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Temporal Patterns of Fos Expression in the Dentate Gyrus after Spontaneous Seizures in a Mouse Model of Temporal Lobe Epilepsy

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Identifying the brain regions and neuronal cell types that become active at the time of spontaneous seizures remains an important challenge for epilepsy research, and the involvement of dentate granule cells in early seizure events continues to be debated. Although Fos expression is commonly used to evaluate patterns of neuronal activation, there have been few studies of Fos localization after spontaneous seizures. Thus, in a pilocarpine model of recurrent seizures in C57BL/6 mice, Fos expression was examined at multiple time points after spontaneous seizures to follow the temporal and spatial patterns of Fos activation. By 15 min after the beginning of a spontaneous behavioral seizure, Fos labeling was evident in dentate granule cells. This labeling was particularly striking because of its wide extent and relatively uniform appearance in the granule cell layer. At later time points, from 30 min to 4 h after a spontaneous seizure, Fos labeling was also detected in interneurons within the dentate gyrus and in widespread regions of the temporal lobe. Interestingly, the timing of Fos activation appeared to differ among different types of GABAergic interneurons in the dentate gyrus, with labeling of parvalbumin neurons along the base of the granule cell layer preceding that of GABA neurons in the molecular layer. The findings in this mouse model are consistent with previous suggestions that spontaneous seizures in temporal lobe epilepsy may result from a periodic breakdown of the normal filter functions of the dentate gyrus and a resulting increase in hypersynchronous activity of dentate granule cells.

Key words: **pilocarpine; hippocampus; granule cells; interneurons; immunohistochemistry; GABA neurons**

Introduction

Temporal lobe epilepsy is characterized by spontaneous seizures that originate focally and then spread within the limbic system and beyond. What specific brain regions are active during the initiation of the seizure activity and what conditions trigger the spontaneous seizures are among the most persistent and perplexing questions in the epilepsy field.

The dentate gyrus is commonly considered to be a gate that prevents excessive activity from entering other hippocampal regions (Heinemann et al., 1992; Lothman et al., 1992). Previous studies have suggested that a breakdown of this gate, with activation of dentate granule cells, could be a critical event in the development of seizure activity within the temporal lobe (Collins et al., 1983; Heinemann et al., 1992). Support for this idea *in vivo* has come primarily from studies of acute induced seizures in which maximal activation of the dentate gyrus occurs (Stringer and Lothman, 1989; Stringer and Sowell, 1994). Evidence for similar pervasive activation of the dentate granule cells at the time of spontaneous seizures is lacking.

Labeling of c-*fos* and its protein product Fos has been used extensively to evaluate neuronal activation after electrically and chemically induced seizures (Dragunow and Robertson, 1987; Morgan et al., 1987; Barone et al., 1993; Woldbye et al., 1996; Willoughby et al., 1997; Motte et al., 1998; Mihaly et al., 2001). In contrast, there have been surprisingly few studies of Fos activation after spontaneous seizures, particularly in models of recurrent seizures that develop after status epilepticus. In two such reports, little or no increase in Fos expression was detected in granule cells at 1 h after a spontaneous seizure in pilocarpine-treated rats during the chronic period (Mello et al., 1996; Harvey and Sloviter, 2005). Instead, strong labeling of interneurons in the dentate gyrus was observed in one of the studies, and this led the investigators to suggest that granule cells may be quiescent at the onset of spontaneous seizures, attributable in part to their inhibition by activated interneurons (Harvey and Sloviter, 2005). These provocative findings emphasize the need for evaluating patterns of neuronal activation after spontaneous seizures in additional models of temporal lobe epilepsy, including mouse models, because there could be differences in the circuits that are activated.

Although C57BL/6 mice are generally resistant to neuronal damage after kainate-induced seizures (Schauwecker and Steward, 1997), these mice show substantial cell loss in the hilus and

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several other brain regions after pilocarpine-induced status epilepticus (Houser et al., 2002). Importantly, C57BL/6 mice consistently develop spontaneous seizures within the first few weeks after status epilepticus. Fos expression was thus examined at multiple time points after the spontaneous seizures to follow the temporal and spatial patterns of Fos activation. The specific goals were to determine whether spontaneous seizures in these mice were associated with increased Fos expression in dentate granule cells and to compare the patterns of Fos labeling in granule cells and interneurons in the dentate gyrus after the spontaneous seizure.

Parts of this work have been published previously in abstract form (Peng et al., 2003).

Materials and Methods

Animals and pilocarpine treatment. Young adult (6–8 weeks of age) C57BL/6 male mice (20–27 g; Harlan, Indianapolis, IN) were used in this study. Sustained seizures were induced in experimental animals by the administration of pilocarpine, a muscarinic cholinergic agonist, and the protocols have been described previously (Peng et al., 2004). Briefly, 30 min before pilocarpine administration, animals were injected with a low dose of the cholinergic antagonist methyl scopolamine nitrate (1 mg/kg, i.p.) to reduce peripheral cholinergic effects. Animals in the experimental group then received an injection of pilocarpine hydrochloride (320–340 mg/kg, i.p.; Sigma, St. Louis, MO) to induce status epilepticus. Diazepam (5 mg/kg, i.p.; Abbott Laboratories, Chicago, IL) was administered at 3 h after the onset of status epilepticus to stop or limit behavioral seizures. Control animals received an identical series of injections, except that the pilocarpine was replaced with a similar volume of sterile saline. After the pilocarpine injection, experimental animals were monitored for a minimum of 5 h to assess the severity and length of the behavioral seizures.

After recovery from status epilepticus, the behavior of pilocarpine-treated mice was continuously monitored by direct observation or videotape 5 d/week, using infrared light during the dark phase (6:00 P.M. to 6:00 A.M.). All animal use protocols conformed to National Institutes of Health guidelines and were approved by the University of California, Los Angeles, Chancellor's Animal Research Committee and the Veterans Administration Institutional Animal Care and Use Committee.

Behavioral outcomes. Spontaneous behavioral seizures were observed in all pilocarpine-treated animals. The spontaneous seizures typically consisted of periods of freezing, clonic movements of the forelimbs, rearing, or rearing and falling (stage 3–5 limbic seizures) (Racine, 1972), and these seizures were often either followed or preceded by a brief (10–20 s) generalized motor seizure. The total duration of the observed behavioral seizure was typically ~1 min and no longer than 2 min. For this study, only mice that had clearly observable behavioral seizures (Racine's scale 4 and/or 5), observed directly or on videotape, were used for analysis of Fos expression.

The frequency of spontaneous seizures varied among the animals. Some of the mice remained seizure-free for several days, others had frequent seizures, and some exhibited clustered seizures, with as many as 10 seizures in 1 h. Thus, criteria were established to ensure that a mouse perfused after a behavioral seizure had not experienced a spontaneous seizure during the previous 24 h and had not had an additional seizure during the interval before perfusion. One mouse that experienced several seizures in the 24 h before the final seizure was used for comparison.

Tissue preparation. After a minimum of 2 weeks of survival, spontaneous seizures were identified, and animals were prepared for analyses at a series of time points after the spontaneous seizures. A total of 24 epileptic and 16 control mice were included in this study. The mice were deeply anesthetized with sodium pentobarbital (90 mg/kg, i.p.) and perfused through the ascending aorta with 4% paraformaldehyde in 0.12 M phosphate buffer, pH 7.3. Animals were perfused at 15 min ($n = 4$, including one animal that experienced multiple seizures before perfusion), 30 min ($n = 6$), 1 h ($n = 4$), 2 h ($n = 3$), 4 h ($n = 2$), 8 h ($n = 1$), and 18–24 h ($n = 4$) after spontaneous behavioral seizures. One to three control mice were perfused along with the pilocarpine-treated mice at each time point. The survival time after pilocarpine treatment was 2–14 weeks. The longer time was required to meet the established criterion of a 24 h seizure-free period before the last seizure. The extended survival times also permitted analyses of Fos labeling at shorter and longer times after status epilepticus.

After perfusion, the brains were maintained *in situ* at 4°C for 1 h and then removed from the skull and postfixed in the same fixative for 1 h. After thorough rinsing in phosphate buffer, the brains were cryoprotected in a 30% sucrose solution, blocked in the coronal plane, frozen on dry ice, and sectioned at 30 μm on a cryostat. Forebrain sections containing the rostral half of the hippocampus were sectioned in the coronal plane. Near the middle of the hippocampus (~2.18 mm posterior to bregma) (Franklin and Paxinos, 1997), the brain blocks were reoriented, and the caudal half of the hippocampus was sectioned horizontally. Sections at 300 μm intervals were mounted on slides and stained with cresyl violet for general morphological study. The remaining sections were stored in a cryoprotectant solution at −20°C until processing.

Immunohistochemistry for Fos protein. To reveal Fos expression levels in the hippocampal formation after spontaneous seizures, free-floating sections were processed for Fos immunohistochemistry with avidin–biotin peroxidase methods. Two antisera to Fos, raised in rabbit and goat, were used to confirm the results on adjacent sections. Fos antiserum raised in goat recognizes a peptide sequence at the N terminus of human c-Fos p62 that is identical to the corresponding mouse sequence (catalog #sc-52-G; Santa Cruz Biotechnology, Santa Cruz, CA) and is non-cross-reactive with the other members of Fos family such as FosB, Fra1, and Fra2. The Fos antiserum from rabbit was raised against residues 4–17 of human Fos (Ab-5; Oncogene Research Products, San Diego, CA). These antisera produced nearly identical results, except for a slightly higher background with the rabbit antiserum. For consistency, all single Fos labeling that is shown in this study was obtained with the Fos antiserum produced in goat. The Fos antiserum from rabbit was used for double labeling of Fos and parvalbumin or Fos and glutamate decarboxylase 67 (GAD67).

Before immunohistochemical processing, sections were incubated in 1% H_2O_2 for 30 min to reduce endogenous peroxidase-like activity. After a rinse in 0.1 M Tris-buffered saline (TBS), pH 7.3, sections were incubated in 10% normal serum (normal rabbit serum for goat anti-Fos and normal goat serum for rabbit anti-Fos) in TBS containing 0.3% Triton X-100 and avidin (200 μl/ml) for 3–4 h to reduce nonspecific binding. The sections were incubated with a Fos antiserum overnight at room temperature (RT): Fos antiserum raised in goat (sc-52-G; 1:1000), diluted in TBS containing 2% normal rabbit serum and biotin (200 μl/ml) or Fos antiserum from rabbit (Ab-5; 1:25,000) diluted in TBS containing 2% normal goat serum and biotin (200 μl/ml). After rinsing, the sections were incubated in biotinylated secondary antiserum (1:1000; Vector Laboratories, Burlingame, CA) at RT for 1 h: rabbit anti-goat IgG for the sections incubated in goat anti-Fos or goat anti-rabbit IgG for the sections incubated in rabbit anti-Fos. After a thorough rinse, the sections were incubated in avidin–biotin peroxidase complex (1:200; Vectastain Elite ABC; Vector Laboratories) in TBS for 1 h. To visualize the peroxidase labeling, sections were processed with 0.06% diaminobenzidine tetrahydrochloride (DAB) and 0.006% H_2O_2 diluted in 0.075 M PBS for 12 min, and immunolabeling was enhanced by incubation in 0.003% osmium tetroxide in PBS for 30 s. Additional sets of sections were processed with a glucose oxidase–DAB–nickel method (Shu et al., 1988) to intensify the labeling. After rinsing, sections were mounted on gelatin-coated slides, dehydrated, and coverslipped.

Immunohistochemistry for neuron-specific nuclear protein. To determine the severity of cell loss in the dentate gyrus and other hippocampal regions of the experimental animals, both coronal and horizontal sections from control and experimental mice were processed immunohistochemically for a neuronal marker [neuron-specific nuclear protein (NeuN)]. The protocol was similar to that for Fos immunohistochemistry, with replacements of appropriate normal serum (horse), primary antiserum (1:1000; mouse anti-NeuN; catalog #MAB377; Chemicon International, Temecula, CA), and secondary antiserum (1:200; biotinylated horse anti-mouse IgG from Vector Laboratories).

Double-immunofluorescence labeling. To determine the chemical identity of the neurons that express Fos protein after spontaneous seizures, double-immunofluorescence labeling for Fos and several other cell markers were performed. Antiserum to the protein product of *Prox1*, a homeobox gene that is selectively expressed in granule cells of the postnatal rodent brain (Pleasure et al., 2000), was used to identify dentate granule cells. Antisera to parvalbumin and GAD67 were used to label GABAergic interneurons along the inner border of the granule cell layer and the dentate molecular layer, respectively.

For Fos and Prox1 double-immunofluorescence labeling, the sections were treated with 10% normal donkey serum (NDS) in 0.1 M TBS containing 0.3% Triton for 2 h, incubated in a mixture of goat anti-Fos (sc-52-G; 1:1000) and rabbit anti-Prox1 (1:30,000; catalog #AB5475; Chemicon International) diluted with TBS containing 2% NDS at RT for three nights. After thorough rinsing in TBS, sections were incubated in a mixture of fluorescent dye-labeled secondary antisera at RT for 2 h: donkey anti-goat IgG labeled with Alexa Fluor 555 was used to visualize Fos labeling, and donkey anti-rabbit IgG conjugated to Alexa Fluor 488 (1:500; both from Molecular Probes, Eugene, OR) was used for visualizing Prox1.

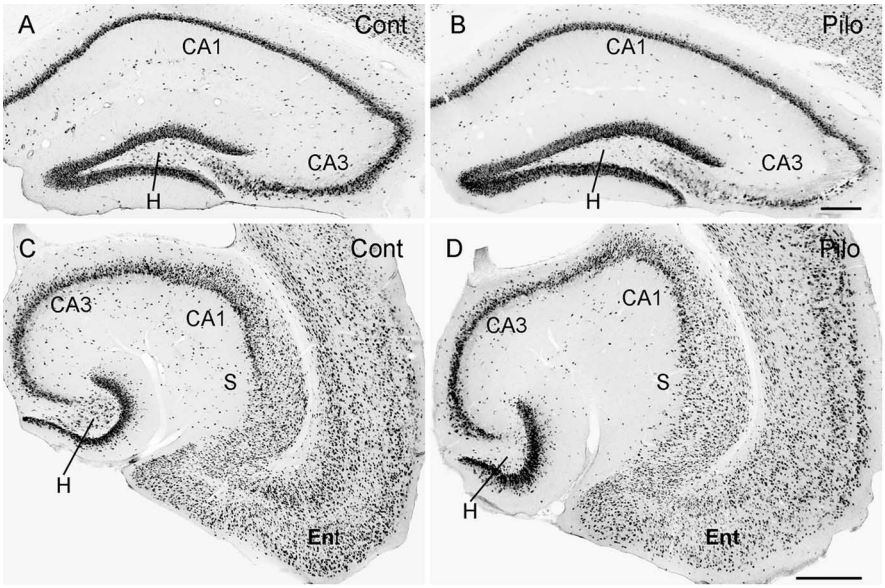

Figure 1. ***A–D***, NeuN labeling in dorsal (***A***, ***B***; coronal) and ventral (***C***, ***D***; horizontal) sections of the hippocampal formation in control (***A***, ***C***; Cont) and pilocarpine-treated (***B***, ***D***; Pilo) mice. ***B***, ***D***, In the pilocarpine-treated mice, cell loss is most obvious in the hilus (H) of the dentate gyrus (***B***, ***D***) and in the pyramidal cell layer of CA3 (***B***). Neurons are relatively well preserved in other hippocampal regions that include CA1, subiculum (S), and entorhinal cortex (Ent). Scale bars: (in ***B***, ***D***) ***A–D***, 200 μm.

For Fos and GAD67 or Fos and parvalbumin immunofluorescence labeling, sections were incubated in 10% NGS to block nonspecific binding sites with or without 0.3% Triton X-100 (for parvalbumin or GAD67, respectively) for 2 h. Sections were then incubated in a solution containing rabbit anti-Fos (Ab-5; 1:30,000) and either mouse anti-GAD67 or mouse anti-parvalbumin (1:2000; catalog #MAB5406 and #MAB1572, respectively; both from Chemicon International) in TBS with 2% NGS at RT for three nights. After thorough rinsing in TBS, sections were incubated in a mixture of goat anti-mouse IgG conjugated to Alexa Fluor 488 and goat anti-rabbit IgG conjugated to Alexa Fluor 555 (both 1:500; Molecular Probes) at RT for 2 h.

After the incubations, all sections were rinsed in TBS for at least 20 min, mounted on slides, and coverslipped with the antifade medium Prolong Gold (Molecular Probes).

Data analysis. Single Fos immunolabeling was analyzed, and digital images were obtained with a Zeiss (Thornwood, NY) Axioplan 2 microscope equipped with an AxioCam digital camera system and AxioVision 3.0 software. Qualitative analyses of Fos labeling were conducted for all animals. In addition, semiquantitative analyses of Fos-labeled cells in the granule cell layer were performed for control mice and pilocarpine-treated mice that had not experienced behavioral seizures for 18 h or longer before perfusion. The average number of Fos-labeled cells in the granule cell layer was determined in sections from six control mice that were processed in a single run. Comparisons of numbers of Fos-expressing cells in the granule cell layer were then conducted in three pairs of closely matched control mice and pilocarpine-treated mice that had not exhibited a spontaneous seizure for the preceding 18–24 h. Each pair of animals in the latter group had the same date of pilocarpine treatment and perfusion, and sections from the three pairs of animals were processed for Fos immunohistochemistry in the same run. Images were obtained using a 10× objective and analyzed with the NIH ImageJ (version 1.33) program. The contour of the granule cell layer was outlined in each section, a threshold was established that detected virtually all Fos-labeled neurons in the granule cell layer of the pilocarpine-treated animals (in which labeling was often lighter than that in control mice), and automatic cell counts were then performed with this threshold for all sections. Data were analyzed with Student's *t* test.

Double-labeled sections were scanned, and digital images were obtained with a Zeiss LSM 510 META confocal microscope. Confocal images were analyzed with Zeiss LSM 5 Image Examiner software. The presence of Fos labeling in parvalbumin- and GAD67-immunopositive interneurons and Prox1-immunoreactive dentate granule cells was evaluated at each time point.

Results

Hippocampal cell loss in pilocarpine-treated mice

As previously reported, C57BL/6 mice are susceptible to hippocampal cell loss after pilocarpine-induced status epilepticus (Houser et al., 2002; Peng et al., 2004). The patterns of neuronal loss in the hippocampal formation were similar to those observed in pilocarpine-treated rats (Obenaus et al., 1993). Extensive neuronal loss was found in the hilus and adjacent regions of CA3 in all pilocarpine-treated mice in this study (Fig. 1*B*,*D*). In most animals, the hilar cell loss was greater in the rostral (Fig. 1*B*) than in the caudal (Fig. 1*D*) regions of the dentate gyrus. Dentate granule cells were generally well preserved, except in three of the 24 pilocarpine-treated mice in which there were small patches of cell loss in the granule cell layer. Cell loss in CA1 and more distal regions of CA3 (CA3c) varied among animals with some mice having very little neuronal loss and others showing substantial loss in the pyramidal cell layer of these regions.

Fos expression in control mice and pilocarpine-treated mice without recent seizures

In control mice, scattered cells with strong-to-moderate Fos labeling were consistently observed throughout most regions of the hippocampal formation (Fig. 2*A*,*B*). However, the abundance of labeled cells varied among the animals, and representative sections from mice with intermediate (Fig. 2*A*) and high (Fig. 2*B*) levels of Fos labeling are illustrated. In the dentate gyrus, scattered Fos-labeled cells were present in the granule cell layer. In comparable levels of coronal sections from six control mice, processed with identical conditions, a maximum of 64 and a minimum of 12 (mean = 32.2 ± 4.8) Fos-labeled cells were observed

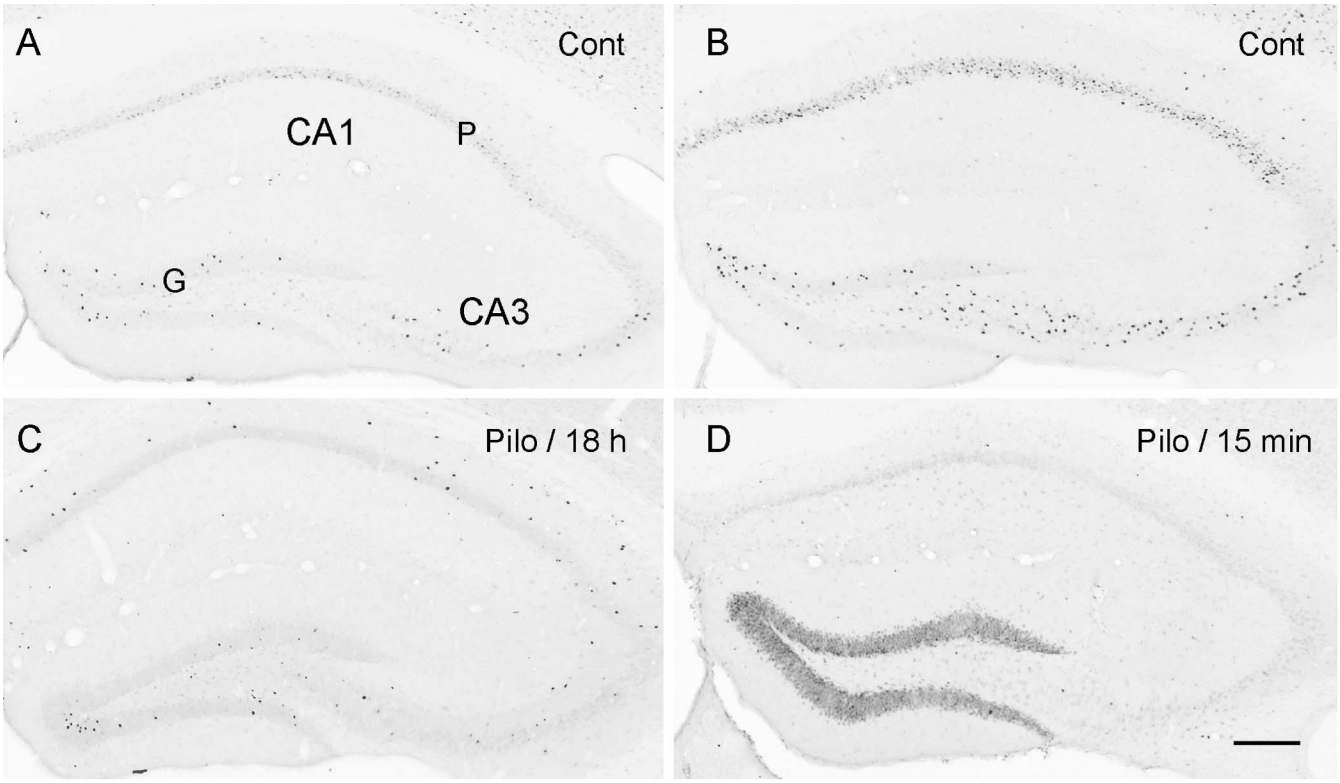

Figure 2. Fos expression in the dorsal hippocampus of control mice (***A***, ***B***; Cont) and pilocarpine (Pilo)-treated mice at 18 h (***C***) and 15 min (***D***) after a spontaneous seizure. ***A***, ***B***, In control mice, the extent of Fos labeling varies among animals and moderate (***A***) and maximal (***B***) levels of labeling are illustrated. Despite the variation in numbers of Fos-labeled neurons, the distribution patterns are very similar. Scattered Fos-labeled cells are present in the granule cell layer (G) of the dentate gyrus and the pyramidal cell layer (P) of CA1 and CA3 in the control mice. ***C***, In a pilocarpine-treated mouse that was seizure-free for the preceding 18 h, only scattered Fos-labeled cells are evident. Labeling appears similar to that in control mice, although Fos-labeled cells in the granule cell and pyramidal cell layers are less abundant than in some control animals. ***D***, In contrast, at 15 min after a spontaneous seizure, prominent Fos labeling extends throughout the granule cell layer. Few labeled neurons are evident in the hippocampal regions at this time. Scale bar: (in ***D***) ***A–D***, 200 μm.

in the granule cell layer on each side of a coronal section (mean density, 33.0 ± 5.1 Fos-labeled cells per 100,000 μm^2). The strong labeling of a small number of granule cells in control tissue could reflect activity of individual granule cells that, as recent studies have demonstrated, are sufficient to activate CA3 neurons and thus convey information to the hippocampus (Henze et al., 2002).

In control mice, very few Fos-labeled cells were observed in the hilus, and virtually no labeled cells were evident in the molecular layer. Some strongly labeled cells were evident in the pyramidal cell layer of CA3 and CA1, and some cells with moderate labeling were occasionally observed in stratum oriens. The overall distribution pattern of Fos labeling in the dentate gyrus and other hippocampal areas was similar to that described previously in normal (control) rats (Dragunow, 1988) and mice (Bertaina-Anglade et al., 2000).

In pilocarpine-treated mice that had not experienced spontaneous seizures for at least 18 h before perfusion, the distribution of Fos-labeled cells in the hippocampus was similar to that observed in control animals (Fig. 2*C*). However, the abundance of Fos-labeled cells in the granule cell and pyramidal cell layers appeared to be lower in pilocarpine-treated mice that had not experienced a recent seizure than in normal control mice. Quantitative data from three pairs of mice (control mice and mice at 18–24 h after spontaneous seizures) demonstrated an average of 30 ± 3.5 Fos-labeled cells per 100,000 μm^2 in the granule cell layer of controls and 8.1 ± 2.6 Fos-labeled cells in a similar area of the pilocarpine-treated animals ($p < 0.05$).

Fos expression in epileptic mice

Time course of changes of Fos immunolabeling in the hippocampal formation after spontaneous behavioral seizures

Distinct changes in the patterns of Fos labeling were observed in the hippocampus from 15 min to 4 h after a spontaneous seizure. The changes were similar at rostral (Fig. 3) and caudal (Fig. 4) levels of the hippocampal formation. Coronal sections of the dentate gyrus illustrate changes primarily in the granule cell layer (Fig. 3), and horizontal sections of the caudal hippocampal formation (Fig. 4) allow comparison of labeling in different regions of the hippocampal formation.

At 15 min after the onset of the spontaneous seizure, increased Fos expression was evident in the dentate granule cell layer (Figs. 2*D*, 3*B*, 4*B*). At this short interval, Fos immunoreactivity was not increased in other regions of the dentate gyrus (Fig. 3*B*) or hippocampus (Figs. 2*D*, 4*B*). The vast majority of granule cells demonstrated light-to-moderate Fos labeling so that the entire dentate granule cell layer was homogeneously labeled. When Fos labeling was compared at different rostral–caudal levels, the density of labeling in the granule cells was slightly, but consistently, higher in the caudal (temporal) levels than in the rostral (septal) ones (data not shown). Interestingly, at this time point, fewer

strongly labeled cells were evident in the dentate gyrus (Fig. 3, compare *A*, *B*) and other hippocampal regions of the pilocarpine-treated mice than in these regions in control mice. The only other region in which a slight increase in labeling was noted at this early time point was the parasubiculum (Fig. 4*B*). A clear increase in Fos labeling in neurons throughout the granule cell layer was evident in all three animals studied at 15 min after a spontaneous seizure (when no other seizures were observed during the preceding 24 h). The pattern of labeling in Figure 3*B* is representative of that in all mice examined at this short postseizure interval.

At 30 min after a spontaneous seizure, very strong Fos immunoreactivity was observed in cells throughout the granule cell layer in all six animals at this time point (Figs. 3*C*, 4*C*). When sections from these mice were processed in parallel with those at other time points, granule cell labeling was consistently highest at this interval, as illustrated in Figures 3*C* and 4*C*. Increased Fos labeling was also evident in other regions of the hippocampal formation, including CA3, CA1, subiculum, presubiculum, parasubiculum, and entorhinal cortex (Fig. 4*C*). Among the latter regions, the parasubiculum exhibited consistently high levels of Fos expression. In addition to the heavily labeled neurons in the granule cell layer, some nearby cells in the molecular layer and hilus were strongly labeled (Figs. 3*C*, 4*C*, 5*A*,*B*).

At 1 h after spontaneous seizures, strong Fos labeling was evident in most dentate granule cells. However, compared to that at 30 min, Fos labeling in many granule cells was slightly decreased (Figs. 3*D*, 4*D*). Fos immunoreactivity in the other hippocampal regions remained similar to that observed at 30 min after seizures (Fig. 4*D*). Distinct labeling of the granule cell layer was observed in all four animals studied at this time point. However, the proportion of heavily labeled to moderately labeled cells varied among animals.

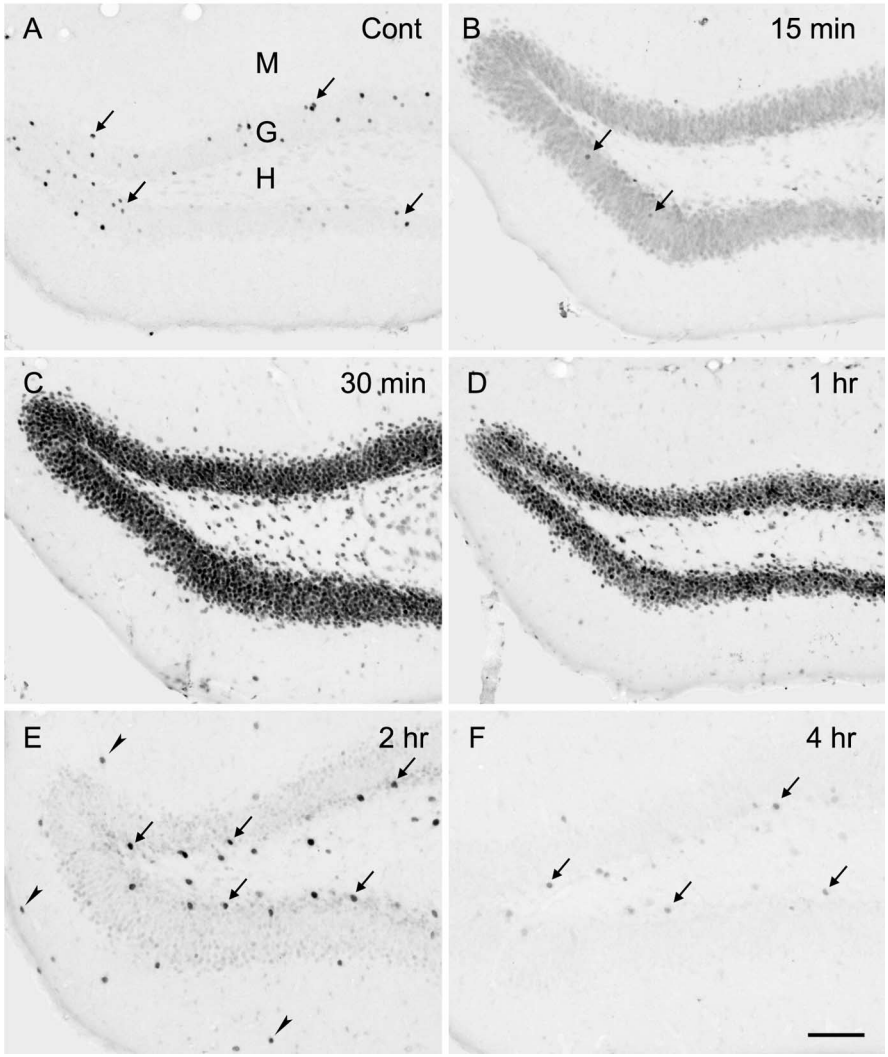

Figure 3. ***A–F***, Fos expression in the dorsal dentate gyrus of a control mouse (***A***; Cont) and pilocarpine-treated mice at different time points after spontaneous seizures (***B–F***). ***A***, In a control animal, strong Fos labeling is evident in a small number of scattered cells (arrows) in the granule cell layer (G). Few labeled cells are present in the hilus (H) and molecular layer (M). ***B***, By 15 min after the onset of a spontaneous seizure, moderate Fos labeling is present in most granule cells, but the number of scattered, strongly labeled cells (arrows) is low. ***C***, At 30 min after a spontaneous seizure, very strong Fos immunoreactivity is present throughout the granule cell layer and within the hilus. ***D***, At 1 h after a spontaneous seizure, many cells in the granule cell layer are strongly labeled, but others show moderate levels of labeling. ***E***, At 2 h after a spontaneous seizure, low Fos immunoreactivity is present in the granule cell layer, but strong Fos labeling is evident in large cells along the inner border of the granule cell layer (arrows) and in the molecular layer (arrowheads). ***F***, At 4 h after a spontaneous seizure, Fos labeling in the granule cell layer is very low, but some large cells in the subgranular zone (arrows) show moderate Fos labeling. Scale bar: (in ***F***) ***A–F***, 100 μm.

At 2 h after spontaneous seizures, Fos labeling in the dentate granule cell layer was decreased to a level below that of the animals at 15 min after seizures, but some lightly labeled cells remained (Figs. 3*E*, 4*E*). In contrast to the lower Fos expression in the granule cell layer, some large cells in other areas, such as the border between the granule cell layer and hilus, exhibited strong Fos immunoreactivity. This pattern was observed in all three of the animals studied at the 2 h time point. Strong Fos labeling was also observed in cells within the hilus and CA3; however, in the other hippocampal regions, Fos expression was lower than at 30 min and 1 h after spontaneous seizure (Fig. 4*E*).

At 4 h after spontaneous seizure, Fos labeling throughout much of the granule cell layer had decreased to control levels, but scattered Fos-labeled cells within the granule cell layer were fewer than in control sections (Fig. 3, compare *F*, *A*). In contrast, some large moderately labeled cells were evident in the subgranular region and in the hilus (Figs. 3*F*, 4*F*). This pattern was present in the two animals studied at 4 h after a spontaneous seizure. In the mice at 8 h or longer after seizures, the number of Fos-labeled cells throughout the hippocampal formation was lower than that in control mice. However, Fos labeling in the thalamus and cerebral cortex was generally similar to that in controls at these later time points (data not shown).

The labeling patterns were quite consistent among mice at similar time points after spontaneous seizures, despite differences in the lengths of time after pilocarpine treatment. For example, in mice studied at 30 min after spontaneous seizures, very

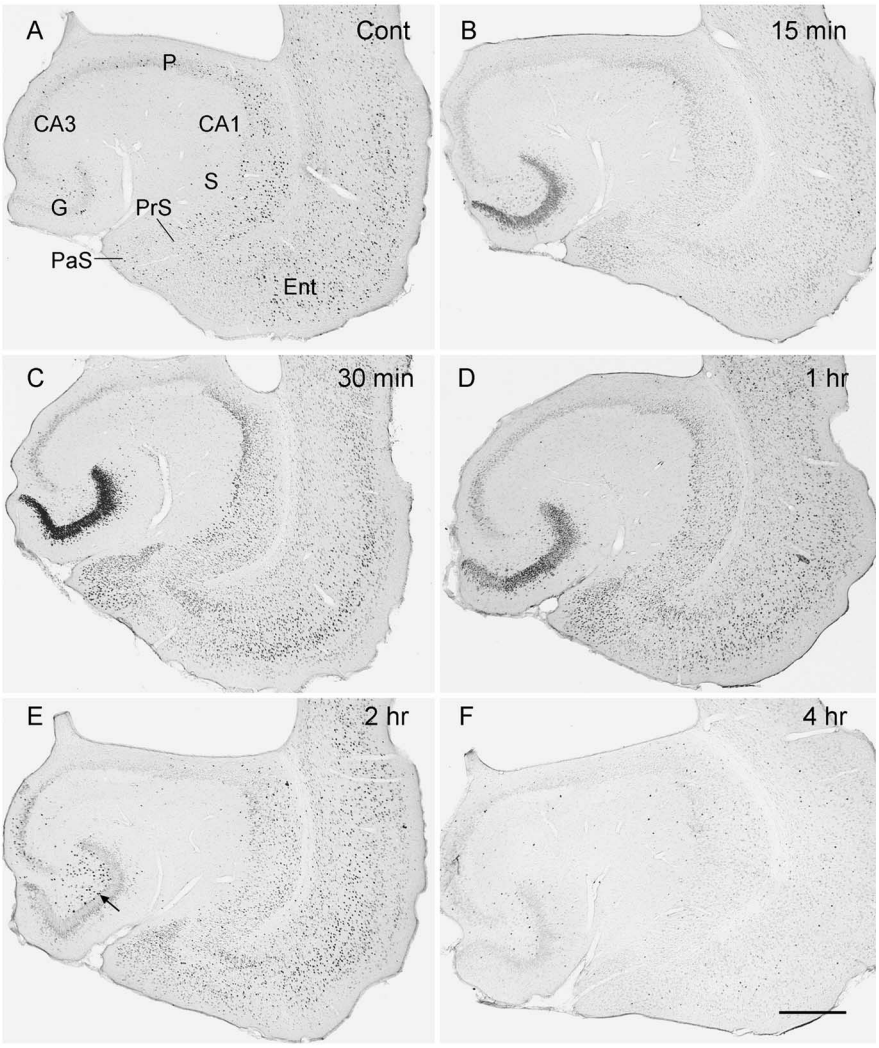

Figure 4. ***A–F***, Fos expression in the ventral hippocampal formation of a control mouse (***A***; Cont) and pilocarpine-treated mice (***B–F***) at different intervals after spontaneous seizures. ***A***, In control animals, Fos immunoreactivity is detected in scattered cells in the granule cell layer (G) of the dentate gyrus, pyramidal cell layer (P) of CA3 and CA1, subiculum (S), presubiculum (PrS), parasubiculum (PaS), and entorhinal cortex (Ent). ***B***, At 15 min after a spontaneous seizure, elevated Fos immunoreactivity is evident in the dentate granule cell layer, but the number of scattered, strongly labeled cells, evident in the control tissue, is reduced throughout many other regions of the hippocampal formation. ***C***, At 30 min after a spontaneous seizure, Fos labeling is very strong in the dentate granule cell layer. Increased Fos labeling is also evident in most other regions of the hippocampal formation. ***D***, At 1 h after a spontaneous seizure, Fos immunoreactivity remains high in most hippocampal regions. Distinct labeling is present in the dentate granule cell layer, although the intensity of labeling is lower than at 30 min. ***E***, By 2 h after a seizure, Fos immunoreactivity in the granule cell layer is substantially lower than that observed after 30 min and 1 h. However, labeled cells are evident in the hilus, and some strongly labeled large cells are evident along the inner border of the granule cell layer (arrow). ***F***, By 4 h after a spontaneous seizure, Fos labeling is low throughout the hippocampal formation, and the number of scattered Fos-labeled cells appears lower than in control animals (compare with ***A***). Scale bar: (in ***F***) ***A–F***, 200 μm.

similar labeling patterns were observed in those at 3 weeks (Fig. 4*C*) and 14 weeks (Fig. 5*A*) after status epilepticus.

One factor that could have contributed to the consistency in labeling was the continuous monitoring for behavioral seizures during the 24 h before the last observed seizure and the exclusion of mice that experienced additional seizures during this time. One mouse with multiple seizures during the previous 24 h (with a 3 h interval between the last two seizures) was studied at 15 min after the last seizure for comparison with the experimental group described above that had experienced a single seizure. This animal, rather than having selective granule cell labeling, as observed in the other animals in the 15 min group, exhibited strong labeling of interneurons in the dentate gyrus and light-to-moderate labeling of granule cells (Fig. 6). The interneuron labeling closely resembled that observed in animals at longer (2 h or more) postseizure intervals (Figs. 3*E*, 4*E*). The strong interneuron labeling was presumed to result from Fos activation in these neurons by earlier seizures.

Early seizure-related activation of granule cells

Prox1 was used as a marker for granule cells in double-labeling studies to determine (1) whether Fos labeling at early time points was confined to granule cells and (2) whether Fos-labeled cells in the molecular layer and hilus were granule cells.

At 15 and 30 min after spontaneous seizures, the pattern of Fos and Prox1 labeling were very similar, suggesting that the vast majority of Fos-labeled cells were granule cells (Fig. 7*A–C*). Double-labeled cells were densely packed in the granule cell layer, as expected for dentate granule cells. In addition, numerous Fos-labeled cells in the hilus and molecular layer (Fig. 7*A*,*D*) were also labeled for Prox1 (Fig. 7*B*,*C*,*E*,*F*). These findings demonstrate that early activation of granule cells is not limited to those in the granule cell layer but also includes dispersed granule cells in the molecular layer as well as hilar granule cells, some of which could have been generated after the episode of status epilepticus (Scharfman et al., 2002).

At the early time points, strong Fos labeling appeared to be confined to granule cells. At 15 min after a seizure, essentially all Fos-labeled cells were labeled for Prox1. At 30 min after a spontaneous seizure, a few cells at the base of the granule cell layer showed very light Fos labeling that contrasted with the strong labeling of the adjacent granule cells (Fig. 7*G*). These lightly Fos-labeled cells showed no Prox1 labeling (Fig. 7*H*,*I*), thus confirming that they were not granule cells.

At 1 h after the spontaneous seizure, numerous Fos-labeled cells that did not label with Prox1 were detected at the base of the granule cell layer (Fig. 7*J–L*). At 2 h after a spontaneous seizure, cells at the inner border of the granule cell layer and in the molecular layer showed strong labeling for Fos but showed no Prox1 expression (Fig. 7*M–O*). This labeling sequence suggested a progression from granule cell to non-granule cell Fos activation.

Delayed seizure-related Fos expression in interneurons

Two markers were used to identify GABAergic interneurons in the dentate gyrus. Parvalbumin clearly labeled many interneurons along the inner border of the granule cell layer but labeled relatively few interneurons in the molecular layer. Thus, GAD67 was used to identify GABAergic interneurons in the latter region.

Each marker was used in double-labeling studies with Fos to identify activated GABAergic interneurons.

At 15 min after spontaneous seizures, no Fos labeling was evident in parvalbumin- or GAD67-labeled dentate interneurons (Fig. 8*A*,*B*). By 30 min after a seizure, some parvalbumin-labeled cells along the inner border of the granule cell layer remained free of Fos labeling, but others showed very low levels of Fos labeling (Fig. 8*C*). No Fos immunoreactivity was evident in the GAD67-labeled neurons in the molecular layer (Fig. 8*D*). By 1 h after seizures, most of the parvalbumin-labeled cells in the dentate gyrus showed moderate-to-strong Fos immunoreactivity (Fig. 8*E*), and some GAD67-labeled cells in the molecular layer were also slightly to moderately labeled for Fos (Fig. 8*F*). By 2 h after spontaneous seizures, strong Fos immunoreactivity was evident in most parvalbumin- and GAD67-labeled interneurons in the dentate gyrus (Fig. 8*G*,*H*). In contrast, dentate granule cells were only lightly labeled at this time point (Fig. 8*G*,*H*).

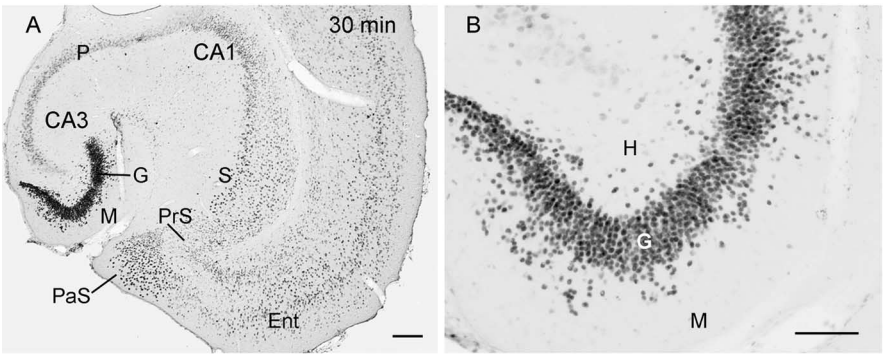

Figure 5. Fos expression in the hippocampal formation at 30 min after a spontaneous seizure in a mouse at 14 weeks after pilocarpine-induced status epilepticus. ***A***, In this long-term animal, the pattern of Fos labeling at 30 min is very similar to that in a mouse at a shorter interval (3 weeks) after pilocarpine treatment (compare with Fig. 4*C*). In addition to strong Fos labeling of the granule cell layer (G), numerous labeled cells are evident in the pyramidal cell layer (P) of CA3 and CA1, subiculum (S), parasubiculum (PaS), presubiculum (PrS), and entorhinal cortex (Ent). ***B***, At higher magnification of the dentate gyrus, strong labeling is present in the granule cell layer and in small dispersed cells that resemble granule cells in the molecular layer (M) and hilus (H). Scale bars: ***A***, 200 μm; ***B***, 100 μm.

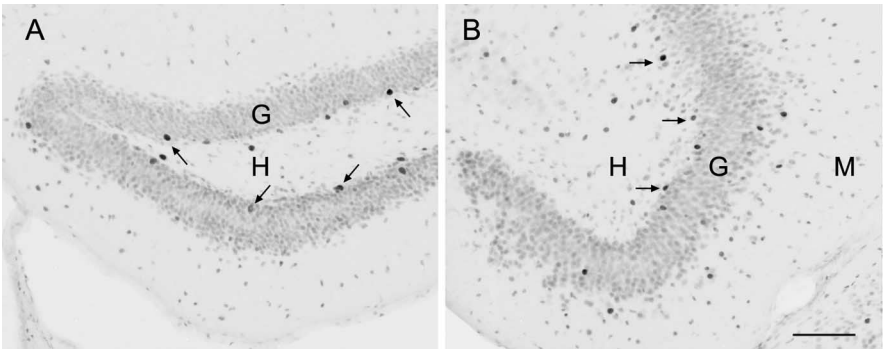

Figure 6. ***A***, ***B***, Fos expression in the dorsal (***A***) and ventral (***B***) dentate gyrus at 15 min after a spontaneous seizure in a mouse that experienced multiple seizures during the preceding 24 h. The majority of granule cells are moderately labeled. In addition, large, darkly labeled neurons (arrows) are present along the border between the granule cell layer (G) and hilus (H). This contrasts with the labeling in mice at 15 min after a single seizure in which relatively homogenous labeling of only granule cells was present (Figs. 3*B*, 4*B*). The strong labeling of presumptive interneurons is similar to that observed in mice at 2 h after a single seizure (Fig. 3*E*), and this would be consistent with the 3 h interval between the last two seizures in this mouse. M, Molecular layer. Scale bar: (in ***B***) ***A***, ***B***, 100 μm.

Discussion

This study identified a distinct sequence of Fos expression in neurons of the hippocampal formation after spontaneous behavioral seizures in a mouse pilocarpine model of recurrent seizures, and three major findings emerged. First, dentate granule cells showed early and extensive Fos labeling after a spontaneous seizure in this model. Second, strong Fos activation in interneurons of the dentate gyrus occurred at later time points than that in granule cells. Finally, the timing of increased Fos expression appeared to differ among different types of GABA neurons in the dentate gyrus.

Dentate granule cells show early Fos activation after spontaneous seizures

One of the most striking findings in this study was the early, selective Fos labeling of dentate granule cells. Increased Fos expression was detected as early as 15 min after the onset of a spontaneous seizure and appeared to be restricted to dentate granule cells at this time.

These findings contrast with previous reports of a general lack of increased Fos expression in granule cells after chronic seizures in the rat pilocarpine model (Mello et al., 1996; Harvey and Sloviter, 2005). The reasons for the differences in labeling patterns are unclear but could be related to the different intervals at which the animals were studied after spontaneous seizures or to species differences in the types and severity of seizures. Such differences, when elucidated, could provide new insights into multiple mechanisms and network alterations involved in temporal lobe epilepsy.

In the current study, the early Fos labeling in granule cells was not confined to the granule cell layer but included numerous granule cells within the hilus. Many of these neurons could be newly generated granule cells that have been identified previously in the rat hilus after pilocarpine-induced status epilepticus (Parent et al., 1997; Scharfman et al., 2000). In a previous study in the rat, hilar granule cells exhibited increased Fos expression at 4 h after a spontaneous seizure (the earliest time studied), thus demonstrating that the newly generated neurons had become integrated into the network (Scharfman et al., 2002). The current findings are in agreement with these observations but suggest that, in the mouse, such hilar granule cells may be activated along with other granule cells at very early stages of spontaneous seizures.

The localization of Fos is commonly used to indicate neuronal activation that includes increased metabolic activity of the neurons and, presumably, the firing of action potentials (for review, see Herrera and Robertson, 1996; Herdegen and Leah, 1998). Nevertheless, it remains possible that the Fos activation could reflect only a depolarization of the granule cells and associated calcium entry that could be sufficient to activate c-*fos* and subse-

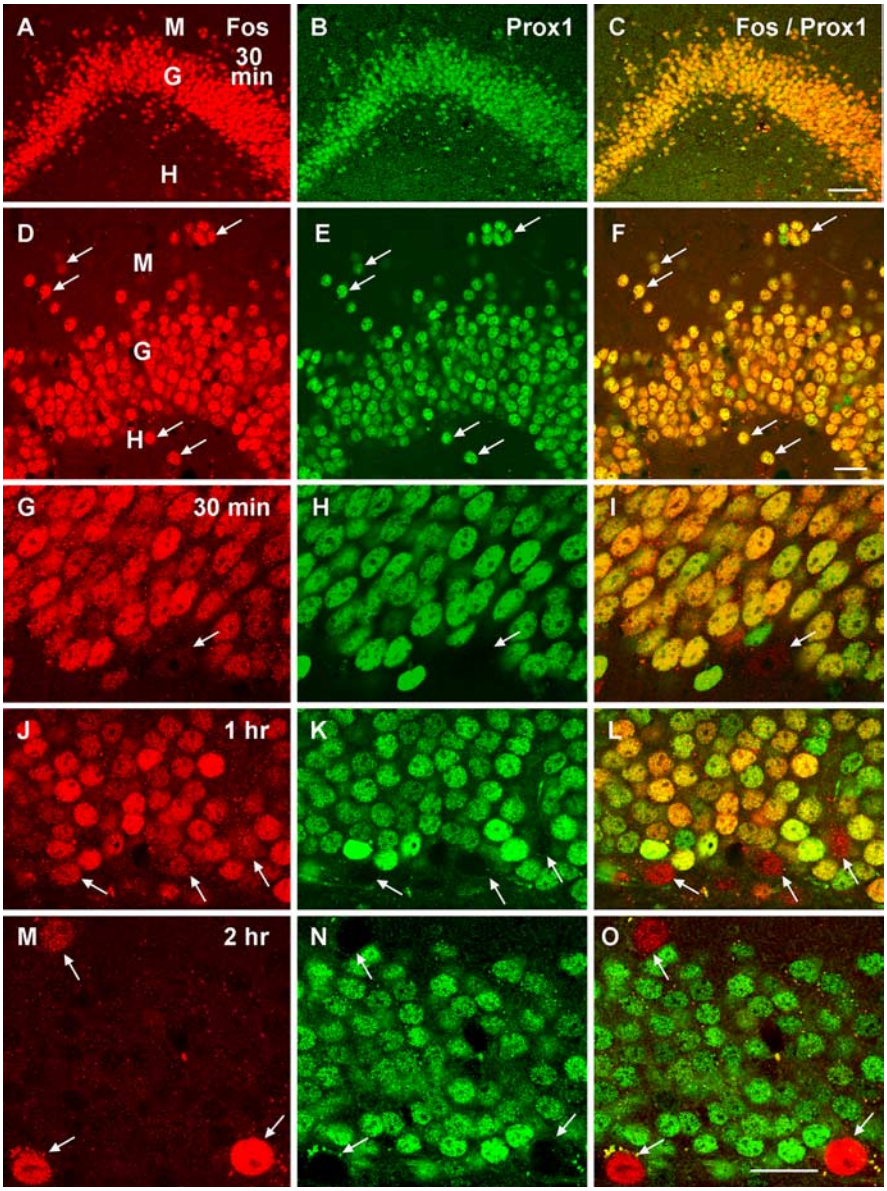

Figure 7. Comparisons of Fos and Prox1 labeling in the dentate gyrus at progressively longer times after spontaneous seizures with confocal microscopy. ***A–C***, At 30 min after a spontaneous seizure, the patterns of Fos and Prox1 labeling are very similar, and extensive colocalization is evident in these low-magnification views of the ventral dentate gyrus. ***D–F***, At higher magnification of the region in ***A–C***, Fos-labeled cells in the granule cell layer as well as in the molecular layer (M; arrows) and hilus (H; arrows) are labeled for the granule cell marker Prox1. ***G–I***, In the dorsal dentate gyrus, nearly all Prox1-labeled cells are also strongly to moderately labeled for Fos at 30 min after a spontaneous seizure. Only light Fos labeling is evident in a large non-Prox1 cell (arrow) at the inner border of the granule cell layer (G). ***J–L***, At 1 h after a spontaneous seizure, several Fos-labeled cells (arrows) along the base of the granule cell layer do not show Prox1 labeling and are presumed to be interneurons. ***M–O***, At 2 h after spontaneous seizures, strong Fos labeling is present in large non-Prox1 cells (arrows) at the base of the granule cell layer, but very little Fos labeling is evident in the Prox1-labeled granule cells. Scale bars: (in ***C***) ***A–C***, 100 μm; (in ***F***) ***D–F***, 50 μm; (in ***O***) ***G–O***, 20 μm.

quently its associated protein Fos (Greenberg et al., 1986; Sheng et al., 1990; Labiner et al., 1993). However, several current findings appear to be more consistent with granule cell firing than with depolarization only. First, the labeling of granule cells increased from moderate to strong levels between 15 and 30 min after the beginning of the spontaneous seizure. It seems unlikely that such increases in the intensity of labeling would be associated with a limited depolarization without a progression to granule cell firing. A recent preliminary report of increased granule cell firing at the onset of spontaneous seizures in the rat supports this suggestion (Bower and Buckmaster, 2004) (but see Harvey and Sloviter, 2005). Also, after the initial Fos activation in granule cells, other regions of the hippocampal formation, including CA3, CA1, subiculum, parasubiculum, and entorhinal cortex, showed increased Fos labeling, and this would be consistent with increased activation of subsequent links in the hippocampal circuitry. An association between Fos labeling and granule cell activity in this model is also supported indirectly by a lack of increased Fos expression in dentate granule cells in a mouse model of audiogenic seizures in which seizure-induced neuronal activation is limited primarily to brainstem structures (Klein et al., 2004).

The early appearance of Fos labeling in dentate granule cells, 15 min after the onset of the behavioral seizure, raises the possibility that granule cell activation occurs at or possibly before the initiation of the seizures. Some time is required for synthesis and expression of the Fos protein after the rapid activation of c-*fos*, and this interval has been estimated to be between 20 and 40 min (Woldbye et al., 1996). Delays of 30–90 min are commonly used when examining Fos activation after experimental manipulations (Morgan et al., 1987; Dragunow, 1988; Bertaina-Anglade et al., 2000; Mirzaeian and Ribak, 2000). Thus, the detection of Fos labeling as early as 15 min after the first observable sign of the seizure would be consistent with granule cell activation before or near the time of seizure initiation. Preseizure Fos expression in dentate granule cells has been described previously after systemic administration of kainate and was considered to be associated with an increase in high-frequency action potentials in the region (Willoughby et al., 1997).

Fos activation occurs in numerous granule cells after spontaneous seizures

A particularly interesting feature of the early Fos labeling was its widespread appearance throughout the granule cell layer. Although such extensive Fos labeling might be expected after the strong stimulation produced by most electrically or chemically induced seizures, the pattern would not necessarily be expected in association with the relatively brief behavioral seizures in the current mouse model.

The factors responsible for the change from Fos expression in isolated granule cells in control conditions to simultaneous labeling of large numbers of granule cells near the time of spontaneous seizures are unknown. However, mossy fiber sprouting and the resulting excitatory interconnections among granule cells has been proposed to contribute to increased synchrony of neuronal firing in many temporal lobe epilepsy models (Tauck and Nadler,

1985; Cronin and Dudek, 1988; Sutula et al., 1988). The excitability of these reorganized circuits could be increased periodically by fluctuating conditions that could have widespread effects in the granule cell layer, such as increased levels of extracellular potassium (Patrylo and Dudek, 1998; Hardison et al., 2000), altered GABAergic inhibition (Cronin et al., 1992; Kobayashi and Buckmaster, 2003; Sayin et al., 2003; Peng et al., 2004), including zinc-induced failure of inhibition (Buhl et al., 1996; Coulter, 2000; Cohen et al., 2003), and nonsynaptic factors that might include increased numbers of functional gap junctions among the granule cells (Schweitzer et al., 1992; Pan and Stringer, 1996; Traub et al., 2004). Demonstration of such influences at the time of spontaneous seizure initiation in this model would be of considerable interest.

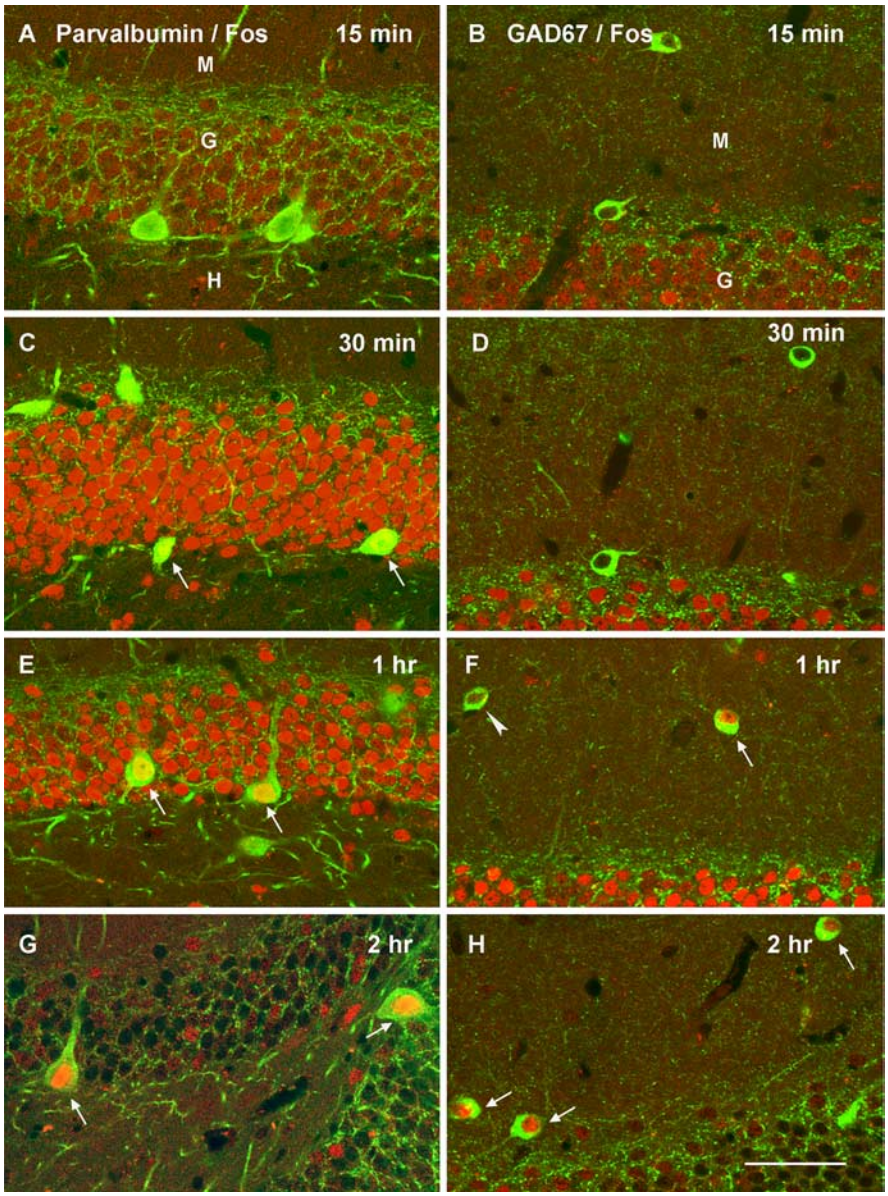

Figure 8. ***A–H***, Progressive changes in Fos labeling of parvalbumin-containing neurons in the subgranular zone (***A***, ***C***, ***E***, ***G***) and GAD67-labeled neurons in the molecular layer (***B***, ***D***, ***F***, ***H***) at different time points after spontaneous seizures. ***A***, ***C***, ***E***, ***G***, In parvalbumin-labeled neurons (green), Fos labeling (red) is not evident at 15 min (***A***), is slight at 30 min (***C***, arrows), strong at 1 h (***E***, arrows), and very strong at 2 h (***G***, arrows) after spontaneous seizures. ***B***, ***D***, ***F***, ***H***, GAD67-labeled cells (green) in the molecular layer show no Fos immunoreactivity (red) at 15 min (***B***) and 30 min (***D***) after spontaneous seizures. Moderate (arrowhead) to strong (arrow) Fos labeling is evident at 1 h (***F***) after a spontaneous seizure. ***H***, Most GAD67-labeled cells (arrows) in the dentate molecular layer show strong Fos labeling at 2 h. M, Molecular layer; G, granule cell layer; H, hilus. Scale bar: (in ***H***) ***A–H***, 50 μm.

Fos activation is delayed in interneurons after spontaneous seizures

In the current mouse model, Fos activation occurred at slightly later postseizure times in interneurons than in granule cells. A similar sequence of Fos labeling has been noted in previous studies of Fos activation after seizures induced acutely by electrical or chemical stimulation (Dragunow et al., 1992; Woldbye et al., 1996). Thus, the granule cell to interneuron sequence of labeling does not appear to be related solely to alterations in the current epilepsy model. A likely explanation for the sequential labeling patterns is that the dynamics of Fos expression vary among neuronal cell types. Different Fos activation times could be related to differences in the intrinsic characteristics of the cell types such as either their repertoire of receptors or their content of calcium channels and calcium-binding proteins that regulate internal calcium stores and, thus, Fos activation (Lerea et al., 1992; Ghosh et al., 1994). Because of such factors, as well as the limited temporal resolution of Fos activation, it is currently not possible to relate directly the sequence and timing of Fos activation to the sequence of neuronal activity in different cell types. One possibility is that, despite increased neuronal activity in many cell types and regions, granule cells expressed Fos more readily and rapidly than other neurons. However, a relationship between the sequence of Fos labeling among cell types and that of neuronal activity cannot be ruled out. The Fos labeling in both granule cells and interneurons occurred early and within a relatively short period ($<$1 h). Thus, although clearly speculative, the sequence of Fos labeling could be associated with strong initial activation of granule cells, followed by strong activation of interneurons involved in feedback inhibition of the granule cells, such as the parvalbumin-containing interneurons, and then by increased activity of GABA neurons in the molecular layer that participate in feedforward inhibition (Han et al., 1993; Freund and Buzsaki, 1996).

The observed differences in the temporal patterns of Fos activation among different types of hippocampal neurons reemphasize basic questions about the relationship between Fos activation and neuronal activity (Labiner et al., 1993). The precise firing patterns that are most effective in activating Fos remain ill-defined. Likewise, it is unknown whether different types of neurons have different thresholds for Fos activation. Such information is needed for a more complete interpretation of the present findings. Nevertheless, the fact that the timing of Fos activation

differs among neuronal cell types *in vivo* after spontaneous seizures makes these issues particularly relevant.

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