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Cell Damage and Neurogenesis in the Dentate Granule Cell Layer of Adult Rats After Pilocarpine- or Kainate-Induced Status Epilepticus

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ABSTRACT: Dentate granule cells are generally considered to be relatively resistant to excitotoxicity and have been associated with robust synaptogenesis after neuronal damage. Synaptic reorganization of dentate granule cell axons, the mossy fibers, has been suggested to be relevant for hyperexcitability in human temporal lobe epilepsy and animal models. A recent hypothesis suggested that mossy-fiber sprouting is dependent on newly formed dentate granule cells. However, we recently demonstrated that cycloheximide (CHX) can block the mossy-fiber sprouting that would otherwise be induced by different epileptogenic agents and does not interfere with epileptogenesis in those models. Here, we investigated cell damage and neurogenesis in the dentate gyrus of pilocarpine- or kainate-treated animals with or without coadministration of CHX. Dentate granule cells were highly vulnerable to pilocarpine induced-status epilepticus (SE), but were hardly damaged by kainate-induced SE. CHX pretreatment markedly reduced the number of injured neurons after pilocarpine-induced SE. Induction of SE dramatically increased the mitotic rate of KA- and KA + CHX-treated animals. Induction of SE in animals injected with pilocarpine alone led to 2–7-fold increases in the mitotic rate of dentate granule cells as compared to 5- and 30-fold increases for pilocarpine + CHX animals. We suggest that such increased mitotic rates might be associated with a protection of a vulnerable precursor cell population that would otherwise degenerate after pilocarpine-induced SE. We further suggest that mossy-fiber sprouting and neurogenesis of granule cells are not necessarily linked to one another. *Hippocampus 2000;10:169–180.*

KEY WORDS: cell damage; cell division; epilepsy; dentate gyrus; mossy fiber sprouting.

INTRODUCTION

Since 1825, as first observed by Bouchet and Cazauvieilh, atrophy of the hippocampal formation has been centrally involved in the pathogenesis of temporal lobe epilepsy (TLE) (Bouchet and Cazauvieilh, 1825). In the pioneer studies of Mouritzen-Dam (1979, 1980), dentate hilar and granule cells of patients with epilepsy were reported to be the most vulnerable neuronal populations in the hippocampal region. The same studies suggested that the number of generalized convulsions and the duration of the epileptic disorder could be associated with the degree of such neuronal loss. In subsequent studies, a substantial and preferential cell loss was reported in other regions of the hippocampal formation, while the dentate granule cell layer was considered to be relatively preserved (Babb et al., 1984a,b). Similar results were also described in many animal models of epilepsy (Ben-Ari et al., 1981; Olney et al., 1983; Freund et al., 1991; Cavazos et al., 1994). More recent studies of the fate of dentate granule cells after status epilepticus using electron microscopy analysis, however, have indicated that these cells might be considered a highly vulnerable neuronal population (Sloviter et al., 1996; Mello et al., 2000).

Little over a decade ago, the suggested role played by dentate granule cells in the pathogenesis of TLE, regardless of their resistance or vulnerability, was tentatively associated with the sprouting of mossy fibers (Tauck and Nadler, 1985; Sutula et al., 1988; Babb et al., 1991; Wuarin and Dudek, 1996), and more recently, to the neurogenesis of dentate granule cells (Parent et al., 1997, 1998; Gray and Sundstrom, 1998). The birth of new neurons in the hippocampal complex of adult rodents has long been shown to take place in the subgranular region of the dentate gyrus (Altman and Das, 1965). These cells have been ultrastructurally confirmed as neurons (Kaplan and Hinds, 1977) and have been shown to extend axons into the mossy-fiber pathway up to the CA3 hippocampal area (Stanfield and Trice, 1988). In a recent paper, Parent et al. (1997) demonstrated increased neurogenesis of dentate granule cells after pilocarpine-induced SE and suggested that the newly generated cells would be important in the genesis of dentate mossy-fiber sprouting (MFS). While most authors tend to suggest that such a reorganization might underlie seizures (Tauck and Nadler, 1985; Cronin and Dudek, 1988; Sutula et al., 1989; Babb et al., 1991; Spencer and Spencer, 1994; Masukawa et al., 1997), it was recently shown that spontaneous recurrent seizures are still observed when MFS is prevented by pretreatment with cycloheximide (CHX), a

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protein synthesis inhibitor (Longo and Mello, 1997, 1998, 1999). From the hypothesis raised by Parent et al. (1997), a possible way to explain the above reported blockade of MFS by CHX could be via a blockade of SE-induced neurogenesis by CHX.

Here we aimed to investigate whether treatment with CHX would alter the increased rate of neurogenesis and the development of supragranular MFS after SE. In order to do that, we evaluated cell proliferation on the granule cell layer by treating the animals with bromodeoxyuridine (BrdU, a marker of mitotic activity) and MFS onto the supragranular layer with neo-Timm's staining. In addition, we tried to assess whether CHX could influence SE-induced damage to the progenitor cell population in the subgranular zone of the dentate gyrus. All experiments (cell proliferation, cell damage, and mossy-fiber sprouting) were carried out either with or without the simultaneous administration of CHX. Results were compared across the different experimental models (kainate × pilocarpine) and conditions (with × without CHX).

TABLE 1.

*Experimental Groups**

	2.5 h	8 h	24 h	48 h	72 h	7 days
PILO	9	4	4	5	–	6
PILO + CHX	6	–	4	4	–	5
KA	6	4	5	4	–	6
KA + CHX	4	–	3	3	3	5
Saline (S)	–	–	–	–	–	7
S + CHX	–	–	–	–	–	5

*Number of animals per experimental group for each time-point studied. Animals sacrificed from 2.5–72 h were used for silver impregnation histological procedure and at 7 days for neuronal proliferation (BrdU staining). –, time-points for which no data were assessed.

MATERIALS AND METHODS

Animals and Drug Treatment

Male Wistar rats weighing 150–250 g were housed in groups of 6–7, on a standard light-dark cycle, with rat chow pellets and tap water continuously available. They were allowed 48 h of rest before the start of the experiment. Seizures were induced by i.p. injections of pilocarpine hydrochloride (320–350 mg/kg, Merck, Darmstadt, Germany), or kainic acid (10 mg/kg, Sigma, St. Louis, MO). Scopolamine methyl bromide (1 mg/kg, i.p., Sigma) was injected 30 min before pilocarpine to reduce its peripheral effects. Approximately 30 min after pilocarpine and 2 h after kainate injection, most of the animals had entered status epilepticus (SE). All animals were injected with thionembutal (25 mg/kg, i.p.) 90 min after SE onset to reduce mortality. Based on the different latency from drug administration to the behavioral onset of SE (which is approximately 120–150 min for KA as compared to 20–30 min for pilocarpine), cycloheximide was administered 1 h after KA injection (thus, approximately 1 h prior to onset of SE) or 50 min prior to the injection of pilocarpine (thus approximately 1 h prior to onset of SE). At the used dose of 1 mg/kg, CHX blocks ribosomal protein synthesis for more than 12 h (Ch'ih et al., 1977) and the supragranular MFS after pilocarpine- or intrahippocampal kainate-induced SE (Longo and Mello, 1997, 1998, 1999).

Cell Damage

Detection of cell injury was performed with a silver staining technique on a total of 22 rats for the pilocarpine group, 14 for the CHX-pilocarpine group, and 19 and 13 for the kainate and CHX-kainate groups, respectively (see Table 1). In order to determine the profile of neuronal injury, animals were sacrificed at 2.5 h (n = 25), 8 h (n = 8), 24 h (n = 16), 48 h (n = 16), or 72 h (n = 3) after SE onset (for numerical distribution, see Table 1). At the above-mentioned times, animals were deeply anesthetized (thionembutal, 50 mg/kg, i.p.) and were perfused through the left ventricle according to the procedures of Gallyas et al. (1990, 1993) and van den Pol and Gallyas (1990). Perfusion procedures took into account the techniques developed by Cammermeyer (1961, 1967, 1978) to avoid "dark" cell artifacts. Details on this procedure have been previously reported (Mello and Covolan, 1996). In brief, under deep anesthesia, the animals had their chests opened and the perfusion through the left ventricle of the heart was started within less than 1 min from chest opening, with heart frequency still above 100/min. The perfusion buffer was used for only 2 min, and was then followed by the perfusion fixative. The buffer solution contained 0.1% (w/v) Tris, 0.59% (w/v) calcium chloride, and 0.09% (w/v) sodium chloride. Composition of perfusion fixative was 2% (v/v) paraformaldehyde, 2.5% (v/v) glutaraldehyde, 0.1% (w/v) Tris, 0.59% (w/v) calcium chloride, and 0.36% (w/v) sodium chloride, with the final solution pH adjusted to 7.5. Removal of brains from the skull did not take place at the end of perfusion but only 24 h later, in order to avoid "dark" cell artifacts (see above). This procedure is an essential and effective measure in avoiding the staining of neurons that might occur simply due to mechanical strain to the brain if it were to be removed from skull right after perfusion. Once removed from the skull, brains were immersed in fixative for at least 1 week before sectioning.

On the day prior to sectioning, brains were immersed overnight in perfusion buffer containing 30% sucrose solution. Cryostat coronal brain sections (64 μm thick) were washed in two changes of 1% acetic acid to remove the fixative. These sections were consecutively dehydrated in 50%, 75%, and 100% 1-propanol (5 min each), and then kept in 98% 1-propanol containing 1.2% (v/v) sulfuric acid for 16 h at 56°C. After rehydratation in 75%, 50%, and 25% 1-propanol and in distilled water, the sections were treated with 3% acetic acid for 10 min to reduce background staining, immediately followed by color development in a solution containing 10% (w/v) Na_2CO_3, 0.2% (w/v) NH_4NO_3, 0.2% (w/v) $AgNO_3$, 2% (w/v) silicotungstic acid, and 0.35% (v/v) formaldehyde for approximately 10 min. Development was terminated by washes in 1% acetic acid for 30 min. Sections were examined by light microscopy for the presence of silver-impregnated cells. Identification of damaged areas was made using cresyl violet-stained

adjacent sections (64 μm) corresponding to the rat brain map of Swanson (1992).

BrdU Labeling

Seven days after SE induction, 22 rats (see Table 1) received four injections of bromodeoxyuridine (BrdU; 50 mg/kg, i.p., Sigma-Aldrich; dissolved in 0.007 N NaOH in 0.9% NaCl) during a period of 24 h to label mitotic cells (6-h interval between each pulse, starting at approximately 2:00 PM). Control animals injected with saline (n = 7) or saline plus cycloheximide (n = 5) were also subject to four BrdU injections, in the same way as above. Twenty-four hours after the last BrdU injection, animals received an anesthetic overdose (thionembutal, 50 mg/kg, i.p.) and were transcardiacally perfused with normal saline followed by 2% paraformaldehyde. Coronal cryostat sections (24 μm thick) were obtained through the septo-temporal extent of the hippocampus. For BrdU immunostaining, we used the Zymed® BrdU staining kit.

Tissue Fixation and Neo-Timm Staining

To assess patterns of supragranular mossy fiber sprouting, neo-Timm staining was performed in pilocarpine- (n = 5), pilocarpine-CHX- (n = 5), kainate- (n = 5), and kainate-CHX- (n = 5) treated rats killed 60 days after SE. After an anesthetic overdose (thionembutal, 50 mg/kg, i.p.), animals were perfused as follows: 25 ml of Millonig's buffer; 50 ml of sodium sulfide fix 0.1% in Millonig's buffer; 100 ml of glutaraldehyde 3%; or 200 ml of sodium sulfide fix 0.1% in Millonig's buffer. The brains were removed and immersed in 15% sucrose overnight. Cryostat-frozen coronal sections 40 μm thick were processed for neo-Timm staining. A detailed description of the neo-Timm staining technique can be found elsewhere (Mello et al., 1993).

Semiquantitative Neuronal Analyses

For the assessment of neuronal damage, sections were analyzed by an observer "blind" to the experimental condition of each animal. Examination of the total number of silver-stained granule cells was focused on three levels of the rostro-caudal hippocampal extension, corresponding to 2.45 mm (level 28), 4.45 mm (level 34), and 5.65 mm (level 38) from bregma (Swanson, 1992). Because a determination of the absolute, unbiased number of argyrophilic neurons was not necessary for the purposes of this study, a profile-sampling method was used (for a recent discussion, see Popken and Farel, 1997). Cell counts were made at a magnification of 200× in both the suprapyramidal and infrapyramidal blades of the dentate gyrus on the three levels above mentioned. Every round silver-stained cell body profile larger than 5 μm in diameter (to exclude glial cells) in the granular and infragranular cell layers of a given section was counted. Neurons in the infragranular layer of the dentate gyrus were also counted separate from those in the granular layer itself (see Fig. 1). Fusiform profiles, possibly hilar interneurons, adjacent to the infragranular layer were not counted. Results are reported as the mean average number of silver-stained neurons in the granule cell layer per 64-μm-thick coronal brain section.

Counts of BrdU-positive nuclei in the dentate granule cell layer (GCL) were performed in three sections per animal (at the same levels described above). Small BrdU-positive nuclei that appeared to be glial cells (less than 5 μm in diameter) were excluded from the analysis. This procedure was identical to that described by Parent et al. (1997). The number of proliferative cells for each level was analyzed statistically with analysis of variance (ANOVA) followed by Fisher's post hoc test. A significance level of 5% was assumed. To allow independent further interpretation of the multiple comparisons of these data, Table 1 lists all *P* and F values.

The intensity of sprouting in the supragranular layer was evaluated by a subjective graduation score suggested by Tauck and Nadler (1985) that varies from 0 (no sprouting) to 3 (most intense), by two independent observers "blind" to the experimental condition of each animal. Hippocampal sections corresponding to 2.45 mm, 4.45 mm, and 5.65 mm from bregma were analyzed for each animal. Scores attributed to each animal represent the mean value given for the three sections by the two observers. Statistical significance levels were obtained by the Kendall analysis test.

RESULTS

Neuronal Damage After Pilocarpine-Induced SE

Cell damage was clearly evident in the granule cell layer 2.5 h after the onset of pilocarpine-induced SE (Fig. 1A), being maximal between 2.5–8 h after SE. At the earliest time point assessed (2.5 h), injured cells in the GCL were 10–40 times more frequently seen in the infrapyramidal than in the suprapyramidal blade. This huge difference tended to persist even by 8 h after SE, mostly due to an increase of the number of stained neurons in the suprapyramidal blade (see Table 2). Damaged dentate granule cells were not only more frequently seem in rostral (septal) levels of the hippocampal formation but also for a more protracted time period. Brain sections taken from middle levels of the hippocampal complex (corresponding to plate 34 in Swanson, 1992) had the least intense damage in the GCL at all time points studied. By 24 h, silver-stained neurons were much less frequent than what was seen 8 h after SE onset (see Table 2). By this time, a large number of dark puncta, suggestive of terminal degeneration of granule and hilar cells, had substituted silver-stained neurons previously seen in the GCL, in the inner third of the molecular layer, and in the hilar region (Fig. 2B). Yet, many dentate granule cell bodies, lacking clearly connected dendrites or axons, could still be visualized in the GCL 24 h after pilocarpine-induced SE. After 48 h of SE onset, staining of the GCL was characterized only by a marked band of dark puncta with no clearly defined cell bodies (not shown).

Neuronal Damage After Kainate-Induced SE

Overall damage to dentate granule cell neurons in animals subjected to KA-induced SE was less intense than that found for

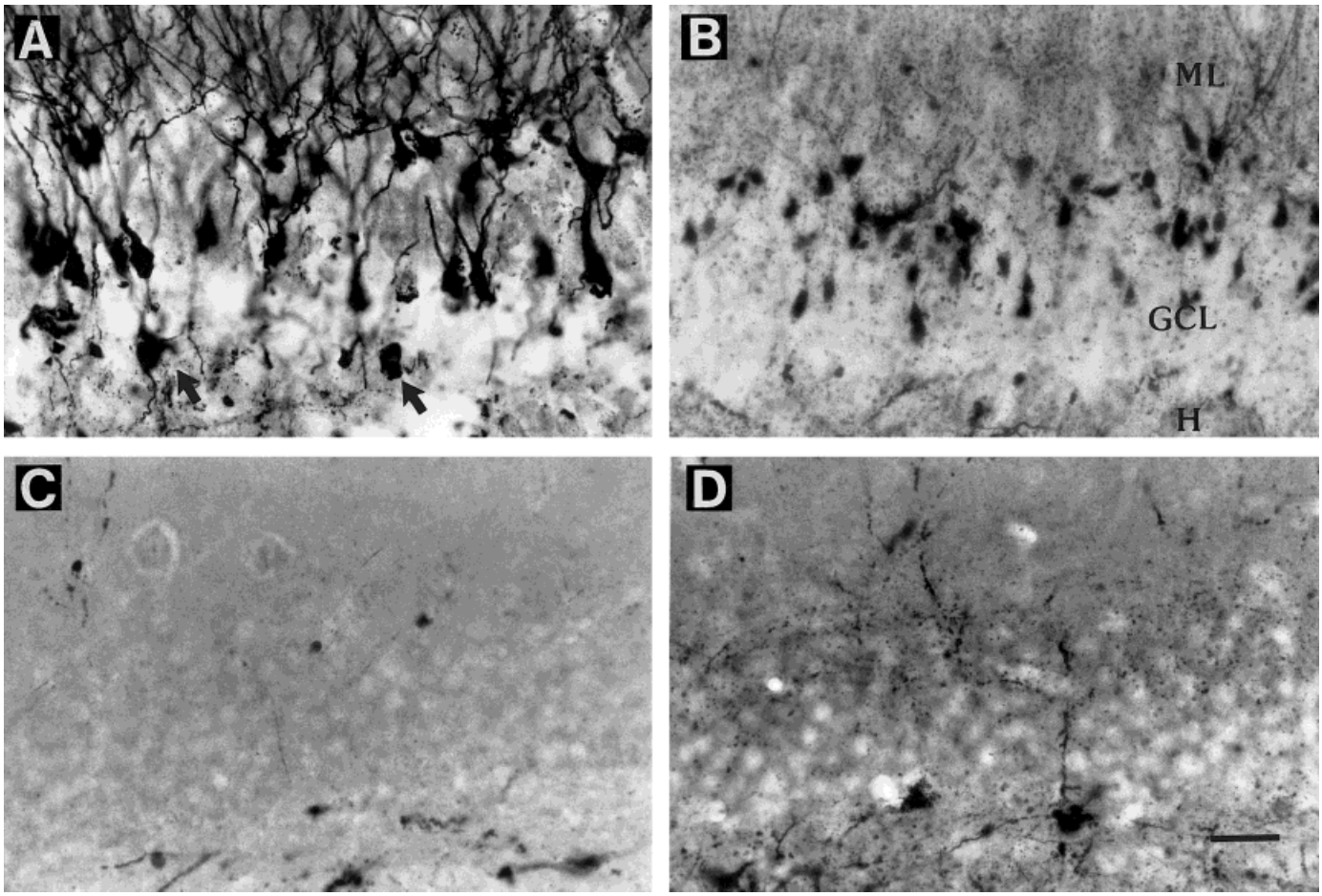

FIGURE 1. High-magnification photographs depicting the time course of pilocarpine (PILO) (A and B) or PILO-CHX (C and D) seizure-induced cell damage (silver impregnation) in hippocampal dentate granule cells. A: At 2.5 h, intense labeling of dentate granule cells. Arrows indicate degenerating cells in the innermost portion of the GCL. B: At 24 h, less intense cell damage and the presence of a marked band of dark puncta occupying the GCL and the inner third of the molecular layer. C: Labeling pattern of dentate GCL at 2.5 h after SE onset in PILO-CHX-treated animals, with very few damaged cells. D: An PILO-CHX-treated animal 24 h after SE onset. Scale bar, 50 μm. GCL, granule cell layer; H, hilus; ML, molecular layer.

pilocarpine-treated rats (for comparison, see Fig. 2A,C for animals perfused 8 h after SE onset, and Table 2). Nevertheless, the general pattern of neuronal injury in the dentate gyrus was qualitatively similar to that reported for pilocarpine. As observed for pilocarpine-treated animals, damage was more prominent in the infrapyramidal rather than the suprapyramidal blade of GCL for kainate-treated animals. Furthermore, silver-stained granule cells were observed only in the more septal and temporal portions of the hippocampal complex (corresponding to levels 28 and 38, respectively, in Swanson, 1992). Kainate-treated animals had a pattern of damage which was less intense, progressing over a longer period of time as compared to damage seen after pilocarpine-induced SE. Damage was most intense by 48 h after SE onset. The maximal dentate neuronal damage for a given blade (suprapyramidal or infrapyramidal) and septo-temporal level (rostral, middle, and caudal) but irrespective of time was at least 3 times less intense for kainate than for pilocarpine (see Table 2).

Neuronal Damage After SE in Cycloheximide-Treated Rats

Dentate granule cell damage after pilocarpine- or kainate-induced SE was either dramatically reduced or abolished by pretreating the animals with CHX. Figure 1 shows the silver staining pattern of dentate granule cells 2.5 h (Fig. 1C) and 24 h (Fig. 1D) after SE onset. Neuronal damage in CHX-treated animals, when present, had a longer delay to show up (see Table 2 and Fig. 2E for pilocarpine-CHX- and Fig. 2G for CHX-kainate-treated animals 24 h after SE onset). The maximal dentate neuronal damage for a given blade (suprapyramidal or infrapyramidal) and septo-temporal level (rostral, middle, and caudal) but irrespective of time was 2–11 times less intense for animals treated with cycloheximide and pilocarpine than for animals subjected only to pilocarpine (see Table 2). Silver-stained dentate granule cells were not seen in KA-CHX-treated animals, even after very long time intervals (e.g., 72 h after SE onset; see Table 2).

TABLE 2.

*Mean Number of Damaged Dentate Granule Cells After SE Onset**

	PILO		PILO-CHX		KA		KA-CHX	
Time	IP	SP	IP	SP	IP	SP	IP	SP
Level 28								
2.5 h	106.6 ± 31.3	2.4 ± 1.5	0	0	0	0	0	0
8 h	79.7 ± 11.8	43.7 ± 36.4	–	–	0.2 ± 0.2	0	–	–
24 h	37.2 ± 37.2	0	58.0 ± 53.6	2.3 ± 2.3	0	0	0	0
48 h	0	0	16.5 ± 14.0	9.7 ± 9.7	19.7 ± 11.4	2.5 ± 1.7	0	0
72 h	–	–	–	–	–	–	0	0
Level 34								
2.5 h	9.6 ± 5.4	1.1 ± 0.6	0	0	0	0	0	0
8 h	9.7 ± 6.4	10.5 ± 5.9	–	–	0.2 ± 0.2	0	–	–
24 h	0	0	0	0	0	0	0	0
48 h	0	0	0	0	0	0	0	0
72 h	–	–	–	–	–	–	0	0
Level 38								
2.5 h	42.7 ± 22.1	4.2 ± 2.3	4.7 ± 4.7	0	0	0	0	0
8 h	53.2 ± 48.0	56.2 ± 50.1	–	–	0	0	–	–
24 h	0	0	0	0	5.8 ± 5.8	4.6 ± 2.9	0	0
48 h	0	0	0	0	19.7 ± 13.7	7.0 ± 13.7	0	0
72 h	–	–	–	–	–	–	0	0

*Data represent mean ± SE of degenerating cells in the dentate granule cell layer in four experimental groups: pilocarpine (PILO), pilocarpine-cycloheximide (PILO-CHX), kainate (KA), and kainate-cycloheximide (KA-CHX). Cell damage was assessed in three levels of the hippocampal rostro-caudal axis (levels 28, 34, and 38, corresponding to the map of Swanson, 1992) in five time-points after SE onset: 2.5, 8, 24, 48, and 72 h. The infrapyramidal and suprapyramidal blades of the dentate gyrus are represented by IP and SP, respectively. 0, lack of damaged cells; –, time points for which no data were assessed.

Damage to the Subgranular Layer vs. Granular Proper

In the most damaged neuronal segment of the GCL, the infrapyramidal blade of the rostral level of the hippocampus, an additional counting of injured neuronal profiles was performed (Table 3). For that area, quantitative assessment of stained cell bodies in the GCL was also performed, separating neurons located in the subgranular zone of the GCL (close to the hilus) from neurons located in the GCL proper. For this distinction, cells were considered part of the subgranular GCL if they were round and located at one cell body diameter or less from the hilar border (see arrows, Fig. 1A). According to these criteria, degenerating cells in the subgranular zone comprised approximately 10% of the total number of degenerating neurons for a given time point and experimental group. The maximal dentate neuronal damage for the subgranular zone (irrespective of time) was reduced by 4 times by the application of cycloheximide to animals subject to pilocarpine-induced SE and zeroed in kainate-treated animals (see Table 3).

Mitotic Rate After Pilocarpine- or Kainate-Induced SE

The effects of SE induction on the mitotic rate of cells in the dentate granule cell layer were monitored by BrdU mitotic labeling in pilocarpine- or kainate-treated animals, some of which also had been coinjected with cycloheximide. Our own preliminary results and a previous report (Parent et al., 1997) indicated that 7 days after SE induction was an adequate time point to study SE-induced granule-cell neurogenesis.

As previously reported, a small number of BrdU-positive nuclei in the subgranular zone of the GCL were encountered in control animals (Parent et al., 1997, 1998, 1999). These labeled cells were more conspicuous in the infrapyramidal blade of GCL. Results from saline-injected controls did not differ from those in saline-CHX-treated animals (Fig. 3, Table 4). In animals subject to SE, on the other hand, BrdU-immunoreactive (IR) cells were quite conspicuous and tended to form large clusters. For pilocarpine animals subject to SE, counting of BrdU-IR profiles in this region indicated a 3–7-fold increase when compared to control animals, depending on whether counting was performed on the rostral, medium, or caudal levels. For kainate-treated animals, status epilepticus caused an even higher increase ($P < 0.01$; Table 4) in the number of BrdU-IR cells in this region. Clusters of BrdU-IR cells, present in pilocarpine- (Fig. 2B) and kainate-treated (Fig. 2D) animals, were formed preferentially near the subgranular zone, but many of them were distributed throughout the granule cell layer. Despite the clear quantitative difference, no qualitative differ-

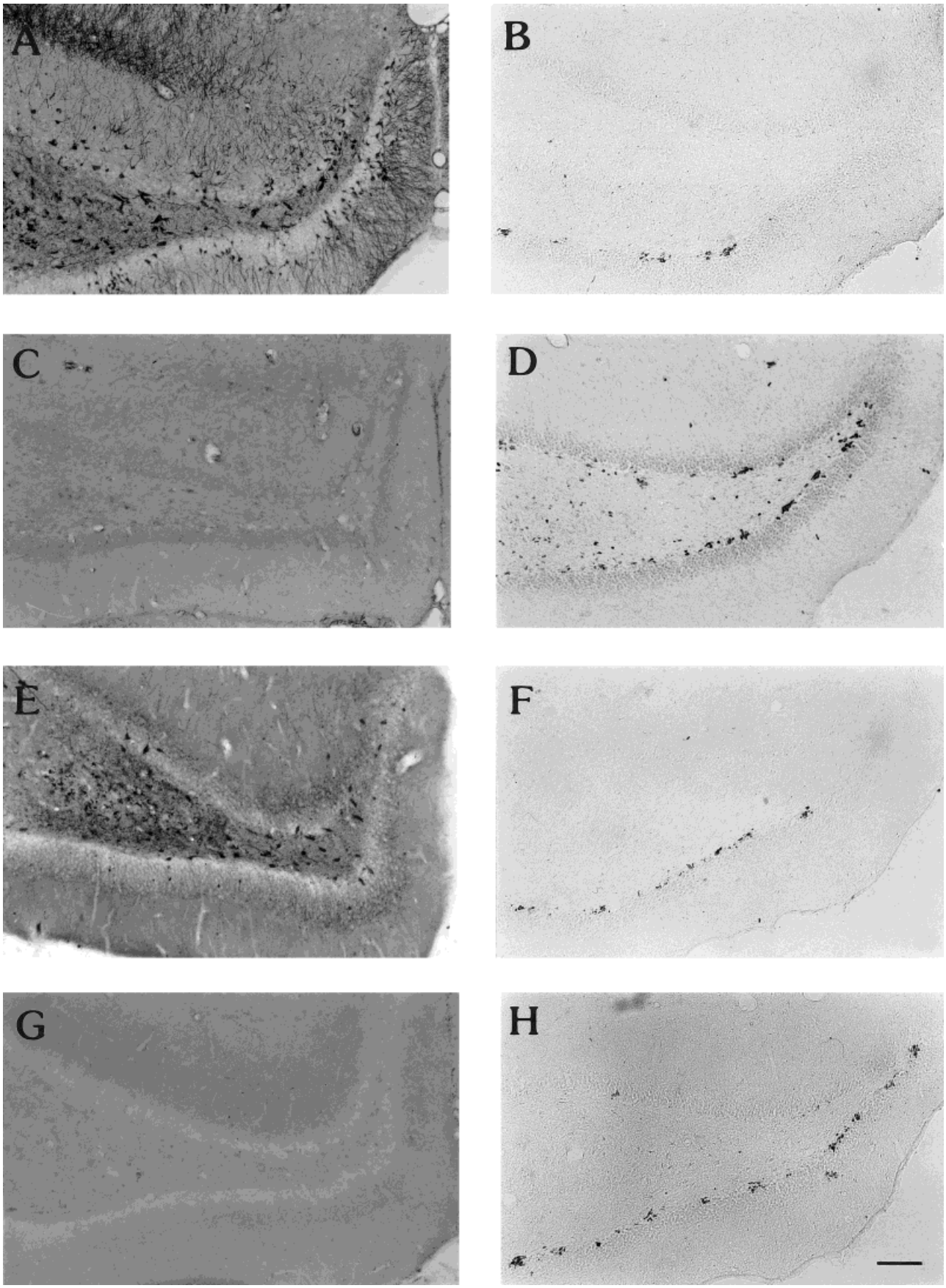

FIGURE 2

TABLE 3.

*Mean Number of Degenerating Neurons Per Section**

	2.5 h	8 h	24 h	48 h	72 h
PILO					
GCL	106.6	79.7	37.2	0	–
SUBG	13.8	6.2	2.5	0	–
	13.0%	7.8%	6.7%	0	–
KA					
GCL	0	0	0	79	–
SUBG	0	0	0	11	–
	0	0	0	13.9%	–
PILO-CHX					
GCL	0	–	58.0	16.5	–
SUBG	0	–	3.5	1.5	–
	0	–	8.0%	9.0%	–
KA-CHX					
GCL	0	–	0	0	0
SUBG	0	–	0	0	0
	0	–	0	0	0

*Number of degenerating cells in the entire width of the dentate granule cell layer (GCL) in the four experimental groups: pilocarpine (PILO), pilocarpine-cycloheximide (PILO-CHX), kainate (KA), and kainate-cycloheximide (KA-CHX). Cell damage was assessed at three levels of the hippocampal rostro-caudal extension (levels 28, 34, and 38, corresponding to Swanson, 1992) in five time-points after SE onset: 2.5, 8, 24, 48, and 72 h. SUBG, subgranular region of the dentate gyrus; 0, lack of degeneration; –, time points for which no data were assessed.

ence was observed in BrdU immunostaining for the rostral, middle, and caudal levels of hippocampal formation.

Mitotic Rate in CHX-Treated Animals Subjected to Pilocarpine- or KA-Induced SE or to Saline Injections

To assess the possible contribution of newly formed dentate granule cells to the aberrant mossy-fiber sprouting that ensues after pilocarpine-induced SE, cycloheximide, which has been shown to block pilocarpine-induced mossy-fiber sprouting (Longo and Mello, 1997), was coadministered with either pilocarpine or kainic acid. The hypothesis was that if aberrant mossy-fiber sprouting is dependent on newly formed granule cells, and as cycloheximide blocks SE-induced aberrant mossy-fiber sprouting, then the above hypothesis could be further supported if cycloheximide prevented SE-induced increases in mitotic rate (no new granule cells), therefore preventing SE-induced mossy-fiber sprouting. In other words, cycloheximide would lead to blockade of SE-induced aberrant mossy-fiber sprouting because it blocked SE-induced granule cell neurogenesis. Much to our surprise, the number of BrdU-IR nuclei in the GCL after pilocarpine-induced SE in CHX-treated animals was more prominent than in animals subjected to pilocarpine-induced SE in the absence of CHX (see Fig. 3 and compare Fig. 2F vs. 2B). Two to eight more BrdU-immunolabeled nuclei were encountered in the dentate granule cell layer when animals were subjected to pilocarpine-induced SE in the presence rather than in the absence of CHX ($P < 0.05$; Table 4). Table 4 reveals an even higher increase in the number of BrdU-IR nuclei in the CHX-pilocarpine group when compared to controls (CTRL-CHX; $P < 0.01$). Animals subjected to KA-induced SE in the presence of CHX had increases in the number of BrdU-IR profiles which were very similar to those already reported for KA-treated animals (compare Fig. 2H and 2D) and significantly higher when compared to control (KA-CHX vs. CTRL-CHX, $P < 0.01$). Therefore, coadministration of CHX altered the number of BrdU-IR profiles in animals subjected to pilocarpine- but not kainate-induced SE.

Mossy-Fiber Sprouting

For both kainic acid- and pilocarpine-treated animals, induction of SE invariably led to the development of supragranular MFS. This newly formed aberrant circuit was significantly more intense for pilocarpine-treated (mean score of 2.3) than for kainate-treated (mean score of 1.4) animals at all levels assessed (Kendall test, $P < 0.05$). A tendency for MFS to be more intense at more caudal levels was observed for all groups. Confirming our previous results (Longo and Mello, 1997, 1999), treatment of animals with CHX significantly attenuated or abolished MFS (mean score of 0.5) that would otherwise be induced by pilocarpine (Kendall test, $P < 0.01$). In the same way, treatment of animals with CHX significantly attenuated or abolished MFS (mean score of 0.4) that would otherwise be induced by kainic acid (Kendall test, $P < 0.03$). Examples of such findings are depicted in Figure 4.

FIGURE 2. Photomicrographs of silver-stained (left) or BrdU-labeled (right) hippocampal dentate cells in pilocarpine- or kainate-treated animals. A and C indicate, respectively, silver-stained cells from pilocarpine- and kainate-treated animals perfused 8 h after SE onset. E and G indicate, respectively, silver-stained cells from CHX-pilocarpine- and CHX-kainate-treated animals perfused 24 h after SE onset. B, D, F, and H indicate, respectively, BrdU-IR dentate cells from pilocarpine-, kainate-, CHX-pilocarpine-, and CHX-kainate-treated animals 7 days after SE onset. B: A relatively low number of proliferating cells in the GCL after pilocarpine-induced SE, as compared to D, F, and H. This is in contrast to the high number of damaged granule cells after the same drug treatment in A. C: Note the lack of damaged cells after kainate treatment. D: Seven days later, a high number of BrdU-positive cells can be seen after kainate injection. E and F: Administration of CHX led to a reduced amount of cell damage in the dentate granule cell layer after pilocarpine treatment (as compared to A), and to an increased mitotic rate (as compared to B). Scale bar, 100 μm.

DISCUSSION

Previous studies with silver staining have shown the vulnerability of dentate granule cells in several situations, such as cortical injury (van den Pol and Gallyas, 1990), biccuculine-induced SE (Gallyas et al., 1990), volkesin injection (Gallyas et al., 1990), and

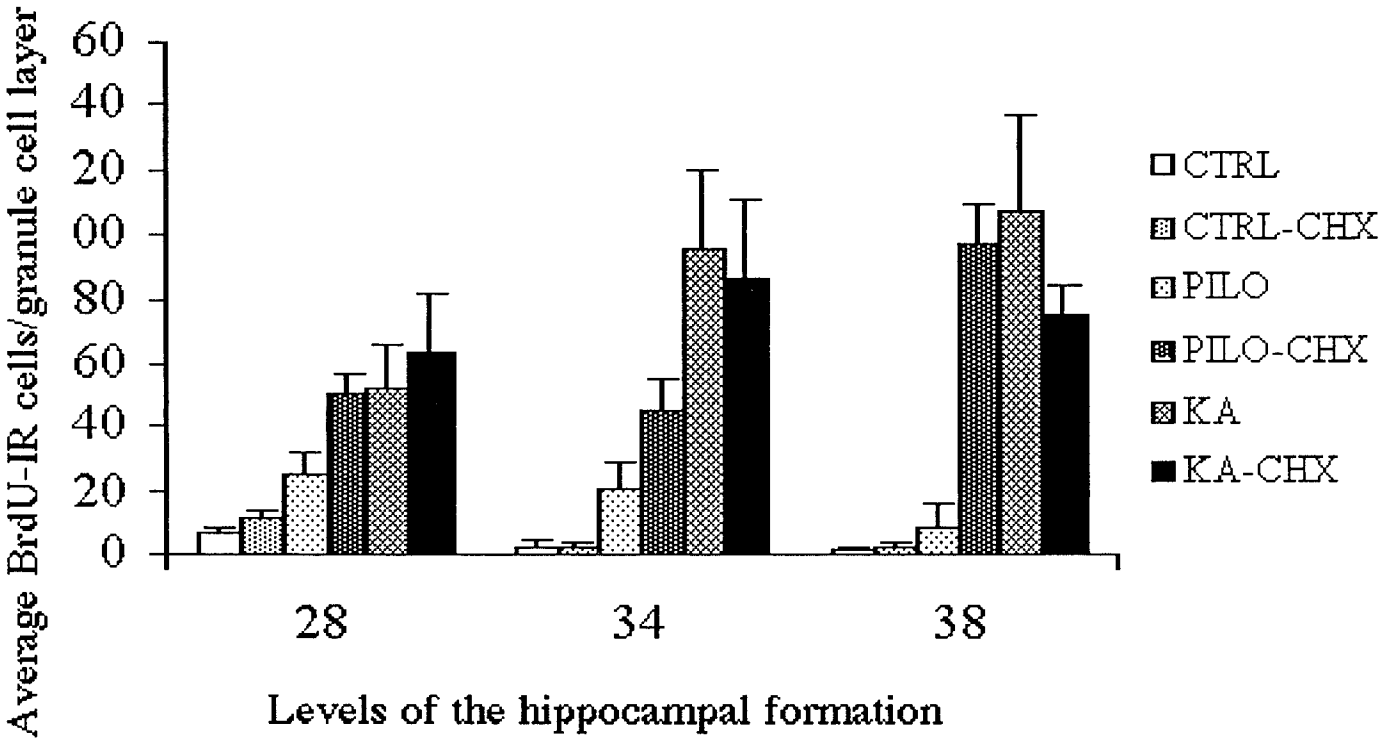

FIGURE 3. Quantification of BrdU-immunoreactive (IR) cells in three levels of dentate gyrus. Numbers of BrdU-IR cells were determined 7 days after drug treatment. Numbers are totals per dentate granule cell layer (means ± SE). Statistical analysis is given in Table 4. Experimental groups: Control (CTRL); control-cycloheximide (CTRL-CHX); pilocarpine (PILO); pilocarpine-cycloheximide (PILO-CHX); kanic acid, (KA); kanic acid-cycloheximide (KA-CHX).

after adrenalectomy (Sloviter et al., 1993a,b). Neuronal cell-counting in pilocarpine epileptic rats (Mello et al., 1992) and in patients with temporal lobe epilepsy (Mouritzen-Dam, 1980, 1982; Babb et al., 1984a; deLanerolle et al., 1989; Mathern et al., 1994) also demonstrated such loss. In all of the above papers, even though present, granule cell loss was reported as a rather lesser event when compared to that of other areas. Relative preservation of dentate granule cells and the vulnerability of hilar cells after pilocarpine-induced SE was also reported by Obenaus et al. (1993), using a silver impregnation method. Recently, however, dentate granule cells were shown to be vulnerable to the epileptic activity triggered by repetitive stimulation of the perforant pathway (Sloviter et al., 1996). In a previous study, we reported pilocarpine-induced damage of dentate granule cells with ultrastructural features of terminal degeneration 48 h after SE onset (Mello et al., 2000). Our current findings do not allow the assumption that all dentate granule cells are in fact degenerating, but do clearly demonstrate the vulnerability of dentate granule cells in rats submitted to pilocarpine-induced SE. The major advantage of the silver-staining procedure used in the current study lies in this abil-

TABLE 4.

Statistical Analysis of BrdU Immunoreactive Cell Comparisons: Levels of Significance*

	CON vs. CON-CHX	CON vs. PILO	CON vs. KA	CON-CHX vs. PILO-CHX	CON-CHX vs. KA-CHX	PILO vs. PILO-CHX	KA vs. KA-CHX
Level 28 (F, 5.96; *P*, 0007)	NS	0.0097	0.0045	0.0006	0.0238	0.0249	NS
Level 34 (F, 7.95; *P*, 0001)	NS	0.0384	0.0018	0.0039	0.0078	NS	NS
Level 38 (F, 10.32; *P*, 0001)	NS	NS	0.0023	0.0011	0.0001	0.0028	NS

*Statistical analysis of number of BrdU-positive cell comparisons was performed with factorial ANOVA and Fisher's post hoc test. $P \leq 0.05$ was considered to represent statistical significance. Overall F and *P* from ANOVA are given in the first column. NS, not significant.

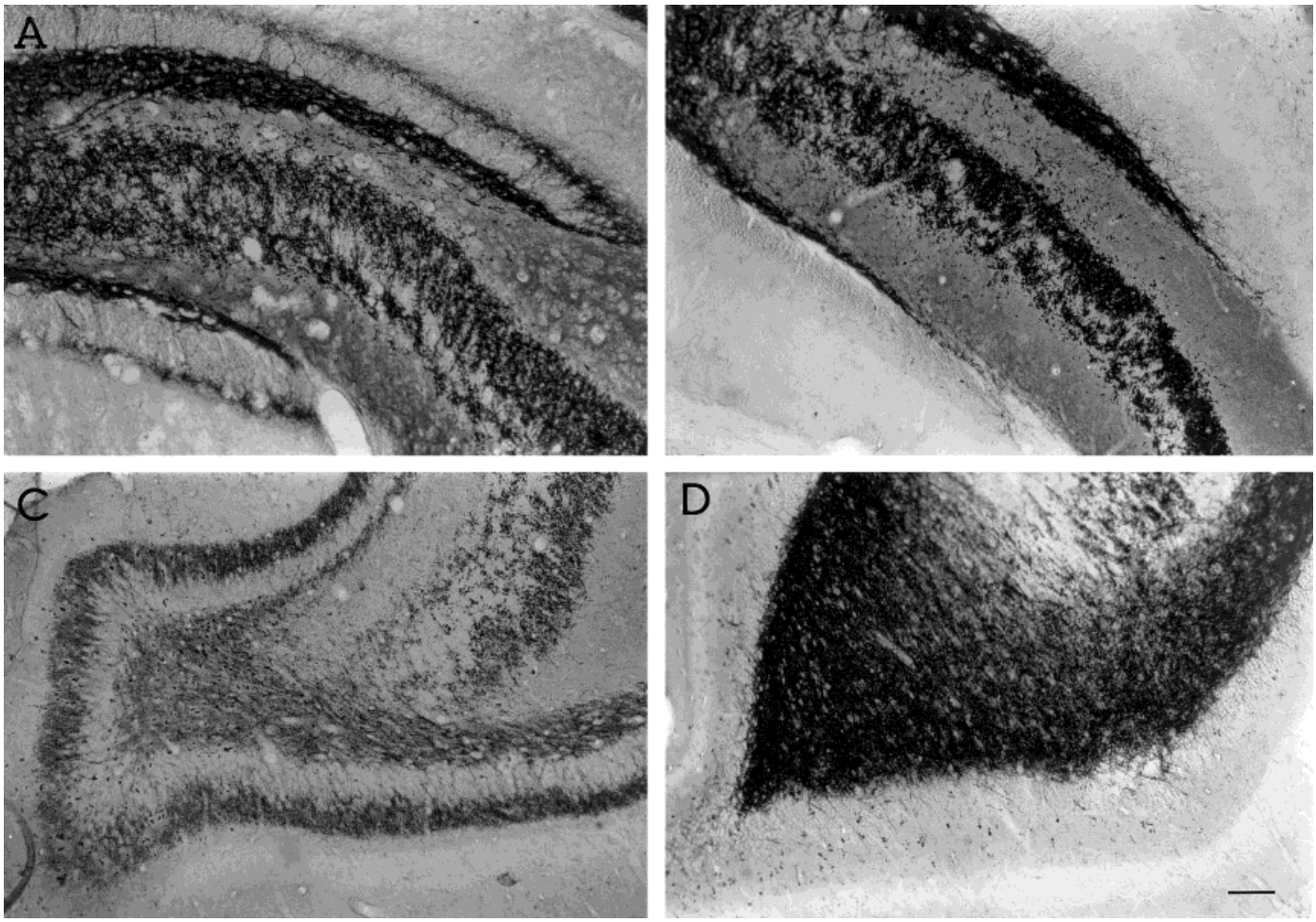

FIGURE 4. Photomicrographs showing mossy-fiber sprouting staining in chronic epileptic rats injected with pilocarpine- or kainic acid. A and B: Neo-Timm-stained sections from the midportion of the hippocampus. A: A kainate-treated animal demonstrates the aberrant reorganization of granule cell mossy fibers into the inner molecular layer. B: Note the lack of supragranular MFS in a kainate-CHX-treated rat. C and D: Neo-Timm staining of sections from ventral dentate gyrus of pilocarpine-treated animals. The robust MFS observed in C (a pilocarpine-treated animal) contrasts with the lack of such a staining in D (a pilocarpine-CHX-treated animal). All micrographs are at the same magnification. Scale bar, 300 μm.

ity to demonstrate substantial populations of injured dentate granule cells early after SE onset. The precise mechanisms determining the enhanced affinity of injured neurons to the silver stains have not been resolved (Gallyas et al., 1992). However, this method can selectively detect traumatized neurons within minutes after injury induction (van den Pol and Gallyas, 1990).

From the original work of Ben-Ari et al. (1981) with the kainate model of epilepsy, to our own results with the pilocarpine model (Mello et al., 1996a), the entorhinal cortex has been considered an early structure to be activated in or prior to status epilepticus. The already-known pathways from the entorhinal cortex to the hippocampal complex have recently been expanded with the recent description of a projection from the deep layers (IV–VI) of the entorhinal cortex to the inner molecular layer of the dentate gyrus (Deller et al., 1996). This pathway follows the characteristic orientation of the perforant pathway and is located preferentially in the infrapyramidal blade of the dentate gyrus. Thus, early activation of the more excitable portions of the entorhinal cortex (Jones, 1993) would tend to preferentially activate neurons in the infrapyramidal rather than suprapyramidal GCL. This hypothesis would be supported by our current findings of an initial preferential damage of neurons in the infrapyramidal rather than in the suprapyramidal GCL early after pilocarpine-induced SE. In fact, c-Fos mapping of the brain areas activated after a convulsive dose of pilocarpine shows an initial activation of the deep layers of the entorhinal cortex, which is later followed by the activation of the superficial layers (Mello et al., 1996b). In kainate-treated animals, however, another anatomical circuit was proposed by Ben-Ari et al. (1981) that could explain the lack of damage in the dentate GCL in that model. The epileptiform activity that starts in the entorhinal cortex (layers III and II) propagates to the hippocampal formation through projections to both the CA3 and CA1 fields of Ammon's horn (for revision, see Steward and Scoville, 1976).

Postnatal generation of dentate granule cells has long been established, and approximately 85% of this cell population is pro-

duced after birth (Bayer, 1980), with a substantial (35–43%) increase in the number of dentate granule cells between 1 month and 1 year of age in the rat (Bayer and Yackel, 1982). In the adult rat, the postnatal generated granule cells arise in the subgranular zone, migrate into the granule cell layer, and express the neuronal marker neuron-specific enolase (Altman and Bayer, 1990; Cameron et al., 1993). The newborn granule cells are capable of extending axonal projections to the pyramidal CA3 region (Stanfield and Trice, 1988) and have ultrastructural features associated with neurons (Kaplan and Hinds, 1977). Our result of increased mitotic rate of dentate granule cells following limbic-induced seizures confirms data reported by Parent et al. (1997) and extends their findings for the kainic acid model as well. In both experimental models, BrdU-labeled cells were distributed in the subgranular zone in clusters, and some of them could be seen throughout the granule cell layer.

The increased neurogenesis in the dentate gyrus following limbic-induced seizures is in opposition to the seizure-induced cell damage in the same area. A comparison of our current findings on neuronal injury and neurogenesis under different epileptogenic agents and chemical influence (cycloheximide) might help in the understanding of this subject. Animals subject to pilocarpine-induced SE had the most intense dentate granule cell injury and the smallest increase in neurogenesis. Conversely, animals with small or absent damage to the granule cells up to 24 h after SE onset had the highest increases in mitotic rate. It is tempting to suggest that the epileptic activity triggered by pilocarpine might lead to both damage and neurogenesis of this cell population. In the instances where both events are present, such as in pilocarpine-induced SE, it is possible that injury of a portion of the precursor granule cell population in the subgranular zone leads to a smaller base for cell mitosis. Protection of this cell population, such as that conferred by cycloheximide early after pilocarpine-induced SE, leads to a much higher mitotic rate. Indeed, our findings suggest that cycloheximide protects cells in the subgranular zone (some of which might be part of the granule cell precursor population) from injury. Conversely, after kainate-induced SE, where such injury is not present or is minimal, addition of cycloheximide does not change SE-induced increases in mitotic rate.

Recently it was demonstrated that cycloheximide effectively blocks both kainate-induced neurotoxicity (Schreiber et al., 1993) and kindling-induced apoptosis of subgranular-located cells (Bengzon et al., 1997). It is therefore possible that the observed subsequent increases in the mitotic rate of dentate granule cells due to cycloheximide after pilocarpine-induced SE were due to a protection to a population of granule cell precursors achieved by blocking the de novo protein synthesis required for apoptosis of these cells. Our results of a further increase in dentate BrdU labeling after pilocarpine-induced SE in CHX-pretreated animals are consistent with the idea that the mitotic rate after limbic-induced seizures is regulated by the number of available progenitor cells. The lack of cell damage early after kainic acid-induced SE supports this finding, since the CHX or non-CHX pretreatment did not modify the dentate mitotic rate.

Both pilocarpine and systemic kainic acid produce seizures with many similarities to human temporal lobe epilepsy. Mossy-fiber sprouting has long been demonstrated to occur in both models and in patients with epilepsy as well (Tauck and Nadler, 1985; Sutula et al., 1989; Babb et al., 1991, 1992; Mello et al., 1993; Wuarin and Dudek, 1996). However, we do not yet know the relative contribution of this change to the development of seizure activity. In their work, Parent et al. (1997) hypothesized that aberrant axon growth arises from newly born dentate granule cells. As already mentioned, previous reports from our group (Longo and Mello, 1997, 1998, 1999), as well as the current data, indicate the capacity of cycloheximide to block SE-induced supragranular MFS. Based on the work by Parent et al. (1997), a possible hypothesis would be that cycloheximide prevented neurogenesis, thus preventing mossy-fiber sprouting. Contrary to this hypothesis, our current findings show that cycloheximide may not affect (in the kainate model), or may even promote (in the pilocarpine model), further increases in granule cell neurogenesis. Therefore, we may hypothesize that the increased mitotic rate of dentate granule cells in the pilocarpine and kainate models per se is not sufficient to generate supragranular MFS. Further support for this has recently been reported (Parent et al., 1999). Accordingly, aberrant mossy-fiber sprouting triggered by pilocarpine-induced SE also develops in X-irradiated rats (Parent et al., 1999). In that work, whole-brain irradiation inhibited granule-cell neurogenesis but not seizure-induced mossy-fiber sprouting in adult rats (Parent et al., 1999). We do not yet know through which mechanism cycloheximide is able to block SE-induced mossy-fiber sprouting, but it clearly is not dependent on the blocking of dentate granule cell neurogenesis.

A previous study in surgical material from children with extrahippocampal seizures did not indicate an association between aberrant mossy fiber sprouting and hilar neuron loss (Mathern et al., 1994). However, that study was the first to speculate that *newly* differentiating granule cells could contribute to aberrant mossy-fiber sprouting, which received further support based on results with the pilocarpine model in rats (Parent et al., 1997). Recently, Mathern et al. (1997) compared the total number of dentate granule cells in adult patients with temporal lobe epilepsy and autopsy cases. The result was a small increase (16%), though not statistically significant, in the number of dentate granule cells in patients with extrahippocampal mass lesions when compared to autopsy cases Patients with hippocampal sclerosis, on the other hand, presented a dramatic reduction in the number of dentate granule cells. Considering that both groups had approximately the same seizure duration and that patients with mass lesions did not have hippocampal damage such as did patients with hippocampal sclerosis, this increased number of dentate granule cells could at least, in part, correspond to neuronal mitosis in this region. It is important to note that the above discussion is highly speculative: it was not discussed by the authors, and the increase in granule cell number was not statistically significant (Mathern et al., 1997).

Although increased hippocampal dentate granule cell neurogenesis (Parent et al., 1997, 1998; Gray and Sundstrom, 1998) and abnormal synaptogenesis (Tauck and Nadler, 1985; Mello et al., 1993; Wuarin and Dudek, 1996) are prominent features of animal models of temporal lobe epilepsy, these might represent independent events, as suggested by our current findings and those of others (Parent et al., 1999). Thus, the functional roles played by

newborn granule cells remain to be defined. In fact, the relative "resistance" of dentate granule cells observed in previous studies based on cell counting, (Ben-Ari et al., 1981; Olney et al., 1983; Freund et al., 1991; Cavazos et al., 1994) leads us to hypothesize that cells newly generated after SE do survive and substitute for cells that eventually die after SE. Further studies addressing the electrophysiological properties of dentate granule cells born after SE induction might help in understanding their potential contribution to epileptogenesis.

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