Reduced Inhibition and Increased Output of Layer II Neurons in the Medial Entorhinal Cortex in a Model of Temporal Lobe Epilepsy

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Temporal lobe epilepsy is the most common type of epilepsy in adults, and its underlying mechanisms are unclear. To investigate how the medial entorhinal cortex might contribute to temporal lobe epilepsy, we evaluated the histology and electrophysiology of slices from rats 3–7 d after an epileptogenic injury (pilocarpine-induced status epilepticus). Nissl staining, NeuN immunocytochemistry, and *in situ* hybridization for GAD65 mRNA were used to verify the preferential loss of glutamatergic neurons and the relative sparing of GABAergic interneurons in layer III. From slices adjacent to those that were used for anatomy, we obtained whole-cell patch recordings from layer II medial entorhinal cortical neurons. Recordings under current-clamp conditions revealed similar intrinsic electrophysiological properties (resting membrane potential, input resistance, single spike, and repetitive firing properties) to those of controls. Spontaneous IPSCs were less frequent (68% of controls), smaller in amplitude (57%), and transferred less charge (51%) than in controls. However, the frequency, amplitude, and rise time of miniature IPSCs were normal. These findings suggest that after epileptogenic injuries the layer II entorhinal cortical neurons receive less GABA_A receptor-mediated synaptic input because presynaptic inhibitory interneurons become less active. To investigate the possible consequences of reduced spontaneous inhibitory input to layer II neurons, we recorded field potentials in the dentate gyrus, their major synaptic target. At 5 d after pilocarpine-induced status epilepticus the spontaneous field potentials recorded *in vivo* were over three times more frequent than in controls. These findings suggest that an epileptogenic injury reduces inhibition of layer II neurons and results in excessive synaptic input to the dentate gyrus.

Key words: pilocarpine; GABA; interneurons; IPSCs; GAD; dentate gyrus

Introduction

The entorhinal cortex holds a pivotal position linking neocortical and hippocampal regions, and it is likely to play an important role in normal brain function (Van Hoesen, 1982; Witter et al., 1989) and in pathophysiological mechanisms (Braak and Braak, 1992). Layer II neurons of the entorhinal cortex provide the predominant excitatory synaptic input to the hippocampal dentate gyrus (Segal and Landis, 1974; Steward and Scoville, 1976; Schwartz and Coleman, 1981; Ruth et al., 1982). Under normal conditions the activity of layer II neurons is controlled by GABA (Finch et al., 1988; Jones, 1994; Funahashi and Stewart, 1998). When GABA_A receptors are blocked, the entorhinal cortex initiates epileptiform activity (Jones and Lambert, 1990; Menendez de la Prida and Pozo, 2002). Bear et al. (1996) and Scharfman et al. (1998) reported that in models of temporal lobe epilepsy, unlike controls,

layer II neurons respond to synaptic activation with dramatically prolonged repetitive depolarizations, multiple action potentials, and little evidence of an IPSP in normal bathing medium. Therefore, in patients and models of temporal lobe epilepsy a hyperexcitable entorhinal cortex might deliver excessive, synchronous, excitatory synaptic input to the dentate gyrus.

Clinical evidence also suggests that the entorhinal cortex is involved in temporal lobe epilepsy. Spontaneous seizures have been recorded in the hippocampus and entorhinal cortex of patients (Spencer and Spencer, 1994) and models of temporal lobe epilepsy (Bertram, 1997). In patients the stimulation of the entorhinal cortex evokes responses in the hippocampus that resemble spontaneous interictal spikes (Rutecki et al., 1989; Wilson et al., 1990); surgical resection of anterior temporal lobe structures including the entorhinal cortex is an effective treatment (Siegel et al., 1990; Goldring et al., 1992), and MRI studies show that the entorhinal cortex is shrunken (Bernasconi et al., 1999, 2000, 2001; Salmenperä et al., 2000; Jutila et al., 2001). The loss of layer III neurons in the medial entorhinal cortex is a common neuropathological feature in patients (Du et al., 1993) and models of temporal lobe epilepsy (Schwob et al., 1980; Clifford et al., 1987; Du and Schwarcz, 1992; Du et al., 1995; Ribak et al., 1998).

To investigate the mechanisms underlying layer II neuron hyperexcitability, we used anatomical and electrophysiological

Received April 16, 2003; revised July 29, 2003; accepted Aug. 1, 2003.

This work was supported by National Institutes of Health—National Institute of Neurological Disorders and Stroke Grants NS12151 and NS39110. M.K. is a recipient of a fellowship from Uehara Memorial Foundation. We thank Dr. J. Huguenard for software and advice on IPSC data analysis and Drs. A. Tobin and N. Tillakaratne for GAD cDNAs.

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methods in brain slices and *in vivo* to evaluate the medial entorhinal cortex of rats that had experienced pilocarpine-induced status epilepticus 3–7 d previously. We verified the preferential loss of glutamatergic neurons and sparing of GABAergic neurons in layer III of the medial entorhinal cortex (Du et al., 1995; Eid et al., 1999) and asked the following questions: (1) Do the intrinsic electrophysiological properties of layer II neurons change in a way that makes them hyperexcitable after an epileptogenic injury? (2) Do layer II neurons receive less spontaneous inhibitory synaptic input after epileptogenic injuries, and, if so, is it attributable to a loss of GABAergic synapses, reduced probability of synaptic release of GABA, or reduced activity of presynaptic interneurons? (3) Is there increased spontaneous, synchronized, synaptic output from layer II of the entorhinal cortex after an epileptogenic injury?

Materials and Methods

Animals. All experiments were performed in accordance with National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and were approved by the Stanford University Institutional Animal Care and Use Committee. A previously described pilocarpine treatment protocol was used (Turski et al., 1989; Buckmaster et al., 2002). Briefly, male Sprague Dawley rats (35–63 d old) were treated with pilocarpine (380 mg/kg, i.p.) 20 min after atropine methylbromide (5 mg/kg, i.p.). Approximately 60% of the treated rats experienced status epilepticus. Diazepam (10 mg/kg, i.p.) was administered 2–3 hr after the onset of status epilepticus and repeated as needed. Rats were examined 3–7 d after status epilepticus ($n = 20$), before the onset of spontaneous seizures. It is likely that most, if not all, of these rats would have developed epilepsy, because >90% of the rats that we video-monitored in previous studies displayed spontaneous, recurrent seizures within 3 months after pilocarpine-induced status epilepticus (Buckmaster et al., 2002; Kobayashi and Buckmaster, 2003). Control groups included age-matched naive rats ($n = 14$) and pilocarpine-treated rats that did not experience status epilepticus ($n = 21$). We found no significant differences in the anatomical or electrophysiological results from the two types of controls.

Slice experiments. The slice experiment methods have been described previously (Kobayashi and Buckmaster, 2003). Briefly, animals were anesthetized deeply with pentobarbital (75 mg/kg, i.p.) and decapitated. Tissue blocks including the entorhinal cortex were removed rapidly and stored for 3 min in modified ice-cold artificial CSF (M-ACSF) (in mM): 230 sucrose, 2.5 KCl, 10 MgSO₄, 1.25 NaH₂PO₄, 26 NaHCO₃, 2.5 CaCl₂, and 10 D-glucose. Horizontal slices (350 μ m) were cut by a microslicer (VT1000S, Leica, Wetzlar, Germany). Slices were incubated at 32°C for 40 min in a submersion-type holding chamber that contained 50% M-ACSF and 50% normal ACSF, pH 7.35–7.40. The ACSF contained (in mM): 126 NaCl, 3 KCl, 2 MgSO₄, 1.25 NaH₂PO₄, 26 NaHCO₃, 2.0 CaCl₂, and 10 D-glucose. After that the slices were placed in ACSF at 32°C for 1 hr. ACSF was aerated continuously with a mixture of 95% O₂/5% CO₂. Slices thereafter were maintained at room temperature until used for recording.

Principal neurons in layer II of the medial entorhinal cortex were identified with the use of Nomarski optics (40 $\times$, Nikon, Tokyo, Japan) and an infrared-sensitive video camera (Hamamatsu Photonics, Hamamatsu, Japan). Whole-cell patch-clamp recordings were obtained in current-clamp or voltage-clamp mode. Electrical signals were amplified by a patch amplifier (Axopatch 1-D, Axon Instruments, Foster City, CA). Pipette solutions had a pH of 7.3 and osmolality of 300 mOsm. The pipette solution for current-clamp recordings contained (in mM): 100 potassium gluconate, 40 HEPES, 20 biocytin, 10 EGTA, 5 MgCl₂, 2 disodium-ATP, and 0.3 sodium-GTP. The composition of the pipette solution for voltage-clamp recordings was (in mM): 120 cesium methanesulfonate, 20 biocytin, 10 HEPES, 8 NaCl, 5 QX-314, 2 magnesium-ATP, 0.3 sodium-GTP, and 0.1 BAPTA. The presence of QX-314 and cesium in the pipette solution precluded the recording of GABA_B receptor-mediated IPSCs. Under voltage-clamp recording conditions the liquid junction potential was 7 mV, and all voltages were corrected

accordingly. We did not correct the relatively small junction potential under the current-clamp recording conditions. Thick-walled borosilicate patch electrodes (4–6 M Ω) were pulled on a micropipette puller (P-97, Sutter Instruments, Novato, CA). Slices were submerged in a recording chamber maintained at 30–31°C and superfused with ACSF at 1.5 ml/min. Seal resistance was >1 G Ω , and only data obtained from electrodes with access resistance of 8–20 M Ω and <20% change during recordings were included in this study. Series resistance was 80% compensated. Voltage-clamp recordings were low-pass filtered at 1–2 kHz and digitized at 8 kHz. Current-clamp recordings were filtered at 4–10 kHz and digitized at 8–20 kHz.

Input resistance was measured from the slope of least-squares regression lines fit to *I*–*V* curves measured at the peak and at the steady state (current pulse amplitude up to 400 pA). The hyperpolarization-induced sag ratio was calculated as the steady-state voltage deflection divided by the peak voltage deflection after a hyperpolarizing current step sufficient to produce a peak voltage deflection of ~ 20 mV. Single action potentials were evoked by brief (5 msec) depolarizing current pulses, and the amplitudes of the action potential and afterdepolarization (ADP) were measured from the positive peak to the resting membrane potential. The amplitudes of the fast afterhyperpolarization (fAHP) and the medium-duration afterhyperpolarization (mAHP) were measured from the negative peak to the resting membrane potential. Repetitive firing in response to long (500 msec) depolarizing current pulses was evaluated by measuring the slope of a least-squares regression line in a plot of the number of spikes versus the amplitude of injected current (up to ~ 400 pA).

Spontaneous and miniature IPSCs were detected at a threshold of three times the SD of baseline noise amplitude by the use of event detection software (kindly provided by Dr. J. Huguenard, Stanford University). Threshold values for spontaneous IPSCs recorded in normal ACSF were 5.7 ± 0.2 pA in 3–7 d post-status epilepticus rats ($n = 22$ cells) and 6.7 ± 0.4 pA in control rats ($n = 24$ cells). Threshold values for miniature IPSC recordings were 4.8 ± 0.2 pA in 3–7 d post-status epilepticus rats ($n = 21$ cells) and 4.8 ± 0.3 pA in control rats ($n = 19$ cells).

Anatomy. Immediately after slicing, the last (most dorsal) slice prepared was placed in 4% paraformaldehyde in 0.1 M phosphate buffer (PB; pH 7.4) at 4°C for at least 24 hr. This slice corresponds to a horizontal section ~ 6 mm below bregma in the rat brain atlas of Paxinos and Watson (1998). After fixation the slices were stored in 30% ethylene glycol and 25% glycerol in 50 mM PB at less than -20°C . Slices were sectioned with a sliding microtome set at 30 μ m. From each slice one section was stained with 0.25% thionine, and one was processed for GAD65 mRNA *in situ* hybridization, using previously described protocols and reagents (Buckmaster and Jongen-Rèlo, 1999; Kobayashi and Buckmaster, 2003). The GAD65 cDNA (kindly provided by Drs. A. Tobin and N. Tillakaratne, University of California at Los Angeles) was ~ 2.4 kb and was isolated from a λ ZapII library from adult rat hippocampus (Erlander et al., 1991). Neuron profiles were counted with a microscope (Nikon) equipped with a 40 $\times$ objective, motorized stage (Ludl Electronic Products, Hawthorne, NY), and Lucivid and Stereo Investigator software (MicroBrightField, Colchester, VT). We evaluated the entire entorhinal cortex, because the border between the medial and lateral parts can be challenging to define. To estimate the density of Nissl-stained neuron profiles, we drew a contour around layer III of the entorhinal cortex. Neuron profiles were counted if their nucleus at least partially fell within the counting frame, but not if it touched the left or lower border of the counting frame. An average of 317 profiles was counted per entorhinal cortex. The counting frame was $100 \times 100 \mu\text{m}$, and the counting grid was $250 \times 250 \mu\text{m}$, so an average of 16% of the total area was sampled randomly and systematically. The density of neuron profiles was calculated by dividing the estimated total number of layer III neuron profiles by the total area of layer III. To measure the density of GAD-positive neuron profiles, we drew one contour around layer III of the entire (lateral and medial) entorhinal cortex and another contour around layers I + II. All of the GAD-positive neuron profiles within the contours were counted. Averages of 289 and 224 profiles were counted in layer III and layers I + II, respectively, and the density of GAD-positive neuron profiles was calculated as described above.

To visualize biocytin-labeled neurons after recording, we fixed and

Figure 1. The medial entorhinal cortex in control (*A, C, E*) and 3–7 d post-status epileptic rats (*B, D, F*). *A, B*, Nissl-stained sections reveal the preferential loss of layer III neurons in the post-status epileptic rat (*B*). Presubiculum and parasubiculum are to the right; lateral entorhinal cortex is to the left. *C, D*, Adjacent sections from the same rats were processed for GAD65 mRNA *in situ* hybridization. Both rats display numerous GAD-positive neuronal profiles in all layers, including layer III. *E, F*, Recorded neurons were identified morphologically by biocytin labeling (red). Counterstaining for NeuN immunoreactivity (green) revealed the layers of the medial entorhinal cortex and the loss of layer III neurons in the post-status rat (*F*). I.d., lamina dissecans.

stored slices as described above and then processed them by using a whole-mount protocol with counterstaining by NeuN immunocytochemistry. Slices were rinsed in 0.5% Triton X-100 and 0.1 M glycine in 0.1 M PB and then placed in a blocking solution containing 0.5% Triton X-100, 2% goat serum (Vector Laboratories, Burlingame, CA), and 2% bovine serum albumin in 0.1 M PB for 4 hr. Slices were incubated in mouse anti-NeuN serum (1:100; MAB377, Chemicon, Temecula, CA) in blocking solution overnight. Slices were rinsed in 0.5% Triton X-100 and 0.1 M glycine in 0.1 M PB and then incubated with the fluorophores Alexa 594 streptavidin (5 μg/ml) and Alexa 488 goat anti-mouse (10 μg/ml; Molecular Probes, Eugene, OR) in blocking solution overnight. Slices were rinsed in 0.5% Triton X-100 and 0.1 M glycine in 0.1 M PB, mounted on slides, and coverslipped with Vectashield (Vector Laboratories). Slices were examined, and images were obtained with a confocal microscope (LSM 5 Pascal, Zeiss, Oberkochen, Germany).

In vivo field potential recording. Dentate gyrus field potentials were obtained by using methods described previously (Buckmaster and Dudek, 1997). Briefly, rats were anesthetized (1.2 gm/kg urethane, i.p.) and placed in a stereotaxic apparatus. Body temperature was maintained with a heating pad with feedback control. Holes were drilled in the skull at the following coordinates (in mm, relative to bregma): −4.6 and 2.6 lateral for recording and −7.6 and 4.6 lateral for stimulating. Electrode

depths were determined by optimizing responses to stimulation. Recording electrodes were glass micropipettes broken to an inner diameter of ~15 μm and filled with 0.9% sodium chloride. Field potentials were amplified and recorded (Axoclamp-2B and pClamp, Axon Instruments). A bipolar concentric electrode (SNEX-100, Rhodes Medical Instruments, Woodland Hills, CA) activated perforant path fibers in the angular bundle (0.1 Hz, 150 μsec duration), and the stimulus intensity was set to evoke a maximal amplitude field EPSP recorded with the electrode tip in the hilus of the dentate gyrus.

After determining the optimal depth of the stimulating electrode and recording evoked responses, we recorded spontaneous field potentials for at least 10 min. Afterward, evoked responses were checked to verify that conditions had not changed. In some experiments the recording electrode was maintained in the dentate gyrus while the stimulating electrode was replaced with a patch pipette (broken to an inner diameter of ~100 μm) so that the tip of the pipette occupied the optimal position for stimulating the angular bundle. Fine tubing was inserted into the patch pipette so that the internal pipette solution could be changed. Spontaneous field potentials were recorded from the dentate gyrus while 0.9% sodium chloride control solution or a sodium channel blocker (2% lidocaine, Abbott Laboratories, North Chicago, IL) was injected into the patch pipette and leaked into the angular bundle. Spontaneous field potentials were filtered (4–1000 Hz), plotted, and analyzed (Clampfit, Axon Instruments). An investigator who was blind to the experimental groups identified spontaneous field potentials and measured their frequency, duration, and peak-to-peak amplitude.

All chemicals unless otherwise specified were purchased from Sigma (St. Louis, MO). All statistical values are presented as the means ± SEM. Statistical comparisons were performed with an unpaired Student's *t* test. The level of *p* < 0.05 was considered significant.

Results

Preferential loss of excitatory neurons in layer III

Previous studies demonstrated the loss of layer III neurons in the medial entorhinal cortex of patients (Du et al., 1993) and models of temporal lobe epilepsy (Schwob et al., 1980; Clifford et al., 1987; Du and Schwarcz, 1992; Du et al., 1995; Ribak et al., 1998). To determine whether this had occurred 3–7 d after status epilepticus in our rats, we evaluated tissue collected during slice preparation for electrophysiological recording. All of the rats that had experienced at least 2 hr of status epilepticus (*n* = 13), but none of the controls (*n* = 26), demonstrated reduced density of Nissl-stained neuron profiles in layer III of the most medial part of the medial entorhinal cortex (Fig. 1*A,B*). The estimated number of Nissl-stained neuron profiles in the entorhinal cortex after status epilepticus was 60% of controls (1385 ± 113 vs 2309 ± 64 profiles; *p* < 0.0001, Student's *t* test). The area of layer III of the entorhinal cortex 3–7 d after status epilepticus was 90% of controls (0.80 ± 0.02 vs 0.88 ± 0.02 mm²; *p* < 0.02, Student's *t* test).

Figure 2. Layer III neuron profile densities in sections of the entire (medial + lateral) entorhinal cortex of control and 3–7 d post-status epileptic rats. In these scatter plots the horizontal lines indicate the means. The density of Nissl-stained neuron profiles was reduced significantly after status epilepticus ($**p < 0.0001$, Student's *t* test). However, the density of GAD65 mRNA-positive neuron profiles was similar in control and post-status epileptic rats.

The density of Nissl-stained neuron profiles in layer III of the entorhinal cortex was 66% of controls (1741 ± 118 vs 2657 ± 86 profiles/mm²; $p < 0.0001$, Student's *t* test; Fig. 2).

Previous studies demonstrated that in models of temporal lobe epilepsy parvalbumin- and GABA-immunoreactive interneurons in layer III of the medial entorhinal cortex were relatively spared as compared with the vulnerable pyramidal neurons (Du et al., 1995; Eid et al., 1999). To determine whether layer III interneurons were spared after status epilepticus in our rats, we evaluated sections processed for *in situ* hybridization of GAD65 mRNA. GAD-positive neurons were present in all layers of the entorhinal cortex (Köhler et al., 1985), even in layer III of the medial entorhinal cortex in rats that had experienced status epilepticus (Fig. 1C,D). The density of GAD-positive neuron profiles in layer III after status epilepticus (455 ± 17 profiles/mm²; $n = 12$) was similar to controls (432 ± 10 profiles/mm²; $n = 25$; Fig. 2). The density of GAD-positive neuron profiles in layers I + II after status epilepticus (352 ± 14 profiles/mm²) was similar to controls (335 ± 10 profiles/mm²). These findings demonstrate relative sparing of GABAergic interneurons and preferential loss of glutamatergic neurons in layer III of the medial entorhinal cortex 3–7 d after pilocarpine-induced status epilepticus.

Normal intrinsic electrophysiological properties of layer II neurons

Previous studies have reported that layer II neurons of the entorhinal cortex are hyperexcitable in chronic models of temporal lobe epilepsy (Bear et al., 1996; Scharfman et al., 1998). To determine whether this hyperexcitability is attributable to changes in intrinsic electrophysiological properties, we evaluated responses to current injection (Fig. 3). The identity of the layer II cells was verified with biocytin labeling and counterstaining of neuronal layers with NeuN immunocytochemistry (Fig. 1E,F). All biocytin-labeled cells had a soma positioned in layer II, spiny dendrites, and a large-diameter axon that extended toward the angular bundle.

The resting membrane potential, input resistance, and sag ratio of post-status epilepticus rats were similar to controls and to previously reported values from control rats (Alonso and Klink, 1993; Jones, 1994; Wang and Lambert, 2003) (Table 1). Single action potentials were followed by a fAHP, ADP, and mAHP (Fig. 3B). These afterpotentials play an important role in the regulation of repetitive spike firing (Schwindt et al., 1988; Kobayashi et al., 1997), and they were similar in control and post-status epilepticus rats (Table 1). Long (500 msec) depolarizing current

Figure 3. Typical subthreshold and firing properties of layer II neurons in the medial entorhinal cortex of a 3–7 d post-status epilepticus rat. *A*, Hyperpolarizing current injection elicited a prominent sag in the response of the membrane potential. *B*, Single action potentials were elicited by a brief intracellular current injection in the same neuron. Inset shows a time- and voltage-expanded trace. An action potential was followed by fast afterhyperpolarization (fAHP), afterdepolarization (ADP), and medium-duration afterhyperpolarization (mAHP). *C*, Repetitive firing was induced by a long (500 msec) intracellular current injection in the same neuron, which displayed slight spike adaptation and no bursting. The number of evoked spikes was plotted against the depolarizing current pulse amplitude for all of the neurons in the post-status epilepticus sample. The slope of least-squares regression lines through the 0–400 pA current range was measured.

pulses evoked slightly adapting trains, not bursts, of action potentials that were similar in control and post-status epilepticus rats (Fig. 3C, Table 1). These findings suggest that the intrinsic electrophysiological properties of layer II neurons in the medial

Table 1. Intrinsic electrophysiological properties of layer II principal cells in the medial entorhinal cortex

	Control	<i>n</i>	3–7 d Post-status	<i>n</i>
Resting membrane potential (mV)	-59.1 ± 0.9	18	-57.9 ± 0.6	20
Input resistance				
Peak (M Ω)	96 ± 12	14	85 ± 8	18
Steady (M Ω)	78 ± 14	14	61 ± 9	18
Sag ratio (%)	66 ± 4	14	61 ± 3	18
Single action potential				
Threshold (mV)	-46.1 ± 0.7	18	-45.5 ± 0.5	20
Amplitude (mV)	102.5 ± 2.9	18	96.9 ± 2.6	20
Half-duration (msec)	0.9 ± 0.0	18	0.9 ± 0.0	20
fAHP ^a amplitude (mV)	1.7 ± 1.2	18	1.6 ± 0.9	20
ADP ^b amplitude (mV)	6.3 ± 0.9	18	6.0 ± 0.6	20
mAHP ^c amplitude (mV)	-1.7 ± 0.2	18	-2.1 ± 0.2	20
mAHP half-duration (msec)	54.8 ± 5.3	18	64.1 ± 3.2	20
Repetitive firing				
Slope (spikes/nA)	45 ± 4	9	40 ± 3	18

Values represent mean $\pm$ SEM. ^aFast afterhyperpolarization; ^bafterdepolarization; ^cmedium-duration after hyperpolarization. There were no significant differences in the values of control versus 3–7 d post-status epileptic rats (*t* test).

entorhinal cortex are not responsible for their hyperexcitability after an epileptogenic treatment. Similarly, in another model of temporal lobe epilepsy the neurons in deep layers of the entorhinal cortex display hyperexcitable evoked responses but have normal intrinsic electrophysiological properties (Fountain et al., 1998).

Reduced spontaneous inhibitory postsynaptic currents of layer II neurons

In a chronic model of temporal lobe epilepsy layer II neurons of the medial entorhinal cortex responded to stimulation of deep layers with a barrage of EPSPs and spikes; however, in the presence of glutamate receptor blockers, stimulation close to the cell evoked monosynaptic IPSPs of normal amplitude (Bear et al., 1996). To evaluate GABA_A receptor-mediated synaptic inputs to layer II neurons of the medial entorhinal cortex, we recorded spontaneous IPSCs (sIPSCs) in control (*n* = 24 cells) and post-status epileptic rats (*n* = 22 cells; Fig. 4). sIPSCs were recorded at a holding potential of 0 mV, which is near the reversal potential of glutamatergic postsynaptic currents. Analysis of sIPSCs revealed that their average frequency was reduced to 68% of controls (19.3 ± 1.5 vs 28.5 ± 2.3 Hz; *p* < 0.01, Student's *t* test),

charge transfer was reduced to 51% of controls (11.3 ± 1.9 vs 22.1 ± 3.0 pC/sec; *p* < 0.01, Student's *t* test), amplitude was reduced to 57% of controls (21.1 ± 2.5 vs 37.3 ± 3.5 pA; *p* < 0.001, Student's *t* test), but 10–90% rise time was similar to controls (1.8 ± 0.1 vs 2.0 ± 0.1 msec). These findings suggest that after status epilepticus the layer II neurons in the medial entorhinal cortex receive abnormally low levels of spontaneous inhibitory synaptic input.

Reduced frequency of sIPSCs could be attributable to a loss of inhibitory synapses, reduced probability of synaptic release of GABA, and/or reduced activity of presynaptic interneurons. To help to distinguish among these possibilities, we evaluated miniature IPSCs (mIPSCs) recorded in the presence of 1 μ M tetrodotoxin, 25 μ M CNQX, and 50 μ M D-APV. Analysis of the mIPSCs revealed no significant differences in the frequency, amplitude, and 10–90% rise times in control and post-status epileptic rats (Fig. 5). These findings indicate that a loss of inhibitory synapses and reduced probability of synaptic release of GABA are unlikely mechanisms to account for the reduction in sIPSCs in layer II neurons of the medial entorhinal cortex after an epileptogenic injury.

Figure 4. Spontaneous IPSCs (sIPSCs) of layer II medial entorhinal cortical neurons recorded in slices from a control (A1) and 3–7 d post-status epileptic rat (A2). *Aa*, *Ab*, Middle and bottom traces show time-expanded views of the regions indicated by the bars under the top traces. *B*, Cumulative probability plots of inter-event interval, amplitude, and 10–90% rise time of sIPSCs in each cell in the control (black lines) and 3–7 d post-status epileptic groups (gray lines). Note that most curves of the inter-event interval in 3–7 d post-status epileptic rats are shifted to the right (lower frequency), and those of amplitude are shifted to the left (lower amplitude) as compared with controls. *C*, Frequency, charge transfer, amplitude, and 10–90% rise time of sIPSCs in control and 3–7 d post-status epileptic rats. Error bars indicate SEM. The frequency, charge transfer, and amplitude of sIPSCs in 3–7 d post-status epileptic rats were significantly less than those in controls. ***p* < 0.01; ****p* < 0.001 (Student's *t* test).

Increased output from layer II of the entorhinal cortex after status epilepticus

Layer II neurons of the medial and lateral entorhinal cortex project to the middle and outer thirds of the dentate gyrus molecular layer, respectively (Steward and Scoville, 1976), where their axon terminals

account for at least 85% of the synaptic contacts formed with dendritic spines (Nafstad, 1967; Matthews et al., 1976), and they generate large-amplitude EPSPs in granule cells (Andersen et al., 1966). To evaluate the possible consequences of reduced inhibition of layer II neurons, we recorded spontaneous field potentials in the dentate gyrus of urethane-anesthetized rats 5 d after pilocarpine treatment. In these experiments the control rats were treated identically as the status epilepticus rats, except they received only 200 mg/kg pilocarpine and did not experience status epilepticus. With a stimulating electrode placed in the angular bundle to activate perforant path fibers (0.1 Hz, 150 μ sec duration), the stimulus intensity (1.1 ± 0.2 vs 1.0 ± 0.3 mA) and maximum amplitude of the evoked field EPSPs (17.4 ± 1.1 vs 15.2 ± 1.5 mV) were similar in control ($n = 6$) and post-status epilepticus rats ($n = 8$), and only a single population spike could be elicited (Fig. 6A). However, the frequency of spontaneous field potentials recorded in the dentate gyrus was over three times higher in post-status epilepticus rats (0.39 ± 0.03 vs 0.12 ± 0.03 Hz; $p < 0.0001$, Student's t test; Fig. 6B). The duration and peak-to-peak amplitude of the spontaneous field potentials were similar in control and post-status epilepticus rats (0.85 ± 0.18 vs 1.05 ± 0.16 sec and 1.8 ± 0.1 vs 1.6 ± 0.2 mV, respectively). To test whether the spontaneous field potentials were generated by the entorhinal cortex, we attempted to block them by focally applying lidocaine into the stimulating electrode site. In three of three post-status epilepticus rats 2% lidocaine, but not 0.9% sodium chloride, reversibly blocked the spontaneous field potentials recorded in the dentate gyrus (Fig. 6C). These findings suggest that conduction along the perforant path is necessary for generating spontaneous field potentials in the dentate gyrus and that the entorhinal cortex delivers excessive, synchronous, synaptic input to the dentate gyrus after epileptogenic injury.

Discussion

The principal findings of this study are that 3–7 d after an epileptogenic treatment that preferentially killed excitatory neurons in layer III of the medial entorhinal cortex but spared inhibitory interneurons, (1) the intrinsic electrophysiological properties of layer II neurons did not change significantly, (2) layer II neurons received less spontaneous GABA_A receptor-mediated synaptic input, but their mIPSCs were normal, and (3) synchronized, spontaneous, synaptic output from layer II of the entorhinal cortex to the dentate gyrus increased.

Preferential loss of excitatory neurons in layer III of the medial entorhinal cortex

Previous studies have demonstrated the preferential loss of layer III neurons of the medial entorhinal cortex in patients (Du et al., 1993) and models of temporal lobe epilepsy. The models include

Figure 5. Miniature IPSCs (mIPSCs) of layer II medial entorhinal cortical neurons recorded in CNQX/*D*-APV/TTX in slices from a control (A1) and a 3–7 d post-status epilepticus rat (A2). Aa, Ab, Middle and bottom traces show time-expanded views of regions indicated by the bars under the top traces. B, Cumulative probability plots of inter-event interval, amplitude, and 10–90% rise time of mIPSCs in each cell in the control (black lines) and 3–7 d post-status epilepticus groups (gray lines). C, Frequency, amplitude, and 10–90% rise time of mIPSCs in control and 3–7 d post-status epilepticus rats. Error bars indicate SEM. There were no significant differences between control and post-status epilepticus rats (Student's t test).

rats treated with kainic acid, pilocarpine, amino-oxyacetic acid, or electrical stimulation (Schwob et al., 1980; Clifford et al., 1987; Du and Schwarcz, 1992; Du et al., 1995) and monkeys with alumina gel injections into the temporal lobe (Ribak et al., 1998). In the medial entorhinal cortex a majority of GABAergic interneuron somata are located in layer II and the superficial part of layer III, and, of all of the layers, layer II contains the highest density of GABAergic axon terminals, which synapse with GABA-negative somata (Köhler et al., 1985). Previous studies reported that, despite the loss of Nissl-stained layer III neurons in the medial entorhinal cortex, parvalbumin- and GABA-immunoreactive interneurons in this region were relatively spared (Du et al., 1995; Eid et al., 1999). Our results with another marker for inhibitory interneurons (*in situ* hybridization for GAD65 mRNA) corroborate previous reports and extend them by demonstrating similar densities of GAD-positive neurons in layers I + II of the entorhinal cortex, which is the location of the somata and most of the dendrites of layer II neurons. The mechanisms underlying the vulnerability of glutamatergic neurons and the protection of GABAergic interneurons are unclear. Nevertheless, these findings indicate that inhibitory interneurons survive; therefore, interneuron loss is an unlikely mechanism of layer II hyperexcitability 3–7 d after an epileptogenic injury.

Figure 6. Dentate gyrus field potentials recorded in control (A1, B1) and 3–7 d post-status epilepticus rats (A2, B2, C). *A*, The maximum amplitude of the field EPSP evoked by perforant path stimulation was similar in control and post-status epilepticus rats, and only a single population spike was evoked. *B*, The frequency of spontaneous field potentials was increased significantly after status epilepticus, but the individual events were similar in duration and amplitude. *C*, Spontaneous field potentials were blocked reversibly by focal application of 2% lidocaine (second arrow) to the stimulation electrode site (perforant path). After the lidocaine was washed out of the pipette, the spontaneous field potentials returned. Focal application of 0.9% sodium chloride (first arrow) did not block the spontaneous field potentials.

Layer II neurons generate excessive, synchronous output

At 5 d after status epilepticus the spontaneous field potentials recorded in the dentate gyrus were over three times more frequent, and they could be blocked by focal application of lidocaine to the angular bundle. These findings suggest that layer II neurons of the entorhinal cortex generate excessive, synchronous output to the dentate gyrus after an epileptogenic injury. Because the increased frequency of spontaneous field potentials precedes the onset of spontaneous seizures, it is not just a side effect of epilepsy and instead might contribute to epileptogenic processes. Our findings reveal that, during the latent period while mossy fibers in the dentate gyrus are sprouting axon collaterals and forming new synaptic contacts, granule cells receive excessive, synchronous, excitatory, synaptic input. Patterns of afferent input can have long-lasting effects on the organization of neuronal circuits during development (Wiesel and Hubel, 1965) and after injuries (Carmichael and Chesselet, 2002). Therefore, increased synchronized output of the entorhinal cortex in pre-epileptic patients and models might affect synaptic reorganization in the dentate gyrus. These findings also suggest that an increased frequency of spontaneous field potentials alone is not sufficient to trigger spontaneous seizures, because rats were not observed to experience motor convulsions during the latent period. Similarly, rats treated with amino-oxyacetic acid to produce layer III neu-

ron loss in the medial entorhinal cortex displayed spontaneous field potentials in the hippocampus and entorhinal cortex, but they were not observed to develop spontaneous seizures (Scharfman et al., 1998). Therefore, other changes besides or in addition to increased synchronous output from layer II neurons appear to be necessary to produce temporal lobe epilepsy.

The spontaneous field potential oscillations included dentate EEG spikes. Dentate EEG spikes are generated by synchronous EPSPs of granule cells, and the EPSPs are produced by the synchronous discharge of layer II neurons in the entorhinal cortex (Bragin et al., 1995). It has been proposed that dentate spikes inhibit output from the hippocampus back to the entorhinal cortex (Bragin et al., 1995). If this is the case, the increased frequency of spontaneous field potentials might be anti-epileptic. However, in kainate-induced chronically epileptic rats the dentate EEG spikes are more frequent and larger in amplitude than in controls (Buckmaster and Dudek, 1997), and there is a progressive increase in the frequency and amplitude of dentate EEG spikes at the onset of spontaneous seizures (Hellier et al., 2000). In patients with temporal lobe epilepsy the stimulation of the entorhinal cortex evokes a large-amplitude field potential spike in the hippocampus (Wilson et al., 1990) that closely resembles spontaneous interictal spikes (Rutecki et al., 1989), which occur nearly simultaneously in the entorhinal cortex and hippocampus (Spencer and Spencer, 1994). These findings raise the possibility that, after epilepsy develops, output from layer II neurons might trigger seizures. Perhaps in the presence of the typical neuropathology of mesial sclerosis (including hilar neuron loss and mossy fiber sprouting), excessive, synchronized, synaptic inputs from the entorhinal cortex together with reduced inhibition (de Lanerolle et al., 1989; Obenaus et al., 1993; Williamson et al., 1999; Kobayashi and Buckmaster, 2003; Sayin et al., 2003) and increased recurrent excitation in the dentate gyrus (Nadler et al., 1980; Sutula et al., 1989; Wuarin and Dudek, 1996) occasionally combine to surpass the seizure threshold.

Reduced inhibition might cause excessive, synchronous activity of layer II neurons

Like previous studies of models of temporal lobe epilepsy (Bear et al., 1996; Fountain et al., 1998; Scharfman et al., 1998), we found no evidence to support the hypothesis that the hyperexcitability of the entorhinal cortex is attributable to changes in the intrinsic neuronal electrophysiology. Therefore, we evaluated synaptic mechanisms. Anatomical (Dolorfo and Amaral, 1998) and functional evidence (Biella et al., 2002) suggests that layer II neurons normally interact via associative connections. Reducing synaptic inhibition in associative networks synchronizes neuronal discharge (Miles and Wong, 1987) and facilitates its spread (Chagnac-Amitai and Connors, 1989). In slices the entorhinal cortex initiates epileptiform discharges at bicuculline concentrations sufficient to block GABA_A receptors >50% (Menendez de la Prida and Pozo, 2002). It is possible, therefore, that the increased frequency of spontaneous dentate field potentials was attributable, at least in part, to reduced spontaneous inhibition of layer II neurons in the medial entorhinal cortex. However, it is not clear whether the increased synchronous activity of layer II neurons was initiated in layer II, required interaction with deep layers of the entorhinal cortex (Jones and Lambert, 1990; Dickson and Alonso, 1997; Lopantsev and Avoli, 1998; Stewart, 1999), or was relayed from an afferent to the entorhinal cortex.

Mechanisms of reduced inhibitory synaptic input of layer II neurons

It has been proposed that in models of temporal lobe epilepsy the layer II neurons of the medial entorhinal cortex become hyperexcitable because presynaptic inhibitory interneurons become “disconnected” from their normal excitatory afferent input after layer III neuron loss (Bear et al., 1996; Eid et al., 1999). If this hypothesis were correct, one would expect layer II neurons to receive less spontaneous inhibitory synaptic input after layer III neuron loss. In addition, if presynaptic interneurons survived and maintained their synaptic contacts with layer II neurons and if those synapses maintained normal probabilities of synaptic release, one would expect that the frequency of mIPSCs would be unaffected by layer III neuron loss. Our findings of reduced sIPSC frequency and amplitude but normal mIPSCs in layer II neurons 3–7 d after pilocarpine-induced layer III neuron loss are consistent with reduced activity of presynaptic inhibitory interneurons. However, from the available data we cannot distinguish among various potential mechanisms, including disconnection from afferents, that might reduce interneuron activity.

The “dormant basket cell” hypothesis was proposed originally as a mechanism of hyperexcitability in the dentate gyrus, whereby inhibitory interneurons become disconnected from their proposed normal excitatory synaptic input (Sloviter, 1994). However, unlike the entorhinal cortex, in the dentate gyrus of patients and models of temporal lobe epilepsy there is significant loss of GABAergic interneurons (de Lanerolle et al., 1989; Obenaus et al., 1993) and a significant reduction not only of sIPSCs but also mIPSCs and monosynaptic evoked IPSPs (Williamson et al., 1999; Kobayashi and Buckmaster, 2003; Sayin et al., 2003). These findings suggest that reduced inhibition of dentate granule cells is attributable, at least partially, to the loss rather than to the inactivity of interneurons. The mechanisms of reduced inhibition, therefore, appear to be different in the dentate gyrus and in the medial entorhinal cortex, even in rats treated identically and evaluated at the same time point in the epileptogenic process (Kobayashi and Buckmaster, 2003). These observations underscore the potential complexity of temporal lobe epileptogenesis. The underlying mechanisms may be multiple, diverse, regionally specific, and synergistic.

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