimulation of hippocampal slices isolated from kainate-treated rats produces multiple population spikes in a small percentage of slices. Following blockade of GABA_A receptors, a greater percentage of these slices exhibits multiple population spikes, and bursts of EPSPs are unmasked (Cronin et al. 1992; Patrylo and Dudek 1998; Tauck and Nadler 1985; Wuarin and Dudek 1996). By contrast, in both physiological buffer and following GABA_A receptor blockade, hilar stimulation of slices from normal rats produces a single antidromic population spike in most slices. Although hilar-evoked short-latency granule cell field PSPs (Figs. 3B, 4, and 5) have not been reported in slices isolated from KA-treated rats, two groups (Okazaki et al. 1999; Wuarin and Dudek 1996) did observe a greater incidence of hilar-evoked EPSP/Cs recorded in single granule cells in slices isolated from KA-treated rats compared with controls. Also similar to our results (Fig. 5), these hilar-evoked EPSP/Cs were abolished with a combination of AMPA/KA and NMDA receptor antagonists (Okazaki et al. 1999; Patrylo and Dudek 1998). Hilar stimulation evoked responses in the granule cell layer of slices isolated from humans with limbic epilepsy also were
remarkably similar to findings presented in our study. Masukawa et al. (1992) reported that, in physiological buffer, hilar stimulation evoked multiple population spikes in acute slices from 5 of 12 patients with limbic epilepsy; by contrast, hilar stimulation of 1 control evoked a single population spike without accompanying PSPs. Recording from single granule cells, Franck et al. (1995) reported that, in physiological buffer, hilar stimulation evoked burst discharges in slices isolated from one of nine patients with limbic epilepsy; following partial blockade of GABA_A receptors, slices from five of nine patients displayed burst discharges. Thus numerous similarities in the expression of granule cell hyperexcitability exist between long-term hippocampal slice cultures and acute slices isolated from epileptic human/rat hippocampus.

MORPHOLOGICAL ABNORMALITIES. Some of the morphological abnormalities reported here also have been described in animal models and humans with limbic epilepsy. Despite deafferentation, hippocampal slice cultures have been reported to retain relatively normal cytoarchitecture and synaptic connectivity (Daily et al. 1994; Frotscher and Heinrich 1993, 1995; Gahwiler et al. 1997; Li et al. 1993, 1994; Robain et al. 1994; Stoppini et al. 1993, 1997; Zimmer and Gahwiler 1987). However, recent studies (Gahwiler et al. 1997; Pavlidis and Madison 1999) estimate that these normal synaptic connections are increased 10 times relative to acute slices. Similar expansions of normal synapses have been reported in epileptic rat and human hippocampus. Indeed, the most extensively studied synaptic rearrangement in the epileptic rat and human hippocampus is mossy fiber sprouting, an expansion of a small normal projection (de Lanerolle et al. 1989; Houser et al. 1990; Laurberg and Zimmer 1981; Mello et al. 1992; Molnar and Nadler 1999; Okazaki et al. 1995; Represa et al. 1993; Ribak and Peterson 1991; Seress 1992; Sutula et al. 1988, 1989; Tauck and Nadler 1985). A slight mossy fiber expansion is also present in long-term hippocampal slice cultures (Roubort et al. 1999). By analogy, our findings in slice culture suggest that the small CA3 pyramidal cell to dentate granule cell projection found in normal rat (Ishizuka et al. 1990; Li et al. 1994; Scharfman 1993, 1994a,b) may be expanded in the epileptic hippocampus. This hypothesis remains to be addressed in rat and human.

In addition to making normal synapses, neurons in slice culture also may form aberrant synapses, which may include the reciprocal CA1 pyramidal cell to dentate granule cell synapse suggested by our results. Although mossy fiber innervation of CA1 does exist in other species (Gaarskjaer et al. 1982; Laurberg and Zimmer 1980; Steward 1992), this projection has not been documented in normal rat or human. Mossy fiber innervation of CA1/CA2 has been reported, however, in rat following partial neurotoxic destruction of CA3 (Cook and Crutcher 1985) and more importantly, in the human epileptic hippocampus (Babb et al. 1992; Houser et al. 1990; Williamson and Spencer 1994). In a similar fashion, although CA1 pyramidal cell innervation of the dentate gyrus has never been documented, this projection may occur in the epileptic hippocampus. Previous studies documenting synaptic rearrangements of CA1 pyramidal cells in KA-treated rats (Esclapez et al. 1999; Perez et al. 1996) did not report CA1 pyramidal cell innervation of the dentate gyrus. However, the KA model may not represent all changes associated with human temporal lobe epilepsy. Whether CA1 pyramidal cells innervate the dentate gyrus in human epileptic hippocampus remains to be addressed.

While a dominant focus on granule cell hyperexcitability in the epileptic hippocampus has centered on reciprocal synaptic connections between granule cells mediated by sprouted mossy fiber axons, the present results raise a host of alternative sources of excitatory synaptic inputs to the granule cells in a synaptically reorganized hippocampus. Indeed, the list of synaptic rearrangements associated with seizures includes sprouting of granule cells (Houser et al. 1990; Mello et al. 1992; Nadler et al. 1980; Sutula et al. 1988, 1989; Tauck and Nadler 1985), CA1 pyramidal cells (Bausch and McNamara 1999; Bausch et al. 1998; Esclapez et al. 1999; Perez et al. 1996), and possibly GABAergic neurons (Bausch and Chavkin 1997; Davenport et al. 1990; Mathern et al. 1997). Our data suggesting possible CA1 and CA3 pyramidal cell projections to the dentate granule cell layer broadens the spectrum of possible synaptic rearrangements and provides a framework for investigating whether similar rearrangements exist in the epileptic hippocampus of humans.

Hippocampal slice cultures as a model to investigate mechanisms underlying synaptic rearrangements and epileptogenesis

The in vitro long-term hippocampal slice culture model provides a simple system to begin to elucidate the molecular and cellular mechanisms underlying synaptic rearrangements and epileptogenesis. Currently, the only avenue available to investigate these mechanisms is in vivo because acute in vitro slices are short-lived and slice preparation severs many axonal projections. However, in vivo studies are hampered by slow data collection, the inability to control reagent concentration in the brain and unwanted side effects caused by systemically administered reagents. In contrast to in vivo studies, the external environment of slice cultures can be altered easily without unwanted systemic side effects and, unlike acute in vitro slices, hippocampal slice cultures can be maintained for weeks to months. Thus the hippocampal slice culture preparation is an attractive alternative to in vivo and acute in vitro preparations.

Despite the prominent advantages offered by slice cultures, potential disadvantages are also evident. Hippocampal slice cultures are subjected to trauma, cell death, and deafferentation from extrahippocampal regions during slice preparation, are bathed in culture media rather than cerebral spinal fluid, and are covered in a prominent glial layer. Such differences from in vivo conditions may alter neuronal activity/excitability, dendritic and/or axonal morphology, synapse formation, synaptic properties, and the molecular bases for these functional and structural modifications as well as protein expression and/or protein-protein interactions. These disadvantages notwithstanding, slice cultures provide a relatively simple chronic in vitro model of limbic epileptogenesis; it seems likely that some commonalities exist between limbic epileptogenesis in vivo and in this in vitro preparation. Thus while the long-term in vitro hippocampal slice culture model does not obviate the need for in vivo animal models, slice cultures can provide a relatively simple first step in investigating the cellular and molecular mechanisms underlying epileptogenesis and synaptic rearrangements.

In summary, long-term organotypic hippocampal slice cul-
tures provide a chronic in vitro model of limbic epilepsy with striking electrophysiological and morphological similarities to both human and in vivo models. This in vitro model provides a simple, useful system to begin to elucidate the molecular and cellular mechanisms underlying synaptic rearrangements and epileptogenesis, which will bring us one step closer toward developing novel new therapies for temporal lobe epilepsy.

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