Recurrent Excitatory Connectivity in the Dentate Gyrus of Kindled and Kainic Acid–Treated Rats

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Lynch, Michael and Thomas Sutula. Recurrent excitatory connectivity in the dentate gyrus of kindled and kainic acid-treated rats. J. Neurophysiol. 83: 693–704, 2000. Repeated seizures induce mossy fiber axon sprouting, which reorganizes synaptic connectivity in the dentate gyrus. To examine the possibility that sprouted mossy fiber axons may form recurrent excitatory circuits, connectivity between granule cells in the dentate gyrus was examined in transverse hippocampal slices from normal rats and epileptic rats that experienced seizures induced by kindling and kainic acid. The experiments were designed to functionally assess seizure-induced development of recurrent circuitry by exploiting information available about the time course of seizure-induced synaptic reorganization in the kindling model and detailed anatomic characterization of sprouted fibers in the kainic acid model. When recurrent inhibitory circuits were blocked by the GABA_A receptor antagonist bicuculline, focal application of glutamate microdrops at locations in the granule cell layer remote from the recorded granule cell evoked trains of excitatory postsynaptic potentials (EPSPs) and population burst discharges in epileptic rats, which were never observed in slices from normal rats. The EPSPs and burst discharges were blocked by bath application of 1 μM tetrodotoxin and were therefore dependent on network-driven synaptic events. Excitatory connections were detected between blades of the dentate gyrus in hippocampal slices from rats that experienced kainic acid–induced status epilepticus. Trains of EPSPs and burst discharges were also evoked in granule cells from kindled rats obtained after ≥1 wk of kindled seizures, but were not evoked in slices examined 24 h after a single afterdischarge, before the development of sprouting. Excitatory connectivity between blades of the dentate gyrus was also assessed in slices deafferented by transection of the perforant path, and bathed in artificial cerebrospinal fluid (ACSF) containing bicuculline to block GABA_A receptor–dependent recurrent inhibitory circuits and 10 mM [Ca^{2+}]_o to suppress polysynaptic activity. Low-intensity electrical stimulation of the infrapyramidal blade under these conditions failed to evoke a response in suprapyramidal granule cells from normal rats (n = 15), but in slices from epileptic rats evoked an EPSP at a short latency (2.59 ± 0.36 ms) in 5 of 18 suprapyramidal granule cells. The results are consistent with formation of monosynaptic excitatory connections between blades of the dentate gyrus. Recurrent excitatory circuits developed in the dentate gyrus of epileptic rats in a time course that corresponded to the development of mossy fiber sprouting and demonstrated patterns of functional connectivity corresponding to anatomic features of the sprouted mossy fiber pathway.

INTRODUCTION

Mossy fiber axons in the dentate gyrus undergo sprouting and reorganization of their terminal field in experimental models of epilepsy and in the human epileptic temporal lobe (de Lanerolle et al. 1989; Houser et al. 1990; Mikkonen et al. 1998; Represa et al. 1989; Sutula et al. 1988, 1989; Tauck and Nadler 1985). In epileptic rats, sprouted axon collaterals of granule cells project from the hilus of the dentate gyrus into the supragranular layer in both transverse and longitudinal directions along the septotemporal axis (Buckmaster and Dudek 1999; Sutula et al. 1998). Mossy fiber axons from granule cells in the infrapyramidal blade of the dentate gyrus cross the hilus and form synapses in the supragranular layer of the suprapyramidal blade (Sutula et al. 1998). Reorganization of the mossy fiber pathway also occurs in the hilus of the dentate gyrus of epileptic rats, where mossy fibers form more small terminal boutons, and have fewer giant boutons and greater axon length compared with normal rats (Buckmaster and Dudek 1999; Sutula et al. 1998). Axonal remodeling and synaptic reorganization of mossy fiber axons that alters connectivity and local circuits within the hilus and supragranular layer in the dentate gyrus may modify functional properties of the dentate gyrus and influence epileptogenesis, seizure propagation, and memory. Current source density analysis has demonstrated that the terminal field of the sprouted mossy fiber pathway in the supragranular layer of the dentate gyrus is the site of an abnormal inward current that is consistent with synaptic transmission in the sprouted pathway (Golarai and Sutula 1996), but the functional significance of this seizure-induced plasticity is uncertain.

Recurrent excitatory connections are absent or minimal in the normal dentate gyrus, but are prominent in other regions of the hippocampus or in the neocortex that are susceptible to epileptic synchronization (Clai borce et al. 1986; Deuchars and Thomson 1996; Deuchars et al. 1994; MacVicar and Dudek 1980; Miles and Wong 1986). Seizure-induced formation of recurrent excitatory circuits by sprouted mossy fibers in the dentate gyrus could potentially contribute to epileptogenesis by increasing excitatory drive. Because strong systems of inhibition in the dentate gyrus and formation of recurrent inhibitory circuits by sprouted mossy fibers may mask or suppress activity in excitatory circuits, it is not surprising that seizure-induced recurrent excitation may not be detected unless GABA_A receptor–mediated inhibition is blocked, or in conditions such as use-dependent fading of inhibitory postsynaptic potentials/inhibitory postsynaptic currents (IPSPs/IPSCs) (Deisz and Prince 1989; McCarren and Alger 1985). Because sprouting increases with repeated seizures (Cavazos et al. 1991), it would also be anticipated that evidence for recurrent
excitatory circuits would be more easily detectable after many repeated seizures.

In previous studies, focal application of glutamate to the granule cell layer evoked long-latency trains of excitatory postsynaptic potentials (EPSPs) in granule cells from rats that had experienced status epilepticus induced by treatment with kainic acid or pilocarpine, but not in granule cells from normal rats (Molnar and Nadler 1997; Wuarin and Dudek 1996, 1997). Variable latency burst discharges and excitatory postsynaptic currents (EPSCs) are more frequently evoked in granule cells by antidromic activation of the mossy fiber pathway in hippocampal slices from rats that experienced status epilepticus than in controls, which suggests that mossy fiber sprouting could be contributing to recurrent excitation (Cronin et al. 1992; Okazaki et al. 1999; Patrylo and Dudek 1998; Tauck and Nadler 1985). These results are consistent with the development of recurrent excitatory circuitry in the dentate gyrus of rats after status epilepticus, but do not address the critical question of whether sprouted mossy fiber axons form functionally significant monosynaptic recurrent excitatory circuits with other granule cells. Furthermore, it is uncertain if the functional consequences of seizure-induced synaptic reorganization are similar in kindled rats that experience brief repeated seizures and rats that have experienced status epilepticus. In this study, we have used glutamate microstimulation methods and focal electrical microstimulation to exploit the opportunity provided by the kindling model, which induces mossy fiber sprouting with a predictable time course and in a gradually progressive manner, and detailed knowledge now available about the anatomical features of sprouted mossy fiber axon collaterals, to make new inferences about formation of recurrent circuits in dentate gyrus of epileptic rats.

METHODS

Surgical procedures

Adult male Sprague-Dawley rats (250–350 g) were anesthetized with a combination of ketamine (80 mg/kg im) and xylazine (10 mg/kg im) and were stereotactically implanted with an insulated stainless steel bipolar electrode for stimulation and recording. The electrode was implanted in either the perforant path (8.1 mm posterior, 4.4 mm lateral, 3.5 ventral with respect to bregma) or the olfactory bulb (9.0 mm anterior, 1.2 mm lateral, 1.8 mm ventral with respect to bregma) and was fixed to the skull with acrylic. Methods of animal handling and all experimental procedures were approved by the Research Animal Care Committee of the University of Wisconsin.

Kindling procedures

After a 2-wk recovery period following electrode placement, the unrestrained awake animals in the kindling group received twice-daily kindling stimulation (5 days per week) with a 1-s train of 62-Hz biphasic constant-current 1.0-ms square-wave pulses. The stimulation was delivered at the lowest intensity that evoked an afterdischarge according to standard procedures (Cavazos et al. 1991). The electroencephalogram was recorded from the bipolar electrode, which could be switched to the stimulator by a digital circuit for the delivery of the kindling stimulation. The evoked behavioral seizures were classified according to standard criteria (Sutula and Steward 1986) and ranged from class I (behavioral arrest with electrographic afterdischarge) to class V (bilateral tonic-clonic motor activity with loss of postural tone with prolonged afterdischarges, which are comparable to partial complex seizures with secondary generalization). Kindled rats were killed within 24 h after the final kindling stimulation.

Administration of kainic acid

Adult male Sprague-Dawley rats (250–350 g) were injected with kainic acid (9–12 mg/kg ip or sc) and were observed for signs of behavioral seizure activity, which typically consisted of altered responsiveness to environmental stimuli, irregular tonic-clonic movements of the extremities, and alterations in postural tone. The injected rats were observed and returned to their cages after 2–3 h, when the most severe behavioral alterations usually diminished. Previous studies have documented that kainic acid produces initially intense electrographic seizures that gradually diminish and usually cease by 4–5 days (Ben-Ari et al. 1981; Lothman et al. 1981; Sutula et al. 1992) and that are eventually followed by spontaneous recurrent seizures (Calvalheiro et al. 1982; Cronin and Dudek 1988; Pisa et al. 1980). Rats that did not experience status epilepticus in response to the kainic acid injection were not used in this study. Rats were killed 4–8 wk after kainic acid treatment. Kainic acid was obtained from Sigma.

Preparation of hippocampal slices

Rats were decapitated after induction of anesthesia by ether, and the brains were rapidly removed and placed into ice-cold artificial cerebrospinal fluid (ACSF) with the following composition (in mM): 124 NaCl, 4.4 KCl, 1.2 KH2PO4, 2.4 CaCl2, 1.3 MgSO4, 26 NaHCO3, and 10 glucose, which was saturated with 95% O2-5% CO2 at pH 7.4. Transverse hippocampal slices were cut from the septal half of the hippocampus with a vibratome (Technical Products International) at a thickness of 400–450 μm, perpendicular to the septotemporal axis. The slices were allowed to equilibrate for at least 1 h in oxygenated ACSF at 20–22°C, before being transferred to a submersion recording chamber and bathed in ACSF at 31–32°C. In the recording chamber, slices were perfused with ACSF containing 6.2 mM K+ and GABA_A receptor–mediated inhibition was blocked with 10 μM (−)-bicuculline methiodide (Sigma). In some experiments, the voltage-dependent block of N-methyl-d-aspartate (NMDA) receptor channels by Mg2+ was relieved by replacing MgSO4 in the ACSF with Na2SO4 (Mayer et al. 1984; Nowak et al. 1984). We have referred to ACSF with no added MgSO4 as “Mg2+-free”, but such solutions may still contain trace concentrations of Mg2+.

Recording and stimulation methods

Granule cells were impaled with tapered glass micropipettes (100–150 MΩ) filled with 2 M potassium acetate, adjusted to pH 7.4. Granule cells were identified by distinctive physiological criteria such as highly negative resting membrane potential, strong spike frequency adaptation, and absence of a voltage “sag” in response to hyperpolarizing current injection (Fricke and Prince 1984; Lübke et al. 1998; Scharfman 1992; Staley et al. 1992). Granule cells were included in this study when stable impalements were obtained, the resting membrane potential was at least −60 mV, the input resistance was at least 40 MΩ, and the action potential amplitude exceeded 50 mV. Bridge balance was routinely monitored by a conventional bridge circuit used for intracellular recording and current injection. Extracellular recording electrodes (2–10 MΩ) containing 2 M NaCl were positioned within 200 μm of the intracellular electrode. Orthodromic synaptic responses were evoked in granule cells of the dentate gyrus by monopolar constant-voltage stimuli (0.05 ms) delivered by electrodes placed in the stratum molecular of the dentate gyrus in the region of the perforant path. Responses were amplified, digitized, and stored on optical disks for off-line analysis.
Assessment of connectivity in the dentate gyrus by glutamate microstimulation

Excitatory connectivity between granule cells was investigated by determining whether focal application of glutamate in the molecular layer of the dentate gyrus evoked an EPSP in a recorded granule cell during bath application of 10 μM bicuculline to block GABA<sub>A</sub> receptor–dependent IPSPs. In previous studies in the hippocampus and dentate gyrus, application of glutamate microdrops to dendrites and cell bodies under these conditions activated local excitatory synaptic circuits, but not axons, presynaptic terminals, or GABA<sub>A</sub> receptor–dependent inhibitory circuits (Christian and Dudek 1988a,b). Glutamate was applied to the molecular layer of the dentate gyrus through a glass micropipette with a tip of ~20 μm, which was filled with l-glutamic acid (20 mM) in ACSF. Delivery of the glutamate was controlled by a picospritzer (General Valve) that generated positive-pressure pulses (50 ms, 40 psi) at the blunt end of the micropipette. The glutamate solutions were routinely mixed with trace amounts of India ink to monitor micropipette placement and glutamate solution flow. In preliminary experiments, direct application of India ink or ink-stained ACSF did not elicit responses from recorded cells (n = 5), and the distribution of the ink after pressure injection typically indicated diffusion for ~100 μm from the pipette tip. After obtaining a stable granule cell impalement, the micropipette containing glutamate was lowered beneath the surface of the slice at the most distal site in the dentate gyrus (usually either at the blade crest or tip) from the impaled cell, and a single pulse of glutamate was applied to the molecular layer. Subsequent pulses were applied closer to the impaled cell, in steps of ~100 μm. In some slices, glutamate pulses were also applied in the molecular layer of the opposite blade of the dentate gyrus, and in the hilus. After systematic stimulation at locations remote from the recorded cell, glutamate was directly applied adjacent to the impaled cell to verify the integrity of the stimulation and recording procedure. This usually resulted in a large, long-lasting depolarization with repetitive discharges, followed by a refractory period.

Assessment of connectivity in the dentate gyrus by electrical microstimulation

Connectivity between blades of the dentate gyrus was also assessed by electrical microstimulation applied in transverse hippocampal slices transected by razor cuts that removed the CA3<sub>xy</sub>, regions of the hippocampus, the subiculum, and the entorhinal cortex. Perforant path connections between blades were transected by removing the crest of the dentate gyrus (see Fig. 6). The stimulation pulses consisted of constant-voltage 100 μA pulses delivered by a bipolar electrode (100 μm tip separation; World Precision Instruments) to the molecular layer of the infrapyramidal blade while recording from a granule cell in the opposite suprapyramidal blade. Electrical microstimulation experiments were conducted at low stimulus intensities (~10 V), in ACSF with 10 μM (−)-bicuculline methiodide to block GABA<sub>A</sub> receptor–mediated inhibition and 10 mM Ca<sup>2+</sup> to suppress polysynaptic recurrent excitatory and inhibitory circuits (Berry and Pentreath 1976; Crepel et al. 1997; Miles and Wong 1987; Williams and Johnston 1991).

Histological procedures

After recording, hippocampal slices were immersed in an aqueous solution of 10% (vol/vol) Formalin in 0.9% (wt/vol) NaCl, and stored for at least 24 h at 4°C. After cryoprotection and freezing on dry ice, tissue sections of 60 μm thickness were cut on a freezing microtome in the same plane as the transverse hippocampal slice. All sections were mounted on slides, stained with cresyl violet, and juxtaposed with detailed drawings made during recording of sites of stimulation and recording.

RESULTS

Passive membrane properties of granule cells from normal and epileptic rats

Data were collected from 23 control rats, 30 rats that experienced kainic acid–induced status epilepticus, and 21 kindled rats. To assess evolving alterations in connectivity in the dentate gyrus induced by kindling, kindled rats were killed at one of three time points: 1) within 24 h of the first evoked after-discharge (n = 5), before the development of mossy fiber sprouting (Cavazos et al. 1991); 2) at ~1 wk after onset of kindling stimulation (n = 5), when mossy fiber sprouting first becomes detectable by histological methods (Cavazos et al. 1991); or 3) after extensive kindling (30–180 generalized class V seizures; n = 11), when mossy fiber sprouting is well developed (Cavazos et al. 1991; Sutula et al. 1988). The average resting membrane potential of dentate gyrus granule cells from control rats was −72.3 ± 1.5 mV (mean ± SE, n = 46 cells), from kainic acid–treated rats was −72.6 ± 1.3 mV (n = 71 cells), and from kindled rats was −72.7 ± 1.2 mV (n = 50 cells; P = 1.0, ANOVA). Granule cell input resistances were 58.5 ± 3.3 MΩ, 60.4 ± 3.3 MΩ, and 65.5 ± 3.7 MΩ for control, kainic acid–treated, and kindled rats (P = 0.4, ANOVA).

Glutamate microstimulation in the dentate gyrus of normal rats

Application of glutamate to the molecular layer of the dentate gyrus in ACSF containing 10 μM bicuculline failed to evoke EPSPs in 11 granule cells from 7 normal rats (Fig. 1), which confirmed previous observations (Wuarin and Dudek 1988a,b). Glutamate microstimulation in the dentate gyrus of normal rats

![FIG. 1. Focal application of glutamate to the molecular layer of the dentate gyrus in hippocampal slices from normal and kainic acid-treated rats. Top trace: representative example of an intracellular recording in a granule cell from a normal rat in a hippocampal slice bathed in artificial cerebrospinal fluid (ACSF) containing 10 μM bicuculline to block GABA<sub>A</sub> receptor–dependent inhibitory circuitry. Arrow indicates the onset of stimulation with a 50-ms pulse of glutamate applied by a picospritzer. Middle trace: focal glutamate application also failed to evoke excitatory postsynaptic potentials (EPSPs) in 11 granule cells from 7 normal rats (Fig. 1), which confirmed previous observations (Wuarin and Dudek 1988a,b). Bottom trace: focal application of glutamate to the molecular layer of a dentate gyrus in ACSF containing 10 mM bicuculline to block GABA<sub>A</sub> receptor–dependent inhibitory circuitry. Arrow indicates the onset of stimulation with a 50-ms pulse of glutamate applied by a picospritzer.](http://jn.physiology.org/lookup/doi/10.1152/jn.00673.2016)
In these 11 granule cells, which included 4 infrapyramidal granule cells and 7 suprapyramidal cells, glutamate application to the molecular layer of the same blade at distances of 200–800 μm from the impaled cell (n = 11) or to the opposite blade (n = 5) did not evoke an EPSP or any apparent change in ongoing activity recorded by intracellular or extracellular methods.

Because lowering the Mg$^{2+}$ content of the bathing medium in the presence of GABA$_A$ receptor blockade has been shown to enhance excitatory polysynaptic activity in other regions of the hippocampus by relieving Mg$^{2+}$ block of the NMDA receptor (Heinemann et al. 1992; Tancredi et al. 1990; Traub et al. 1994), we also tested for glutamate-evoked responses in ACSF in which Mg$^{2+}$ was omitted. In Mg$^{2+}$-free ACSF with 10 μM bicuculline, glutamate application also failed to evoke EPSPs in 14 of 14 granule cells from 8 normal rats. Glutamate microdrops were applied at ~100-μm intervals along the molecular layer of the dentate gyrus at an average of six sites per slice, usually in the same blade as the impaled cell, but also in the hilus (n = 7) and opposite blade (n = 6). EPSPs were not observed in response to glutamate microapplication at any of these sites in granule cells from normal rats (Fig. 2). These results suggest that enhancement of excitatory transmission by relief of the Mg$^{2+}$ block of the NMDA receptor is not sufficient to increase excitatory connectivity between granule cells in the dentate gyrus of normal rats.

**Glutamate microstimulation in the dentate gyrus of kainic acid–treated rats**

In contrast, application of glutamate microdrops to the molecular layer of the dentate gyrus in hippocampal slices obtained from 14 rats at 4–8 wk after kainic acid–induced status epilepticus evoked trains of EPSPs in 6 of 30 granule cells in ACSF with 10 μM bicuculline (Fig. 1 and Table 1), confirming previous reports (Wuarin and Dudek 1996). Trains of EPSPs were evoked by glutamate application in the same blade as the impaled granule cell at sites from 200–900 μm from the recording site (Fig. 3). These results were observed in granule cells in both the suprapyramidal (n = 4), and infrapyramidal (n = 2) blades. The stimulation sites that evoked EPSPs appeared to be distributed in an irregular patchy distribution relative to the recorded granule cell (Fig. 3). The patchy distribution of the evoked responses is consistent with the irregular distribution of sprouted mossy fiber synaptic terminals in the supragranular layer described in detailed anatomic studies of granule cells filled with biocytin in hippocampal slices and in vivo in kainic acid–treated rats (Buckmaster and Dudek 1999; Sutula et al. 1998). In these studies, sprouted mossy fiber synaptic terminals projected into irregular and randomly distributed regions of the supragranular layer, rather than in the diffuse pattern implied by the distribution of labeled terminals examined by Timm histochemistry at the light microscopic level. The patchy pattern of evoked responses is also consistent with the patchy distribution of labeled terminals.
examined at the ultrastructural level (Okazaki et al. 1995; Ribak et al. 1998; Sutula et al. 1988; Zhang and Houser 1999).

When Mg\(^{2+}\) was removed from the ACSF, glutamate microdrops evoked trains of EPSPs in 8 of 13 granule cells from kainic acid–treated rats \((P < 0.02\) vs. Mg\(^{2+}\)-free conditions in control rats, Fisher’s exact test; Table 1), which suggests that synaptic transmission in circuits that contribute to recurrent excitatory connectivity in the dentate gyrus may involve NMDA receptors. The evoked EPSP trains occurred at variable latency (5–1,000 ms) after glutamate application, and lasted for 250 ms to 15 s, which suggested that the trains were generated by buildup of reverberating network activity (Ayala et al. 1973; Christian and Dudek 1988a; Hablitz 1984; Miles and Wong 1983). The distribution of stimulation sites that evoked EPSPs in Mg\(^{2+}\)-free ACSF was also irregular and patchy relative to the recorded cells (not shown). In 4 of 13 granule cells from kainic acid–treated rats in Mg\(^{2+}\)-free ACSF with 10 mM bicuculline, glutamate microdrops evoked EPSP trains that were accompanied by action potentials or granule cell burst discharges (Fig. 4). Granule cell EPSP trains elicited by glutamate microapplication were detected in extracellular electrodes and, in three of three cases, were blocked by 1 \(\mu\)M tetrodotoxin, indicating that they were network-driven synaptic events and were not due to effects of glutamate on presynaptic terminals (Fig. 4).

Because recent anatomic studies have demonstrated that seizure-induced mossy fiber sprouting from infrapyramidal granule cells can cross the hilus and form terminal boutons in

### Table 1. Microstimulation-evoked responses in hippocampal slices from normal and epileptic rats

<table>
<thead>
<tr>
<th></th>
<th>Number of Rats</th>
<th>Cells With EPSPs in 1.3 mM Mg(^{2+})</th>
<th>Cells With EPSPs in 0 mM Mg(^{2+})</th>
<th>Infra- to Suprapyramidal Pathway (Electrical Stimulation)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>23</td>
<td>0/11</td>
<td>0/14</td>
<td>0/15</td>
</tr>
<tr>
<td>Kainic acid–treated</td>
<td>30</td>
<td>6/30</td>
<td>8/13(^{a,b})</td>
<td>5/18</td>
</tr>
<tr>
<td>Kindled (1 afterdischarge)</td>
<td>5</td>
<td>0/7(^{a})</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kindled (5–8 afterdischarges)</td>
<td>5</td>
<td>4/9(^{a})</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kindled (30–180 class V seizures)</td>
<td>11</td>
<td>4/20</td>
<td>8/12(^{a,e})</td>
<td></td>
</tr>
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EPSPs, excitatory postsynaptic potentials. \(^{a}P < 0.02\) vs. control rats in 0 mM Mg\(^{2+}\). \(^{b}P = 0.01\) vs. kainic acid–treated in 1.3 mM Mg\(^{2+}\). \(^{c}P < 0.05\) vs. control rats in 1.3 mM Mg\(^{2+}\) and 10 mM Ca\(^{2+}\). \(^{d}P = 0.01\) vs. 30–180 class V seizures in 0 mM Mg\(^{2+}\). \(^{e}P = 0.02\) vs. 30–180 class V seizures in 1.3 mM Mg\(^{2+}\). All significance levels obtained by Fisher’s exact test for the comparison of proportions.

FIG. 3. Representative example of the distribution of sites of focal glutamate application in a hippocampal slice from a rat killed 59 days after experiencing kainic acid–induced status epilepticus. The slice was bathed in ACSF containing 10 \(\mu\)M bicuculline and 1.3 mM Mg\(^{2+}\). The location of the recorded granule cell in the suprapyramidal blade of the dentate gyrus is indicated by the arrowhead. Focal application of glutamate to the molecular layer at sites marked by circles (●) evoked trains of EPSPs from 3 of 5 suprapyramidal sites and 1 of 2 infrapyramidal sites. The site corresponding to the bottom trace of Fig. 1 is indicated by the asterisk. Sites of glutamate application that evoked EPSPs were typically distributed in a patchy pattern in the molecular layer relative to the recorded granule cell. Gliosis and neuronal loss are apparent in the hilus and CA3, region (compare to Fig. 2). Calibration bars: 10 mV, 500 ms; scale bar 500 \(\mu\)m.
Glutamate evoked responses in granule cells from kainic acid-treated rats were network-driven field events. In a hippocampal slice bathed in Mg\textsuperscript{2+}-free ACSF with 10 \mu M bicuculline, focal glutamate application at sites remote from the recorded granule cell evoked EPSPs that were occasionally accompanied by action potentials and were also simultaneously recorded by extracellular electrodes (bottom trace of each pair). EPSPs evoked by glutamate application were reversibly blocked by 1 \mu M tetrodotoxin, demonstrating that they were generated by network-driven synaptic events and were not due to a direct effect of glutamate on synaptic terminals. Calibration bars: 10 mV (intracellular), 4 mV (extracellular), 500 ms.

the supragranular layer of the suprapyramidal blade (Sutula et al. 1998), glutamate microdrop application was also used to test for connectivity between blades. In two of five hippocampal slices from kainic acid–treated rats, application of glutamate to the molecular layer of the infrapyramidal blade evoked trains of EPSPs in suprapyramidal granule cells (see Fig. 3). Application of glutamate to the molecular layer of suprapyramidal blade did not evoke a response in three of three infrapyramidal granule cells from kainic acid–treated rats. Application of glutamate to the hilus of hippocampal slices from kainic acid–treated rats did not evoke responses in granule cells in the infrapyramidal blade (n = 3) or the suprapyramidal blade (n = 5) in Mg\textsuperscript{2+}-free ACSF with 10 \mu M bicuculline, in agreement with previous reports (Wuarin and Dudek 1996).

**Glutamate microstimulation in the dentate gyrus of kindled rats**

Evidence for recurrent excitatory circuitry was also assessed in hippocampal slices from rats that experienced brief repeated seizures evoked by kindling. The time course of development of EPSPs evoked by glutamate microstimulation was evaluated at progressive stages of kindling to assess whether functional connectivity developed in the dentate gyrus with a time course corresponding to the time course of development of mossy fiber sprouting, which has been characterized in detail in previous studies (Cavazos et al. 1991; Sutula et al. 1988). In 4 of 20 granule cells in hippocampal slices from kindled rats that experienced 30–180 generalized class V seizures, application of glutamate to the molecular layer of the dentate gyrus evoked trains of EPSPs. As with glutamate-evoked EPSP trains in hippocampal slices from kainic acid–treated rats, granule cell EPSP trains in hippocampal slices from kindled rats were evoked at a variable latency (5–800 ms) and varied in duration (200 ms to 5 s). In Mg\textsuperscript{2+}-free ACSF with 10 \mu M bicuculline, EPSP trains were evoked in 8 of 12 granule cells from kindled rats (Fig. 5, Table 1). Glutamate application to the suprapyramidal blade evoked EPSPs in five of seven suprapyramidal granule cells, and application to the infrapyramidal blade evoked EPSPs in three of five infrapyramidal granule cells at distances ranging from 200 to 700 \mu m from the impaled cell. As in kainic acid–treated rats, systematic application of glutamate pulses in ~100-\mu m steps along the blade revealed that locations along the blade that evoked EPSPs were distributed in an irregular patchy pattern relative to the recorded granule cell (not shown).

Because of the possibility that sprouted mossy fibers may form recurrent excitatory circuits with granule cells, it was of interest to determine whether there was a correlation between the development of excitatory connectivity detected by glutamate microstimulation and the development of mossy fiber sprouting induced by seizures in kindled rats. Kindling is particularly advantageous to address this question, because mossy fiber sprouting develops gradually in response to repeated seizures, and both the time course and the extent of sprouting have been characterized as a function of repeated seizures (Cavazos et al. 1991). Sprouting is not apparent in kindled rats at 24 h after a single afterdischarge, but first becomes apparent at 4–7 days after the initial stimulations that evoke synchronous afterdischarges (Cavazos et al. 1991). In seven granule cells from five kindled rats killed 24 h after a...
single evoked afterdischarge, when sprouting has not yet de-
veloped (Cavazos et al. 1991), glutamate application failed to
evoke a response in Mg$^{2+}$-free bathing medium with 10 μM
bicuculline (Fig. 5; $P = 0.02$ vs. extensively kindled rats,
Fisher’s exact test). In contrast, glutamate application in dis-
inhibited Mg$^{2+}$-free ACSF to the dentate gyrus of kindled rats
killed at 1 wk after five to eight evoked afterdischarges, when
histological evidence of sprouting first becomes apparent
(Cavazos et al. 1991), evoked trains of EPSPs in four of nine
granule cells from five rats (Fig. 5). This observation is
consistent with the possibility that sprouted mossy fibers may be
contributing to increased excitatory connectivity by forming
recurrent excitatory circuits between granule cells in the den-
tate gyrus.

Electrical microstimulation in the dentate gyrus of normal
and epileptic rats

The ability to evoke EPSPs in granule cells by focal micro-
injection of glutamate into regions of the dentate gyrus depends
not only on the presence of axonal connections between the
cells, but is also influenced by factors such as the rate of
diffusion of glutamate after pressure injection, the peak local
concentration at sites of glutamate receptors, and rate of glu-
tamate removal from the extracellular space (Goodchild et al.
1982). Because these technical factors influence the latency of
EPSPs evoked by focal microinjection of glutamate, it is not
possible to determine whether the evoked EPSPs are generated
by activation of polysynaptic or monosynaptic circuits. More-
ever, observations that the trains of EPSPs and burst discharges
evoked by glutamate microstimulation occurred at a variable
latency after injection (see Fig. 3) and were blocked by bath
application of tetrodotoxin suggests that these population
events may have been generated by buildup of excitatory
activity spreading in a network of recurrent circuits (Johnston
and Brown 1984; Wong et al. 1986).

Previous studies have not allowed the assessment of the
latency of responses evoked in the reorganized circuitry of the
dentate gyrus (Okazaki et al. 1999; Wuarin and Dudek 1996).
Electrical microstimulation methods were therefore used to
obtain a more reliable estimate of latency of EPSPs evoked by
focal stimulation of the dentate gyrus in hippocampal slices
from epileptic rats. If sprouted mossy fibers that arise from
granule cells in the infrapyramidal blade of the dentate gyrus
form excitatory connections with granule cells in the suprapy-
ramidal blade as suggested by both in vitro and in vivo labeling
of sprouted mossy fiber axons (Sutula et al. 1998), activation of
granule cells in the infrapyramidal blade should evoke an EPSP
in granule cells of the suprapyramidal blade with a latency
compatible with mossy fiber monosynaptic connections
(Doller and Weight 1982; Jonas et al. 1993; Williams and
Johnston 1991). This prediction was tested by stimulating the
infrapyramidal blade while recording from granule cells in the

![FIG. 6. Focal electrical microstimulation demonstrated excitatory connectivity between blades of the dentate gyrus in transected hippocampal slices from kainic acid-treated rats. A: representative example of a transected transverse hippocampal slice from a kainic acid-treated rat containing only the infrapyramidal and suprapyramidal blades of the dentate gyrus, the intervening hilus, a small sector of CA3c, and CA1. The transection removed the crest of the dentate gyrus, which severed perforant path connections between the blades. Note the gliosis and loss of neurons in the hilus and CA3c. Scale bar: 500 μm. B: in ACSF containing 10 μM bicuculline to suppress inhibitory postsynaptic potentials (IPSPs) and 10 mM Ca$^{2+}$ to suppress polysynaptic activity, stimulation of the molecular layer of the infrapyramidal blade (site indicated by arrows in A) with a 100-μs constant-voltage pulse of 7 V evoked an EPSP in a granule cell in the suprapyramidal blade (location indicated by the arrowhead in A). The latency of the EPSP was 2.7 ms (measured from the center of the stimulation artifact to EPSP onset indicated by the arrowhead in the bottom trace). Focal electrical microstimulation evoked EPSPs in 5 of 18 suprapyramidal granule cells in kainic acid-treated rats with an average latency of 2.59 ± 0.36 ms. In contrast, stimulation of the infrapyramidal blade in hippocampal slices from normal rats failed to evoke EPSPs in 15 suprapyramidal granule cells ($P < 0.05$, Fisher’s exact test). Calibration bars: 5 mV, 25 ms (top trace), 2 mV, 8 ms (bottom trace).]
suprapyramidal blade in transected hippocampal slices from normal and kainic acid–treated rats in which perforant path connections to granule cells in each blade were cut (Fig. 6) and polysynaptic activity was suppressed by 10 mM\[Ca^{2+}]_o\) (Berry and Pentreath 1976; Crepel et al. 1997; Miles and Wong 1987).

In transected slices of the dentate gyrus from kainic acid–treated rats containing only the infrapyramidal and suprapyramidal blades, the intervening hilus, and a small sector of CA3, (Fig. 6), which were bathed in ACSF with 10 \(\mu\)M bicuculline to suppress IPSPs and 10 mM Ca\(^{2+}\) to suppress polysynaptic activity, stimulation of the molecular layer of the infrapyramidal blade with a 100-\(\mu\)s constant voltage pulse (<10 V) evoked EPSPs in 5 of 18 suprapyramidal granule cells. EPSP latency (measured from the center of the stimulation artifact to EPSP onset) was 2.59 ± 0.36 ms (Fig. 6). In contrast, stimulation of the infrapyramidal blade in hippocampal slices from normal rats failed to evoke EPSPs in 15 suprapyramidal granule cells (\(P < 0.05\), Fisher’s exact test). These observations are evidence that seizure-induced granule cell axonal reorganization results in the formation of monosynaptic excitatory connections between blades of the dentate gyrus.

**DISCUSSION**

There has been considerable interest in determining whether sprouted mossy fibers induced by repeated seizures form recurrent circuits in the dentate gyrus (McNamara 1999). Paired intracellular recording could provide definitive evidence about formation of recurrent excitatory circuits by demonstrating that current injection that evokes spikes in one granule cell evokes an EPSP or EPSC in another granule cell. Paired recordings in hippocampal regions such as CA3 that are known to have extensive recurrent connections, however, have demonstrated that not more than 5% of neurons form recurrent excitatory circuits (MacVicar and Dudek 1980; Miles and Wong 1986). As a preliminary alternative to laborious paired recording experiments that would be required to conclusively resolve this question, we employed the methods of glutamate microstimulation, which has been used previously in studies of the hippocampus and dentate gyrus (Christian and Dudek 1988a,b; Wuarin and Dudek 1996), and a focal electrical microstimulation technique in transected hippocampal slices, to assess development of excitatory connectivity in dentate gyrus of rats that had experienced seizures. The design of the experiments exploited information about the time course of development of recurrent mossy fiber circuitry induced by kindled seizures (Cavazos et al. 1991), and the detailed information now available about the anatomic features of sprouted mossy fiber axon collaterals in kainic acid–treated rats (Buckmaster and Dudek 1999; Sutula et al. 1998).

Confirming previous anatomic and physiological studies, the experiments did not detect evidence for excitatory connections between regions of the dentate gyrus in normal rats when GABA\(_A\) receptor–dependent recurrent inhibitory circuitry was blocked and excitatory synaptic transmission was enhanced by increasing the NMDA receptor–dependent synaptic current. The experiments provided new evidence that excitatory connections develop in the dentate gyrus of kindled rats in a time course that paralleled the development of mossy fiber sprouting (Cavazos et al. 1991). In addition, the spatial patterns of intrablane and interblade connectivity in kainic acid–treated rats corresponded to patterns of arborization of sprouted mossy fiber axons described in anatomic studies (Sutula et al. 1998), and focal electrical microstimulation experiments furthermore demonstrated interblade excitatory connections at a latency consistent with monosynaptic excitatory pathways.

**EPSPs evoked by glutamate microinjection and electrical microstimulation as evidence for recurrent excitatory connections**

The observation that glutamate application in the granule cell and molecular layer can evoke EPSPs in other granule cells of epileptic kindled and kainic acid–treated rats is consistent with the development of recurrent excitatory circuits in response to repeated seizures, but these results are not sufficient to conclude that recurrent excitatory circuits are formed by synapses of sprouted mossy fibers on other granule cells. In the initial description of EPSPs evoked by glutamate microstimulation in kainic acid–treated rats (Wuarin and Dudek 1996), and in our experiments in kindled and kainic acid–treated rats, there was a long and variable latency between glutamate application and the onset of EPSPs. This delay (as long as 1,000 ms after glutamate application in our experiments) may be at least partially explained by technical factors such as the glutamate diffusion, peak local concentration at sites of glutamate receptors, and glutamate removal, but could also be caused by a gradual buildup of polysynaptic excitatory activity spreading in a network of recurrent circuits (Johnston and Brown 1984; Wong et al. 1986). If recurrent circuits formed by sprouted mossy fibers on other granule cells contribute to generation of the trains of EPSPs evoked by glutamate microstimulation, it would be expected that 1) the development of EPSPs should correlate with the time course of mossy fiber sprouting, 2) the spatial distribution of the sites of glutamate application that evoke trains of EPSPs should correspond to projection patterns of sprouted mossy fiber axons, and 3) electrical microstimulation of regions of the dentate gyrus that contain cells of origin of sprouted mossy fibers should evoke EPSPs in granule cells with a latency consistent with a monosynaptic pathway.

**Spatial features of seizure-induced excitatory connectivity in epileptic rats**

When GABA\(_A\) receptor–dependent IPSPs were blocked by bicuculline, EPSPs were evoked in granule cells of the epileptic rat by application of glutamate to sites in the molecular layer of the same blade as well as the opposite blade of the dentate gyrus relative to the recorded granule cell. Although the EPSPs were evoked at distances from ~200–900 \(\mu\)m from the recorded cell within the same blade, the distribution of sites that evoked EPSPs was patchy. This anatomic feature of the responses and the pattern of interblade projection from infrapyramidal to suprapyramidal blade are consistent with the projection patterns of biocytin-filled sprouted mossy fiber axons observed in anatomic studies (Sutula et al. 1998).


**Time course of induction of excitatory connectivity in epileptic rats: relationship to development of mossy fiber sprouting**

In previous studies, spontaneous burst discharges were not observed 2–4 days after kainic acid–induced status epilepticus, but were observed months later after the development of sprouting (Cronin et al. 1992; Patrylo and Dudek 1998; Wuarin and Dudek 1996). In the present study we have further explored the relationship between development of increased excitatory connectivity as assessed by glutamate microdrops in the kindling model, which induces mossy fiber sprouting with a predictable time course and in a gradually progressive manner. Trains of EPSPs were not evoked in dentate gyrus from rats examined 24 h after a single kindled afterdischarge, when sprouting has not yet developed, but were evoked by glutamate application in hippocampal slices from rats examined at ~1 wk after initial kindling stimulation when histological evidence of sprouting first becomes apparent (Cavazos et al. 1991). At 24 h after a single afterdischarge, the NMDA receptor–dependent component of the evoked population EPSP and the evoked population spike have been enhanced by kindling stimulation (Sutula and Steward 1986; unpublished observations), but this seizure-induced enhancement of granule cell synaptic transmission, which precedes development of sprouting, was not accompanied by development of excitatory connectivity in the dentate gyrus. These observations were confirmed by the inability to evoke EPSPs by glutamate microstimulation in hippocampal slices from normal rats exposed to Mg2+-free ACSF, which enhances excitatory transmission by increasing the NMDA receptor–mediated component of synaptic transmission (Herren et al. 1985; Schneiderman and MacDonald 1987; Traub et al. 1994). These previous studies and the present results together suggest that acute seizure-induced increases in synaptic efficacy, which are sufficient to alter susceptibility to burst discharges (unpublished observations), are not sufficient to alter excitatory connectivity in the dentate gyrus. More slowly developing chronic cellular alterations, possibly mossy fiber sprouting, are required.

Mossy fiber sprouting was not directly assessed by histological analysis in our experiments, but the time course of development of excitatory connectivity in kindled rats was comparable to the time course of mossy fiber sprouting in previous studies (Cavazos et al. 1991). Furthermore, previous studies in kainic acid–treated rats with extensive sprouting have not uniformly demonstrated burst discharges when GABA<sub>A</sub> receptor–mediated inhibition is blocked (Cronin et al. 1992; Patrylo and Dudek 1998), and as in our study, glutamate microstimulation does not uniformly evoke EPSPs in the presence of sprouting (Molnar and Nadler 1997; Wuarin and Dudek 1996, 1997). In slices demonstrating evoked EPSPs, the patchy distribution of sites where glutamate evoked responses suggests dependence on underlying local circuitry.

Available anatomic studies of the sprouted pathway demonstrate that hippocampal slices, regardless of orientation, transect the mossy fiber pathway and processes of other neurons in the dentate gyrus and therefore invariably limit attempts to comprehensively assess the structural and functional features of the sprouted pathway (Amaral and Witter 1989; Buckmaster and Dudek 1999; Buckmaster et al. 1996; Sutula et al. 1998). The experiments reported here, although still preliminary, have nevertheless provided new spatial and temporal details about the development of recurrent excitatory circuits in the epileptic dentate gyrus. A complete analysis of the spatial relationship between the pattern of evoked EPSPs and sprouted mossy fiber terminals would likely require in vivo paired recording and detailed anatomic analysis of axonal projections from the site of stimulation.

**Recent excitatory circuits that could contribute to increased excitatory connectivity in the epileptic dentate gyrus**

Although the induction of glutamate-evoked EPSPs in granule cells is strong evidence that recurrent excitatory connections develop in the dentate gyrus of epileptic rats, the identity of circuits that contribute to this seizure-induced connectivity is uncertain. Granule cells have reciprocal connections with polymorphic neurons in the hilus in the dentate gyrus, and also form distinctive giant mossy fiber synapses with “thorny excrescences” or complex spines of mossy cells, which provide excitatory feedback to granule cells (Amaral 1978; Buckmaster et al. 1996; Claiborne et al. 1986; Jackson and Scharfman 1996; Scharfman et al. 1990; Seress and Pokorny 1981). Seizure-induced enhancement of excitatory transmission in this pathway, or in the polysynaptic circuit from the dentate gyrus or entorhinal cortex to CA3 with direct projection from CA3 back to granule cells, could play a role in the generation of the glutamate-evoked EPSPs observed in the present study (Scharfman 1996). However, we were unable to elicit EPSPs in hippocampal slices from normal rats, even in disinhibited Mg2+-free ACSF, which enhances polysynaptic excitatory circuitry in other regions of the hippocampus (Crepel et al. 1997; Tancredi et al. 1990; Traub et al. 1994). Furthermore, because mossy cells, pyramidal neurons in CA3, and other polymorphic neurons in the hilus are especially susceptible to seizure-induced cell death (Ben-Ari 1985; Cavazos et al. 1994; Meldrum et al. 1973; Nadler and Cuthbertson 1980; Sloviter 1983), it seems improbable that enhancement of transmission in these excitatory circuits would generate glutamate-evoked EPSPs in the epileptic hippocampus, which undergoes substantial seizure-induced loss in these regions.

Other possible sources of increased excitatory connectivity include seizure-induced sprouting by surviving mossy cell axons, which might increase polysynaptic innervation to granule cells, and sprouting by mossy fiber axons in the hilus, which has been demonstrated in biocytin-filled granule cells of kainic acid–treated rats (Buckmaster and Dudek 1999; Sutula et al. 1998). Because sprouting by mossy fibers in the hilus increases the number of small synapses (Sutula et al. 1998), which almost exclusively terminate on GABAergic interneurons in normal rats (Acsády et al. 1998), it seems unlikely that reorganization in these polysynaptic circuits would increase excitatory innervation to granule cells (Kotti et al. 1997).

**Monosynaptic excitatory circuits in the dentate gyrus of epileptic rats**

In a further attempt to identify the specific circuit or circuits that contribute to recurrent excitatory connectivity induced by seizures in the dentate gyrus, electrical microstimulation of the molecular layer of the infrapyramidal blade was used to more...
accurately measure the latency of the recurrent connections that form between blades of the dentate gyrus. These measurements were performed in transected slices of the hippocampus that contained only the infrapyramidal and suprapyramidal blades, the intervening hilus, and a small sector of CA3b. In these slices (see Fig. 6), severing the perforant path connection between blades by removing the crest of the dentate gyrus eliminated the possibility of antidromic back-propagation from the infrapyramidal to suprapyramidal blade, and the small remaining sector of CA3 after transection reduced the possibility of activation of polysynaptic circuits through the CA3 network (Scharfman 1994–1996). In addition, polysynaptic activity was further reduced by bath application of ACSF with 10 μM bicuculline to block IPSPs and 10 mM [Ca2+]o, which suppresses polysynaptic activity (Berry and Pentreath 1976; Crepel et al. 1997; Miles and Wong 1987). Under these recording conditions in kainic acid–treated rats, low-intensity electrical stimulation of the molecular layer of the infrapyramidal blade evoked EPSPs in granule cells of suprapyramidal blade at a latency of 2.6 ms, which were not observed in normal rats. This latency indicates monosynaptic transmission and is consistent with the possibility that sprouted mossy fibers form functional recurrent excitatory circuits between blades of the dentate gyrus in epileptic rats.

Net functional effects of seizure-induced recurrent neuronal circuits

Synapses formed by sprouted mossy fibers on dendrites of granule cells would result in recurrent excitatory circuits that could increase excitatory drive and promote epileptogenesis. Conversely, recurrent inhibitory circuits formed by mossy fiber synapses on inhibitory interneurons would be expected to enhance inhibition. Ultrastructural analysis of asymmetric synapses formed by sprouted mossy fibers has provided evidence that both types of synapses are formed, but the relative abundance of these functionally distinct circuits has not been formally assessed (Franck et al. 1995; Kotti et al. 1997; Okazaki et al. 1995; Ribak and Peterson 1991; Ribak et al. 1998; Zhang and Houser 1999). Under normal physiological conditions, activity in recurrent excitatory circuits is balanced by activity in inhibitory circuits and may be masked or suppressed by recurrent inhibition (Crepel et al. 1997; Miles and Wong 1987; Scharfman 1996), except for brief periods when dynamic alterations in afferent activity or use-dependent fading of inhibition may disrupt the balance. Synchronous excitatory activity that overwhelms inhibition and generates a seizure is a relatively uncommon event in the presence of strong systems of recurrent of inhibition. More complete understanding of the contribution of seizure-induced circuit reorganization to epileptogenesis will need to resolve two critical questions: 1) what is the relative abundance of seizure-induced inhibitory and excitatory circuits in regions of the brain that are sites of seizure initiation, and 2) what are the dynamic processes that result in reduction of inhibition and are permissive for epileptic synchronization?

Conclusions

The time course of development, patterns of excitatory connectivity, and the latency of excitatory connections demonstrated in these experiments support the possibility that sprouted mossy fibers form monosynaptic recurrent circuits with other granule cells that contribute to recurrent excitation in the epileptic dentate gyrus. Paired intracellular recordings will be necessary to provide unequivocal physiological evidence for seizure-induced formation of functional granule cell–to–granule cell connections. Furthermore, because anatomic evidence has demonstrated that seizure-induced sprouting produces marked septotemporal divergence in the normally lamellar projection of the mossy fiber terminal field (Sutula et al. 1998) and preparation of hippocampal slices invariably alters the circuitry of the hippocampus, in vivo recordings of pairs of granule cells may be required to fully assess the extent and functional significance of mossy fiber sprouting. Despite these limitations, the data presented here provide evidence that seizure-induced mossy fiber sprouting is associated with the development of aberrant monosynaptic recurrent excitatory circuitry that may be more extensive than previously appreciated.

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