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Up-Regulation of GAD65 and GAD67 in Remaining Hippocampal GABA Neurons in a Model of Temporal Lobe Epilepsy

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ABSTRACT

In the pilocarpine model of chronic limbic seizures, subpopulations of glutamic acid decarboxylase (GAD)-containing neurons within the hilus of the dentate gyrus and stratum oriens of the CA1 hippocampal region are vulnerable to seizure-induced damage. However, many γ-aminobutyric acid (GABA) neurons remain in these and other regions of the hippocampal formation. To determine whether long-term changes occur in the main metabolic pathway responsible for GABA synthesis in remaining GABA neurons, the levels of mRNA and protein labeling for the two forms of GAD (GAD65 and GAD67) were studied in pilocarpine-treated animals that had developed spontaneous seizures. Qualitative and semiquantitative analyses of nonradioactive in situ hybridization experiments demonstrated marked increases in the relative amounts of GAD65 and GAD67 mRNAs in remaining hippocampal GABA neurons. In addition, immunohistochemical studies demonstrated parallel increases in the intensity of terminal labeling for both GAD65 and GAD67 isoforms throughout the hippocampal formation. These increases were most striking for GAD65, the isoform of GAD that is particularly abundant in axon terminals. These findings demonstrate that, in a neuronal network that is capable of generating seizures, both GAD65 and GAD67 are up-regulated at the gene and protein levels in the remaining GABA neurons of the hippocampal formation. This study provides further evidence for the complexity of changes in the GABA system in this model of temporal lobe epilepsy. J. Comp. Neurol. 412:488–505, 1999.

Indexing terms: glutamate decarboxylase; hippocampus; rats; pilocarpine; seizures

The discovery of two isoforms of glutamate decarboxylase (GAD), the synthesizing enzyme for γ-aminobutyric acid (GABA), has led to many questions about their differential functions. The two isoforms (GAD65 and GAD67) each are capable of synthesizing GABA, but they are encoded by two different genes (Erlander et al., 1991) and differ substantially in their interactions with the cofactor pyridoxal 5′-phosphate and their regulatory control (Erlander et al., 1991; Kaufman et al., 1991; Martin et al., 1991a,b; Rimvall and Martin, 1992; Martin and Rimvall, 1993; Rimvall et al., 1993), as well as their predominant intraneuronal localization (Kaufman et al., 1991; Esclapez et al., 1994). Such differences suggest that the two GAD isoforms could have different functional roles and could be regulated differently in response to physiological activity (Erlander and Tobin, 1991; Martin and Rimvall, 1993; Esclapez et al., 1994).

One suggestion is that GAD65 may be particularly important for local control of GABA synthesis at synaptic sites, whereas GAD67 may be responsible for maintaining baseline levels of GABA for both neurotransmitter and metabolic functions (Erlander and Tobin, 1991; Esclapez et al., 1994). Thus, it might be expected that both GAD isoforms would be regulated in response to alterations in GABA neuron activity. However, despite considerable evidence for activity-dependent regulation of GAD67 (Litwak et al., 1990; Drengler and Oltmans, 1993; Benson et al.,

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1994; Drengler et al., 1996), evidence for such regulation of GAD65 is limited. In several earlier lesion and pharmacological studies in which both GAD65 and GAD67 mRNAs were examined, up-regulation of GAD67 mRNA generally was greater than that of GAD65 mRNA (Soghomonian and Chesselet, 1992; Soghomonian et al., 1992; Delfs et al., 1996). However, increased GAD65 mRNA levels have been found in some specific brain regions and in selected groups of neurons (McCarthy et al., 1995; Laprade and Soghomonian, 1997). Furthermore, a recent study demonstrated a preferential up-regulation of GAD67 mRNA by discrete stressful stimuli in several hypothalamic nuclei. In contrast, GAD65 mRNA was up-regulated preferentially by prolonged stress stimulation (Bowers et al., 1998). These data suggested that the biosynthesis of the two GAD isoforms could be under the control of different duration-dependent stimuli.

The hippocampal formation is an ideal region in which to examine the regulation of the two forms of GAD, because both GAD mRNAs are expressed prominently in the region, suggesting that a dual system for GABA synthesis is necessary for normal GABAergic function in this brain region (Houser and Esclapez, 1994b). Both GAD isoforms can be localized immunohistochemically throughout the hippocampal formation (Fukuda et al., 1998; Sloviter et al., 1996), and, although both forms are present in axon terminals, GAD65 is particularly abundant at these locations, as it is in other regions of the central nervous system (Esclapez et al., 1994).

A model of spontaneous recurrent seizures was selected for the current studies of GAD regulation because it represents a naturally occurring (although pathophysiologic) condition in which neuronal activity is modified. Furthermore, the level of functional activity of GABA neurons in the hippocampal formation in epilepsy is of considerable interest. Although the hippocampal formation is a region that frequently exhibits hyperexcitability and seizure activity in chronic models of epilepsy, the functional state of GABA neurons in the region continues to be debated. Some investigators have suggested that GABA neurons in the hippocampal formation may be less active in seizure-prone animals (Sloviter, 1991; Bekenstein and Lothman, 1993). However, other investigators recently have demonstrated that many of the GABA neurons that remain in these animals receive afferent input and are active electrophysiologically during the chronic stage (Esclapez et al., 1997; Rempe et al., 1997).

In view of the apparent close correlation between the level of functional activity and GAD mRNA and protein levels in other brain regions (Litwak et al., 1990; Drengler and Oltmans, 1993; Benson et al., 1994), it may be anticipated that increased activity of GABA neurons in the hippocampal formation would be associated with increased levels of regulatable GAD mRNAs and proteins. Likewise, low levels of activity of GABA neurons might be associated with a decrease or lack of change in the expression of these mRNAs and proteins.

Therefore, in pilocarpine-treated animals with chronic limbic seizures, we used in situ hybridization and immunohistochemical methods to determine whether long-term changes occur in the levels of mRNAs and proteins for the two GAD isoforms in remaining hippocampal GABA neurons and whether GAD65 and GAD67 isoforms are regulated similarly. Preliminary reports of some of these findings have been published previously (Houser and Esclapez, 1994a, 1996).

MATERIALS AND METHODS

Experimental animals

Sustained seizures were induced in young adult male Sprague-Dawley rats (150–200 g; Harlan Sprague-Dawley, Indianapolis, IN) by the administration of pilocarpine, a muscarinic cholinergic agonist. The injection protocols were similar to those described previously (Turski et al., 1983; Cavalheiro et al., 1987; Obenaus et al., 1993). A low dose of the cholinergic antagonist methyl scopolamine nitrate (1 mg/kg, i.p.; Sigma, St. Louis, MO) was administered 30 minutes prior to pilocarpine injection to minimize peripheral cholinergic effects (Baez et al., 1976; Turski et al., 1983). Animals then received a single injection of pilocarpine hydrochloride (350 mg/kg, i.p.; Sigma). After the injection, animals were placed in an observation box and monitored for a minimum of 5 hours to assess the severity and length of the induced behavioral seizures, which have been described previously (Turski et al., 1983; Cavalheiro et al., 1991). The animals were then observed periodically in the vivarium for general behavior and spontaneous seizures for a period of three months. Seven rats that initially showed robust behavioral seizures for 3–4 hours or longer after pilocarpine injection and later developed spontaneous, recurrent seizures and five uninjected control animals were included in this study. All animal-use protocols conformed to Veterans Administration (VA) and National Institutes of Health guidelines and were approved by the VA Animal Research Committee, West Los Angeles VA Medical Center, and the University of California-Los Angeles Chancellor's Animal Research Committee.

Tissue preparation

Rats were deeply anesthetized with sodium pentobarbital (60 mg/kg) and perfused through the ascending aorta with 150–200 ml (1 ml/g body weight) of a fixative solution of 4% formaldehyde (freshly depolymerized from paraformaldehyde) in 0.12 M phosphate buffer, pH 7.3. After perfusion, the brains were left in situ for one hour at 4°C. They were then removed from the skull, postfixed in the same fixative for one hour at room temperature (RT), rinsed in sodium phosphate buffer for 1.5 hours, and immersed in a cryoprotective solution of 20% sucrose in the same phosphate buffer overnight at 4°C. Blocks of forebrain that contained the hippocampus were quickly frozen on dry ice. Sections (30-μm-thick) were cut on a cryostat, rinsed in 0.01 M phosphate-buffered saline, pH 7.4 (1 × PBS), collected sequentially in tubes containing an ethylene glycol-based cryoprotective solution (Watson et al., 1986; Lu and Haber, 1992), and stored at −20°C until histologic processing. Before use, both PBS and cryoprotective solutions were treated with 0.05% diethylpyrocarbonate (DEPC) and autoclaved to inactivate RNase activity.

Every tenth section was stained with cresyl violet to determine the general histologic characteristics of the tissue and the location of the sections within the rostral-caudal extent of the hippocampal formation. From each animal, adjacent selected sections were processed for nonradioactive in situ hybridization histochemistry with GAD67 and GAD65 cRNA probes and for immunohisto-

chemistry with antibodies that are specific for each GAD isoform.

In situ hybridization

Probe synthesis. The rat GAD67 and GAD65 probes used in this study were digoxigenin-labeled riboprobes obtained by in vitro transcription of two previously described GAD cDNAs. The GAD67 cDNA (2.7 kb) was a subclone of the GAD67 clone isolated from a λgt-11 rat whole brain library using a feline GAD67 cDNA (Kaufman et al., 1986; Tillakaratne et al., 1992). The GAD65 cDNA (2.4 kb) was isolated from a λ *zap*II rat hippocampus library quote (Erlander et al., 1991). GAD67 and GAD65 cDNAs, each containing the entire coding region, were subcloned into the Bluescript transcription vector (SK polylinker; Stratagene Cloning Systems, La Jolla, CA) in both orientations to obtain antisense and sense RNA probes. The transcription was carried out with the nonradioactive RNA labeling kit (Boehringer Mannheim, Indianapolis, IN), as described previously (Esclapez et al., 1993). Briefly, the recombinant plasmids containing the GAD67 cDNA insert were linearized with the restriction enzyme *Sal*I and transcribed with T3 RNA polymerase. The plasmids containing the GAD65 cDNA insert were linearized with *Xba*I and transcribed with T7 RNA polymerase. The transcripts labeled during the transcription with digoxigenin-11-UTP were fragmented by limited alkaline hydrolysis in order to obtain probes of approximately 160 nucleotides in length.

The labeling efficiency of the digoxigenin-labeled probes for both GAD mRNAs was determined each time by direct immunological detection on dot blots with a nucleic acid detection kit (Boehringer Mannheim). The intensity of the signal for each probe was compared with a serial dilution of digoxigenin-labeled control RNA of known concentration. Only antisense and sense GAD67 and GAD65 probes with comparable signal intensity (comparable labeling efficiency), as determined in dot blots, were used for in situ hybridization.

Hybridization and detection. Free-floating sections were processed for GAD67 and GAD65 in situ hybridization as described previously (Esclapez et al., 1993). To enhance penetration of the probes, sections were pretreated with the following solutions: 0.02 N HCl for 10 minutes; 0.01% Triton X-100 in 1 × PBS for three minutes; and 0.25 μg/ml proteinase K in 50 mM Tris buffer, 5mM ethylene diamine tetraacetate (EDTA), pH 7.4, for 10 minutes. After these pretreatments, sections were incubated for one hour at RT in a prehybridization solution containing 50% formamide, 750 mM NaCl, 25 mM EDTA, 25 mM piperazine-N,N′-bis (2-ethanesulfonic acid) (PIPES), 0.2% sodium dodecyl sulfate (SDS), 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin, 250 μg/ml poly A, and 250 μg/ml salmon sperm DNA. Sections were then incubated for 16 hours at 50°C in the hybridization solution that consisted of the prehybridization solution with the addition of 0.2 ng/μl digoxigenin-labeled RNA probe, 100 mM dithiothreitol (DTT), and 4% dextran sulfate. After hybridization, sections were rinsed in a 4 × saline sodium citrate solution (1 × SSC: 150 mM NaCl, 60 mM Na citrate, pH 7.0) containing 10 mM sodium thiosulfate, and treated with ribonuclease A (50 μg/ml in 0.5 M NaCl, 10 mM sodium thiosulfate, 1 mM EDTA, 10 mM Tris-HCl buffer, pH 8.0) for 30 minutes at 37°C. Low- to high-stringency washes were performed with decreasing concentrations of SSC, ending with an incubation in 0.1 × SSC, 10 mM sodium thiosulfate for 30 minutes at 55°C. Sections were then processed for immunodetection of the digoxigenin label by means of a nucleic acid detection kit (Boehringer Mannheim). The sections were rinsed twice in 100 mM Tris-HCl buffer, 150 mM NaCl, pH 7.5; incubated for one hour in the same buffer containing 0.5% blocking reagent and 0.3% Triton X-100; and then incubated overnight at 4°C in alkaline phosphatase-conjugated sheep antibodies to digoxigenin diluted 1:1,000 in the same buffer containing 0.3% Triton X-100. On the following day, the sections were rinsed thoroughly and incubated in a chromogen solution containing nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) reagents (for details, see Esclapez et al., 1993).

The amount of time in the chromogen solution were determined according to two different protocols. In one series of experiments, sections from control and pilocarpine-treated animals were incubated in the chromogen solution until optimal staining was achieved for each GAD mRNA in each of the two animal groups. Optimal staining was defined as a maximum number of specifically stained neurons (maximum sensitivity) with a low background of general tissue staining and no nonspecific staining of cell bodies. For each probe, the optimal color-reaction times were similar for all sections belonging to the same animal group (control or pilocarpine-treated animals) but very different among sections from the two groups. For a probe concentration of 0.2 ng/μl (the concentration chosen for these experiments), the optimal color-reaction times for most hippocampal regions of control animals were approximately four hours when processed with GAD67 or GAD65 probes. Optimal color-reaction times always were shorter (approximately two hours for GAD65 and three hours for GAD67) for sections from pilocarpine-treated animals, despite the fact that sections from both groups of animals were processed in parallel and under identical experimental conditions. Levels of labeling in relation to the color-reaction times have been discussed previously and are considered to reflect differences in the relative amounts of mRNA (Höltke and Kessler, 1990; Esclapez et al., 1993); neurons with higher levels of mRNA are labeled after a shorter time in the chromogen solution than neurons with lower levels of mRNA when all other conditions are the same. These observations prompted us to conduct more detailed studies to compare directly the differences in levels of labeling of GAD mRNA-containing neurons between pilocarpine-treated rats and control rats.

For these studies, sections from control and pilocarpine-treated animals that were processed for GAD65 or GAD67 mRNA were incubated for identical times in the chromogen solution. For each GAD mRNA, series of sections from control and pilocarpine-treated animals that were processed identically were removed from the chromogen solution at two time intervals (e.g., 2 hours and 4 hours) to compare the labeling intensities among sections from the two animal groups at each interval. The differences in the levels of labeling were determined subsequently by qualitative and quantitative analyses of the intensity of the color-reaction product of GAD mRNA-containing hippocampal neurons.

In all experiments, the color reaction was stopped by rinsing the sections in 10 mM Tris-HCl, pH 8.0, with 1 mM EDTA. Sections were then mounted on gelatin-coated

slides, dried, and coverslipped in an aqueous mounting medium (Crystal/Mount; Biomeda, Foster City, CA).

Immunohistochemistry

Antibodies. The polyclonal antiserum K2, described by Kaufman et al. (1991), was used to localize GAD67. This antiserum was generated in rabbit after injection of GAD67 that was produced in a bacterial expression system from a cloned feline GAD cDNA (Kaufman et al., 1986; Kobayashi et al., 1987) and purified by sodium dodecyl sulfate polyacrylamide gel electrophoresis (Kaufman et al., 1991). This antiserum recognizes primarily rat GAD67 on Western blots (Kaufman et al., 1991) and tissue sections (Esclapez et al., 1994).

The monoclonal antibody GAD-6, obtained by Chang and Gottlieb (1988), was used to localize GAD65. This antibody was produced by a GAD-6 hybridoma line obtained after immunization of a mouse with rat brain GAD. It specifically recognizes rat GAD65 on Western blots (Chang and Gottlieb, 1988; Kaufman et al., 1991) and tissue sections (Esclapez et al., 1994).

Immunohistochemical methods. Sections from control and pilocarpine-treated animals were processed in parallel for GAD67 or GAD65 immunohistochemistry (IHC) by means of unlabeled K2 or GAD-6 antibodies, respectively, and standard avidin-biotin-peroxidase immunolabeling methods (Vectastain Elite ABC, Vector Laboratories, Burlingame, CA), as described previously (Esclapez et al., 1994). The sections were rinsed for 30 minutes in 0.02 M potassium PBS (16.5 mM K_2HPO_4, 3.5 mM KH_2PO_4, 150 mM NaCl, pH 7.4; KPBS). Some sections were then incubated for 1 hour at RT in the same buffer containing 3% normal goat serum (GAD67-IHC) or normal horse serum (GAD65-IHC); other sections were incubated in the same normal serum solutions with the addition of 0.3% Triton X-100. After these steps, sections processed for GAD67-IHC were incubated for 20 hours at RT in K2 antiserum diluted 1:4,000 in KPBS containing 1% normal goat serum. Sections processed for GAD65-IHC were incubated similarly in GAD-6 monoclonal antibody diluted 1:100 in KPBS containing 1% normal horse serum. After these incubations, sections were rinsed for 30 minutes in KPBS; incubated for one hour at RT in biotinylated goat anti-rabbit immunoglobulin G (IgG) or horse anti-mouse IgG, diluted 1:200 in KPBS containing 1% normal goat or horse serum; rinsed in KPBS for 30 minutes; and incubated for one hour at RT with an avidin-biotin-peroxidase solution prepared in KPBS according to the manufacturer's recommendations. After a 30-minute rinse in 0.075 M PBS, pH 7.3, sections from control and pilocarpine-treated rats were processed for the same time (15 minutes) in 0.06% 3–3′-diaminobenzidine-HCl and 0.006% H_2O_2 diluted in PBS. The sections were rinsed in PBS for 30 minutes, treated in 0.05% osmium tetroxide for 30 seconds, rinsed thoroughly in PBS, mounted on gelatin-coated slides, dehydrated, and coverslipped with Permount.

Data analysis

Qualitative studies of the levels of labeling for the two forms of GAD and their mRNAs as well as semiquantitative studies of the labeling intensity of GAD mRNA-containing neurons were conducted in the hippocampal formation to identify differences between control and experimental animals. The quantitative analysis of the labeling intensity of GAD mRNA-containing neurons was performed by densitometric measurements of the intensity of the nonradioactive in situ hybridization signals with an image analyzing system (AIC IM 3000; Analytical Imaging Concepts, Inc., Irvine, CA) according to previously described methods (Larsson et al., 1991; Larsson and Hougaard, 1993, 1994a,b). The image analyzing system used in our study included a personal computer-based computer with an image-processing board containing a 512 × 512 × 8 bit frame buffer, an analog RGB video monitor (Trinitron Color Video monitor; Sony, Park Ridge, NJ), and a monochrome CCD camera (CCD-72 series camera; Dage MTI, Michigan City, IN) that was connected to a Zeiss Axioplan microscope (Zeiss, Thornwood, NY). Image acquisition, processing, and analysis for the densitometric measurements were controlled by using the image AIC software (version 3.00; Analytical Imaging Concepts).

All image acquisitions and subsequent densitometric analyses were performed under the same conditions of light illumination with the microscope light source stabilized at the isobestic wavelength of the NBT-BCIP reaction product (585 nm). Prior to conducting the measurements, the system was calibrated by a set of neutral density filters (Kodak-Eastman, Rochester, NY) to automatically convert gray level values to optical density (OD) units. The correlation between the gray level and the OD values was linear ($r^2 = 0.97$; $y = 0.0039 \times +0.0635$) over the range of 0–0.8 OD in which results were obtained. In addition, the system was calibrated for morphometric measurements that allowed automatic conversion of bit-plane (pixel) units into μm^2.

For each control and pilocarpine-treated animal group, four series of sections that had been processed for GAD65 or GAD67 mRNAs and incubated in the chromogen solution for two different time intervals (2 hours and 4 hours) were analyzed in parallel. A first analysis was conducted to determine differences in the labeling intensity among labeled neurons from different regions of the hippocampal formation, including CA1, CA3, and the dentate gyrus. This analysis was performed on selected sections from both control and pilocarpine-treated animals. Statistical analysis with nonparametric tests (Mann-Whitney U-test) did not indicate differences in neuronal labeling intensity among these three main regions of the hippocampal formation in the series of sections from each group. Thus, for the additional quantitative analyses of the labeling intensity, no distinctions were made between the hippocampal regions.

For each series of sections from control and pilocarpine-treated rats, quantitative data were obtained from all GAD mRNA-containing neurons of the hippocampus on both sides in two or three sections from each animal. Image acquisitions of three or four microscopic fields were necessary to analyze the labeling intensity of all GAD-mRNA-containing neurons from each hippocampus with a ×5 objective. For each hippocampal field image, the labeled area (cytoplasm) of GAD mRNA-containing neurons was selected by computerized object threshold procedures. The average gray level values of labeled neurons and of background, as well as the corrected gray level value (average gray level value of labeled neurons minus average gray level value of background) and its corresponding OD value and standard deviations, were calculated automatically by using the image analysis software. All steps involved in these procedures have been described in detail

(Larsson et al., 1991). The mean intensity of labeling that corresponded to the mean corrected gray level value obtained from the total number of hippocampal fields was calculated for each series of control and pilocarpine-treated rats. Corresponding OD values and standard errors (S.E.M.) were determined. Statistical analyses of differences in the intensity of labeling for GAD65 and GAD67 mRNAs at the two time intervals in the chromogen solution (two hours and four hours) between control and pilocarpine-treated animals were performed with a mixed-model analysis of variance (ANOVA) and Student's t-test.

RESULTS

In pilocarpine-treated animals with spontaneous recurrent seizures, a marked and consistent cell loss was observed in the hilus of the dentate gyrus and, to a lesser extent, in the CA3c pyramidal cell layer (Fig. 1A; compare with control, Fig. 1B). A similar pattern of cell loss has been described previously in this pilocarpine model (Turski et al., 1986; Obenaus et al., 1993). The loss of hilar neurons contrasted with the relatively good preservation of dentate granule cells and pyramidal cells of CA1 and most of CA3 (Fig. 1A). Furthermore, in adjacent sections processed for in situ hybridization for GAD65 or GAD67 mRNAs, a discrete but significant decrease in the number of GAD mRNA-containing neurons was observed consistently in the hilus of all chronic pilocarpine-treated animals (Fig. 1C; compare with control, Fig. 1D). Despite this marked hilar cell loss, many GAD mRNA-containing neurons were present in the hippocampal formation (Fig. 1C). These remaining neurons included GAD mRNA-containing neurons in all hippocampal regions and neurons in the dentate gyrus, such as those in the molecular layer, neurons along the inner border of the granule cell layer (presumed basket cells), and a few neurons in the deep hilus (Fig. 1C).

Increased GAD mRNA labeling in remaining hippocampal neurons of chronic pilocarpine-treated rats

Remaining GAD mRNA-containing neurons in the hippocampal formation of chronic pilocarpine-treated rats exhibited marked differences in the levels of GAD mRNA labeling compared with the levels in control animals. Differences in the intensity of labeling were evident among sections from all pilocarpine-treated and control rats for both GAD mRNAs.

Qualitative analysis. Clear increases in the intensity of neuronal labeling for GAD65 and GAD67 mRNAs were observed consistently in all pilocarpine-treated animals compared with control animals. Such increases were quite prominent for GAD65 mRNA, and the clearest indication of these increases was provided when sections were processed for a relatively short time (2 hours) in the chromogen solution. After short color-reaction times (2 hours), marked differences in the labeling intensity were observed among sections from pilocarpine-treated and control rats (compare Fig. 2A with Fig. 2B). In sections from pilocarpine-treated rats, remaining GAD mRNA-containing neurons within the hippocampal formation were labeled distinctly, and virtually all of these neurons exhibited a strong level of labeling (Fig. 2A). Such strong labeling was observed for GAD65 mRNA-containing neurons in the hippocampus as well as in the dentate gyrus, where the strongly labeled neurons included those along the inner border of the granule cell layer and the remaining neurons in the deep hilus (Fig. 2A). In sections from control animals, many GAD65 mRNA-containing neurons were detected within the hippocampal formation and were highly concentrated in the hilus of the dentate gyrus, but these neurons were lightly labeled for GAD65 mRNA (Fig. 2B). Thus, after short times in the chromogen solution, the strong intensities of labeling for GAD65 mRNA-containing neurons in pilocarpine-treated animals contrasted with relatively low levels of labeling of GAD65 mRNA-containing neurons in control animals (compare Fig. 2A with Fig. 2B).

After longer color-reaction times (4 hours), differences in the levels of labeling among sections from pilocarpine-treated and control rats also were observed (compare Fig. 3A with Fig. 3B). However, these differences were less marked than when sections were processed for a shorter time in the chromogen solution. In sections from control rats, after the longer incubation time, virtually all labeled neurons in the hippocampus and dentate gyrus displayed an intense reaction product for GAD65 mRNA (Fig. 3B), and this level of labeling clearly was higher than that in sections processed in parallel but for only two hours in the chromogen solution (compare Fig. 3B with Fig. 2B). In sections from pilocarpine-treated animals, the entire population of labeled neurons within the hippocampal formation exhibited very strong levels of labeling for GAD65 mRNA (Fig. 3A). The intensities of labeling of GAD65 mRNA-containing neurons were higher than those observed at shorter color-reaction times (compare Fig. 3A with Fig. 2A). However, the labeling intensities for the more heavily labeled neurons appeared to have reached saturation levels. Despite the strong labeling observed in all sections, labeling intensities of GAD65 mRNA-containing neurons in control animals were lower than those in pilocarpine-treated animals (compare Fig. 3A with Fig. 3B). In addition, the size of many of these GAD65 mRNA-containing neurons within the different layers of the dentate gyrus as well as CA1–CA3 appeared larger than that of labeled neurons in control specimens (compare Fig. 3A with Fig. 3B).

An increase in neuronal labeling also was detected for GAD67 mRNA in hippocampal neurons of pilocarpine-treated rats (Fig. 4A). However, differences in the intensity of labeling for GAD67 mRNA-containing neurons among sections from pilocarpine-treated and control rats appeared less than those observed for GAD65 mRNA (Fig. 4). After a short time in the chromogen solution (2 hours), relatively small numbers of GAD67 mRNA-containing neurons were detected throughout the hippocampal formation of control rats, and these neurons were lightly labeled for GAD67 mRNA (data not shown). In pilocarpine-treated animals, most of the remaining GABA neurons were labeled but exhibited a low intensity of labeling (data not shown). When sections were processed for an intermediate time (3 hours) in the chromogen reaction, neurons distributed throughout all layers of the dentate gyrus and CA1–CA3 hippocampal regions exhibited light-to-moderate levels of labeling for GAD67 mRNA in control rats (Fig. 4B). In sections from pilocarpine-treated rats, the majority of the neurons showed moderate-to-high intensities of labeling for GAD67 mRNA (Fig. 4A). After a longer color-reaction time (4 hours), levels of labeling for both pilocarpine-treated and control rats increased, and GAD67 mRNA-

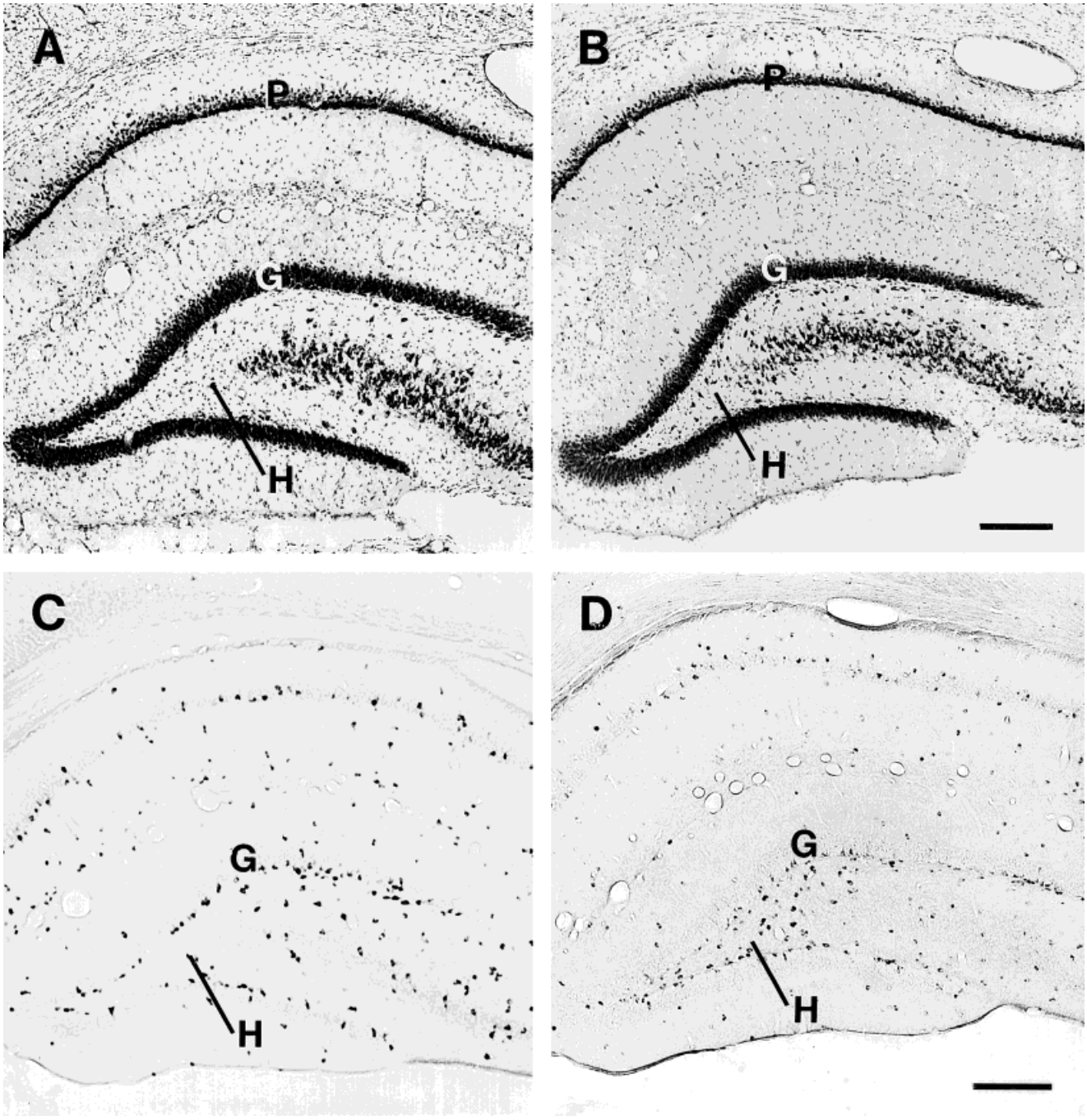

Fig. 1. Differential vulnerability of hippocampal neurons in pilocarpine-treated animals with chronic limbic seizures, as indicated by cresyl violet-stained (A,B) and glutamic acid decarboxylase 65 (GAD65) mRNA-labeled (C,D) sections from pilocarpine-treated (A,C) and control (B,D) rats. **A:** In a pilocarpine-treated rat, a marked reduction in the number of neurons is observed in the hilus (H) of the dentate gyrus (compare A with B). This cell loss contrasts with a good preservation of the granule cells (G) of the dentate gyrus and pyramidal cells (P) of the hippocampus. **B:** In a control rat, many neurons are present in the hilus (H). **C:** In a pilocarpine-treated rat, the number of GAD65 mRNA-containing neurons in the hilus (H) of the dentate gyrus is reduced compared with the control animal (compare C with D), but many GAD65 mRNA-containing neurons remain within the hippocampal formation. In the dentate gyrus, these remaining neurons are located mainly along the inner border of the granule cell layer (G), but a few labeled neurons also are observed in the hilus (H). **D:** In a control rat, GAD65 mRNA-containing neurons are distributed within all layers of the hippocampal formation and are highly concentrated along the inner border of the granule cell layer (G) and in the hilus (H). The sections in C and D were processed for two hours and 3.5 hours, respectively, in the chromogen solution. Scale bars = 250 μm in B (also applies to A) and D (also applies to C).

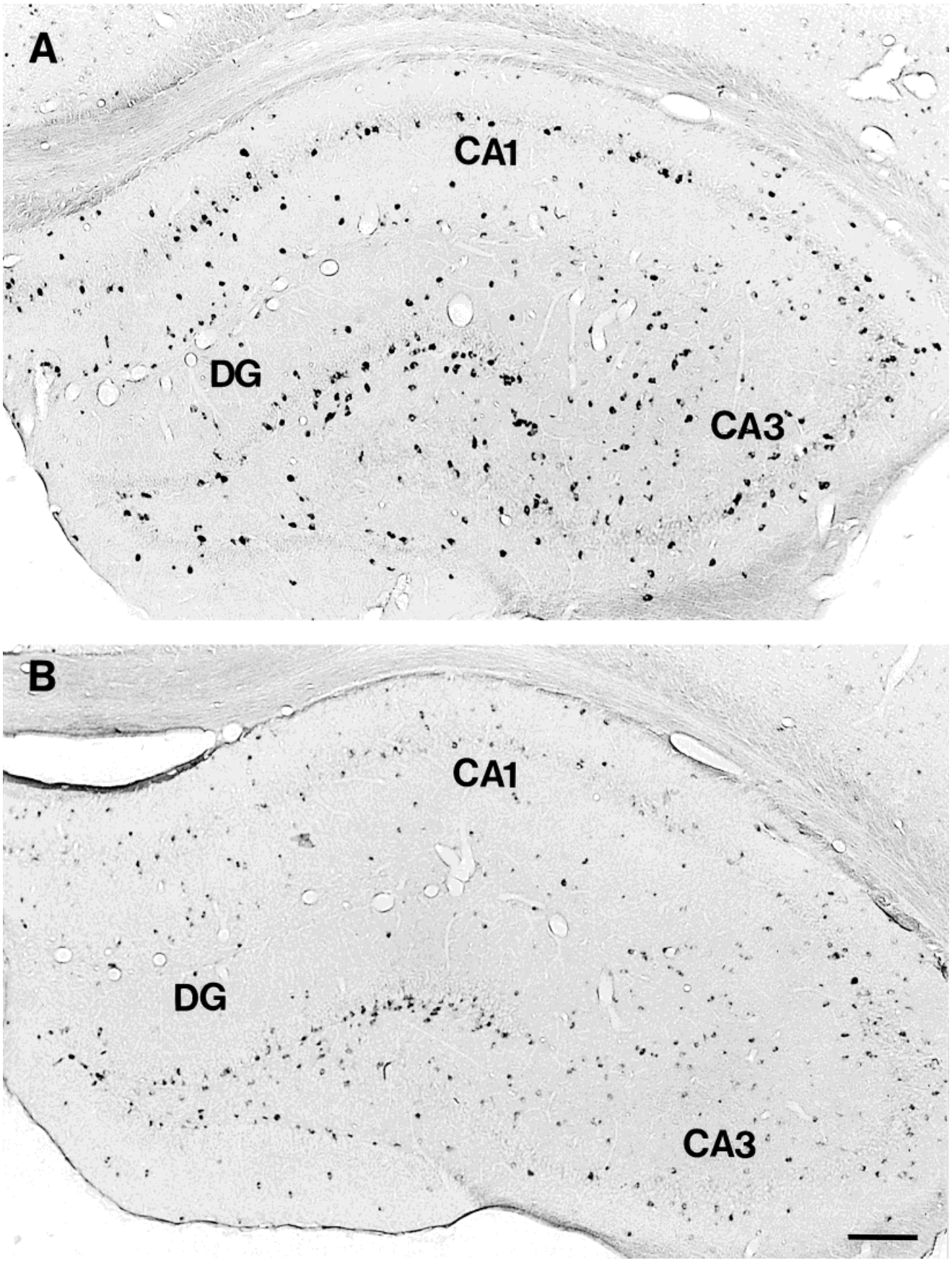

Figure 2

containing neurons in both groups exhibited moderate-to-strong levels of labeling. At the longer time, differences between the two animals groups were less apparent.

In pilocarpine-treated animals, the hippocampal formation was the only brain region within the forebrain sections surveyed that exhibited consistent changes in the levels of labeling for GAD65 and GAD67 mRNAs. No differences were observed in regions like the reticular nucleus of the thalamus, but increases in labeling intensity for GAD65 or GAD67 mRNA occasionally were detected in other brain regions, such as the cerebral cortex (Fig. 5). However, in contrast to the hippocampal formation, such changes were not observed in all pilocarpine-treated animals. In addition, when present, the increases in labeling intensity always were less pronounced than those in the hippocampus and dentate gyrus.

Quantitative analysis. Because differences were observed consistently in the hippocampal formation, we conducted semiquantitative analyses to determine the relative extent of the increases in labeling intensity for GAD65 and GAD67 mRNAs in this region of the pilocarpine-treated rats. The quantitative data showed that, at all time intervals, the mean intensities of labeling of GAD65 mRNA-containing neurons (Fig. 6A) as well as GAD67 mRNA-containing neurons (Fig. 6B) were higher in the pilocarpine-treated animals than in control animals (Table 1, Fig. 6). Statistically significant increases ($P < 0.001$) in the mean intensity of labeling were observed for both GAD mRNAs (76% for GAD65 mRNA; 52% for GAD67 mRNA) when sections were processed for a short time in the chromogen solution (Table 1, Fig. 6A). When sections were processed for a longer time in the color reaction, increases in the mean intensities of neuronal labeling were observed for both GAD65 and GAD67 mRNA in control rats as well as pilocarpine-treated rats (Table 1). However, the differences in neuronal labeling among sections from pilocarpine-treated and control rats were fewer than those found in sections that were processed for the shorter color-reaction time (Table 1). Also, after this amount of time in the chromogen solution, the labeling intensities for GAD65 mRNA in the control and experimental groups were no longer significantly different. The discrepancies in the findings at short and long color-reaction times reflect the fact that, after the longer time, the labeling intensities for the GAD65 and GAD67-containing neurons already had reached saturation levels in the pilocarpine-treated rats. Thus, the semiquantitative analysis underestimated differences between the two animal groups at the longer time.

Fig. 2. Comparison of labeling for GAD65 mRNA in coronal sections of the hippocampal formation from chronic pilocarpine-treated (A) and control (B) rats that were processed for a short time (two hours) in the chromogen solution. **A:** In a section from a pilocarpine-treated rat, many GAD65 mRNA-labeled neurons are evident throughout all layers of the dentate gyrus (DG) and CA1–CA3, and all of these neurons exhibit strong levels of labeling for GAD65 mRNA. **B:** In a control rat section that was processed in parallel and for the same amount of time in the chromogen solution, many GAD65 mRNA-containing neurons are present within the hippocampal formation. However, these neurons are labeled lightly for GAD65 mRNA. After this short color-reaction time, differences in levels of labeling for GAD65 mRNA among sections from pilocarpine-treated and control animals are particularly striking, with levels of labeling in pilocarpine-treated rats that are significantly higher than those in control rats (compare A with B). Scale bar = 200 μm.

Increases in immunohistochemical labeling for both GAD isoforms in chronic pilocarpine-treated animals

The immunohistochemical labeling for GAD65 and GAD67 in the hippocampal formation of control rats will be described briefly to compare this labeling to that of pilocarpine-treated rats. In control animals, both the GAD65 antibody and the GAD67 antiserum labeled neuronal cell bodies and terminals within the hippocampal formation. However, in most hippocampal regions, the antibody to GAD65 labeled terminal fields particularly well (Fig. 7B,D), whereas the antiserum to GAD67 labeled cell bodies most readily (Fig. 8B). For both GAD isoforms, labeled terminals were present in the cell body layer as well as in all dendritic regions of the hippocampal formation (Figs. 7B,D, 8B). In addition, for both GAD isoforms, labeled terminals were concentrated most highly within the pyramidal cell layer of the hippocampus and around the outer part of the granule cell layer of the dentate gyrus (Figs. 7D, 8B). Furthermore, extensive fields of GAD65-containing terminals were observed within the dendritic layers of the hippocampal formation. High densities of GAD65-labeled terminals were present in the outer one-third of the molecular layer of the dentate gyrus and at the border between the stratum lacunosum-moleculare and the stratum radiatum of the hippocampus, whereas lower densities were observed in the other layers (Fig. 7D). GAD67-containing cell bodies were observed within all layers of the hippocampal formation (Fig. 8B). In contrast, with the processing methods used in this study, only a few GAD65-containing cell bodies were detected in most layers of the hippocampus (Fig. 7B). However, in the hilus of the dentate gyrus, both GAD65- and GAD67-immunolabeled cell bodies were evident.

In the pilocarpine-treated rats, a clear increase in GAD65 immunoreactivity was observed in virtually all regions of the hippocampal formation (Fig. 7A,C). This increase in GAD65 immunoreactivity was due primarily to an increase in the labeling of GAD65-containing terminals. Except for differences in the levels of labeling, the overall laminar patterns of labeling for GAD65-containing terminals generally were similar in pilocarpine-treated and control rats. However, two major differences were observed consistently. The levels of labeling were increased in the inner molecular layer of the dentate gyrus to a much greater extent than in the middle part of this layer. In addition, labeling in the stratum lacunosum moleculare was increased more than that in the other layers of the hippocampus (Fig. 7C). However, a mild-to-moderate shrinkage of this layer was observed in all pilocarpine-treated animals and presumably contributed to the increased immunoreactivity in this region. In addition to distinct increases in GAD65 immunoreactivity, slight increases in labeling for GAD67-containing terminals were observed in the hippocampal formation. Such increases were most prominent in the pyramidal cell layer of the hippocampus and in the inner one-third of the molecular layer (Fig. 8A). Remaining GAD67-containing cell bodies were labeled distinctly in all layers of the hippocampal formation. (Fig. 8A), but no obvious differences in the intensity of labeling of these neurons were detected between pilocarpine-treated and control rats. No clear differences in immunolabeling intensity for GAD65 and GAD67 were detected in other brain regions, except occasionally in

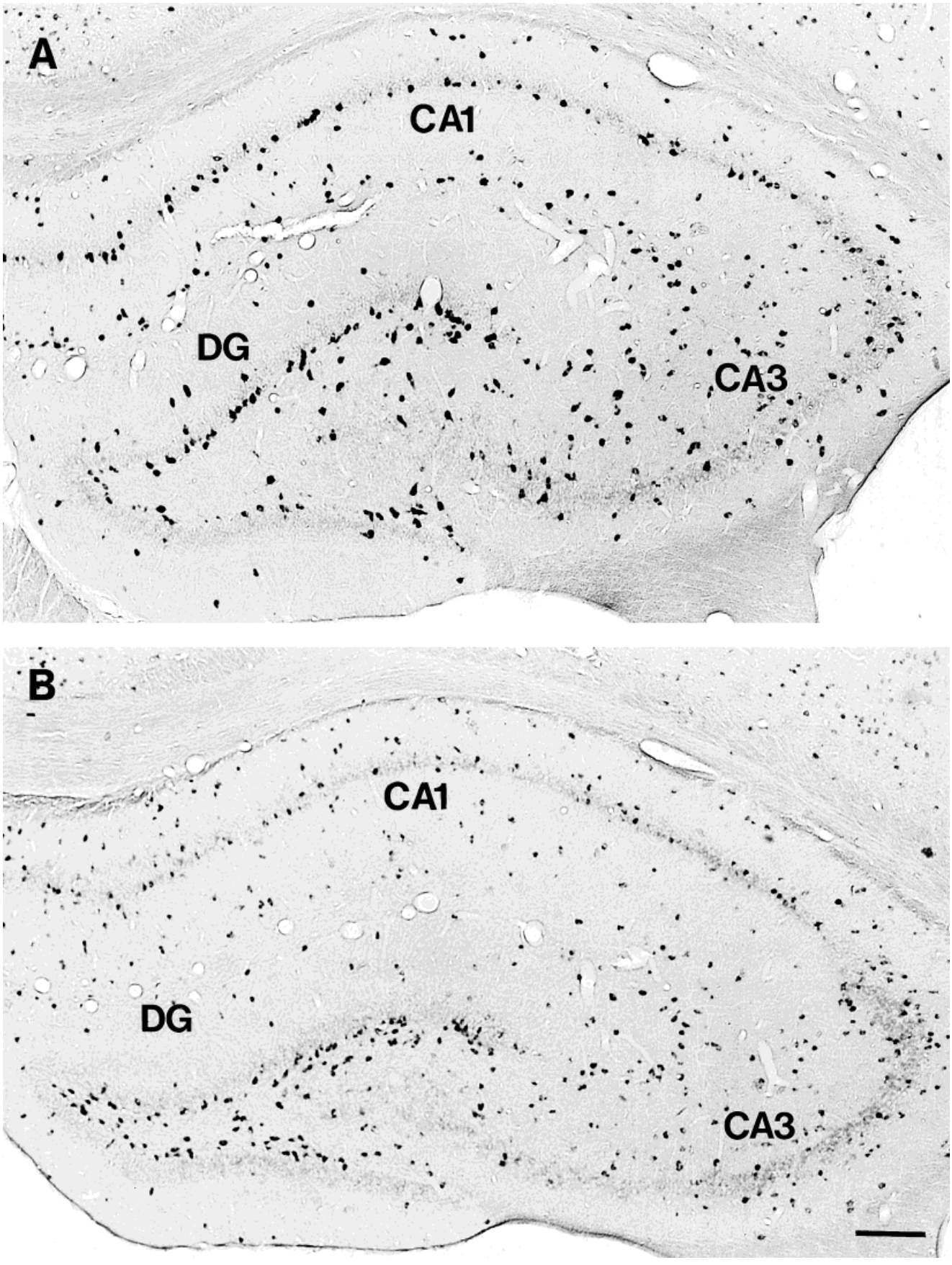

Figure 3

the cerebral cortex of some pilocarpine-treated animals, as described for the GAD mRNAs.

DISCUSSION

This study demonstrates that the two isoforms of GAD are up-regulated at both gene and protein levels in remaining GABA neurons of the hippocampal formation in pilocarpine-treated rats with chronic limbic seizures. There were marked increases in the relative amounts of GAD65 and GAD67 mRNAs in all hippocampal GAD mRNA-containing neurons, as demonstrated by qualitative and semiquantitative analysis of nonradioactive in situ hybridization data. In addition, there were parallel increases in immunohistochemical terminal labeling for both GAD65 and GAD67 isoforms, with particularly striking increases for GAD65.

These findings provide further evidence for multiple changes in the GABA system in a model of temporal lobe epilepsy. Clear loss of some populations of GAD-containing neurons has been described previously in the hilus of the dentate gyrus and in the stratum oriens of CA1 in this and similar models (Obenaus et al., 1993; Houser and Esclapez, 1996; Buckmaster, 1998; Morin et al., 1998), and such loss of GAD-containing neurons is confirmed in the present study. Thus, in at least two specific regions of the hippocampal formation, a high proportion of GABA neurons is damaged. However, substantial numbers of GABA neurons remain in these and other regions of the hippocampal formation, and these neurons are the focus of the present study. The discussion below will examine some methodological issues related to the interpretation of the data and will then consider the regulation of the two GAD isoforms and the functional significance of the up-regulation of the GAD mRNAs and proteins.

Methodological considerations

Our data demonstrate that, within the hippocampal formation, the intensities of labeling for GAD mRNA-containing neurons were consistently stronger in pilocarpine-treated rats than in control animals, and we view these findings as an indication of increased levels of GAD mRNAs in the hippocampal GABA neurons of pilocarpine-treated animals with chronic limbic seizures. This interpretation is consistent with previous biochemical studies, which have shown that differences in the intensity of labeling with nonradioactive hybridization methods reflect differences in the relative amounts of mRNA among neurons, just as different concentrations of silver grains, when using radioactive methods, indicate different amounts of mRNA (Höltke and Kessler, 1990; Esclapez et al., 1993). In the forebrain sections that were examined, the hippocampal formation was the only brain region to display consistent differences in the intensities of GAD mRNA labeling between control and pilocarpine-treated animals. In other regions, such as the reticular nucleus of the thalamus, levels of neuronal labeling were similar in the two animal groups. These findings argue against the suggestion that differences in labeling intensities between the two groups of animals reflect variability of labeling from section to section.

In addition, the present study confirms that nonradioactive in situ hybridization combined with computer-assisted measurements of intensities of labeling (gray level values) can be use for quantification of relative amounts of mRNA, as demonstrated previously by several groups that used similar systems of detection (Larsson and Hougaard, 1990, 1993, 1994a,b; Larsson et al., 1991; Guiot and Rahier, 1995; Zhang et al., 1995). The quantitative analysis of gray level values, as suggested by the qualitative data, demonstrated that the mean intensities of labeling of hippocampal neurons were significantly higher for GAD65 and GAD67 mRNAs in the pilocarpine-treated animals than in control rats. Even though mean intensities of neuronal labeling can be measured with this technique, they reflect relative levels of mRNA that may not be related directly to absolute concentrations of mRNA, because the enzymatic and immunohistochemical methods for mRNA detection are not linear reactions. However, measurements of gray level values allow quantitative analysis of relative differences in intensities of neuronal labeling (Larsson et al., 1991).

Our data also illustrate that the sensitivity of the methods for quantitative and qualitative discrimination of differences in neuronal labeling between control and pilocarpine-treated rats is dependent on the time of chromogen development, and this must be determined for each probe and detection system, as described previously (Larsson et al., 1991; Zhang et al., 1995). For both GAD mRNAs, marked differences in neuronal labeling among sections from control and pilocarpine-treated rats were evident when sections were processed for the shortest color-reaction time selected for use in this study (2 hours in the chromogen solution). Such differences were much less evident for both GAD mRNAs when sections were processed for an extended time in the chromogen solution (four hours under the conditions of the present study). After the extended times, the labeling intensities for GAD65 mRNA in the control and experimental groups were no longer significantly different, although small differences still were observed for GAD67 mRNA. This indicated that the labeling intensities for the GAD mRNA-containing neurons in the pilocarpine-treated animals had already reached, or were approaching, saturation levels.

The optimal times of color development for which differences of labeling intensities could be detected were very similar for the two GAD isoforms, and this was expected, because similar levels of mRNA for GAD65 and GAD67 are found in the hippocampal formation of control rats (Houser and Esclapez, 1994b; present results); the lengths of the

Fig. 3. Comparison of labeling for GAD65 mRNA in coronal sections of the hippocampal formation from chronic pilocarpine-treated (A) and control (B) rats that were processed for a standard color-reaction time (four hours). **A:** In a section from a pilocarpine-treated rat, the entire population of labeled neurons within the hippocampal formation exhibits very strong levels of labeling for GAD65 mRNA. In addition, the sizes of many of these GAD65 mRNA-containing neurons, within the different layers of the dentate gyrus (DG) as well as in CA1–CA3 regions, appear larger than those in a control specimen (compare A with B). **B:** In a section from a control animal, many neurons are labeled strongly for GAD65 mRNA. This labeling is significantly higher than that observed in the adjacent section processed in parallel for 2 hours in the chromogen solution (compare B with Fig. 2B). Despite this relatively strong labeling, the levels of labeling as well as the sizes of GAD65 mRNA-containing neurons are slightly less than the levels in pilocarpine-treated rats (compare B with A). At longer times in the chromogen solution, the differences in the levels of labeling in pilocarpine-treated and control rats are less obvious than in sections processed for 2 hours (compare A and B with Fig. 2). Scale bar = 200 μm.

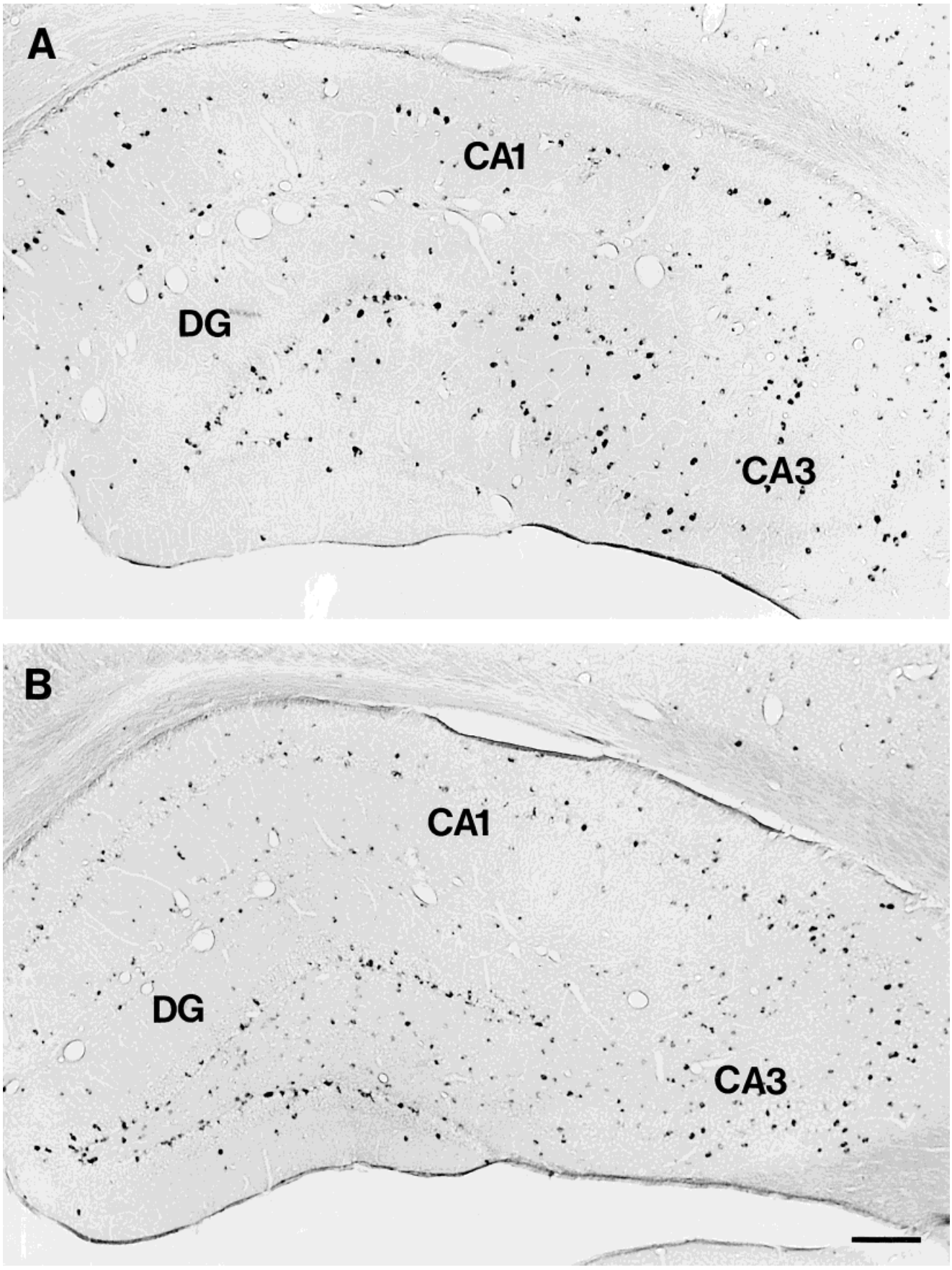

Fig. 4. Comparison of labeling for GAD67 mRNA in coronal sections of the hippocampal formation from chronic pilocarpine-treated (A) and control (B) rats processed for an intermediate color-reaction time (three hours). **A:** In a section from a pilocarpine-treated rat, the majority of neurons distributed throughout all layers of the dentate gyrus (DG) and CA1–CA3 are labeled strongly for GAD67 mRNA. **B:** In a control section that was processed in parallel, neurons exhibit light-to-moderate labeling for GAD67 mRNA. Thus, a slight increase in the labeling for GAD67 mRNA is observed in pilocarpine-treated rats (compare A with B). Scale bar = 200 μm.

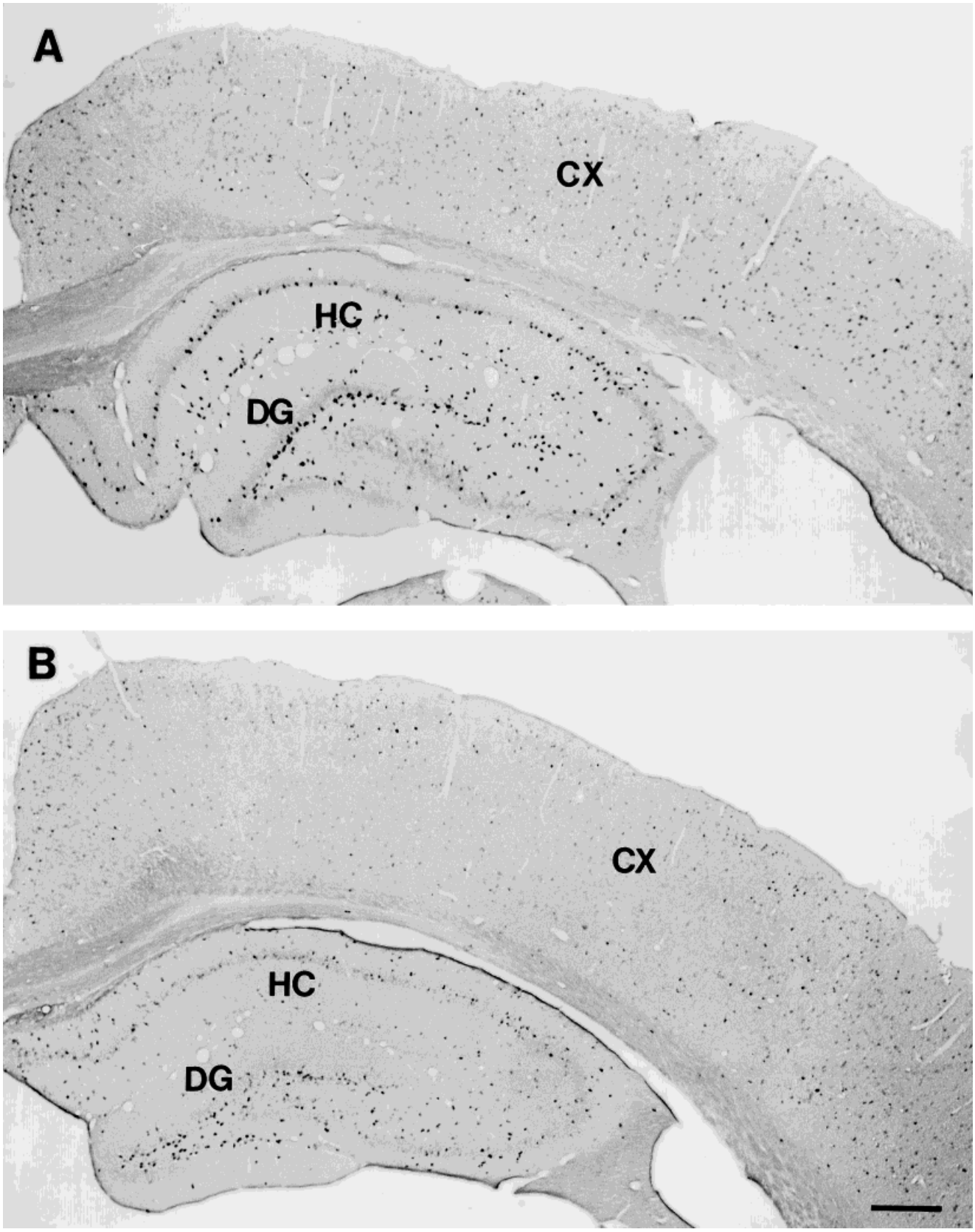

Fig. 5. Comparison of labeling for GAD65 mRNA in coronal sections of the cerebral cortex and the hippocampal formation from pilocarpine-treated (A) and control (B) rats processed for 2 hours in the color reaction. **A:** In a pilocarpine-treated rat, high levels of labeling for GAD65 mRNA are observed for the neurons in the hippocampus (HC) and dentate gyrus (DG), whereas moderate levels of labeling are found in neurons of the cerebral cortex (CX). A strong increase in labeling is found consistently in the hippocampal formation of pilocarpine-treated rats (compare A with B). A slight increase in labeling was detected in the cerebral cortex of this rat, but this was not detected in all pilocarpine-treated rats (compare A with B). **B:** In a section from a control rat, GAD65 mRNA-containing neurons are labeled distinctly in the hippocampus (HC) and dentate gyrus (DG). In contrast, many GAD65 mRNA-containing neurons in the cerebral cortex (CX) are labeled only lightly after this short color-reaction time. Scale bar = 400 µm.

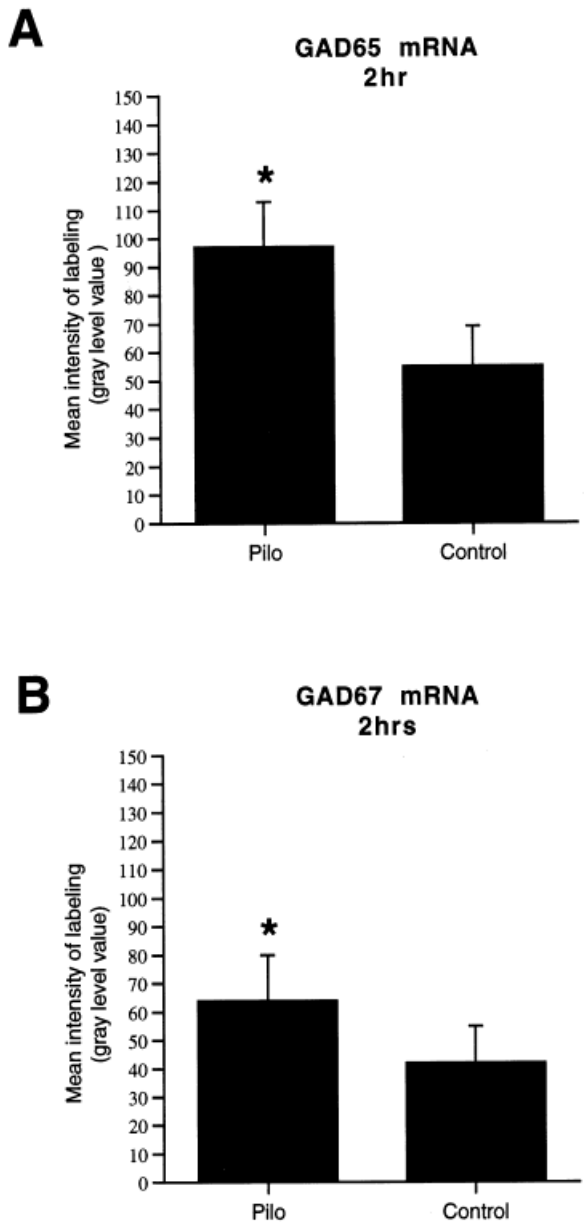

Fig. 6. Histograms comparing the mean intensities of labeling for GAD65 (**A**) and GAD67 (**B**) mRNAs in pilocarpine-treated and control rats obtained from sections processed for 2 hours in the chromogen solution. Statistically significant increases (asterisks; $P < 0.001$) in the mean intensity of neuronal labeling are found for both GAD65 (A) and GAD67 (B) mRNAs in the pilocarpine-treated animals when sections are processed for this short time in the chromogen solution. Error bars indicate S.E.M.

probes and the incorporation levels of digoxigenin-labeled UTP during transcription are similar for the two GAD RNAs (Esclapez et al., 1993); and all other experimental conditions, including the concentrations of the probes, were identical. These methodological considerations allowed us to compare the relative differences in neuronal labeling among sections from control and pilocarpine-treated rats for GAD65 and GAD67 mRNAs and, thus, to determine increases of mRNA levels for both GAD65 (76%) and GAD67 (52%) in hippocampal GABA neurons from pilocarpine-treated rats.

TABLE 1. Comparison of the Mean Intensity of Labeling for GAD 65 and 67 mRNAs in Chronic Pilocarpine-Treated and Control Rats Obtained from Sections Processed for Two Different Lengths of Time in the Chromogen Solution[1]

Time	Mean intensity of labeling in pilocarpine-treated rats-Gray-level value (OD value)	Mean intensity of labeling in control rats-Gray level value (OD value)	Percent difference in pilo-carpine vs. control rats	*T* values	*P* values
GAD65 mRNA					
2 hours	97 ± 16 (0.31)	55 ± 14 (0.15)	+76%	14.21	<0.001
4 hours	116 ± 13 (0.38)	107 ± 14 (0.35)	+8%	1.51	NS
GAD67 mRNA					
2 hours	64 ± 16 (0.18)	42 ± 13 (0.10)	+52%	12.04	<0.001
4 hours	86 ± 16 (0.27)	73 ± 15 (0.22)	+18%	6.53	<0.01

[1]GAD, glutamate decarboxylase; OD, optical density; NS, not significant.

Increased expression of the two GAD mRNAs

This is the first demonstration of the long-term up-regulation of both GAD mRNAs in the remaining GABA neurons of the hippocampal formation in the pilocarpine model of recurrent seizures. However, increased expression for GAD67 mRNA (Feldblum et al., 1990; Schwarzer and Sperk, 1995) and GAD65 nRNA (Schwarzer and Sperk, 1995) have been described previously in two kainate models of chronic seizures. Thus, such changes appear to be common features of GABA interneurons in animals with spontaneous limbic seizures.

The increase in expression levels for GAD65 as well as GAD67 mRNAs in the chronic pilocarpine-treated rats was of particular interest. Previous studies have demonstrated that the levels of GAD67 mRNA can be regulated in several brain regions after lesions and pharmacological alterations (Caboche et al., 1991, 1992; Soghomonian and Chesselet, 1992; Soghomonian et al., 1992; McCarthy et al., 1995; Delfs et al., 1996; Drengler et al., 1996; Falkenberg et al., 1997). Regulation of GAD65 mRNA levels has been observed less commonly under such experimental conditions (Soghomonian and Chesselet, 1992; Soghomonian et al., 1992). These results, combined with studies showing that the two GAD enzymes differ in their affinity for the cofactor pyridoxal-phosphate (Kaufman et al., 1991), have led to suggestions that GAD65 activity may be regulated by cofactor availability, whereas GAD67 activity may be regulated primarily through changes in gene expression (Rimvall et al., 1993; Rimvall and Martin, 1994). However, recent pharmacological studies have demonstrated that the GAD65 isoform also can be regulated at the gene-expression level in some subpopulations of GABA neurons in other brain regions that included the striatum and several hypothalamic nuclei (Laprade and Soghomonian, 1995a,b, 1997; McCarthy et al., 1995; Bowers et al., 1998). Our data provide strong evidence that, under physiological (pathophysiological) conditions, both GAD65 and GAD67 can be regulated at the gene-expression level.

Increased expression of GAD proteins

In addition, our data demonstrate an increase in immunolabeling of GAD-containing terminals in all layers of the hippocampal formation in pilocarpine-treated animals. Our interpretation of this finding is that the increased levels of immunoreactivity reflect increased levels (concen-

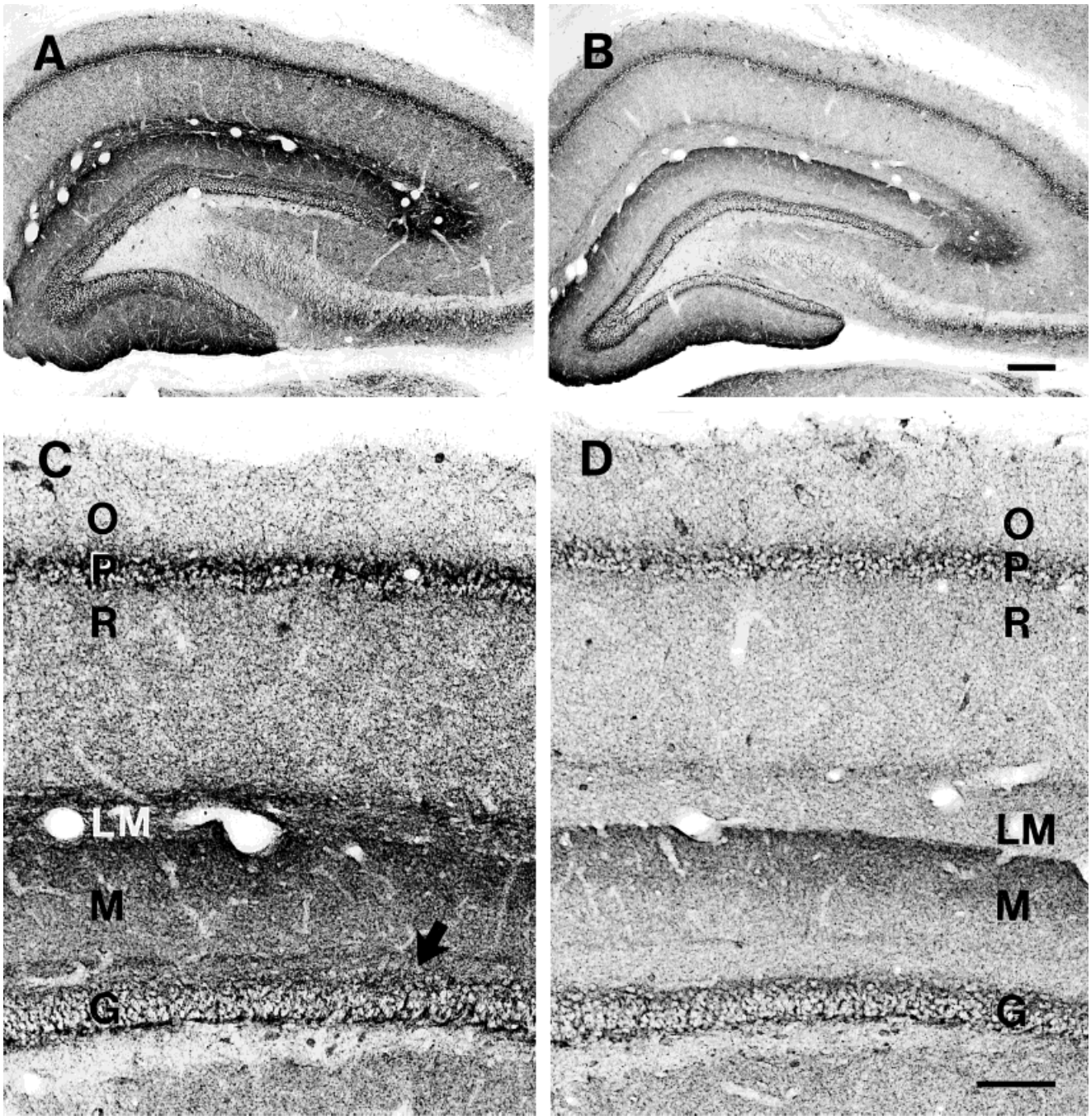

Fig. 7. Comparison of immunohistochemical labeling for GAD65 in coronal sections of the hippocampal formation from pilocarpine-treated (A,C) and control (B,D) rats. **A:** GAD65 immunoreactivity is increased in virtually all regions of the hippocampal formation in pilocarpine-treated animals (compare A with B). **B:** In control animals, GAD65 immunoreactivity, which is concentrated primarily in terminals, is present in cell body layers as well as in all dendritic regions of the hippocampal formation. **C:** In pilocarpine-treated rats, GAD65 immunoreactivity is increased in all layers of the hippocampus, including the stratum oriens (O), stratum pyramidale (P), and stratum radiatum (R), as well as in the molecular layer (M) of the dentate gyrus. Labeling is increased to a greater extent in the inner molecular layer (arrow) than in the middle molecular layer. Likewise, the increase in labeling is greater in the stratum lacunosum-moleculare (LM) than in the other layers of the hippocampus, and shrinkage of this layer also is evident. **D:** In control rats, the highest densities of GAD65-labeled terminals are found in the pyramidal cell (P) and granule cell (G) layers as well as in the outer one-third of the molecular layer (M). A few GAD65-labeled neurons also are evident in all layers of the hippocampal formation. Scale bars = 200 μm in B (also applies to A); 100 μm in D (also applies to C).

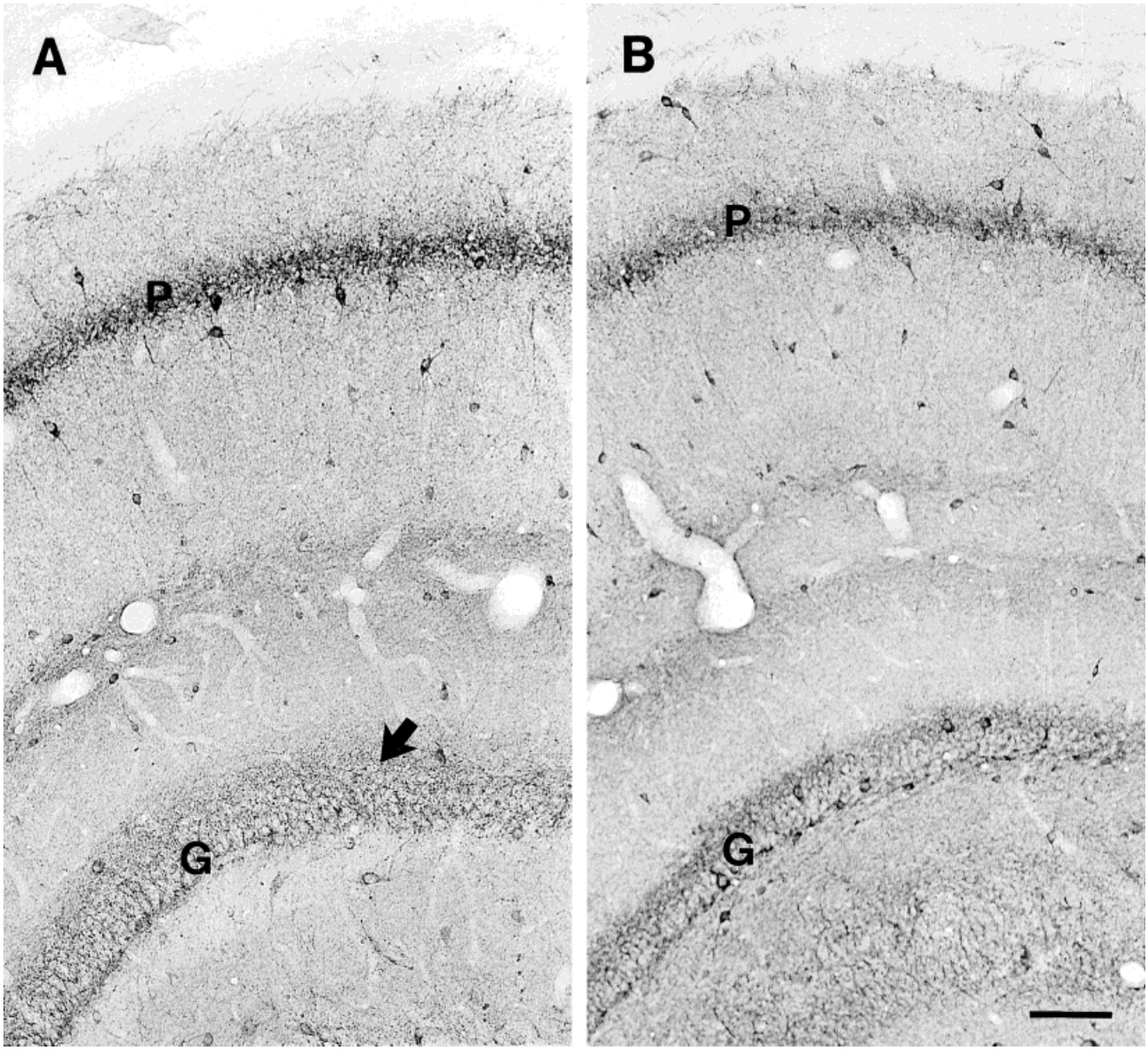

Fig. 8. Comparison of immunohistochemical labeling for GAD67 in coronal sections of the hippocampal formation from pilocarpine-treated (A) and control (B) rats. **A:** GAD67 immunoreactivity is slightly increased in chronic pilocarpine-treated animals (compare A with B). This increase is observed mainly in the pyramidal cell (P) layer of the hippocampus and in the inner one-third of the molecular layer (arrow). **B:** In control rats, GAD67-containing terminals are most abundant in cell body layers that include the pyramidal cell (P) and granule cell (G) layers but are also present in all dendritic regions of the hippocampal formation. Despite relatively similar patterns of distribution for GAD65- and GAD67-labeled terminals, the density (quantity) of GAD67-labeled terminals is much lower than that of GAD65-labeled terminals (compare Fig. 7 with Fig. 8). In contrast, many more GAD67-labeled than GAD65-labeled cell bodies are observed throughout the different layers of the hippocampal formation (compare A and B with Fig. 7). Scale bar = 100 μm.

trations) of GAD proteins in the terminals. This is based primarily on the fact that there is a strong parallel between the increased intensity of terminal labeling and the levels of mRNA for the two GAD isoforms. In particular, the distinct increase in the labeling of GAD65-containing terminals is well correlated with the substantial increase in the expression of GAD65 mRNA.

An alternate explanation for the increased levels of GAD immunoreactivity in the hippocampal formation of pilocarpine-treated animals could be an increase in the density (number) of GAD-containing terminals rather than an increase in the amount of protein in the terminals, and some of our results are consistent with this suggestion. For example, differences in the patterns of labeling for GAD-containing terminals were observed in the molecular layer of the dentate gyrus and stratum lacunosum moleculare of control and pilocarpine-treated animals. In the pilocarpine-treated animals, the levels of labeling for GAD65 and GAD67 are increased substantially more in the inner one-third of the molecular layer and in the lacunosum

moleculare than in the other layers. These differences could reflect an increase in the numbers of terminals in these regions due to possible axonal reorganization of surviving GABA neurons, similar to the sprouting of mossy fibers (Nadler et al., 1981; Cronin and Dudek, 1988; Sutula et al., 1988; Mello et al., 1993). Such axonal reorganization of GABA neurons has been suggested by previous immunohistochemical studies of the Kainate model and human temporal lobe epilepsy tissue (Davenport et al., 1990; Mathern et al., 1995). It is difficult presently to distinguish axonal reorganization of remaining GABA neurons from increased levels of GAD in existing terminals, and both types of changes may occur in the pilocarpine-treated animals.

The strong up-regulation of GAD65 and GAD67 mRNAs and the increased levels of the respective GAD proteins are likely to be responsible for increases in GABA synthesis. Indeed, elevated GABA levels have been observed in the hippocampal formation of pilocarpine-treated animals during the chronic stage (Cavalheiro et al., 1994).

Functional significance of GAD65 and GAD67 up-regulation

One possible explanation for the increased expression of GAD mRNAs and proteins in GABA neurons of the hippocampal formation is that these neurons are part of a functional network in which neuronal activity is increased. Recent electrophysiological studies have shown that, in the pilocarpine model as well as in two other models of spontaneous recurrent limbic seizures, many GABA neurons in CA1 remain functionally connected with their excitatory afferents (Esclapez et al., 1997; Rempe et al., 1997) and that spontaneous interictal activity can be recorded synchronously in interneurons and pyramidal cells (Esclapez et al., 1997). Thus, increased activity of the principal cells could lead to increased activation of interneurons that are part of a feed-back inhibitory circuit. Likewise, increased excitation from extrinsic sources may influence the GABAergic interneurons directly in a feed-forward manner. In both instances, increased activity of the GABA neurons could stimulate increases in GAD mRNA and protein synthesis.

Previous studies involving lesion or functional deafferentation have provided evidence for activity-dependent regulation of GAD67 gene expression in the rat and monkey central nervous system. Levels of GAD67 mRNA were decreased in GABA neurons of the monkey visual cortex when neuronal activity was abolished in one eye by intraocular injection of tetrodotoxin (Benson et al., 1994). Conversely, in rats, lesions of the climbing fiber inputs to the cerebellar Purkinje cells that dramatically increase the spike activity of these GABA neurons produce an increase of GAD67 mRNA levels and GAD activity in Purkinje cells (Litwak et al., 1990; Drengler and Oltmans, 1993; Drengler et al., 1996). Other investigators have observed up-regulation of various peptides in some of the same populations of GABA neurons in the hippocampus in similar, seizure-prone animals and also have interpreted such changes as due to increased activity of these neurons during the chronic period (Sperk et al., 1992; Schwarzer et al., 1995).

The present study suggests that activity-dependent regulation of GAD65 mRNA also occurs, and this could have important functional consequences in seizure-prone regions. GAD65 in axon terminals, as discussed above, may constitute a reservoir of GAD that can become functionally active in response to local influences and might be particularly important in the synthesis of GABA at synaptic sites (Kaufman et al., 1991; Martin and Rimvall, 1993; Esclapez et al., 1994). One might speculate that a long-term modification of the neuronal network could lead to increased activity of excitatory afferents on principal cells, and local GABA neurons might be required to maintain increased GABA synthesis at their synaptic sites to control incoming excitatory activity. For such local control, GAD65 may be particularly important, and, consistent with these ideas, this isoform is highly up-regulated in the chronic, seizure-prone animals.

Such adaptive changes could allow the GABA system to prevent excessive firing of the principal cells most of the time. This suggestion is supported by electrophysiological studies that have demonstrated functional inhibition in several regions of the hippocampal formation in animals with chronic, spontaneous seizures (Soltesz and Mody, 1994; Coulter et al., 1996; Buckmaster and Dudek, 1997; Esclapez et al., 1997; Rempe et al., 1997). However, because the inhibitory system is altered, it could be prone to failure on some occasions. Because a loss of some groups of GABA neurons has occurred, the remaining, possibly reorganized GABA neurons are unlikely to provide precisely the same types of control as the original population. Thus, they may not be able to compensate fully for the damaged neurons. Furthermore, the need for the remaining GABA neurons to maintain high levels of activity to control the increased excitability of the region over long periods of time could make these neurons more prone to failure than GABA neurons in the normal hippocampus. Thus, periodically, the inhibitory control could break down, and seizure activity could occur.

The present findings emphasize the remarkable functional plasticity of GABA neurons in the hippocampal formation and provide strong evidence for the up-regulation of both GAD65 and GAD67 mRNAs and associated proteins under the pathophysiological conditions that exist in a model of temporal lobe epilepsy during the chronic stage. We propose that the observed increases in GAD mRNAs and protein expression in GABA neurons throughout the hippocampal formation are activity-dependent and may be an indication that remaining GABA neurons are highly active.

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