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Hippocampal Granule Cell Activity and c-Fos Expression during Spontaneous Seizures in Awake, Chronically Epileptic, Pilocarpine-Treated Rats: Implications for Hippocampal Epileptogenesis

BRIAN D. HARVEY AND ROBERT S. SLOVITER*
Department of Pharmacology; Department of Neurology; the Graduate Program in Neuroscience, University of Arizona College of Medicine, Tucson, Arizona 85724

ABSTRACT

The process of postinjury hippocampal epileptogenesis may involve gradually developing dentate granule cell hyperexcitability caused by neuron loss and synaptic reorganization. We tested this hypothesis by repeatedly assessing granule cell excitability after pilocarpine-induced status epilepticus (SE) and monitoring granule cell behavior during 235 spontaneous seizures in awake, chronically implanted rats. During the first week post-SE, granule cells exhibited diminished paired-pulse suppression and decreased seizure discharge thresholds in response to afferent stimulation. Spontaneous seizures often began during the first week after SE, recruited granule cell discharges that followed behavioral seizure onsets, and evoked c-Fos expression in all hippocampal neurons. Paired-pulse suppression and epileptiform discharge thresholds increased gradually after SE, eventually becoming abnormally elevated. In the chronic epileptic state, interictal granule cell hyperinhibition extended to the ictal state; granule cells did not discharge synchronously before any of 191 chronic seizures. Instead, granule cells generated only low-frequency voltage fluctuations (presumed "field excitatory postsynaptic potentials") during 89% of chronic seizures. Granule cell epileptiform discharges were recruited during 11% of spontaneous seizures, but these occurred only at the end of each behavioral seizure. Hippocampal c-Fos after chronic seizures was expressed primarily by inhibitory interneurons. Thus, granule cells became progressively less excitable, rather than hyperexcitable, as mossy fiber sprouting progressed and did not initiate the spontaneous behavioral seizures. These findings raise doubts about dentate granule cells as a source of spontaneous seizures in rats subjected to prolonged SE and suggest that dentate gyrus neuron loss and mossy fiber sprouting are not primary epileptogenic mechanisms in this animal model. J. Comp. Neurol. 488:442–463, 2005.

Indexing terms: epilepsy; status epilepticus; hippocampus; dentate gyrus; c-Fos

Hippocampal neuron loss and synaptic reorganization (mossy fiber sprouting) are characteristic features of human temporal lobe epilepsy with hippocampal sclerosis (Meldrum and Bruton, 1992; Engel, 2001). The sclerotic hippocampus is a suspected source of epileptic seizures in human patients (Falconer, 1974; Spencer, 1998, 2002; Wennberg et al., 2002), and the extensive loss of hippocampal pyramidal neurons in many human epileptic hippocampi (Sommer, 1880; Margerison and Corsellis, 1966) has logically focused attention on the remaining dentate granule cells as a likely source of focal seizures (Tauck and Nadler, 1985; Sloviter, 1987; Sutula et al., 1988; Babb et al., 1991). In experimental animals subjected to prolonged status epilepticus (SE), potentially

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*Correspondence to: Robert S. Sloviter, Department of Pharmacology, University of Arizona College of Medicine, 1501 N. Campbell Avenue, Tucson, AZ 85724-5050. E-mail: sloviter@u.arizona.edu

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epileptogenic granule cell hyperexcitability has been hypothesized to occur as a result of hilar mossy cell loss (Sloviter, 1987, 1991b, 1994), dysfunction of surviving mossy cells (Santhakumar et al., 2000; Scharfman et al., 2001), or the synaptic reorganization (mossy fiber sprouting) that follows hilar neuron loss (Tauck and Nadler, 1985).

According to the mossy fiber sprouting hypothesis of hippocampal epileptogenesis, an initial loss of vulnerable hilar mossy cells denervates granule cell dendrites of the dentate inner molecular layer (Nadler et al., 1980; Laurberg and Zimmer, 1981; Longo et al., 2003). The loss of mossy cell input to granule cells is hypothesized to trigger the formation of new and abnormal recurrent excitatory connections among normally unconnected granule cells (Tauck and Nadler, 1985; Sutula et al., 1988; Babb et al., 1991; Wuarin and Dudek, 1996). This newly interconnected granule cell network is therefore conceived of as a malfunctional "syncytium" that either initiates seizures spontaneously (Babb et al., 1991; Wuarin and Dudek, 1996, 2001), or responds abnormally to afferent excitation (Patrylo and Dudek, 1998; Okazaki et al., 1999; Nadler, 2003). This hypothesis of "excitatory" mossy fiber sprouting is appealing, in part, because a gradual process of synaptic reorganization might explain the "latent period" between an initial insult and the subsequent emergence of clinical seizures (Lhatoo et al., 2001; White, 2002).

An alternate view, "inhibitory" mossy fiber sprouting (Sloviter, 1992), suggests that the same loss of vulnerable hilar mossy cells that denervates granule cell dendrites also denervates inhibitory interneurons (Laurberg and Zimmer, 1981; Seress and Ribak, 1984; Deller et al., 1994) and that the reinnervation of inhibitory neurons by aberrant mossy fibers may explain the abnormally increased interictal granule cell paired-pulse inhibition observed in anesthetized, chronically epileptic rats (Sloviter, 1992; Buckmaster and Dudek, 1997; Wu and Leung, 2001). Although ultrastructural studies have confirmed the formation by aberrant mossy fibers of synaptic connections with both granule cells (Represa et al., 1993; Okazaki et al., 1995; Wenzel et al., 2000; Buckmaster et al., 2002; Cavazos et al., 2003) and inhibitory interneurons (Kotti et al., 1997; Wenzel et al., 2000; Buckmaster et al., 2002; Cavazos et al., 2003), the possible effects of cell loss and synaptic reorganization on granule cell excitability remain unclear (Elmer et al., 1997; Longo and Mello, 1999; Nissinen et al., 2001) because it has not been demonstrated in awake, chronically epileptic animals that dentate granule cells become a source of seizure discharges. Although dentate granule cells are reportedly chronically hyperinhibited in anesthetized epileptic rats (Sloviter, 1992; Buckmaster and Dudek, 1997; Wu and Leung, 2001), it has been hypothesized that, in the unanesthetized state, inhibition collapses just before a seizure occurs (Buhl et al., 1996; McNamara, 1999; Coulter, 2000) and that this reveals an underlying hyperexcitability (Cronin et al., 1992) that results in granule cell seizure discharges. Thus, the assumption that dentate granule cells constitute a primary epileptogenic focus in rats subjected to prolonged SE has long been the conceptual underpinning of many experimental epilepsy studies (Tauck and Nadler, 1985; Sloviter, 1987, 1992; Sutula et al., 1988; Buhl et al., 1996; Wuarin and Dudek, 1996). Therefore, in awake, chronically implanted rats made epileptic by an episode of pilocarpine-induced status epilepticus (Cavalheiro et al., 1991), we assessed granule cell excitability at different stages of the epileptogenic process, recorded granule cell activity during 235 spontaneous seizures, and assessed the patterns of c-Fos expression after spontaneous seizures.

MATERIALS AND METHODS

Sixty-four male Sprague-Dawley rats (300–400 g; Harlan Sprague Dawley, Indianapolis, IN) were treated in accordance with the guidelines of the National Institutes of Health for the humane treatment of animals, and the methods used were approved by the University of Arizona Institutional Animal Care and Use Committee.

Pilocarpine-induced SE

Rats were given atropine methylbromide subcutaneously (sc) to prevent deleterious, peripherally induced cholinergic effects (1 mg/kg sc in saline vehicle; Sigma, St. Louis, MO). This was followed 30 minutes later by pilocarpine hydrochloride (250–320 mg/kg sc in saline vehicle; Sigma). Controls received saline injections. After 3 hours of continuous SE, rats were given urethane (0.8 g/kg sc) to suppress the behavioral seizures and aid survival. All animals were given saline periodically by sc injection until recovery. Apple slices were provided as a source of food and water in a combined form until the animals resumed eating and drinking.

Electrophysiological experiments were performed in nine saline-treated, chronically implanted control animals, 19 chronically implanted rats that survived pilocarpine-induced SE and became epileptic (10 rats implanted >60 days after SE, eight of which survived for anatomical analysis, and nine rats implanted prior to SE, four of which were perfusion fixed 4, 7, 8, or 28 days after SE to assess mossy fiber sprouting in the early post-SE period). Additional unimplanted pilocarpine-treated rats (n = 23; six perfusion fixed 9 days post-SE, and 17 perfusion fixed >2 months post-SE) were analyzed for c-Fos expression 1 hour after spontaneous seizures, as described below. Additional rats (n = 4) were perfusion fixed immediately after SE to evaluate SE-induced c-Fos expression.

Implantation of chronic stimulating and recording electrodes for awake recording

With rats under buprenorphine (0.05 mg/kg sc) analgesia and chloral hydrate (350 mg/kg sc) or urethane (1.25 g/kg sc) anesthesia, the surgical area was shaved and then cleaned with povidone-iodine. A midline incision exposed the skull, and six stainless-steel screws were inserted into the skull to stabilize the electrodes and provide an electrical ground for recording. Bipolar stainless-steel stimulating electrodes (Rhodes Medical Instruments, Summerland, CA) were placed bilaterally in the angular bundles of the perforant pathway (~4.5 mm lateral from the midline suture and immediately rostral to the lambdoid suture). Unipolar recording electrodes, fabricated from Teflon-coated 0.003-inch-diameter stainless-steel microwires (7910; A-M Systems, Inc., Carlsborg, WA) were lowered into the brain bilaterally (~2 mm lateral from the midline, ~3 mm caudal to bregma, and 3.5 mm below the brain surface). Final tip locations in the granule cell layer were reached by optimizing the potentials evoked by perforant pathway stimulation (Andersen et al., 1966). Several an-

imals were implanted with one recording electrode in the granule cell layer and one in the contralateral CA1 pyramidal cell layer. A layer of dental acrylic cement attached the electrodes to the screws and skull. Plastic connectors (Ginder Scientific, Ottawa, Ontario, Canada) were fitted to the electrodes and then embedded in acrylic cement to form a mechanically stable cap. The scalp was restored around the cap using a single staple at the caudal end of the incision. Antibacterial gel was applied at the surgical margin, and animals were kept warm and monitored closely until they were ambulatory.

Electrophysiological and video monitoring methods

Electrophysiological testing of awake animals utilized stimuli (0.1 msec duration) generated by a Master 8 stimulator and stimulus isolation unit (Armon MicroProcessor Instruments, Jerusalem, Israel). Potentials were amplified and recorded digitally at a 10-kHz sampling rate (AD Instruments, Mountain View, CA). Animals undergoing physiological testing and observation were video monitored continuously for seizure behavior. Video data were recorded with digital cameras (OrangeMicro, Anaheim, CA) and stored on microcomputers (Apple Computer Inc., Cupertino, CA). Video recordings were triggered and time-stamped with motion detection and capturing software (Ben Software, London, United Kingdom) for integration with the electrophysiological data.

Quantitative analysis of spontaneous granule cell layer activity

To compare the relative magnitudes of spontaneous, high-frequency events recorded in the granule cell layers during the early (<30 days post-SE) and late (30–276 days post-SE) epileptic states, we subjected each spontaneous seizure event to power spectrum analysis (LabView, National Instruments, Austin, TX), which quantifies events according to their frequencies. We also performed a joint time frequency analysis (JTFA; Cohen, 1995; Qian and Chen, 1996) of all spontaneously recorded events associated with spontaneous behavioral seizures, which describes the temporal relationship between the onset of the behavioral seizures and the high-frequency events recorded bilaterally in the granule cell layers during the clinical seizures.

Anatomical methods

Rats were anesthetized with urethane (1.25 g/kg ip) and perfused through the heart with saline for 2 minutes, 0.01% or 0.1% sodium sulfide in 0.1 M phosphate buffer (the low-sulfide concentration was used if c-Fos immunocytochemistry was anticipated), pH 7.4, for 1 minute, saline for 1 minute, followed by 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, for 10 minutes. After storage of intact rats overnight at 4°C, brains were removed from the skull and placed in perfusate. Horizontal and coronal sections 40 μm thick were cut on a Vibratome in 0.1 M Tris (hydroxymethyl) aminomethane (Tris) buffer, pH 7.6. Sections were mounted on gelatin- and chrom alum-coated slides for subsequent Nissl (cresyl violet) staining, Timm staining (Sloviter, 1982), or staining of acutely degenerating neurons with Fluoro-Jade B (Schmued and Hopkins, 2000). After staining, slides were dehydrated in graded ethanols and xylene and then coverslipped with Permount or DPX [solution of distyrene, a plasticizer (BPS-butylpthalatestyrene), and xylene] for Fluoro-Jade B.

For immunocytochemistry, sections were mounted on Superfrost Plus slides, air dried for 10 minutes, placed in 0.1 M Tris buffer, pH 7.6, and processed as previously described (Sloviter et al., 2003). Antisera used included rabbit anti-c-Fos (Ab H125; 1:5,000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-c-Fos (Ab-5; 1:50,000 dilution; Oncogene Research Products, San Diego, CA), guinea pig antivesicular glutamate transporter 1 (Ab5905; 1:50,000; Chemicon, Temecula, CA), mouse antiparvalbumin (P3171; 1:1,000,000; Sigma), mouse antisomatostatin (V1169; 1:300; Biomeda, Foster City, CA), and mouse anti-NeuN (MAB377; 1:10,000; Chemicon). Images were acquired digitally on a E800M microscope (Nikon) with C5180 camera (Hamamatsu, Middlesex, NJ) in Adobe Photoshop 7.0.

Neuronal counting methods

To determine the approximate extent of hilar neuron loss in the dorsal hippocampal region of the recording sites in pilocarpine-treated rats, we selected three nonconsecutive Nissl-stained sections of the dorsal hippocampus. The numbers of surviving hilar neurons were counted in three sections from each animal and compared with the numbers of cells counted in matched sections from three similarly implanted control animals. Hilar neurons were defined as neuronal somata present within the region of the dentate gyrus containing the hilar mossy fiber axon collateral plexus, as illustrated in the Timm-stained section shown in Figure 1C1. Thus, neurons lying between the two blades of the granule cell layer, but within area CA3c, including the basal and apical dendrites of area CA3c, were excluded from the counting procedure. We present these numbers not as precise measurements of the number of neurons present but as estimates of the extent of hilar neuron loss in each animal.

RESULTS

Granule cell excitability in the acute and chronic postinjury periods

Previous in vivo studies in anesthetized rats have consistently reported granule cell hyperexcitability and disinhibition in the acute period following SE (Tauck and Nadler, 1985; Sloviter, 1992; Klitgaard et al., 2002; Kobayashi and Buckmaster, 2003), and both in vivo and in vitro studies have reported granule cell paired-pulse suppression, rather than hyperexcitability, during the chronic epileptic state (Cronin et al., 1992; Sloviter, 1992; Buckmaster and Dudek, 1997; Wu and Leung, 2001). We emphasize at the outset that responses to paired-pulse stimulation do not provide direct measures of synaptic γ-aminobutyric acid (GABA)-mediated inhibition, but serve in awake animals as a technically feasible indicator of the ability of a population of granule cells to suppress subsequent population responses and to reflect the threshold for transition from normal to epileptiform behavior as afferent stimulus frequency increases. The interpretation of altered paired-pulse responses in the rat dentate gyrus has been described in detail previously after treatment with the $GABA_A$ receptor antagonist bicuculline (Sloviter, 1991a) or by treatment with substance P-saporin conjugate, which selectively eliminates hippocampal inhibitory

interneurons without involving significant principal cell loss or detectable mossy fiber sprouting (Martin and Sloviter, 2001).

To assess granule cell excitability and granule cell behavior in each awake animal at sequential stages of the epileptogenic process, we implanted stimulating and recording electrodes in normal animals. Granule cell responses to paired-pulse perforant path stimulation were recorded prior to administration of pilocarpine (n = 9) or saline (n = 3). Granule cell activity was then monitored continuously during pilocarpine-induced SE, after which granule cell responses to perforant path stimulation were assessed daily for the first week after SE, and weekly thereafter. Granule cells generated seizure discharges continuously and bilaterally in all nine rats throughout the 3 hours of pilocarpine-induced behavioral SE. One day after the end of SE, recording of spontaneous activity detected spontaneous granule cell layer population spikes that exhibited the same qualitative characteristics as the granule cell potentials evoked by perforant pathway stimulation. These spontaneous population spikes were not seen in implanted rats prior to SE, or in saline-treated controls. In addition to these spontaneous population discharges that occurred in the first days after SE, afferent stimulation of the perforant pathway consistently evoked granule cell responses that were hyperexcitable compared with the responses evoked in the same animals before SE (Fig. 1). Before SE, all nine rats exhibited single population spikes and paired-pulse suppression at interpulse intervals of 20-40 msec in response to 0.1–1.0-Hz perforant path stimuli (Fig. 1A1). One day after SE, the same animals exhibited multiple population spikes and a loss of paired-pulse suppression (Fig. 1A2) in response to identical afferent stimulation. Granule cell hyperexcitability, as well as a reduced threshold for conversion to epileptiform discharges, persisted throughout the first week after SE (Fig. 1B).

The impairment of granule cell paired-pulse suppression, which persisted for weeks after SE, was most sensitively reflected by the simultaneous assessment of ipsilateral and contralateral granule cell responses to unilateral perforant path stimuli. Before SE, afferent stimulation (0.1 Hz, with an interpulse interval of 20 msec) evoked ipsilateral paired-pulse suppression (full second spike suppression; Fig. 1B1). The same unilateral stimuli evoked only "field excitatory postsynaptic potentials" (fEPSPs; Andersen et al., 1966) in the contralateral granule cell layer, indicating the responsiveness of normal granule cells to relatively weak excitatory commissural stimuli (Fig. 1B1). However, 1 day after SE, identical afferent stimuli in the same animal evoked ipsilateral and contralateral granule cell population spikes, indicating increased granule cell excitability immediately after SE (Fig. 1B2). One week post-SE, afferent stimulation in the same animal evoked responses that indicated partial restoration of paired-pulse suppression and persistent ipsilateral and contralateral granule cell hyperexcitability (Fig. 1B3) compared with the pre-SE responses to identical stimuli (Fig. 1B1).

Timm staining was performed on three rats 4, 7, or 8 days after SE, at a time when granule cells were hyperexcitable. Timm staining revealed that mossy fiber sprouting was not detectable 4 days post-SE (data not shown). At 7 and 8 days post-SE, loss of the normal mossy cell-derived, Timm-positive projection to the inner molecular layer (arrows in Fig. 1C1) was apparent (compare arrowheads and asterisk in Fig. 1D1,2), and sparse mossy fiber sprouting was detectable in the dentate molecular layer 7 and 8 days post-SE (Fig. 1D2; the number of hilar cells remaining in the dorsal hippocampal region of the animal shown in Figure 1C2,D2 revealed a 64.4% ± 2.3% loss of hilar neurons).

Granule cell paired-pulse suppression, which was assessed repeatedly in the six remaining epileptic animals, continued to recover gradually during the weeks after SE and reached an approximately pre-SE extent of suppression ~14–28 days after SE (Fig. 2). However, we emphasize that we do not represent a restoration of paired-pulse suppression to be synonymous with a physiological restoration of normal network inhibition in all its manifestations. These results, indicating persistent granule cell hyperexcitability after SE and a gradual recovery of paired-pulse suppression contrast with the results of Dudek and colleagues, who reported that initial granule cell hyperexcitability caused by kainate-induced SE recovered during the first few days after SE, before mossy fiber sprouting (Hellier et al., 1999). These authors reached this conclusion by evaluating the granule cell field responses to a single stimulus pair delivered on each test day. Therefore, we recorded granule cell responses to a series of identical perforant path stimuli delivered at 1 Hz. This was done to assess and compare the responses to the first stimulus pair with those evoked by identical subsequent stimuli.

During the period 1–7 days post-SE, perforant path stimulation at 1.0 Hz evoked granule cell responses that rapidly (usually within 10 sec) collapsed, causing multiple population spikes, epileptiform discharges (Fig. 3A2,A3), and a behavioral seizure. In the same animal 10 days after SE, the first stimulus pair of a series of 1-Hz stimuli evoked significant second spike suppression (Fig. 3B1), which would have been interpreted as indicating a significant recovery of paired-pulse suppression had it been the only response assessed. However, subsequent stimuli revealed a rapid collapse of highly labile paired-pulse suppression, which resulted in epileptiform granule cell discharges (Fig. 3B2-B5). Three weeks after SE, labile paired-pulse suppression in the same animal still collapsed at an afferent stimulus frequency of 1 Hz (Fig. 3C), which did not produce a collapse of paired-pulse suppression or epileptiform discharges in any of the six control animals tested.

We subsequently identified the weekly test at which the same 1-Hz stimulus paradigm *first* failed to evoke seizure discharges, presumably as a result of the gradually increasing discharge suppression that we observed. The first failure of 1-Hz stimulation to evoke epileptiform discharges occurred 28 days after SE (Fig. 3D). Perfusion fixation and Timm staining at this time confirmed that detectable, but submaximal, mossy fiber sprouting was evident (Fig. 3E–G) in all three animals tested in this manner. Consistently with the partial restoration of granule cell suppression that we observed 28 days post-SE, the Timm-positive terminals present at this relatively early stage of the process of synaptic reorganization surrounded and outlined the somata and dendrites of parvalbumin-positive inhibitory interneurons in the granule cell and molecular layers (Fig. 4). Subsequent weekly evaluations in five epileptic animals monitored both before and during the later chronic epileptic state revealed abnormally increased paired-pulse suppression and abnormally in-

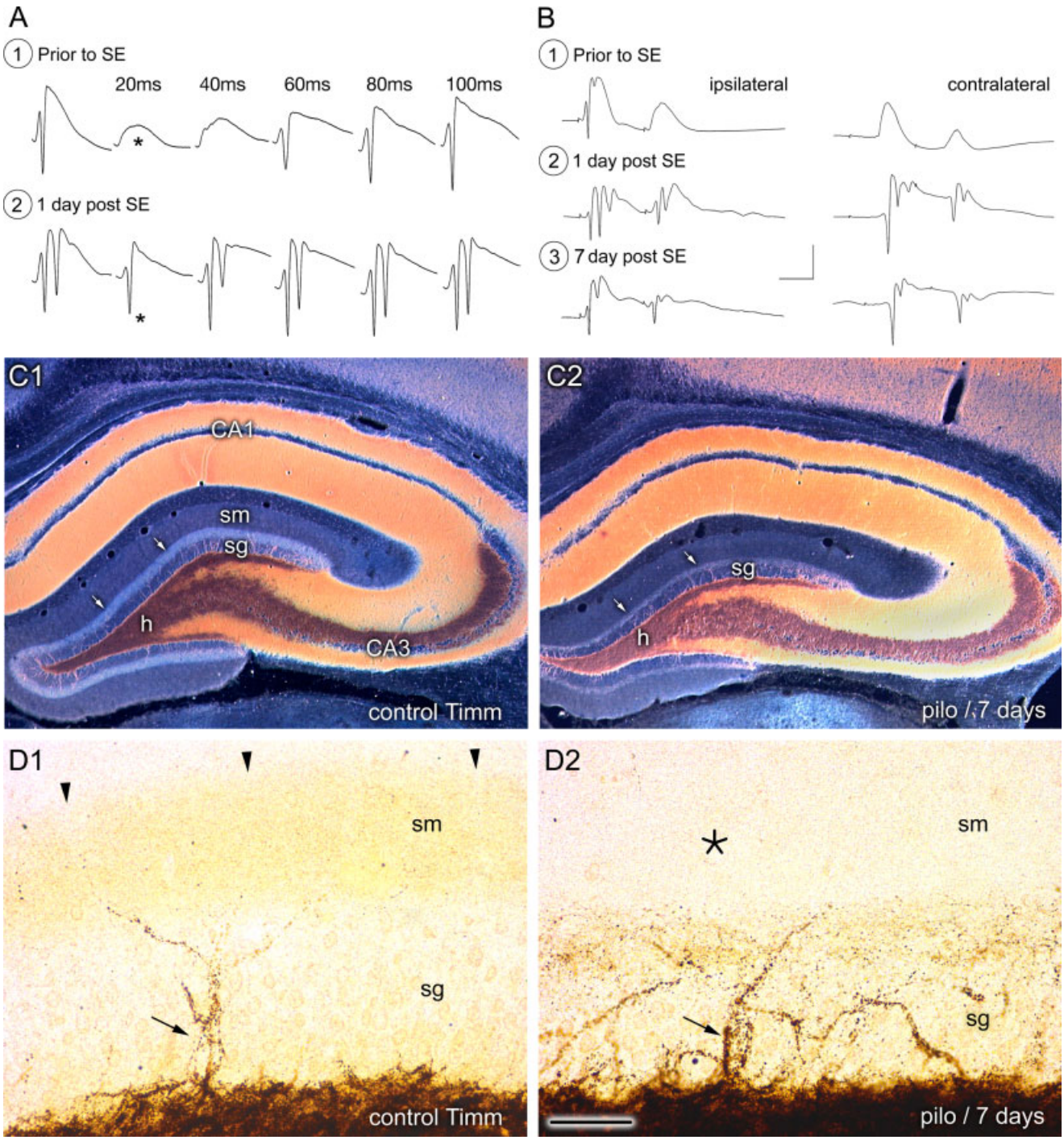

Fig. 1. Persistent granule cell hyperexcitability 1–7 days after status epilepticus (SE). **A:** Granule cell evoked responses to 1.0-Hz paired-pulse stimulation of the perforant pathway before (1) and 1 day after (2) SE. Note that granule cells exhibit hyperexcitability (multiple population spikes in response to single stimuli) and decreased paired-pulse suppression (asterisks) at all interpulse intervals tested (20–100 msec). **B:** Ipsilateral and contralateral granule cell layer responses to unilateral perforant path stimulation. Prior to SE (1), afferent stimuli at 0.1 Hz evoked a single population spike and full paired-pulse suppression at an interpulse interval of 20 msec, and only contralateral field EPSPs. One day post-SE (2), identical afferent stimuli in the same animal evoked abnormal, multiple population spikes ipsilaterally, and similarly hyperexcitable responses contralaterally. Seven days post-SE (3), partial restoration of paired-pulse suppression was evident, but both ipsilateral and contralateral hyperexcitability were still evident. **C:** In this early recovery period, during which restoration of granule cell paired-pulse suppression began, darkfield illumination of Timm-stained glutamatergic axon terminals indicated that the plexus of mossy cell terminals in the inner molecular layer (arrows in C1 and the yellow band in D1) was absent, being replaced by early mossy fiber sprouting in the granule cell layer (arrows in C2; asterisk in D2; stratum granulosum; sg). Note that unstained interneurons (somata of the granule cell layer that are larger than granule cell somata) are targets of Timm-positive terminals in both normal and pilocarpine-treated rats (arrows in **D** point to Timm-positive terminals that outline interneuron somata and dendrites). Calibration bars = 10 mV, 8.5 msec for A; 10 mV, 10 msec in B. C: ×26; D: ×262. Scale bar = 50 μm in D; 500 μm for C.

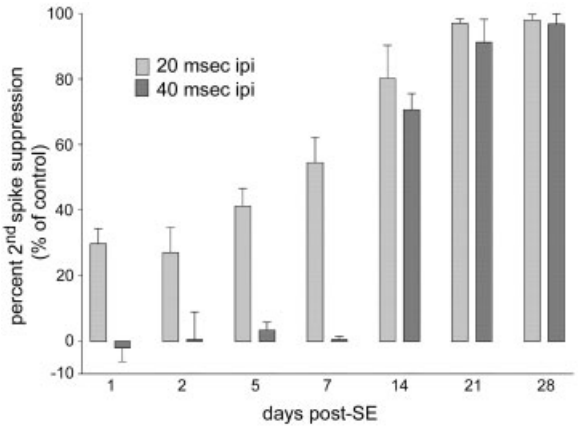

Fig. 2. Gradual recovery of paired-pulse suppression during the month following pilocarpine-induced status epilepticus (SE). The percentage of control paired-pulse suppression in pilocarpine-treated rats was evaluated at interpulse intervals of 20 and 40 msec during the month following pilocarpine-induced SE (n = 6 pilocarpine-treated rats and n = 3 saline-treated controls). The paired pulse index (second spike amplitude divided by first spike amplitude) was calculated for each interpulse interval in each pilocarpine-treated rat. Then, this ratio was divided by the mean of the corresponding value from three control animals. This gave a fraction representing the "% of control value" shown. Note that granule cell paired-pulse suppression recovered slowly and approached control values at approximately 14–28 days after SE (although this is not represented as a restoration of network inhibition in all of its manifestations).

creased seizure discharge thresholds compared with controls. Hilar neuron loss was assessed in all nine animals, and all exhibited hilar neuron loss and mossy fiber sprouting (dorsal hippocampal hilar neuron loss was ~79% in the dorsal hippocampal region from which the physiological recordings were made; 18.1 ± 2.7 hilar neurons per section; range 66–87% fewer hilar neurons than controls; $P < 0.05$, unpaired t-test).

Additional rats (n = 10) were implanted more than 2 months after SE to address the possibility that the presence of implanted electrodes, and repeated stimulus-response testing, could have influenced the epileptogenic process. In all 10 rats that exhibited spontaneous epileptic seizures prior to electrode implantation >2 months post-SE, two characteristics of granule cell excitability were consistently altered compared with responses in six saline-treated controls: 1) abnormally increased paired-pulse suppression of the granule cell population spike was evoked by perforant path stimulation, and 2) an abnormally elevated seizure discharge threshold was evident in response to increasing frequencies of orthodromic stimulation (Fig. 5A,B). In all six saline-treated control animals tested in parallel with these 10 chronically epileptic, pilocarpine-treated rats, paired-pulse stimulation at 0.1–2.0 Hz evoked first population spikes of large amplitudes and second responses that exhibited fully suppressed population spikes at an interpulse interval of 20 msec (Fig. 5A; ratios of second-to-first spike amplitudes = 0). At an interpulse interval of 40 msec, the average suppression of the second population spike in control animals stimulated at 1 Hz was >90% (the ratios of second-to-first spike amplitudes were 0.08% ± 0.01% or 92% ± 1.5% paired-pulse suppression; n = 6). At an interpulse interval of 60 msec, second population spike suppression in controls was ~25% (Fig. 5C). At longer interpulse intervals, second population spike amplitudes in controls exhibited potentiation (Fig. 5A; 80 and 100 msec). In all 10 pilocarpine-treated rats, which were analyzed between 60 and 270 days post-SE, paired-pulse suppression in response to 1-Hz perforant pathway stimulation was significantly increased at all stimulus frequencies and interpulse intervals tested (Fig. 5C).

The threshold for generating granule cell seizure discharges was then determined by recording the responses of granule cells to progressively increasing afferent stimulus frequencies. All six control animals exhibited failure of paired-pulse suppression and conversion to epileptiform behavior either at a stimulus frequency of 1 Hz (60–100 msec interpulse intervals), or, if suppression remained intact at 1 Hz, at a stimulus frequency of 2 Hz (40–80 msec interpulse intervals; arrow in Fig. 5A). In contrast to these 1–2-Hz frequencies at which orthodromic stimuli evoked epileptiform granule cell discharges in all six awake control rats, none of the chronically epileptic rats tested exhibited granule cell seizure discharges in response to afferent stimulation at 1–3 Hz, at any interpulse interval (20–100 msec) tested. Stimulus frequencies of 4–10 Hz were necessary to evoke granule cell seizure activity in all chronically epileptic animals tested.

Histological analysis of eight of these 10 chronically epileptic rats (two rats died prior to perfusion fixation) revealed a highly consistent pattern of extensive hilar neuron loss (Fig. 6B2) and mossy fiber sprouting (Fig. 6C2) but less than extensive loss of hippocampal pyramidal cells (Fig. 6A2). In contrast to the relatively limited hippocampal pyramidal cell loss produced by pilocarpine-induced SE (Sloviter, 2005), all epileptic animals exhibited extensive, bilateral damage to thalamic nuclei (Fig. 6D), the amygdala (Fig. 6E), and the piriform and entorhinal cortices (Fig. 6E). The average loss of dentate hilar neurons in the dorsal hippocampal region from which the recordings were made was ~70% in these pilocarpine-treated animals (saline-treated controls: 85.1 ± 2.6 hilar neurons per section in three dorsal hippocampal sections counted per rat; n = 6; vs. pilocarpine-treated animals: 26.5 ± 3.2 neurons per section; range 50–82% fewer hilar neurons; n = 8; $P < 0.05$, unpaired t-test).

Spontaneous granule cell behavior during early and late epileptic seizures

Although we consistently found granule cells in all chronically epileptic animals to be hyperinhibited (paired-pulse suppression greater than pre-SE values and all rats abnormally resistant to generating granule cell epileptiform discharges in response to afferent stimulation), it has been hypothesized that, just prior to each spontaneous seizure, and in response to excitation at perhaps uniquely effective frequencies (Feng et al., 2003), granule cell inhibition collapses and granule cells generate seizure discharges that precede and cause the spontaneous behavioral seizures that define these animals as epileptic (Buhl et al., 1996; McNamara, 1999; Coulter, 2000). Therefore, we recorded granule cell layer activity bilaterally during 235 spontaneous behavioral seizures in 17 epileptic rats (10 rats implanted >60 days post-SE and seven animals implanted pre-SE, five of which were allowed to survive into the chronic epileptic period). Prior to recording spontaneous granule cell layer activity during epileptic sei-

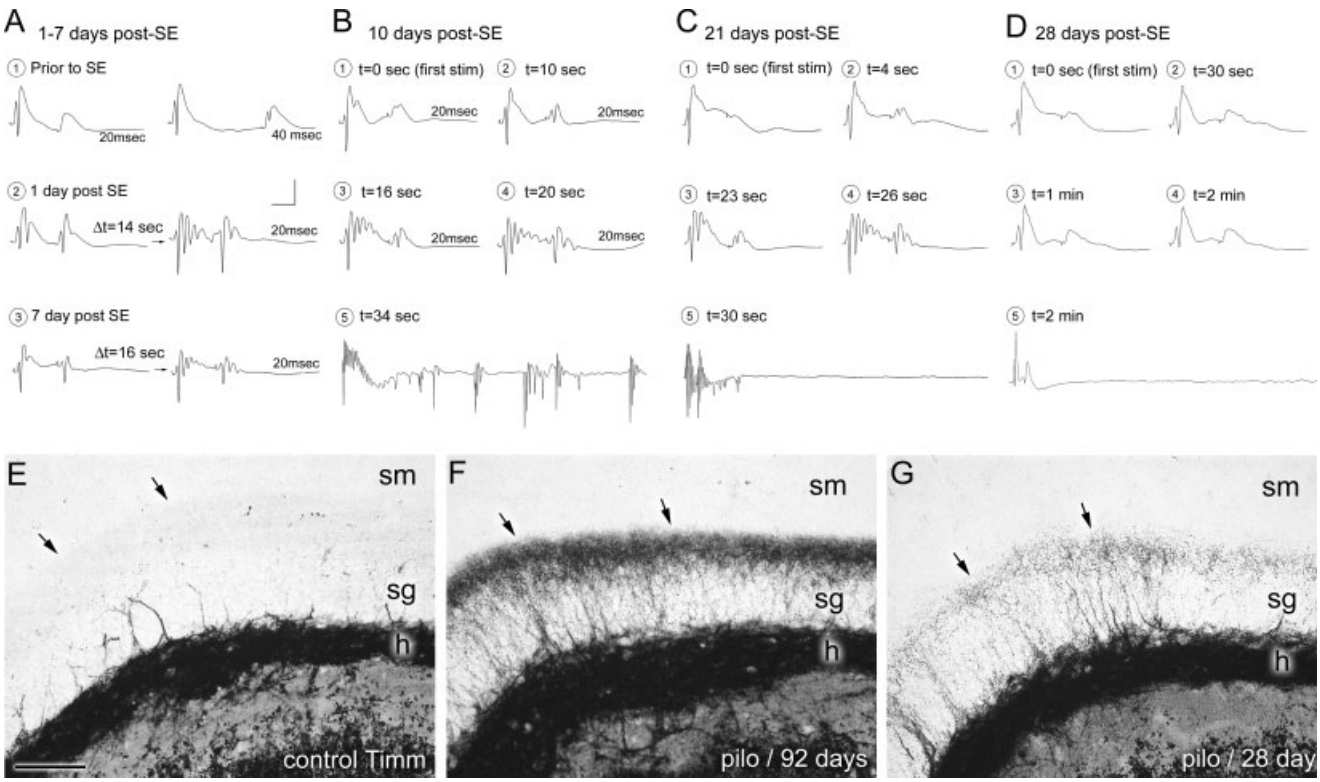

Fig. 3. **A–G:** Restoration of labile granule cell paired-pulse inhibition in the early post-status epilepticus (SE) period is accompanied by mossy fiber sprouting. A1: Prior to SE, continuous 1.0-Hz perforant pathway stimulation at interpulse intervals of 20 or 40 msec evoked stable potentials that did not collapse during 2 minutes of continuous stimulation to result in seizure discharges. A2,3: One and seven days post-SE, identical continuous 1.0-Hz stimuli in the same rat evoked hyperexcitable responses that rapidly collapsed (after 14 seconds 1 day post-SE and after 16 seconds 7 days post-SE), resulting in seizure discharges (arrows in A2,3). B: In the same animal 10 days post-SE, partial recovery of paired-pulse suppression was evident in the response to the first stimulus pair (note paired-pulse suppression in B1), but this suppression was highly labile as reflected by its rapid collapse after 20 seconds of stimulation (B3,4) and conversion to full epileptiform behavior 14 seconds later (B5). C: Three weeks post-SE, the same animal exhibited a further increase in paired-pulse suppression of the second population spike evoked by the first stimulus pair (C1), but this labile suppression collapsed 26–30 seconds after the start of stimulation (C4,5). D: It was not until 28 days post-SE that the same animal exhibited stable responses to 1.0-Hz stimulation for 2 minutes that did not collapse to result in seizure discharges (D1–5). Perfusion fixation and Timm staining in this animal revealed the presence of mossy fiber sprouting in the dentate inner molecular layer (arrows in G), which was greater than that observed in a coprocessed control animal (E) and less than that observed in an identically pilocarpine-treated and coprocessed animal perfusion fixed 92 days post-SE (arrows in F). Calibration bars = 10 mV and 10 msec. ×74. Scale bar = 95 μm in E (applies to E–G.)

zures, we verified the location of the recording electrode tips by recording the characteristic evoked potentials produced by perforant pathway stimulation (Andersen et al., 1966). In addition, we identified the seizure discharge threshold in each animal by increasing afferent stimulus frequency until epileptiform discharges occurred. These evoked discharges were recorded so that the qualitative and quantitative features of evoked granule cell seizure discharges could be compared with the granule cell layer activity recorded in awake epileptic rats during spontaneous behavioral seizures.

Characteristics of seizure discharges evoked in control animals. Epileptiform discharges evoked in control rats by 1–2-Hz stimulation of the perforant pathway contained three distinct components of different frequencies. These discharges included repetitive, negative-going population spikes that contained discharges of two frequencies (Fig. 7A2) and positive-going potentials without superimposed population spikes (Fig. 7A1). The positive-going potentials, which were presumably population "field EPSPs" without superimposed population spikes, exhibited an average width (duration) of 56.7 ± 0.5 msec (calculated from 20 separate epileptiform events in two control animals). Negative-going population spikes at the start of a seizure discharge exhibited a frequency of 348.0 ± 8.5 Hz, followed by slower discharges that exhibited a frequency of 171.7 ± 3.3 Hz. High- and low-frequency events (seizure discharges vs. presumed fEPSPs) could not be easily discriminated unless each trace was expanded and analyzed. That is, when discharges were viewed at a compressed time base typical of EEG recordings (Fig. 7A, compressed trace), low-frequency fEPSPs and high-frequency population spikes could not be easily discriminated, and all recordings could have been assumed to be "seizure" discharges, regardless of whether high-frequency population discharges were present.

Characteristics of evoked seizure discharges in epileptic animals. Epileptiform discharges evoked by high-frequency stimulation (>5 Hz) in epileptic rats implanted >2 months post-SE exhibited both high-frequency seizure

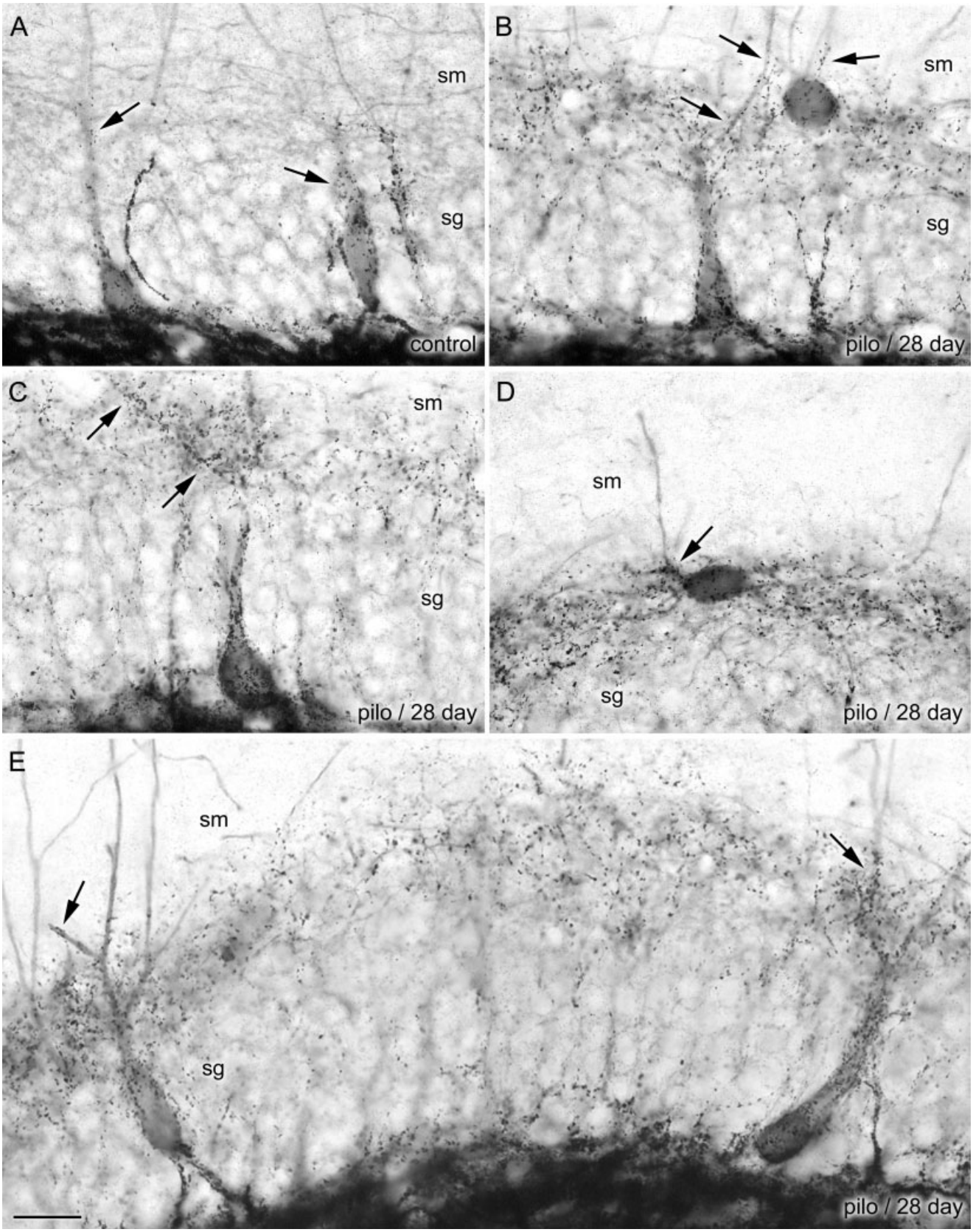

Fig. 4. Parvalbumin-positive inhibitory interneurons are targets of aberrant mossy fiber sprouting at the time of early recovery of granule cell paired-pulse inhibition. **A:** Timm staining in a control rat. Note that Timm-positive terminals surround and outline the somata and proximal dendrites of normal granule cell layer interneurons (arrows), as previously shown (Ribak and Peterson, 1991; Sloviter, 1992), although there are few Timm-positive terminals in the inner molecular layer (sm, stratum moleculare). **B–E:** Timm-stained sections from the animal presented in Figure 3G, which was perfusion fixed 28 days post-SE, the recovery period when 1.0-Hz afferent stimulation first failed to evoke epileptiform discharges (i.e., the epileptiform discharge threshold was increased). Note that aberrant mossy fiber terminals targeted the somata and dendrites of identified inhibitory interneurons of the granule cell and inner molecular layers (arrows; sg, stratum granulosum). A–D: ×340; E: ×354. Scale bar = 26 μm in E; 25 μm for A–D.

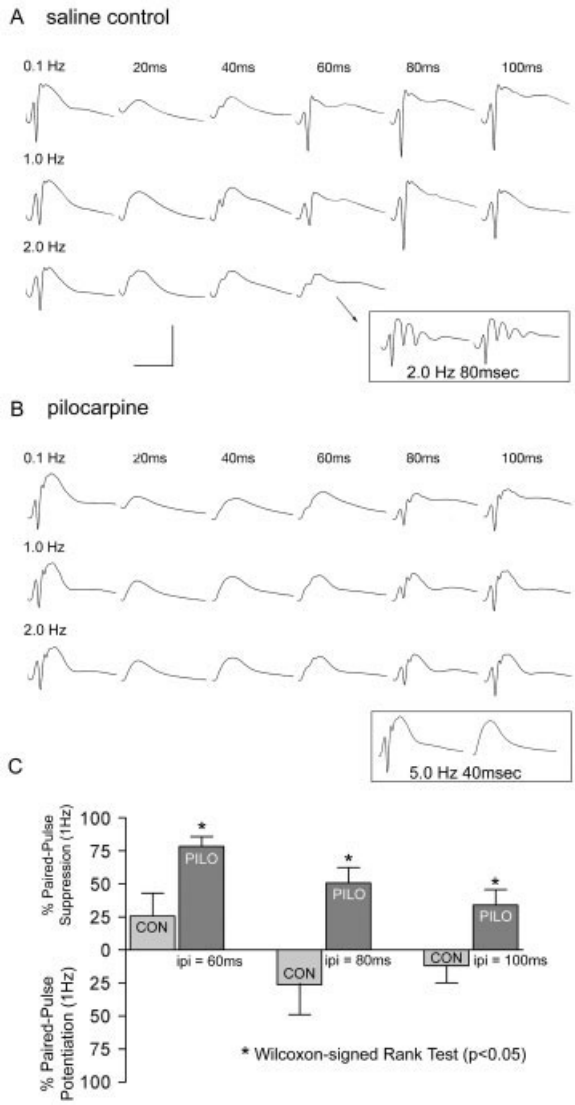

Fig. 5. Dentate granule cell hyperinhibition and elevated seizure discharge threshold in chronically epileptic, pilocarpine-treated rats. **A:** Evoked responses of a saline-treated control animal to paired-pulse perforant pathway stimulation at different frequencies (0.1–2.0 Hz) and interpulse intervals (20–100 msec). Note that paired-pulse suppression in control animals was virtually complete at 20–40-msec intervals and partial at a 60-msec interpulse interval. Granule cells of control animals always generated seizure discharges in response to perforant pathway stimulation at frequencies of 1–2 Hz and interpulse intervals of 40–80 msec. **B:** Increased granule cell paired-pulse suppression in a chronically epileptic rat evaluated 270 days post-SE. Note that, in pilocarpine-treated animals, paired-pulse suppression was virtually complete at 20–60-msec intervals at all stimulus frequencies tested. Note also that 5-Hz stimulation was incapable of evoking granule cell seizure discharges (box). **C:** Increased paired-pulse suppression in chronically epileptic rats (n = 10) compared with controls (n = 6). This bar graph shows that control rats exhibited ~25% paired-pulse suppression at an interpulse interval of 60 msec and potentiation at intervals of 80 and 100 msec in response to 1 Hz perforant pathway stimulation. Conversely, all six chronically epileptic rats exhibited paired-pulse suppression at all intervals tested (20–100 msec) between 60 and 270 days post-SE. Calibration bars = 20 mV, 10 msec.

discharges and presumed fEPSPs (Fig. 7E). However, these animals lacked the highest frequency discharges that were present in controls. Quantitative analysis of 30 separate epileptiform events in each of three epileptic animals showed that evoked seizure discharges in chronically epileptic rats exhibited an initial discharge frequency of 171.0 ± 4.7 Hz and an fEPSP duration of 44.0 ± 0.7 msec. Conversely, in the subsequently evaluated animals in which electrodes were implanted prior to the induction of SE, seizure discharges evoked by 1-Hz perforant pathway stimulation during the first week post-SE exhibited all three frequencies. Thus, the chronic epileptic state was characterized, in part, by a consistent loss of the highest frequency components generated in association with the observed hyperinhibited state.

Characteristics of spontaneous granule cell layer activity in epileptic animals. After verifying the location of the recording electrodes in the granule cell layers, and recording the evoked seizure granule cell discharges described above for later comparison with spontaneous events recorded from the same electrodes, we proceeded to video-monitor behavior and record spontaneous dorsal hippocampal granule cell layer activity bilaterally in anticipation of spontaneous epileptic seizures. We video-recorded 235 spontaneous behavioral seizures in 17 epileptic rats (10 implanted late, seven implanted early). Among these 235 seizures, 44 were recorded during the early post-SE period (<30 days after SE) monitored in seven animals implanted before SE (Fig. 7B,C), and 191 were recorded in 15 rats (10 implanted late, five implanted early) during the chronic epileptic state (>30 days after SE). Among the 191 "late" spontaneous behavioral seizures that we recorded (Fig. 7D), 93 were recorded in 10 animals implanted after rats became epileptic, and 98 late seizures were recorded in the five animals implanted before SE, which were then monitored throughout their epileptic states.

Typical spontaneous seizures involved an initial immobilization, followed by masticatory behaviors, unilateral forepaw clonus, rearing, bilateral eyelid closure, and bilateral forepaw clonus. Other behavioral seizures involved sudden awakening, followed by generalized seizures with or without running behavior. It should be noted that behavioral onset was defined conservatively as minimally involving forelimb clonus and rearing, even though more focal behaviors, including arrest and masticatory movements, often preceded forelimb clonus and rearing. During 44 "early" spontaneous behavioral seizures (Fig. 7B,C), video monitoring showed that granule cell seizure discharges were recruited in 37 (84%) of the 44 spontaneous seizures recorded. Behavioral seizure onsets always preceded the spontaneous events recorded in the granule cell layers (Fig. 8; the mean latency from the behavioral seizure onset to recruitment of granule cell seizure discharge was 12.8 ± 1.8 sec; n = 37; range 1–68 sec), and the granule cells recruited by the generalized behavioral seizure generated both low-frequency potentials without population spikes (Fig. 8B1), and repetitive, high-frequency population spikes (Fig. 8B2). Thus, in the early postinjury period, granule cell seizure discharges were apparently recruited by epileptic discharges that originated elsewhere and caused the behavioral seizures.

Significantly, spontaneous seizures first appeared within a few days after SE. In nine rats monitored immediately after SE (four of which were perfusion fixed for

anatomical analysis 4, 7, 8, and 28 days post-SE and were therefore not monitored during the chronic epileptic state), the first spontaneous behavioral seizures were observed at 2 days (n = 2), 4 days (n = 1), 8 days (n = 1), 9 days (n = 2), 14 days (n = 1), or 24 days (n = 1), or no seizure was observed (n = 1). However, these animals were not monitored continuously after SE and could have had spontaneous seizures at earlier times. Thus, many pilocarpine-treated animals exhibited little or no "latent period" after SE-induced injury and clearly became epileptic before mossy fiber sprouting was evident, as previously noted (Elmer et al., 1997; Longo and Mello, 1999; Nissinen et al., 2000, 2001).

During the chronic epileptic state, in which increased granule cell paired-pulse suppression and elevated granule cell seizure thresholds were observed in response to orthodromic excitation in all 15 rats tested, all 191 spontaneous behavioral seizure onsets preceded activity recorded bilaterally in the granule cell layers (Fig. 8A2). Granule cell layer recordings during these 191 spontaneous behavioral seizures showed that the granule cell layers generated no seizure discharges, i.e., no high-frequency population discharges in 89% of the spontaneous seizures (169 of 191 seizures). In the other 11% of spontaneous seizures, high-frequency discharges were recorded at the end of the recruited activity (the mean latency from the behavioral seizure onset to recruitment of granule cell epileptiform discharge was 25.4 ± 4.0 sec; n = 22 seizures; range 9–88 sec; a significantly longer latency to recruited discharge compared with early spontaneous seizures; $P < 0.004$, two-tailed t-test). In the rats implanted before SE was induced, and in which the 44 early seizures were recorded, 16 of 98 late spontaneous seizures (16%) contained granule cell epileptiform discharges, which were always recorded at the end of the recruited activity. Consistently with the observation that video-recorded behavioral seizures involved relatively few granule cell seizure discharges during the chronic epileptic state, analysis of the granule cell layer activity, independent of the behavioral data, showed that no spontaneous granule cell layer seizure discharges unrelated to behavioral seizures were observed.

Characteristic spectral components of granule cell discharges. We utilized joint frequency time analysis (JTFA; Cohen, 1995; Qian and Chen, 1996) to characterize the recorded events according to their respective frequency bands in preparation for the analysis of spontaneous granule cell layer activity during spontaneous seizures. Granule cell potentials recorded during 142 spontaneous behavioral seizures in seven rats implanted prior to induction of SE were classified as either "early" (<30 days post-SE; n = 44), or "late" (>30 days post SE; n = 98), with 30 days chosen arbitrarily because we mon-

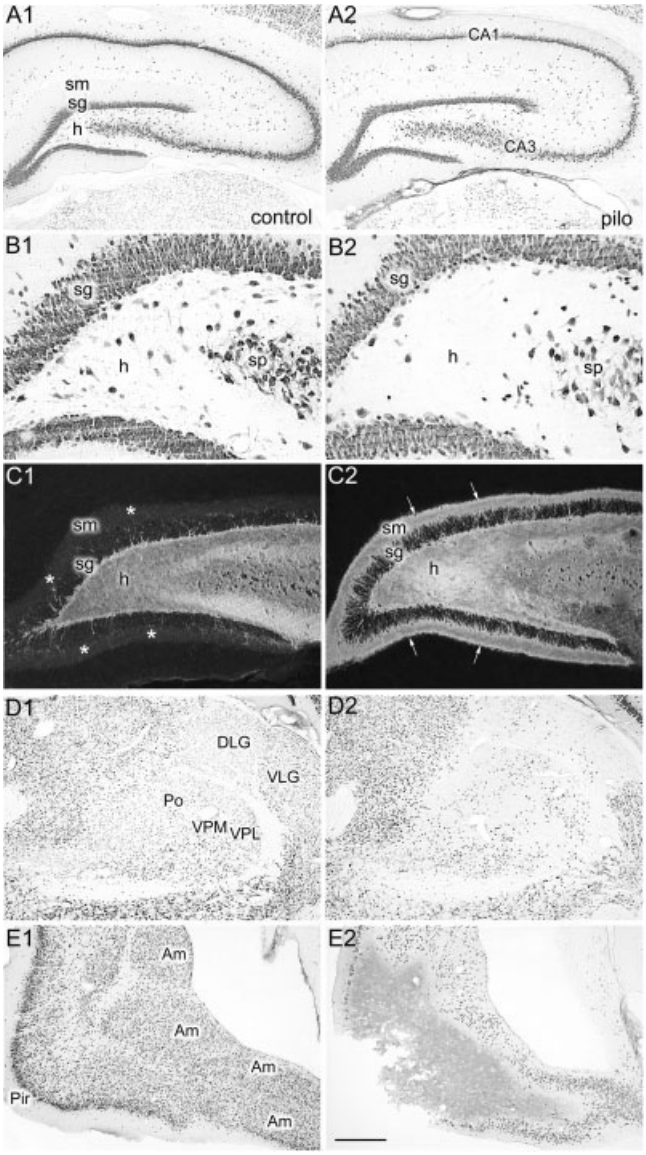

Fig. 6. **A–E:** Pilocarpine status epilepticus (SE)-induced neuronal loss and mossy fiber sprouting in chronically epileptic rats. A1,2: NeuN-immunostained section of the dorsal hippocampus from a control animal and from the chronically epileptic rat shown in Figure 5B (perfusion fixed 276 days post-SE), respectively. Note the relative survival of CA1–CA3 pyramidal cells in this representative epileptic rat. B1,2: Higher magnification of the dentate gyrus in the same sections showing extensive loss of hilar neurons (h). Note loss of hilar neurons (h) but survival of adjoining CA3 pyramidal cells in the stratum pyramidale (sp) of area CA3c. C1,2: Timm-stained sections of the dentate gyrus from the same control and epileptic animals showing the distribution of mossy fibers in the normal dentate gyrus (C1) and after mossy fiber sprouting (C2). Note that the lightly Timm-stained band in the inner stratum moleculare (sm; asterisks) of the normal animal reflects the zinc-containing terminal plexus of hilar mossy cells, which is replaced by zinc-containing granule cell axon collaterals (arrows) in the epileptic animal (C2). The extent of hilar cell loss and mossy fiber sprouting shown is representative of all epileptic, pilocarpine-treated animals described in this study. D1,2: NeuN-immunostained section of the thalamus from a control (D1) and a chronically epileptic (D2) rat 48 days post-SE. Note that, in addition to relatively minor hippocampal cell loss, extensive neuron loss was consistently observed in thalamic nuclei, including the posterior thalamic nucleus (Po), ventral posteromedial nucleus (VPM), ventral posterolateral nucleus (VPL), dorsal lateral geniculate nucleus (DLG), and ventral lateral geniculate nucleus (VLG). E1,2: NeuN-immunostained section of the temporal cortex and associated neuronal nuclei from a control (E1) and a chronically epileptic rat (E2) 48 days post-SE. Note extensive cell loss in the piriform cortex (Pir) and partial survival of various amygdaloid (Am) nuclei. A,D,E: ×12.6; B: ×63; C: ×31.5. Scale bar = 500 μm in E (applies to A,D,E); 100 μm for B; 200 μm for C.

itored animals continuously during the first month to record early spontaneous seizures and then again starting ~6–8 weeks post-SE. Granule cell seizure discharges evoked during the early post-SE period exhibited both high-frequency and lower frequency discharges (333.6 ± 11.8 Hz and 165.2 Hz calculated from 10 discharges), as seen in controls. In addition, when spontaneous behavioral seizures recruited granule cell discharges in the early post-SE period, these discharges exhibited similar high-frequency components (329.5 ± 4.8 Hz and 165.8 ± 12.9 Hz calculated from 10 discharges). Thus, early spontaneous seizures in pilocarpine-treated rats exhibited both of the high-frequency components evoked in normal animals.

Afferent stimuli of increasing frequencies were delivered interictally to all chronically epileptic animals to determine the discharge characteristics of granule cells in the chronic epileptic state. Granule cell seizure discharges superficially similar to those evoked in control animals (Fig. 7A) were evoked in 15 chronically epileptic animals (Fig. 7D). However, these evoked events lacked the highest frequency discharges entirely, leaving only the lower high-frequency events (170.0 ± 0.5 Hz calculated from 10 discharges). Thus, during the chronic epileptic state, spontaneous granule cell recordings exhibited a much more restricted bandwidth of recruited activity than those observed earlier in the same animals, which consisted mainly of low-frequency events corresponding to presumed fEPSPs and single population spikes (Fig. 7D1). In addition, we consistently observed that, when bilateral granule cell afterdischarges were evoked in all 15 chronically epileptic rats by perforant path stimulation, the hippocampal discharges that afferent stimulation evoked nonetheless failed to produce behavioral seizures. This finding is consistent with the conclusion that granule cell discharges, when they occurred, were the result, rather than the cause, of the spontaneous behavioral seizures.

Spectrograms were generated from the JTFA analyses of the granule cell recordings of early spontaneous seizures to determine when the low- and high-frequency components occurred in relation to the onset of each spontaneous behavioral seizure and to provide quantitative data about the relative magnitudes of each frequency com-

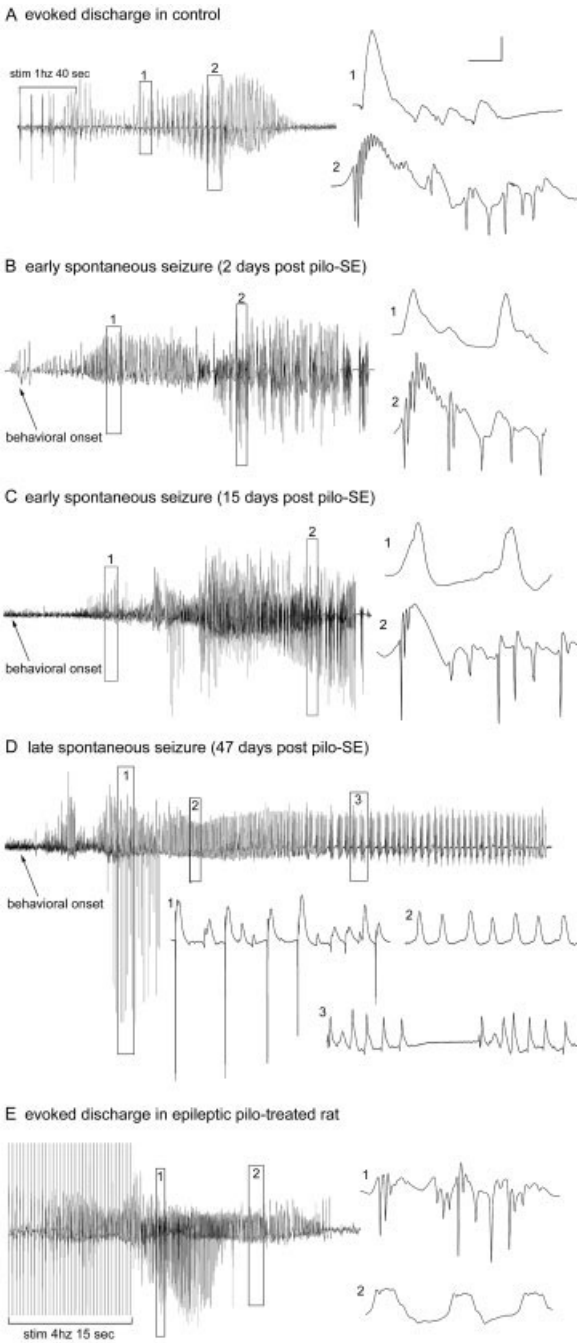

Fig. 7. Evoked and spontaneous granule cell activity recorded from the granule cell layers of chronically epileptic, pilocarpine treated-rats. **A:** Granule cell layer seizure discharges evoked in a control animal (60 days postsaline treatment) by perforant path stimulation at 1 Hz (with paired-pulses 40 msec apart) for 40 sec. Note that evoked granule cell activity generated both low-frequency, positive-going field potentials (expanded box 1) and high-frequency, negative-going population spikes (expanded box 2). **B:** In an epileptic animal 2 days post-SE, spontaneous granule cell layer activity recorded during a spontaneous behavioral seizure exhibited both positive-going field potentials (expanded box 1) and negative-going epileptiform discharges (expanded box 2) that were qualitatively similar to those evoked in the control rat in A. **C:** Thirteen days later, spontaneous granule cell layer activity still exhibited high-frequency, negative-going epileptiform discharges. **D:** Forty-seven days after SE, the spontaneous granule cell layer activity recorded in the same animal during a spontaneous seizure exhibited single population spikes (expanded box 1) and positive-going field potentials (expanded boxes 2 and 3) but no seizure discharges (repetitive population spikes). Note that granule cells generated only single population spikes ~7 seconds after the onset of the observed behavioral seizure (expanded box 1) and then generated only field potentials for the duration of the behavioral seizure. **E:** Evoked granule cell seizure afterdischarges in the same chronically epileptic, pilocarpine-treated rat 45 days post-SE (tested 2 days prior to the activity shown in D), illustrating that, despite the lack of spontaneous seizure discharges in the chronic epileptic state, abnormally high-frequency (4–10Hz) stimulation could evoke granule cell seizure discharges (expanded box 1), as well as positive-going field potentials (expanded box 2). The absence of seizure discharges recorded from the granule cell layers during spontaneous behavioral seizures was a consistent observation in epileptic animals subsequently shown to have dentate hilar cell loss and mossy fiber sprouting. Calibration bars = 3.5 mV, 30 msec in A; 5 mV, 2 sec for B; 4 mV, 30 msec for B1,2; 2.5 mV, 2 sec for C; 3 mV, 100 msec for C1–3; 3 mV, 2 sec for D; 4 mV, 25 msec for D1,2.

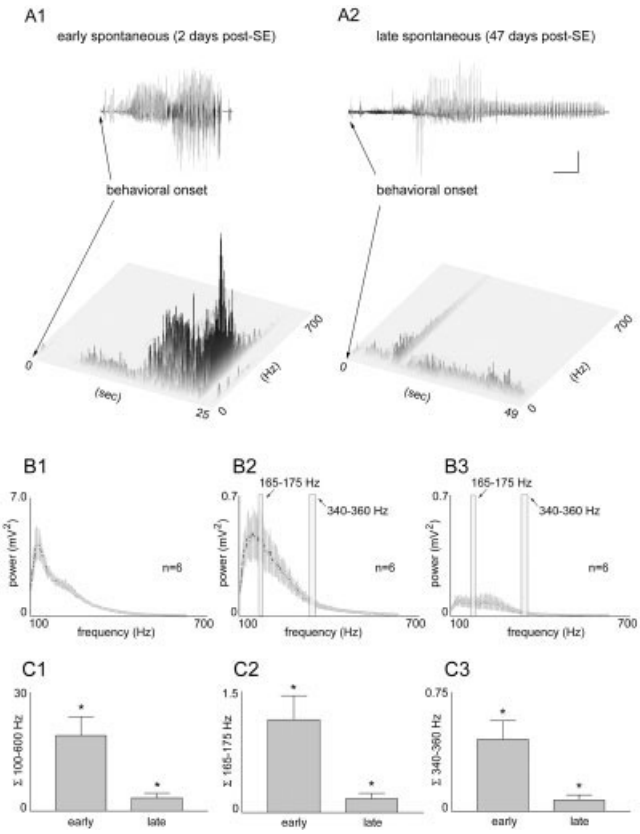

Fig. 8. **A–C:** Quantitative frequency analysis of "early" and "late" spontaneous seizures recorded in the same chronically epileptic, pilocarpine-treated rats. A1,2: Joint time frequency analysis spectrograms of "early" (2 days post-SE) and "late" (47 days post-SE) seizures in the same awake, epileptic animal, indicating when granule cell layer activities of different frequencies occurred in relation to the behavioral seizure onsets. Note that all high-frequency components (100–600 Hz) were greater in magnitude (darkness and height of peaks) in the early vs. late post-status epilepticus (SE) states, indicating a loss of epileptiform discharges over time. Also note that these high-frequency components occurred after, rather than before, the spontaneous behavioral seizure onsets (onset of forepaw clonus and rearing). B1: Averaged power spectrum of six randomly selected, 1-minute-long traces of granule cell activity during SE. B2,3: The six first and last spontaneous seizures during the "early" and "late" spontaneous seizures, respectively, in a typical epileptic pilocarpine-treated rat. Note that all frequencies between 100 and 600 Hz were higher during "early" seizures (B2) than during "late" seizures (B3), indicating the loss of high-frequency epileptiform granule cell discharges in the chronic epileptic state. C1: The average integral taken from the averaged power spectrum (100–600 Hz) in all five chronically epileptic rats that were implanted before SE. Note the significant loss ($P = 0.012$) of all high-frequency components. C2,C3: The average integrals taken from the averaged power spectra of the two high-frequency bands (165–175 Hz and 340–360 Hz) in all five epileptic rats. Note the significant loss ($P = 0.012$ and 0.014, respectively) of both narrow frequency bands. Calibration bars = 5 mV, 4 sec.

ponent. Spectrograms of "early" spontaneous granule cell layer activity typically reflected high-frequency discharges in the frequency range corresponding to epileptiform discharges (Fig. 8A1). However, 72 of 98 late seizures recorded in the same five animals 30–146 days post-SE failed to exhibit the high-frequency discharges (Fig. 8A2) that had been present in the same animals during earlier spontaneous seizures (Fig. 8A1). The mean power spectrum of all early seizures recorded in each rat indicated a wide bandwidth of high-frequency activity (100–600 Hz). Figure 8 shows the characteristics of the spontaneous activity recorded in a typical epileptic rat during: 1) SE (Fig. 8B1), 2) the early period (Fig. 8B2), and 3) the late period (Fig. 8B3). The relative occurrence and duration of granule cell seizure discharges that occurred during the first six spontaneous behavioral seizures in the early period (<30 days post-SE) was highly variable, giving rise to the large standard error within the mean average power spectrum (the shaded region on either side of the lines in Fig. 8B2).

Conversely, during the chronic epileptic state, the mean power spectrum of the last six spontaneous seizures we recorded in each rat indicated a significant reduction in the number of high-frequency events occurring (Fig. 8B3). The absence of components above 200 Hz was consistent with the lack of seizure discharges detected qualitatively during behavioral seizures in the late chronic epileptic period (Fig. 7D). This observation, indicating a decrease in granule cell discharges and discharge frequency over time, was a consistent finding in the chronic epileptic state, and this consistency among different epileptic animals is reflected by the low standard error within the mean average power spectrum (Fig. 8B3). In the five rats in which granule cell layer activity was recorded during 142 spontaneous behavioral seizures, the loss of high-frequency activity was apparent at all frequencies and was highly consistent among animals. Figure 8 shows that there was an 83% loss of total high-frequency activity (Fig. 8C1). Similar losses (86% and 76%) were apparent in the two high-frequency ranges described above (Fig. 8C2 and C3, respectively). Thus, during the chronic epileptic state, granule cells were abnormally resistant to generating epileptiform discharges, both interictally and ictally, and never generated high-frequency epileptiform discharges before the onset of spontaneous seizures.

c-Fos expression as a marker of neuronal excitation

Stimulation-induced c-Fos expression in normal, awake rats. Although granule cell layer recordings from the dorsal hippocampi provided information about granule cell activity in two monitored regions of the dorsal dentate gyrus, they provided no direct information about the behavior of granule cells of the ventral hippocampus. In addition, recent studies suggest that small clusters of hyperexcitable neurons might coexist with strong paired-pulse suppression in epileptic tissue (Bragin et al., 2000). Although unlikely, it is conceivable that all electrode implantations coincidentally failed to detect high-frequency activity from any hyperexcitable neuronal clusters that might exist. Therefore, we assessed c-Fos immunoreactivity 1 hour after spontaneous seizures with the expectation that c-Fos expression might reveal, more globally than focal electrophysiological recording can possibly do, the neuronal populations that discharged when spontaneous seizures occurred. One hour was chosen as the optimal survival period because a recent study by Houser and colleagues showed that c-Fos expression in unimplanted epileptic mice was maximal in both dentate granule cells and hippocampal interneurons 1 hour after seizures (Peng at al., 2003). In addition, we used two polyclonal c-Fos

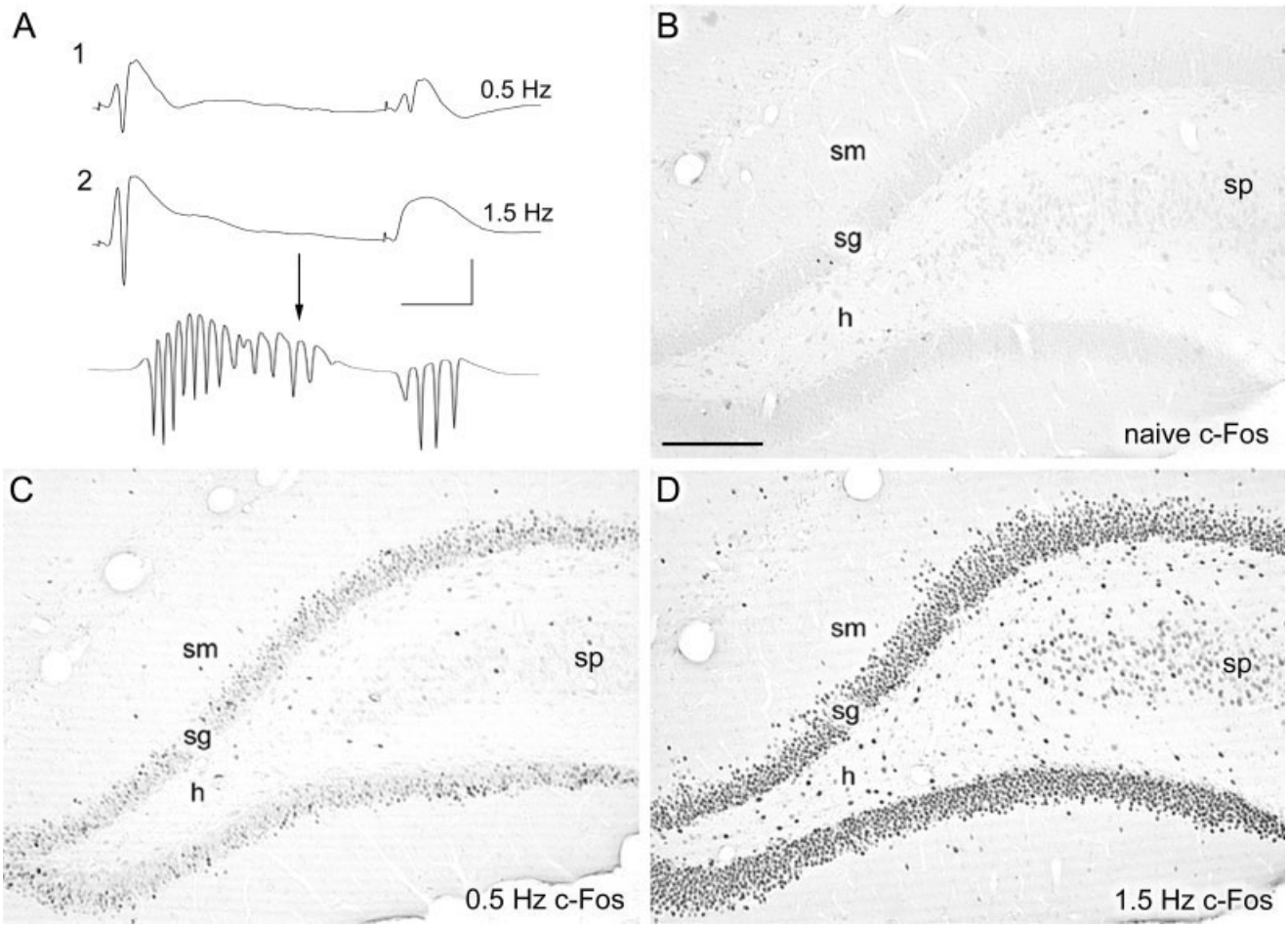

Fig. 9. c-Fos expression as an indicator of hippocampal neuronal activation. **A:** Granule cell responses to stimulation of the perforant pathway at 0.5 Hz for 4 minutes (1) or at 1.5 Hz for 50 sec. The latter treatment evoked seizure discharges that lasted for 7 seconds after the end of stimulation (2). **B:** c-Fos expression in a naive animal showing only scattered c-Fos-positive cells. **C:** One hour after stimulation at 0.5 Hz for 4 minutes (A1), weakly c-Fos-positive cells were evident in the dentate stratum moleculare (sm), stratum granulosum (sg), and hilus (h), but CA3 pyramidal cells of stratum pyramidale (sp) were c-Fos-negative. **D:** Granule cell layer seizure discharges lasting for 7 seconds (A2 above) evoked c-Fos expression 1 hour later in principal cells and interneurons of all hippocampal layers. Calibration bars = 10 mV, 10 msec. ×67. Scale bar = 200 μm in B (applies to B–D).

antisera generated against different amino acid sequences, including the one used by Houser and colleagues, to examine c-Fos expression after spontaneous seizures. To establish the feasibility of using c-Fos expression as a marker of discharging neurons under the experimental conditions of this study, we perfused normal animals 1 hour after one of three treatments: 1) unimplanted naive animals (n = 3), 2) implanted animals that were perforant path stimulated in the awake state at 0.5 Hz for 4 minutes to evoke only single granule cell population spikes (Fig. 9A1; n = 2), and 3) rats stimulated in the awake state at 1.5 Hz to induce a single episode of granule cell afterdischarges lasting for less than 10 seconds (Fig. 9A2; n = 3).

c-Fos expression was minimal in normal rats that were housed alone in a quiet environment for several hours before perfusion fixation, with only a few c-Fos positive nuclei scattered throughout the hippocampus (Fig. 9B). Afferent stimulation at different intensities evoked graded hippocampal c-Fos expression 1 hour after the end of stimulation. Perforant pathway stimulation at 0.5 Hz for 4 minutes, which evoked single population spikes (Fig. 9A1), but no epileptiform discharges, evoked relatively weak, but detectable c-Fos expression in dentate granule cell nuclei throughout the dorsal hippocampus (Fig. 9C). Nuclei of hippocampal CA3 pyramidal cells remained c-Fos negative after 0.5 Hz stimulation (Fig. 9C), presumably reflecting a failure of slow afferent stimulation to excite disynaptic target cells effectively. Conversely, paired-pulse stimulation at 1.5 Hz for ~50 seconds evoked granule cell afterdischarges that lasted for 7 seconds after the end of stimulation and produced intense c-Fos expression in granule cells, pyramidal cells, and interneurons of all hippocampal subregions (Fig. 9D). Thus, we confirmed that c-Fos expression is a relatively sensitive indicator of even briefly discharging hippocampal principal cells and interneurons in awake, chronically implanted rats, as studies under different laboratory conditions have demonstrated previously (Morgan et al., 1987; Shin et al., 1990; Mello et al., 1996; Mirzaeian and Ribak, 2000).

c-Fos expression after "early" spontaneous seizures in unimplanted pilocarpine-treated rats. Initial experiments examined c-Fos expression during SE and 1 hour after spontaneous seizures that developed during the first 2 weeks after SE. Among three rats perfusion fixed at the end of 3 hours of SE, all three exhibited c-Fos expression in all hippocampal neuron populations (Fig. 10B) as well in other cortical and subcortical structures throughout the brain. One hour after early spontaneous seizures (on day 9 post-SE), two of six unimplanted epileptic rats that were observed to have a seizure on day 9 post-SE exhibited similar hippocampal c-Fos expression in all hippocampal neuron populations (Fig. 10C). The other four rats, which had become epileptic prior to day 9 post-SE, but did not have a seizure during the observation period on day 9 post-SE, exhibited c-Fos expression similar to the naive control shown in Figure 9B.

c-Fos expression after "late" spontaneous seizures in chronically epileptic rats. c-Fos expression was assessed in 12 chronically implanted animals that had been monitored in the physiological experiments described above, and were perfusion fixed 1 hour after their last spontaneous seizure (n = 7 implanted >60 days post-SE; n = 5 implanted prior to SE). In 11 of these 12 animals (the remaining atypical rat from this group is described in the following section), the last spontaneous behavioral seizure recorded in each animal failed to recruit dentate granule cell seizure discharges. Among these 11 animals, seven exhibited hippocampal c-Fos expression that was restricted to presumed hippocampal interneurons throughout the dorsal and ventral hippocampus (Figs. 10D–F, 11A), three exhibited c-Fos expression that was not greater than the hippocampal c-Fos immunoreactivity observed in normal animals (Fig. 9B), and one rat exhibited atypical c-Fos expression in only the most ventral granule cells (described in the following section). The identity of c-Fos-positive interneurons was confirmed by colocalization immunofluorescence for c-Fos and vesicular glutamate transporter 1 (vGluT1; Fig. 10E), or c-Fos and the inhibitory neuron markers parvalbumin (PV; Fig. 10F) and somatostatin (data not shown). Virtually all c-Fos-positive nuclei were found to be within neurons immunoreactive for either PV or somatostatin, and vGluT1 immunostaining consistently outlined large, c-Fos-positive, nonprincipal cells in all hippocampal subregions (Fig. 10E). In contrast to the lack of c-Fos expression in hippocampal principal cells after late spontaneous seizures, presumed principal cells in the temporal neocortices and thalami of the same animals were consistently c-Fos-positive (Fig. 11). This finding was consistent with the likelihood that the spontaneous seizures in these animals were apparently of extrahippocampal origin (Mello et al., 1996) and either failed to recruit granule cell discharges or recruited granule cell discharges that consistently followed the behavioral seizure onsets.

The possibility that hippocampal principal cells in chronically epileptic rats were discharging, but incapable of expressing c-Fos in the chronic state, was addressed in three additional chronically epileptic rats that were implanted >60 days post-SE and then given a single 10-Hz 10-second stimulus train 1 hour prior to perfusion. The purpose of this experiment was to determine whether hippocampal principal cell c-Fos expression might be permanently down-regulated in the chronic epileptic state (Morgan et al., 1987; Mello et al., 1996), which would raise the possibility that unmonitored granule cells far from the recording electrodes might be discharging but unable to make c-Fos. One hour after a brief episode of evoked granule cell afterdischarges, all three chronically epileptic rats exhibited c-Fos expression in hippocampal principal cells similar to that shown in an animal perfusion fixed during SE (Fig. 10B), or in an epileptic rat perfusion fixed 1 hour after an early spontaneous seizure that occurred 9 days post-SE (Fig. 10C). This result indicates that the lack of c-Fos expression in hippocampal granule cells and pyramidal neurons of chronically epileptic rats 1 hour after their final spontaneous seizure was unlikely to be due to an inability to express c-Fos.

Atypical c-Fos expression in two chronically epileptic rats. Two atypical animals were relevant to the issue of the ability of hippocampal principal cells to express c-Fos in the chronic epileptic state when forced to discharge, and the possible relationship between mossy fiber sprouting and granule cell hyperinhibition. In one animal implanted with recording electrodes in the granule cell layer and the CA1 pyramidal cell layer, granule cells exhibited an elevated seizure discharge threshold 50 days post-SE, as evidenced by its failure to generate epileptiform seizure discharges until the frequency of perforant path stimulation was raised to 4 Hz (compared with 1–2 Hz in controls). Ten spontaneous seizures were then recorded in this rat during the 24-hour period 56 days post-SE. The first nine behavioral seizures recorded were typical of those detected in chronically epileptic rats, in that the behavioral seizure failed to recruit granule cell seizure discharges. Atypically, however, the tenth spontaneous behavioral seizure in this animal recruited seizure discharges at both the granule cell and the CA1 electrodes, and these discharges began long after the onset of the behavioral seizure (Fig. 12A,B). Because this atypical seizure occurred serendipitously during live observation, we perfused this animal 1 hour later to address the issue of whether rare principal cell seizure discharges that were recruited during the chronic epileptic state were capable of evoking principal cell c-Fos expression. Subsequent c-Fos immunostaining revealed that the brief seizure discharges we recorded (Fig. 12A,B) resulted in c-Fos expression in dorsal hippocampal granule cells and pyramidal cells (Fig. 12C1). Timm staining of alternate sections revealed that this animal exhibited more extensive mossy fiber sprouting in the ventral hippocampus (arrows in Fig. 12D2) than in the dorsal hippocampus (arrows in Fig. 12C2). Consistently with this apparently greater degree of ventral hippocampal mossy fiber sprouting, the ventral dentate gyrus exhibited no detectable granule cell c-Fos expression (arrows in Fig. 12D1) in sections coprocessed with the c-Fos-positive dorsal hippocampal sections. The pattern of c-Fos expression in this atypical animal suggests that, when principal cell populations discharged during the chronic epileptic state, they readily expressed c-Fos. Furthermore, the lack of c-Fos expression in granule cells of the more densely mossy fiber sprouted ventral dentate gyrus contrasted with the presence of c-Fos in CA1 pyramidal/subicular cells of the same ventral hippocampal sections (Fig. 12D1). This observation suggests that, although the spontaneous behavioral seizure apparently failed to recruit the synaptically reorganized granule cells of the ventral hippocampus, CA1–CA3 pyramidal cells in the same sections were nonetheless independently recruited to discharge and participate in the generalized

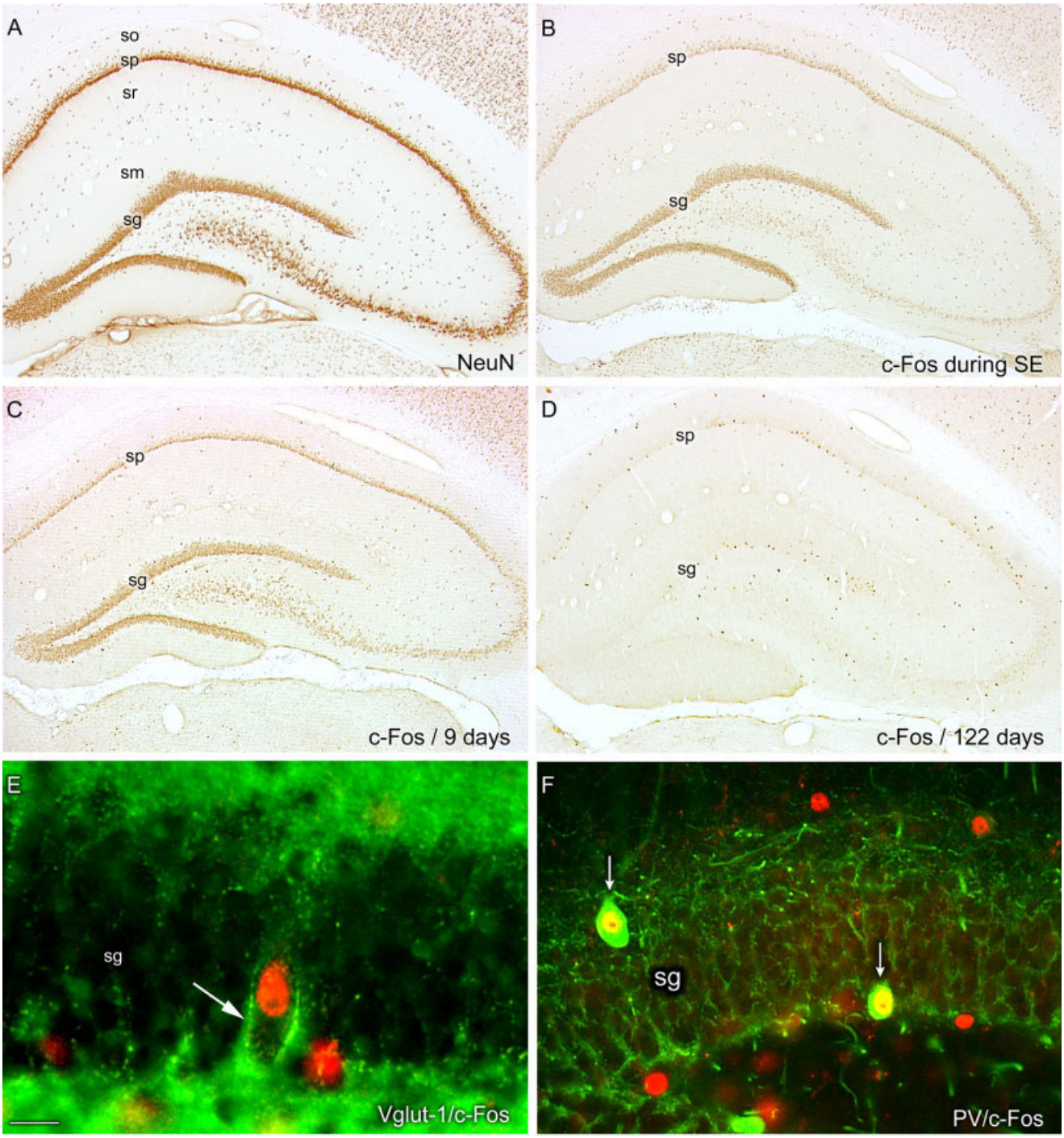

Fig. 10. Hippocampal c-Fos expression 1 hour after early and late spontaneous seizures in chronically epileptic, pilocarpine-treated rats. **A:** NeuN-immunostained section of the dorsal hippocampus of a control animal showing the location of principal cells and interneurons in the normal hippocampus. **B:** c-Fos expression in all hippocampal neurons after 3 hours of status epilepticus (SE). **C:** c-Fos immunoreactivity 1 hour after an "early" spontaneous seizure observed 9 days post-SE. Note that all hippocampal principal cells and numerous interneurons were c-Fos positive. **D:** c-Fos expression 1 hour after a spontaneous seizure that occurred 122 days post-SE. Note that the granule cell layer (sg, stratum granulosum) and the pyramidal cell layers (sp, stratum pyramidale) are c-Fos negative, but that scattered cells are c-Fos positive. **E:** Immunofluorescence colocalization of the excitatory terminal marker vesicular glutamate transporter 1 (vGlut1; green) and c-Fos (red), showing that cells containing c-Fos-positive nuclei have the location and shape of presumed inhibitory interneurons (arrow). **F:** Colocalization of the inhibitory interneuron marker parvalbumin (PV; green) and c-Fos (red), showing that c-Fos-positive neurons include PV-positive interneurons (arrows). A–D, ×26.2; E,F: ×262. Scale bar = 30 μm in E (applies to E,F); 300 μm for A–D.

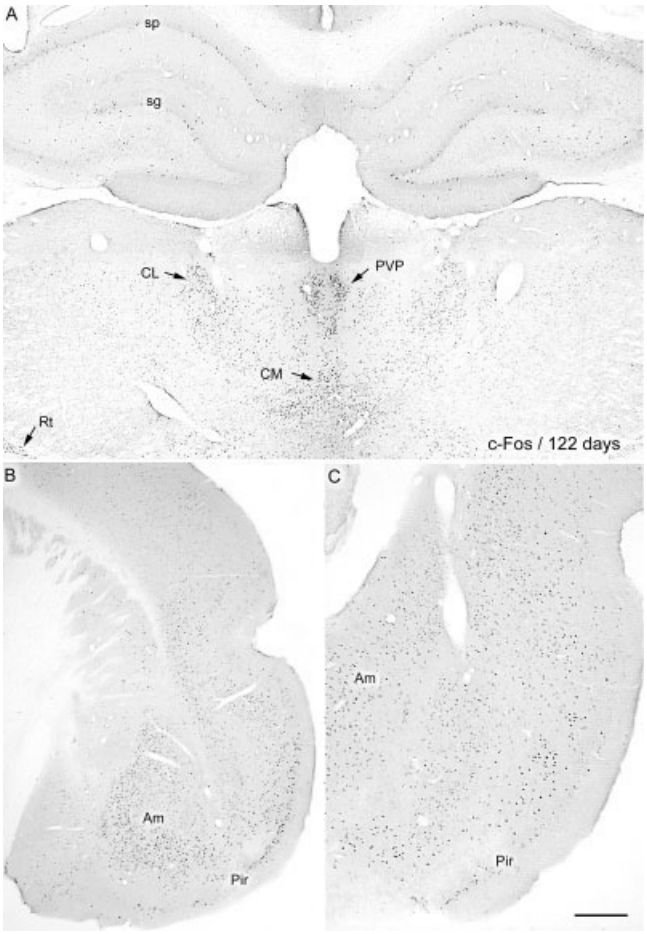

Fig. 11. Cortical and thalamic c-Fos expression 1 hour after a spontaneous behavioral seizure in a chronically epileptic pilocarpine-treated rat. **A:** c-Fos-immunostained section showing c-Fos-positive hippocampal interneurons and c-Fos-positive thalamic nuclei (arrows). **B,C:** c-Fos-immunostained sections of the temporal cortex from the same rat shown in A. Note that there is significant c-Fos expression throughout the piriform cortex (Pir) and in amygdaloid nuclei (Am), which is consistent with an extrahippocampal origin of spontaneous seizures. Rt, reticular nucleus; CM, central medial nucleus; CL, centrolateral nucleus; PVP, paraventricular nucleus. A: ×14.3; B: ×9.7; C: ×19.3. Scale bar = 343 μm in C; 465 μm for A; 690 μm for B.

seizure. This is conjectural, however, and different patterns of c-Fos expression in different septotemporal segments of the hippocampus could also be due to the different topographical projections of several neocortical inputs to the hippocampus (Naber et al., 1999; Shi and Cassell, 1999; Witter et al., 1999).

In the second atypical case encountered, recording electrodes in the dorsal dentate gyri recorded typical granule cell layer potentials during spontaneous seizures, in that they lacked granule cell epileptiform discharges (as shown in Fig. 7D2). Subsequent Nissl, Timm, and c-Fos staining revealed that the dorsal hippocampal region from which the recordings were made exhibited extensive hilar neuron loss (Fig. 13A), dense mossy fiber sprouting (Fig. 13B), and c-Fos expression in interneurons (Fig. 13C), all features typical of other chronically epileptic rats. However, c-Fos-positive granule cells in this animal were visible in the most ventral dentate granule cells of the same sections (arrows in Fig. 13C), as previously reported for an unimplanted pilocarpine-treated rat (Scharfman et al., 2002). This focally c-Fos-positive region of the ventral dentate gyrus also exhibited atypical hilar neuron survival (Fig. 13E) and minimal mossy fiber sprouting (arrows in Fig. 13B) in precisely the same location where granule cells were c-Fos-positive (arrows in Fig. 13C).

c-Fos expression after spontaneous seizures in unimplanted pilocarpine-treated rats. An additional group of unimplanted, pilocarpine-treated rats was used to determine whether c-Fos expression after late spontaneous seizures represented de novo c-Fos expression. This group consisted of 17 chronically epileptic rats that were observed for spontaneous seizures over a 3-day period. Among the 17 epileptic rats observed, 11 exhibited a spontaneous seizure during one of the three observation periods, whereas the six remaining rats did not. Subsequent c-Fos immunostaining revealed that, among the 11 animals that exhibited a spontaneous seizure, six exhibited no hippocampal c-Fos expression greater than naive controls, and five exhibited c-Fos expression primarily in hippocampal interneurons, as shown in Figure 10D. Conversely, all 11 rats exhibited c-Fos expression in the temporal neocortex and thalamus, as shown in Figure 11. Among the six chronically epileptic rats that did not exhibit a spontaneous seizure during the 6-hour period of observation, all six exhibited no c-Fos expression in the hippocampus or temporal neocortex greater than naive control animals processed in parallel (n = 3). These results indicate that the expression of c-Fos in all hippocampal neurons after early spontaneous seizures and the selective expression of hippocampal c-Fos in interneurons after late spontaneous seizures were the result of de novo c-Fos expression after spontaneous seizures, when present, and were not a constantly expressed product of the chronic epileptic state unrelated to the individual spontaneous seizures.

DISCUSSION

The ability to assess granule cell pathophysiology in the same awake animals at different stages of the epileptogenic process has yielded several original findings. First, the acute hilar neuron loss that was produced consistently by granule cell epileptiform discharges during behavioral SE was initially associated with hyperexcitable granule cell responses to afferent stimulation. Granule cell hyperexcitability lasted for several weeks following SE and was characterized by decreased paired-pulse suppression, the appearance of multiple population spikes in response to single afferent stimuli, and reduced thresholds for generating epileptiform discharges in response to perforant pathway stimulation. Although these network-level indices of granule cell hyperexcitability in intact, awake animals cannot be attributed definitively to decreased $GABA_A$ receptor-mediated inhibition, we note that the same three characteristics of granule cell hyperexcitability have been shown to be reproduced in vivo by the $GABA_A$ receptor antagonist bicuculline (Sloviter, 1991a) or by treatment with substance P-saporin conjugate, which selectively eliminates hippocampal inhibitory interneurons, without involving detectable principal cell loss or mossy fiber sprouting (Martin and Sloviter, 2001).

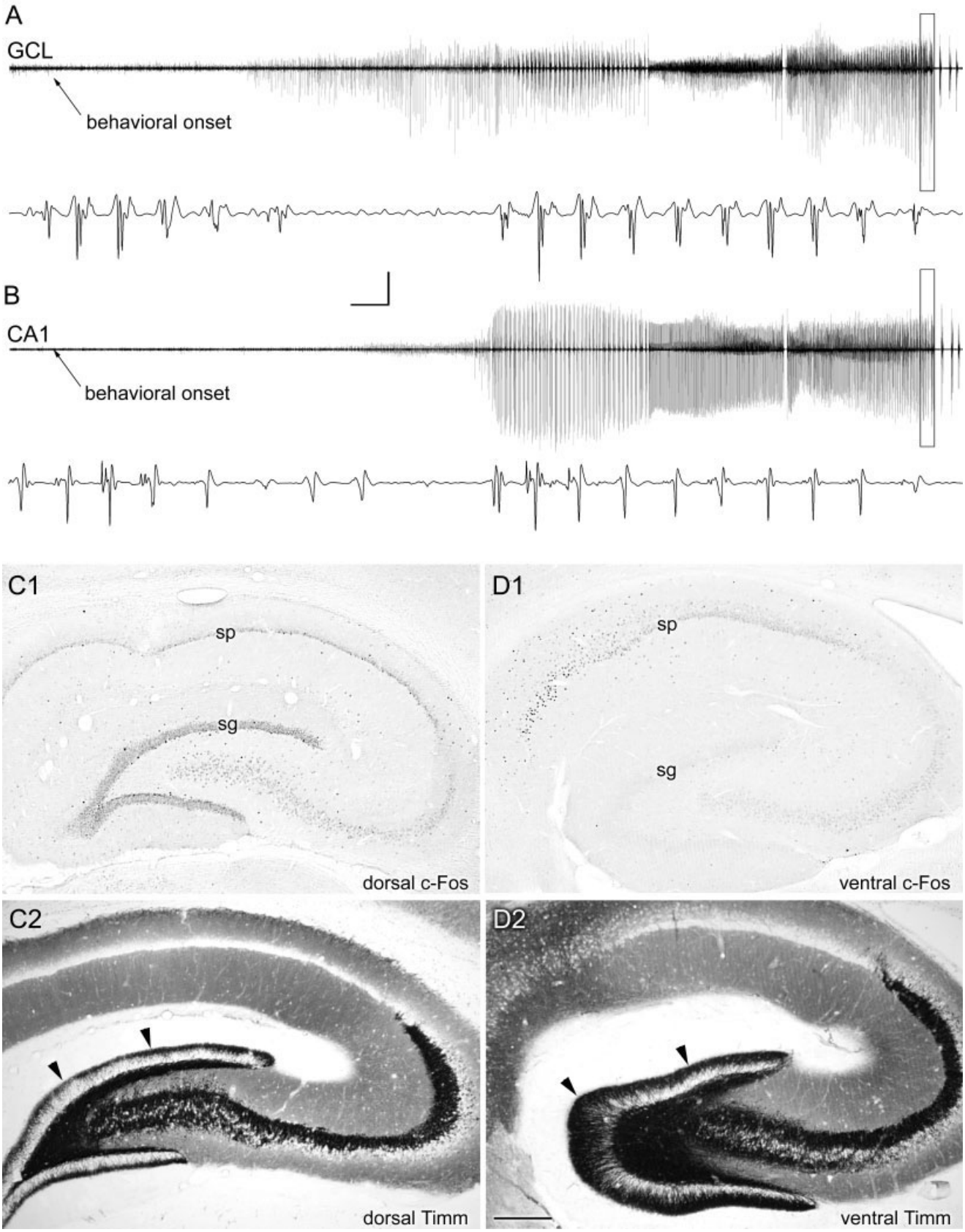

Fig. 12. Atypical recruitment of granule cell and CA1 pyramidal cell discharges during a spontaneous seizure in a chronically epileptic rat. **A,B:** In this animal, perfusion fixed 56 days post-SE, seizure discharges were recorded from both dorsal hippocampal granule cell (GCL) and CA1 pyramidal cell layers beginning approximately 25 seconds after the behavioral seizure onset. **C1:** c-Fos immunostaining of the dorsal hippocampus in a coronal section revealed c-Fos expression in all hippocampal neurons. **D1:** c-Fos immunostaining of the ventral hippocampus in a horizontal section from the same rat revealed c-Fos expression in pyramidal layer neurons but not in the dentate gyrus (sg; stratum granulosum). **C2,D2:** Note that Timm staining revealed less extensive mossy fiber sprouting in the dorsal hippocampus (C2) than in the ventral hippocampus (D2), and a lack of c-Fos expression in the more heavily mossy fiber-sprouted ventral dentate gyrus. Calibration bars = 10 mV, 5 seconds (compressed areas); 6 mV, 75 msec (expanded boxes). ×26. Scale bar = 400 μm in D (applies to C,D).

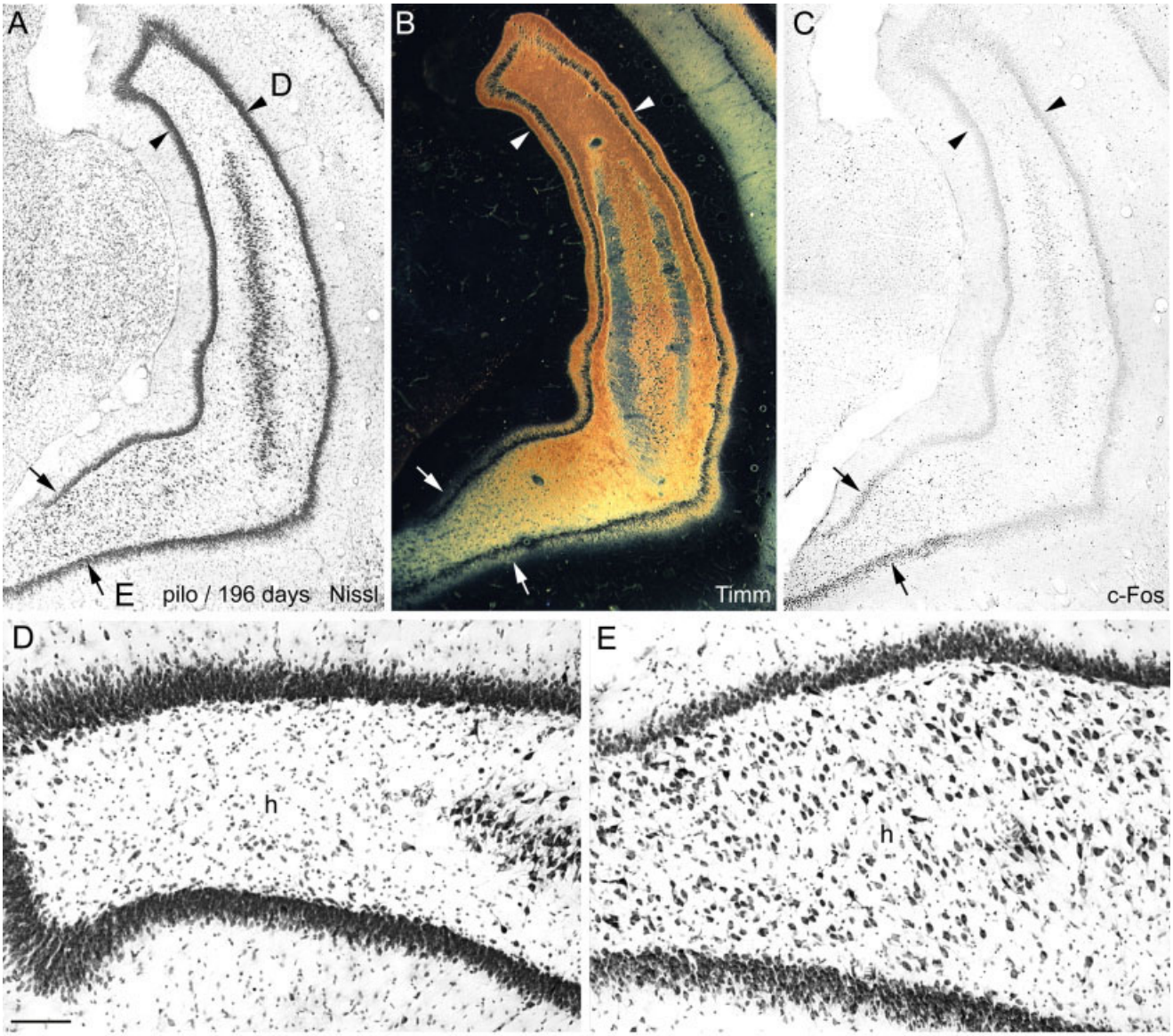

Fig. 13. Atypical c-Fos expression and mossy fiber sprouting in a chronically epileptic, pilocarpine-treated rat 196 days post-SE. **A:** Nissl-stained section of the hippocampus 1 hour after a spontaneous seizure. Note neuronal loss in the hilar region of the dorsal hippocampus between the arrowheads (magnified in D), and neuronal survival in the ventral hilus (area between the arrows, magnified in E). **B:** the adjacent Timm-stained section under darkfield illumination shows dense mossy fiber sprouting where extensive hilar cell loss is evident, but minimal mossy fiber sprouting in the relatively undamaged ventral hippocampus. **C:** c-Fos-immunostained section of the hippocampus from the same rat shown in A,B. Note that ventral dentate granule cells lacking mossy fiber sprouting (arrows) are c-Fos immunopositive, whereas synaptically reorganized granule cells of the dorsal dentate gyrus are c-Fos negative. **D,E:** Nissl-stained sections of the dorsal and ventral dentate gyrus, respectively, showing survival of ventral hilar neurons. A–C: ×24; D,E: ×176. Scale bar = 67 μm in D (applies to D,E); 500 μm for A–C.

Second, the recovery of paired-pulse suppression and the restoration of relatively normal granule cell seizure discharge thresholds paralleled the appearance of early mossy fiber sprouting, which included parvalbumin-positive dentate inhibitory interneurons as apparent targets. Although we note the correlation between the recovery of paired-pulse suppression and the appearance of mossy fiber sprouting that surrounded inhibitory interneurons, a causal relationship between synaptic reorganization and restoration of granule cell inhibition ("inhibitory" mossy fiber sprouting) cannot be inferred on the basis of our functional and structural data. Regardless, granule cells were hyperexcitable immediately after SE, coincident with hilar neuron loss, and then gradually became less, rather than more, excitable as synaptic reorganization progressed.

Third, granule cells monitored in awake, epileptic animals did not discharge synchronously prior to any of 235 spontaneous seizure onsets, which included 44 "early" spontaneous seizures observed in the first 4 weeks post

SE, and 191 spontaneous seizures during the later "chronic" state. Although spontaneous seizures occasionally recruited granule cell epileptiform discharges, no epileptiform discharges occurred before any of the 235 clinical seizure onsets, suggesting that granule cell discharges did not initiate the clinical seizures that developed in this animal model. Even when granule cells were forced by afferent excitation to generate epileptiform discharges, these epileptiform discharges did not cause behavioral seizures in animals that were nonetheless exhibiting frequent spontaneous behavioral seizures. These data, the extensive extrahippocampal damage, and the patterns of c-Fos expression observed after spontaneous seizures (c-Fos-negative principal cells and c-Fos-positive inhibitory interneurons) suggest that the spontaneous seizures were probably of extrahippocampal origin, as initially suggested by Mello and colleagues (1996), although the sites of seizure origin remain to be identified.

Fourth, high-sampling-rate recording, and analysis of spontaneous granule cell layer activity at an expanded time base, revealed that high-amplitude voltage fluctuations (presumed fEPSPs; Andersen et al., 1966) recorded during spontaneous seizures often lacked negative-going population spikes. This suggests that high-amplitude spontaneous activity can be easily mistaken for epileptiform discharges if not acquired at a high sampling rate and analyzed at an expanded time base.

Finally, spontaneous seizures often began prior to mossy fiber sprouting. The lack of silence during the "silent period," which we and others have observed (Cavalheiro et al., 1991; Bertram and Cornett, 1993; Nissinen et al., 2000), suggests that the latent period between injury and clinical seizures, rather than being the necessary consequence of a time-dependent process such as synaptic reorganization, may relate to the number of seizure barriers damaged initially and the time needed for initially sequestered, subclinical discharges to "kindle" through initially undamaged barriers (Goddard et al., 1969; Bertram and Cornett, 1993, 1994).

Gradually increasing granule cell paired-pulse suppression after dentate gyrus cell loss

Our conclusion that granule cells became immediately hyperexcitable after SE and then gradually less excitable and abnormally resistant to generating epileptiform discharges is based on three observations. First, the loss of paired-pulse suppression occurred immediately after injury, as previously reported (Sloviter, 1987, 1991b, 1992; Lowenstein et al., 1992; Wasterlain et al., 1996; Klitgaard et al., 2002; Kobayashi and Buckmaster, 2003). Second, paired-pulse suppression recovered gradually, reaching a pre-SE degree of suppression ~2–4 weeks post-SE. Third, in the later recovery period >1 month post-SE, all epileptic animals exhibited abnormally increased paired-pulse suppression and an abnormally elevated seizure discharge threshold, as previous studies in anesthetized animals have consistently indicated (Sloviter, 1992; Buckmaster and Dudek, 1997; Wu and Leung, 2001). The difficulty we had in evoking granule cell discharges in awake, epileptic rats by afferent stimulation was mirrored by the diminished ability of spontaneous behavioral seizures to recruit granule cell discharges. The paradoxical finding that granule cells became progressively hyperinhibited, rather than hyperexcitable, and were unlikely to be the source of spontaneous seizures is consistent with observations that the sclerotic human hippocampus was more difficult to discharge than the contralateral hippocampus (Cherlow et al., 1977).

Our finding that paired-pulse suppression was restored gradually contrasts with the conclusion by Dudek and colleagues that granule cell paired-pulse suppression recovered within days after SE and, therefore, could not be the result of mossy fiber sprouting onto inhibitory interneurons (Hellier et al., 1999). We attribute this difference to two methodological factors. First, because their animals exhibited only minor (~35%) hilar cell loss, and minimal hilar neuron loss is reliably associated with little or no detectable hyperexcitability (Milgram et al., 1991; Ratzliff et al., 2004; Zappone and Sloviter, 2004), the animals used by Dudek and colleagues did not exhibit the failure of paired-pulse suppression that we originally reported (Sloviter, 1992) and that they attempted to replicate. Second, Dudek and colleagues assessed granule cell inhibition by delivering only one pair of afferent stimuli per test day (Hellier et al., 1999). We replicated their method, but also evaluated responses to subsequent stimuli. This approach showed that the assessment of responses to only one stimulus pair significantly overestimated the strength of paired-pulse suppression present and missed the diminished inhibitory state (Isokawa, 1996) that subsequent stimuli revealed.

Our observations were not model specific. We have also observed qualitatively identical initial hyperexcitability, and gradual restoration of paired-pulse suppression, in kainate-treated rats with extensive hilar cell loss (Harvey and Sloviter, 2002), as well as ictal granule cell hyperinhibition in the chronic epileptic state (unpublished results). Thus, our data in both pilocarpine- and kainate-treated rats indicate that, whenever prolonged SE produces extensive hilar neuron loss, acute granule cell hyperexcitability reliably results, and that the subsequent development of granule cell hyperinhibition is a gradual process that parallels the growth of mossy fiber sprouting (although a causal relationship between synaptic reorganization and restoration of granule cell inhibition cannot be inferred).

c-Fos expression after spontaneous seizures

We utilized c-Fos as a molecular indicator of neuronal activity because of our concern that bilateral recording electrodes in the dorsal hippocampus could not indicate how neurons in other hippocampal regions were behaving. The parallel approaches of focal recording and global c-Fos detection unexpectedly revealed that spontaneous seizures during the chronic state rarely recruited granule cells and resulted in hippocampal c-Fos expression primarily in inhibitory interneurons. Experiments showing that principal cells in chronically epileptic animals readily expressed c-Fos when forced to discharge or in the rare case when granule cell discharges were recruited by spontaneous seizures indicate that the lack of principal cell c-Fos expression after spontaneous seizures reflected their relative quiescence, not their inability to express c-Fos. In addition, the observation that inhibitory interneurons in chronically epileptic rats did not express c-Fos unless a spontaneous seizure had recently occurred indicates that c-Fos expression by interneurons was de novo expression, presumably evoked by direct feed-forward excitation of inhibitory neurons by seizure activity originating outside the hippocampus. However, this mechanism, and the

mechanisms underlying granule cell hyperinhibition, remain to be determined.

It could be hypothesized that hippocampal pyramidal cells may be the source of the spontaneous seizures even if granule cells remain relatively uninvolved. Although pyramidal layer recordings will be needed to address this question directly, the lack of c-Fos expression in hippocampal pyramidal cells, as well as in granule cells, argues against this scenario. Significantly, in one atypical epileptic animal from which CA1 pyramidal cell layer recordings were obtained, a spontaneous behavioral seizure recruited CA1 pyramidal cell discharges, and this animal exhibited c-Fos-positive pyramidal cells 1 hour later. This result suggests that the c-Fos-negative pyramidal cells of other epileptic rats probably reflected a relative lack of involvement of pyramidal cells, although this issue requires further study.

Hippocampal epileptogenesis: human vs. rat

It is difficult to reconcile all of the available human and experimental data. Although the sclerotic human hippocampus is a suspected source of seizures within a network of hyperexcitable temporal lobe nuclei (Bertram et al., 1998; Spencer, 1998, 2002; Wennberg et al., 2002), the sclerotic hippocampus is reportedly hyperinhibited interictally (Cherlow et al., 1977; Colder et al., 1996; Wilson et al., 1998), as we found the dentate gyrus of epileptic rats to be. Therefore, one important issue to resolve is whether human interictal hippocampal hyperinhibition ever collapses, and whether granule cells ever generate epileptiform discharges that initiate clinical seizures. Clearly, we saw no evidence of inhibitory collapse and the generation of granule cell epileptiform discharges prior to the onset of behavioral seizures in pilocarpine-treated rats, but there is probably no compelling reason to assume that normal rats subjected to prolonged SE faithfully represent patients with a neurological disorder that develops within an often initially abnormal brain (Kuks et al., 1993). Our suspicion that the normal rat brain may respond to injuries in ways not identical to perhaps initially abnormal human brains, prevents us from assuming that our results in rats can be seamlessly extrapolated to human patients. Nonetheless, the observation that, when analyzed at a compressed time base, high-amplitude activity recorded during spontaneous seizures (Bertram and Cornett, 1993; 1994; Gorter et al., 2002; Nissinen et al., 2000; Kubova et al., 2004) can lack epileptiform discharges suggests that it may be unknown whether human interictal hippocampal hyperinhibition ever actually collapses and whether human granule cells discharge synchronously. The recent observations that human "hippocampal-onset" seizures only infrequently spread to cause clinical seizures (Wennberg et al., 2002) and that seizures in temporal lobe epilepsy may be triggered by an influence external to the hippocampus (Parish et al., 2004) suggest that some activity recorded from hippocampal depth electrodes may be nonpropagating depolarizations that only superficially resemble propagating epileptiform discharges. On the other hand, recordings made within the hippocampus may be extrahippocampal events generated in nearby structures. Thus, our results in pilocarpine-treated rats raise unanswered questions about the human dentate gyrus as a primary seizure source. Regardless, given that hippocampal cell loss is only one component of the severe and widespread brain damage produced by SE in rats (Honchar et al., 1983; Cavalheiro et al., 1991; Peredery et al., 2000), and given the results of numerous studies suggesting extrahippocampal seizure origins (Du et al., 1995; Bear et al., 1996; Mello et al., 1996; Schwarcz et al., 2000, 2002; Bertram et al., 2001; Roch et al., 2002), synaptically reorganized granule cells cannot be assumed to be "epileptic" unless recordings in awake animals demonstrate it directly.

CONCLUSIONS

In sum, our results in awake rats provide direct evidence against the hypothesis that dentate granule cells become increasingly hyperexcitable as mossy fiber sprouting progresses (Tauck and Nadler, 1985) or that synaptically reorganized granule cells are a likely source of spontaneous seizures in rats subjected to prolonged SE (Wuarin and Dudek, 1996, 2001). These results also raise questions about hypotheses suggesting that hilar mossy cell loss (Sloviter, 1987, 1991b, 1994) or the malfunction of surviving hilar mossy cells (Santhakumar et al., 2000; Scharfman et al., 2001) causes dentate granule cells to become initiators of the spontaneous seizures that define post-SE animals as epileptic. We regard it as a distinct possibility that our results in post-SE animals, which exhibited severe and widespread brain damage, may be minimally applicable to hypotheses about human epilepsy patients, who rarely experience status epilepticus, exhibit far less pathology than post-SE rats, and are otherwise neurologically normal (Sloviter, 2005). In this regard, further development of animal models that involve less severe brain damage but still exhibit spontaneous seizures (Shirasaka and Wasterlain, 1994; Wasterlain et al., 1996) may be particularly fruitful.

Clearly, it must now be determined in awake animals whether any of the currently used animal models involve any hippocampal principal cells as the primary source of spontaneous seizures, which might include subicular neurons (Cohen et al., 2002; Knopp et al., 2005). The possibility that the hippocampus may not play a primary epileptogenic role in animals that have suffered severe and widespread brain damage has significant and unexpected implications for the many experimental studies that have presumed that SE-induced changes in hippocampal gene expression, structure, and function constitute the epileptogenic process. These considerations emphasize the need for a critical reappraisal of current animal models and for the development of experimental preparations that accurately model the relatively focal pathology and pathophysiology of the human neurological condition.

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