

# Increased Levels of Acidic Calponin During Dendritic Spine Plasticity After Pilocarpine-Induced Seizures

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**ABSTRACT:** We have previously shown that, in HEK 293 cells, overexpression of acidic calponin, an actin-binding protein, induces remodeling of actin filaments, leading to a change in cell morphology. In addition, this protein is found in dendritic spines of adult hippocampal neurons. We hypothesized that this protein plays a role in regulating actin-based filaments during dendritic spine plasticity. To assess this hypothesis, the pilocarpine model of temporal lobe epilepsy was selected because an important reorganization of the glutamatergic network, which includes an aberrant sprouting of granule cell axons, neo-synaptogenesis, and dendritic spine remodeling, is well established in the dentate gyrus. This reorganization begins after the initial period of status epilepticus after pilocarpine injection, during the silent period when animals display a normal behavior, and reaches a plateau at the chronic stage when the animals have developed spontaneous recurrent seizures. Our data show that the intensity of immunolabeling for acidic calponin was clearly increased in the inner one-third of the molecular layer of the dentate gyrus, the site of mossy fiber sprouting, and neo-synaptogenesis, at 1 and 2 weeks after pilocarpine injection (silent period) when the reorganization was taking place. In contrast, in chronic pilocarpine-treated animals, when the reorganization was established, the levels of labeling for acidic calponin in the inner molecular layer were similar to those observed in control rats. In addition, double immunostaining studies suggested that the increase in acidic calponin levels occurred within the dendritic spines. Altogether, these results are consistent with an involvement of acidic calponin in dendritic spine plasticity. © 2003 Wiley-Liss, Inc.

**KEY WORDS:** epilepsy; actin; spine formation; drebrin; rat

Abbreviations used: DG, dentate gyrus; G, granule cell layer; GFAP, glial fibrillary acidic protein; H, hilus; IgG, immunoglobulin G; IHC, immunohistochemistry; IML, inner molecular layer; KPBS, potassium phosphate-buffered saline; LTP, long-term potentiation; M, molecular layer; MAP2, microtubule-associated protein; PB, phosphate-buffer; RT, room temperature.

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## INTRODUCTION

Dendritic spines are the main target on which the majority of excitatory glutamatergic synapses have been found in the central nervous system (Gray, 1959; Peters et al., 1976; Parnavelas et al., 1977) and may function as units of synaptic integration (Harris and Kater, 1994; Yuste and Tank, 1996). The shape and density of dendritic spines appear to be influenced by several factors, including age, hormones, neurotrophins, disease, learning, and synaptic activity (Bailey and Kandel, 1993; Horner, 1993; Harris and Kater, 1994). In addition, this plasticity of the spines may be involved in long-term memory storage (Crick, 1982; Bailey and Kandel, 1993). Indeed, long-term potentiation (LTP), a persistent enhancement of synaptic strength that is believed to underlie learning and memory, has been associated with both altered spine morphology (Desmond and Levy, 1988; Fifkova and Morales, 1992; Hosokawa et al., 1995; Toni et al., 1999; Lee and Sheng, 2000) and emergence of new spines (Engert and Bonhoeffer, 1999; Maletic-Savatic et al., 1999).

Dendritic spines are highly enriched with actin filaments (Fifkova and Delay, 1982; Matus et al., 1982), which are thought to be involved in spine plasticity (Smith, 1999; Van Rossum and Hanisch, 1999; Matus, 2000; Rao and Craig, 2000). The identification of the molecular basis responsible for spine plasticity is fundamental in order to understand the mechanisms of synaptic plasticity.

We hypothesized that acidic calponin, an actin-binding protein, is one of the regulators of actin filaments during spine plasticity based on the following observations: (1) electron microscopic studies have shown that this protein is within dendritic spines in adult hippocampal neurons (Agassandian et al., 2000); (2) transfection of acidic calponin into HEK 293 cells induced remodeling of actin filaments, which led to changes in cell morphol-

ogy (Ferhat et al., 2001); and (3) the calponin family stimulates actin polymerization, bundling and stabilization of F-actin filaments (Kake et al., 1995; Kolakowski et al., 1995; Danninger and Gimona, 2000; Ferhat et al., 2001).

In the present study, we tested our hypothesis, relying on a well-characterized experimental model of temporal lobe epilepsy induced by pilocarpine in adult rats. In this model, an important aberrant sprouting of granule cell axons (mossy fibers) (Mello et al., 1993; Okazaki et al., 1995) is reported. In normal conditions, mossy fibers innervate CA3 pyramidal neurons as well as hilar mossy cells and interneurons (Amaral et al., 1990; Ribak and Peterson, 1991; Wenzel et al., 1997; Acsady et al., 1998). After pilocarpine-induced seizures, mossy fibers sprout and form new functional synapses on the granule cell dendrites in the inner molecular layer (IML) of the dentate gyrus (DG) (Okazaki et al., 1995; Okazaki and Nadler, 2001; Buckmaster et al., 2002). Moreover, the sprouting of mossy fibers is associated with a remodeling of dendritic spine shape and density of dentate granule cells (Isokawa, 1998, 2000). Thus, if acidic calponin is involved in spine plasticity, we would expect an increase in this protein during the morphological reorganization observed in pilocarpine-induced seizures. Indeed, our data showed that the acidic calponin levels were strongly increased in the inner one-third of the molecular layer of the DG 1 or 2 weeks after pilocarpine-induced seizures. Furthermore, double immunohistochemical studies for acidic calponin and drebrin, an actin-binding protein specifically localized in dendritic spines, suggested that the increase in acidic calponin levels occurred within dendritic spines of presumed granule cells. These results are consistent with an involvement of acidic calponin in the plasticity of dendritic spines.

## MATERIALS AND METHODS

### Animals

Experiments involving animals were approved by the European Communities Council (86/609/EEC). Adult male Wistar rats weighing 200–290 g (Charles River, France) were injected intraperitoneally (i.p.) with pilocarpine hydrochloride (325–350 mg/kg; Sigma, St. Louis, MO), a muscarinic cholinergic agonist. The injection protocols were similar to those previously described (Turk et al., 1983; Cavalheiro et al., 1987; Obenaus et al., 1993). A low dose of the cholinergic antagonist methylscopolamine nitrate (1 mg/kg, i.p.) was administered 30 min before pilocarpine injection, to reduce the agonist peripheral cholinergic effects (Baez et al., 1976; Turk et al., 1983). Only animals that displayed robust behavioral seizures for 3–4 h were selected in this study. This period of severe sustained seizures was stopped by a single injection of valium (6 mg/kg, i.p.; Sigma), to reduce mortality among these animals. The rats were then observed periodically in the vivarium for general behavior and occurrence of spontaneous seizures for a period of 16 weeks. Pilocarpine-treated animals were studied at several post-injection intervals: during the silent period, when animals displayed an apparent normal behavior (1 and 2 weeks, n =

4 at each interval) and during the chronic stage, when the animals had developed spontaneous recurrent limbic seizures (16 weeks, n = 4). The onset of spontaneous seizure occurrence was 4–6 weeks after pilocarpine injection. Eight age-matched rats from the same litters were used for control experiments.

### Tissue Preparation

The rats were deeply anesthetized with sodium pentobarbital (60 mg/kg, i.p.) and perfused through the heart with a fixative solution (1 ml/g body weight) of 4% paraformaldehyde in 0.12 M phosphate buffer, pH 7.3 (PB). After perfusion, brains were removed from the skull, post-fixed in the same fixative for 1 h at room temperature (RT), rinsed in 0.12 M PB for 1.5 h, and immersed in a cryoprotective solution of 20% sucrose in PB overnight at 4°C. Blocks of brain which contained the entire hippocampal formation were quickly frozen on dry ice and sectioned coronally with a cryostat (40 µm thick). The sections obtained were rinsed in PB, collected sequentially into 1-ml microcentrifuge tubes containing an ethylene glycol-based cryoprotective solution (Watson et al., 1986; Lu and Haber, 1992), and stored at –20°C until used for immunohistochemistry (IHC).

### Primary Antibodies

Antibodies were obtained from various sources. Acidic calponin was detected with a polyclonal antiserum raised in rabbits against the C-terminal of the rat protein. The specificity of this antiserum has been previously assessed by Western blot on the total homogenate of rat hippocampus (Plantier et al., 1999). Drebrin was localized with a mouse monoclonal antibody (M2F6; Shirao and Obata, 1986). The mouse monoclonal antibody against synaptophysin (Chemicon, Temecula, CA), mouse monoclonal antibody against microtubule-associated protein 2 (MAP2; Sigma), and mouse monoclonal antibody against glial fibrillary acidic protein (GFAP; Roche Diagnostics, Meylan, France) were purchased as indicated.

### Immunohistochemistry

Single immunohistochemical labeling for acidic calponin, synaptophysin, and MAP2 was performed with standard avidin-biotin-peroxidase methods (Vectastain ABC kit; Vector Laboratories, Burlingame, CA). Free-floating sections were rinsed for 30 min in PB, incubated in 1% H<sub>2</sub>O<sub>2</sub> (Sigma) for 40 min in PB to block endogenous peroxidase activity, and rinsed for 30 min in 0.02 M potassium phosphate-buffered saline (KPBS; 16 mM K<sub>2</sub>HPO<sub>4</sub>, 3.5 mM KH<sub>2</sub>PO<sub>4</sub>, 150 mM NaCl, pH 7.4). Sections processed for acidic calponin-IHC were preincubated for 1 h at RT in KPBS containing 0.3% Triton X-100 (Sigma) and 3% normal goat serum (Vector) and then incubated overnight at room temperature (RT) in the acidic calponin polyclonal antiserum (1:300) diluted in KPBS containing 0.3% Triton X-100 and 1% normal goat serum. Sections processed for synaptophysin- and MAP2-IHC were preincubated for 1 h at RT in KPBS containing 0.3% Triton X-100 and 3% normal horse serum (Vector) and then incubated overnight at RT in either synaptophysin (1:200) or

MAP2 (1:500) monoclonal antibodies diluted in KPBS containing 0.3%, Triton X-100 and 1% normal horse serum. The following day, sections were rinsed in KPBS, incubated for 1 h at RT in biotinylated goat anti-rabbit IgG (1:200; Vector) or horse anti-mouse IgG (1:200; Vector) in KPBS containing 3% normal goat serum or normal horse serum. Sections were then rinsed in KPBS, incubated for 1 h at RT in the avidin-biotin-peroxidase solution prepared in KPBS according to manufacturer's recommendations. After several rinses in KPBS, sections from control and pilocarpine-treated animals were processed for the same period of time in 0.05% 3-3'-diaminobenzidine-HCl (Sigma) and 0.006% H<sub>2</sub>O<sub>2</sub> diluted in KPBS. The sections were then rinsed in KPBS, mounted on gelatin-coated slides, dried, dehydrated and coverslipped with Permount (Fischer Scientific, Electron Microscopy Sciences, Washington, PA).

### Quantitative Analysis

Semiquantitative analyses of the intensity of immunolabeling for acidic calponin, synaptophysin, and MAP2 were conducted in the granule cell layer (G) and inner molecular layer (IML) of the dentate gyrus to identify differences between control and pilocarpine animals. This analysis of the labeling intensity was performed by optical densitometric measurements of the labeling with an image analyzing system according to previously described methods (Larsson et al., 1991; Larsson and Hougaard, 1993, 1994a,b; Esclapez and Houser, 1999). The image analysis system used in this study included a PC-compatible computer, a Nikon digital camera DXM 1200 connected to a Nikon E800 microscope (Nikon, Melville, NY), a Nikon ACT-1 frame grabber, and NIH Image 1.62 software. All images were acquired with a 40 $\times$  lens, under the same conditions of light illumination, with the microscope light source stabilized, and at a final digitized size of 640  $\times$  480 pixels. The densitometric analysis of labeling was performed using NIH software, which automatically determined the gray level value. For each control and pilocarpine animal, quantitative data were obtained from the hippocampus on both sides in four sections. The analysis of labeling intensity was performed from a total of 16 microscopic fields per region of interest (G and IML). For each of these microscopic fields, the total gray level value was automatically obtained. The gray level value of the corpus callosum was used as a reference value for background. The specific intensity of labeling corresponding to the corrected gray level value was calculated by subtracting the gray level value of the background from the total gray level value. For each region of interest the mean and corresponding standard error to the mean (SEM) intensity of labeling obtained from the total number of microscopic fields were calculated for each series of control and pilocarpine-treated animals.

Statistical analysis of differences in the mean intensity of labeling for acidic calponin, synaptophysin, and MAP2 between control and pilocarpine-treated animals at each interval (1, 2, and 16 weeks) and each region of interest was carried out with a mixed-model analysis of variance (ANOVA) and Student's *t*-test.

### Immunofluorescence

For double-immunofluorescence labeling of acidic calponin/GFAP, acidic calponin/synaptophysin, acidic calponin/MAP2, and acidic calponin/drebrin, sections were rinsed for 30 min in KPBS and preincubated for 1 h at RT in KPBS containing 0.3% Triton X-100 and 1% blocking reagent (Roche diagnostics, Meylan, France). Sections were then incubated overnight at RT in a mixture of acidic calponin antiserum (1:300) and either GFAP (1:100), synaptophysin (1:100), MAP2 (1:500) or drebrin (hybridoma supernatant) monoclonal antibodies diluted in KPBS containing 0.3% Triton X-100 and 1% blocking reagent. After several rinses in KPBS, sections were incubated for 1 h at RT in biotinylated goat anti-rabbit IgG (1:200), rinsed in KPBS and incubated in a mixture of Alexa 488-conjugated goat anti-mouse IgG (1:200, Jackson ImmunoResearch, West Grove, PA) and Cy3-conjugated streptavidin (1:200, Jackson ImmunoResearch) diluted in KPBS. The sections were then mounted on gelatin-coated slides and were dried and coverslipped with Fluoromount G (Southern Biotechnology Associates, Birmingham, AL). The specimens were analyzed with an Olympus fluoview-500 laser scanning microscope. In all cases, no labeling was detected when specific antibodies were replaced with normal rabbit or mouse serum or when primary antibodies were omitted.

## RESULTS

The expression of different proteins of interest was followed in the granule cell and inner molecular layers of the dentate gyrus (DG). In these regions, a major aberrant sprouting of granule cell axons has been observed in pilocarpine-treated animals (Mello et al., 1993; Okazaki et al., 1995). Differences in labeling intensity were observed consistently in sections from all control and pilocarpine-treated animals for acidic calponin, synaptophysin and MAP2 in the granule cell and inner molecular layer of the DG.

### Immunolabeling for Acidic Calponin Is Increased in the Dentate Gyrus After Pilocarpine-Induced Seizures

In control animals (Fig. 1A), the immunohistochemical labeling for acidic calponin was observed in all layers of the dentate gyrus, including the hilus, the granule cell, and molecular layers. In the dendritic layers (hilus and molecular layer), labeling was evenly distributed (Fig. 1A,B). In addition to this diffuse neuronal staining, many astrocytes and cells located along the infragranular border of the DG were labeled for acidic calponin (Fig. 1B).

In pilocarpine-treated animals at 1 week (Fig. 1C,D) and 2 weeks (Fig. 1E,F), the pattern of labeling for acidic calponin was clearly modified, as compared with control rats (Fig. 1A,B). In these pilocarpine-treated animals, the inner one-third of the molecular layer (IML) was strongly labeled for acidic calponin (Fig. 1C-F). This high level of labeling contrasted with the levels of staining observed in the outer two-thirds of the molecular layer.

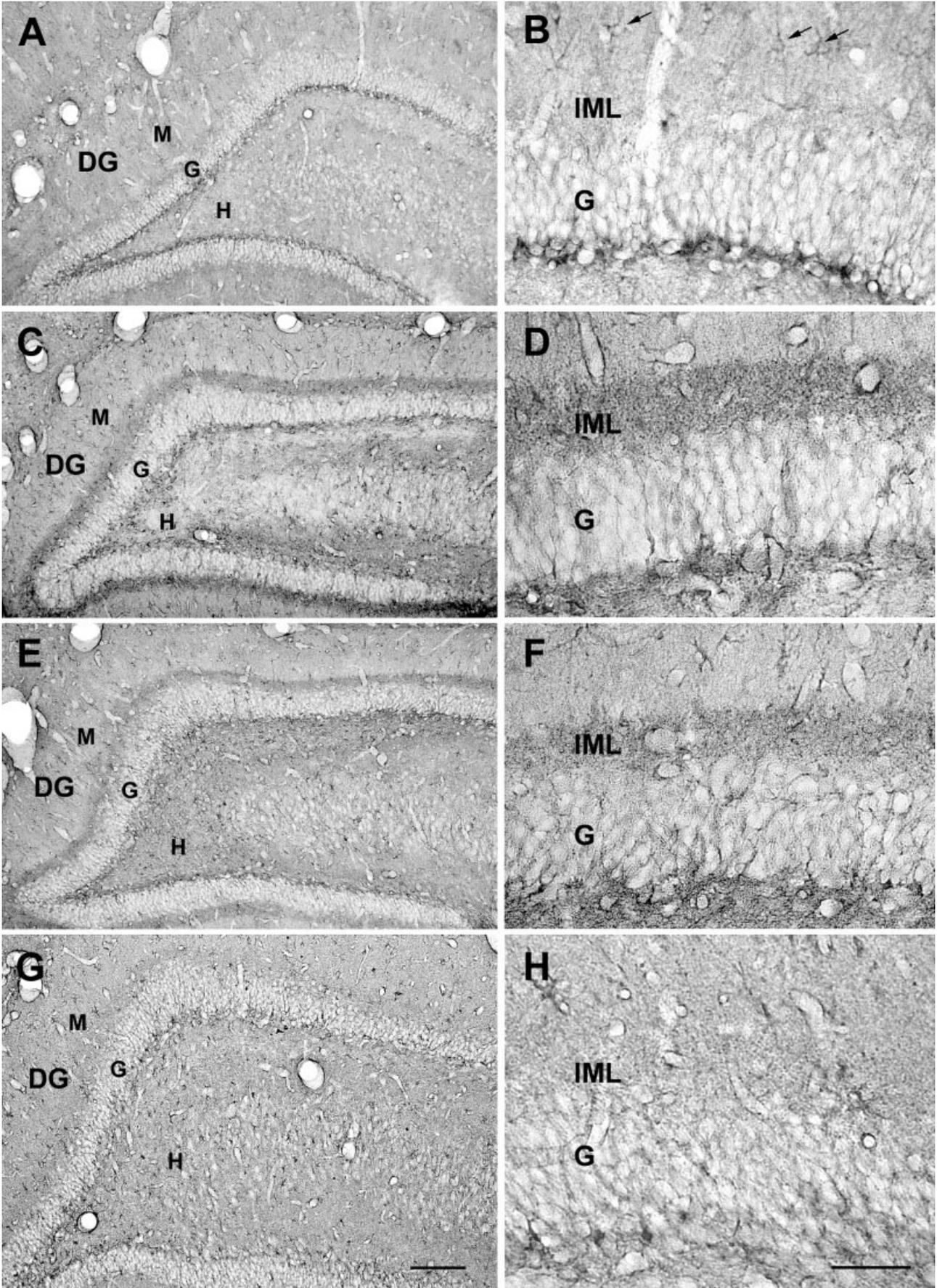


FIGURE 1.

Moderate to high increases in the labeling intensity for acidic calponin were also observed in the granule cell layer and in the hilus. The increases in these layers were mainly observed at 1 week, a time of strong reactive gliosis (Obenaus et al., 1993; see Fig. 5A,B). In contrast, in chronic pilocarpine-treated animals (16 weeks; Fig. 1G), the intensity of labeling for acidic calponin was strongly decreased in the IML of the DG, as compared with pilocarpine rats at 1 (Fig. 1C) and 2 weeks (Fig. 1E). Because in pilocarpine rats, the increased levels of labeling for acidic calponin occurred in the region of intense reorganization, including mossy fiber sprouting (Mello et al., 1993; Okazaki et al., 1995), outgrowth of granule cell dendrites (Parent et al., 1997) and dendritic spine plasticity (Isokawa, 1998, 2000), we further investigated whether acidic calponin expression was co-regulated with the expression of specific markers for both presynaptic (synaptophysin) and postsynaptic (MAP2) elements.

### Immunolabeling Changes for Synaptophysin in the Dentate Gyrus After Pilocarpine-Induced Seizures

Immunolabeling for synaptophysin that labeled axon terminals has been reported previously in the hippocampal formation of adult rats (Masliyah et al., 1991). We describe the staining obtained for synaptophysin in control rats to compare this labeling with that of pilocarpine-treated animals. Briefly, punctiform immunolabeling for synaptophysin was found in all layers of the dentate gyrus (Fig. 2). Within the molecular layer, synaptophysin immunoreactivity showed a trilaminar pattern with the inner and outer part displaying the strongest intensity of staining (Fig. 2A,B). A clear staining surrounding the cell bodies of granule cells was observed (Fig. 2B). The strong staining present in the hilus corresponds to the labeling of  $\gamma$ -aminobutyric acid (GABA)ergic and glutamatergic terminals including mossy fiber terminals (Fig. 3A).

The pattern of labeling for synaptophysin was different in pilocarpine-treated animals at all time intervals examined, as compared with that observed in control rats. One week after pilocarpine injection, the labeling intensity was clearly decreased in the inner one-third of the molecular layer (Fig. 2C,D). In contrast, the labeling intensity in all the other regions of the dentate gyrus including the hilus, the granular cell layer and the outer two-thirds of the molecular layer was relatively well preserved. The pattern of staining for synaptophysin was similar at 2 (Fig. 2E,F) and 16 weeks

(Fig. 2G,H) after pilocarpine injection but differed from that found at 1 week (Fig. 2C,D) and in control animals (Fig. 2A,B). At 2 and 16 weeks, a strong intensity of labeling was present in the inner molecular layer. However, this high level of staining was restricted to the supragranular region of the inner molecular layer (Fig. 2F,H), in contrast to that observed in control animals (Fig. 2B). No major differences in labeling intensity were detected in the hilus, the granular cell layer and the outer molecular layer at 2 and 16 weeks (Fig. 2E–H) compared with control rats (Fig. 2A,B).

### Immunolabeling for Microtubule-Associated Protein 2 Is Increased in the Dentate Gyrus After Pilocarpine-Induced Seizures

The pattern of immunohistochemical labeling for MAP2 in the hippocampal formation of control animals was similar to that previously described (Pollard et al., 1994). Briefly, MAP2 staining was present in all layers of the dentate gyrus including the hilus, granule cell and molecular layers (Fig. 3A,B). MAP2 labeling was evenly distributed in the molecular layer. The soma of granule cells was also clearly labeled for MAP2 (Fig. 3B) as many dendritic processes in the hilus (Fig. 3A). In pilocarpine-treated animals at 1 (Fig. 3C) and 2 weeks (Fig. 3E), a clear increase in MAP2 immunoreactivity was observed in the granular cell and molecular layers of the dentate gyrus (Fig. 3C–F). In addition to this general increase in the level of labeling, the pattern of staining for MAP2 clearly differed in the molecular layer of the dentate gyrus between pilocarpine-treated and control animals. This difference was due primarily to an increase in the labeling intensity, to a much greater extent in the inner one-third of the molecular layer than in the outer-two-thirds of this layer. In contrast, a decreased labeling for MAP2 was observed in the region beneath the granule cell layer as well as in the central part of the hilus including regions near the inner tip of CA3c and the apex (Fig. 3C,E), probably due to the loss of neurons described in this region (Obenaus et al., 1993). In chronic pilocarpine-treated animals (Fig. 3G), the intensity of labeling for MAP2 was strongly decreased in the inner one-third of the molecular layer (Fig. 3H), as compared with pilocarpine-treated rats at 1 (Fig. 3D) and 2 weeks (Fig. 3F).

### Quantitative Analysis of the Changes in Acidic Calponin, Synaptophysin, and MAP2 Levels

Because differences in labeling intensity were observed consistently for acidic calponin, synaptophysin and MAP2 in the inner molecular layer of the dentate gyrus, we conducted semiquantitative analysis in this layer and in the granule cell layer to determine the relative extent of these changes. Quantitative data showed that, in the inner molecular layer of the dentate gyrus, the mean intensities of labeling for acidic calponin were significantly increased in the pilocarpine animals at 1 (70%,  $101.95 \pm 1.05$ ) and 2 weeks (48%,  $88.63 \pm 1.18$ ), as compared with control rats ( $59.88 \pm 1.43$ ) (Fig. 4A, Table 1), whereas no difference was found in pilocarpine-treated animals at 16 weeks ( $59.14 \pm 0.84$ ) (Fig. 4A, Table 1). In the granule cell layer, the intensity of labeling was significantly increased in pilocarpine-treated animals at all time intervals examined (23%,  $57.12 \pm 0.89$  at 1 week, 10%,  $50.99 \pm 0.86$  at 2 weeks

**FIGURE 1.** Acidic calponin immunoreactivity in coronal sections of dentate gyrus from control and pilocarpine-treated animals. **A,B:** In a control rat, immunoreactivity of acidic calponin in the dentate gyrus (DG) is mainly present in astrocytes (arrows) and cells located along the infragranular region of the granule cell layer (G). A diffuse staining is evenly observed in all parts of the molecular layer (M) including the inner molecular layer (IML). **C–H:** Immunoreactivity of acidic calponin in the dentate gyrus of pilocarpine-treated rats at 1 week (C,D), 2 weeks (E,F), and 16 weeks (G,H). In pilocarpine-treated animals at 1 and 2 weeks, acidic calponin immunoreactivity was substantially increased in IML of the DG compared with control (A,B) and to pilocarpine-treated rats at 16 weeks (G,H). Scale bars = 200  $\mu$ m in A,C,E,G; 50  $\mu$ m in B,D,F,H.

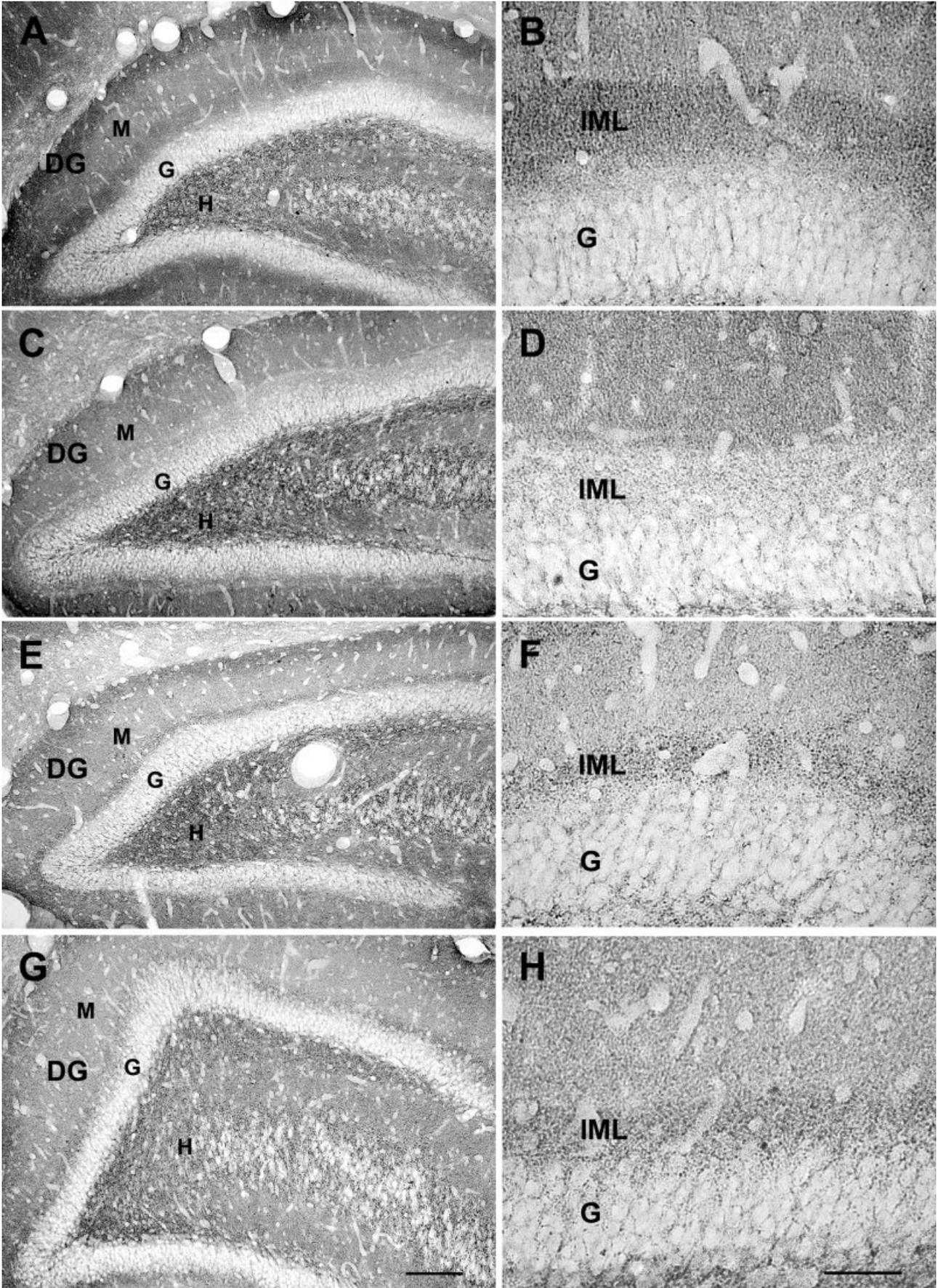


FIGURE 2.

and 12%,  $52.23 \pm 0.58$  at 16 weeks), as compared with control ( $46.53 \pm 1.14$ ), but to a much lower extent than in the inner molecular layer. The intensities of labeling for synaptophysin in the inner molecular layer were significantly decreased in the pilocarpine-treated rats at 1 week (26%,  $81.24 \pm 2.33$ ), and to a much lower extent at 2 (10%,  $92.5 \pm 2.87$ ) and 16 weeks (7%,  $95.28 \pm 2.05$ ), as compared with control ( $102.6 \pm 1.94$ ) (Fig. 4B, Table 1). In addition, the intensities of labeling in this layer were significantly increased at 2 (14%,  $P < 0.01$ ) and 16 (17%,  $P < 0.001$ ) weeks compared with 1 week. No difference in the mean intensity of labeling was found in the granule cell layer of pilocarpine-treated animals at any time interval (1 week  $44.87 \pm 1.03$ ; 2 weeks  $45.97 \pm 1.41$ ; 16 weeks  $46.45 \pm 1.05$ ), as compared with control rats ( $47.43 \pm 1.09$ ) (Fig. 4B, Table 1). The mean intensity of labeling for MAP2 was significantly increased in pilocarpine-treated animals at all time intervals studied, in the inner molecular layer (24%,  $103.1 \pm 2.17$  at 1 week; 22%,  $105.33 \pm 1.74$  at 2 weeks; 6%,  $84.15 \pm 1.38$  at 16 weeks) as well as in the granule cell layer (33%,  $50.58 \pm 1.18$  at 1 week; 15%,  $64.02 \pm 1.57$  at 2 weeks; 15%,  $50.66 \pm 1.64$  at 16 weeks), as compared with control (IML:  $80.01 \pm 1.18$ ; G:  $43.06 \pm 1.07$ ) (Fig. 4C, Table 1). Thus qualitative, like quantitative, analyses showed that the changes in the pattern of labeling for acidic calponin in the molecular layer of the dentate gyrus in pilocarpine-treated animals paralleled those found for MAP2, known as a dendritic marker, at all time intervals examined in this study.

### Immunolabeling for Acidic Calponin Was Absent From Presynaptic Sites But Was Localized in Dendritic Spines

Since acidic calponin is also expressed in glial cells (Ferhat et al., 1996; Plantier et al., 1999; present findings), we first investigated whether the increased labeling intensity for acidic calponin observed in the inner one-third of the molecular layer in pilocarpine-treated animals occurred in glial processes. For this purpose, double labeling for acidic calponin (red) and GFAP (green) was performed in pilocarpine-treated rats at 1 and 2 weeks. In these animals, several glial cells and their processes immunoreactive for

GFAP were also labeled for acidic calponin (Fig. 5A,B). However, in all sections examined, most of the punctiform labeling for acidic calponin in the inner one-third of the molecular layer was not immunoreactive for GFAP (Fig. 5B), suggesting that the increased staining of acidic calponin occurred in neuronal compartments.

Because the punctiform staining of acidic calponin was located in the inner molecular layer, the region of mossy fiber sprouting, we wanted to determine whether this staining revealed presynaptic compartments. Thus, we completed double immunostaining for acidic calponin and synaptophysin, a specific marker for the presynaptic compartment. As illustrated in Figure 5C, both acidic calponin (red) and synaptophysin (green) displayed punctiform patterns. However, these two stains did not co-localize; instead, the two proteins were present in adjacent cellular compartments (Fig. 5C, inset), suggesting a postsynaptic localization of acidic calponin.

To identify the postsynaptic compartment, we performed double labeling for acidic calponin and specific markers for dendritic shafts (MAP2) or for spines (drebrin). Double staining with acidic calponin (red) and MAP2 (green) showed that these proteins were not co-localized (Fig. 5D), as spots labeled for acidic calponin appeared to be attached to dendritic shafts (Fig. 5D, inset). Double labeling for acidic calponin (red) and drebrin (green) showed that in control rats dendritic spines labeled for drebrin in the molecular layer of DG did not contain detectable levels of acidic calponin whereas in pilocarpine-treated rats at 1 and 2 weeks, some spines clearly contained acidic calponin in the inner molecular layer. Indeed, in control animals, many cellular elements were labeled either for acidic calponin or for drebrin in the granule cell and molecular layers of the DG (Fig. 6A,B). The two proteins were never co-localized in the same compartment (Fig. 6B). In pilocarpine-treated animals, many spines labeled only for drebrin and many cellular elements labeled only for acidic calponin (presumed glial processes) were present in the granule cell and molecular layers of the DG (Fig. 6C,D) as in control animals (cf. Fig. 6A,B with C,D). However, in contrast to control animals, some spines labeled for drebrin (Fig. 6C–E) were also labeled for acidic calponin in the pilocarpine-treated animals (Fig. 6C,D,F). These spines double-labeled for the two proteins were observed in the inner molecular layer of the DG (Fig. 6C). No co-localization was observed in the outer two-thirds of the molecular layer. These results indicated that, in pilocarpine-treated animals, acidic calponin protein was present in some dendritic spines of presumed granule cells.

**FIGURE 2.** Synaptophysin immunoreactivity in coronal sections of dentate gyrus from control and pilocarpine-treated animals. A,B: In a control rat, immunoreactivity of synaptophysin in the dentate gyrus (DG) is observed in the granule cell layer (G) and all dendritic layers including the hilus (H) and the molecular layer (M). In the latter layer, the staining was stronger in the inner and outer molecular layers than in the middle molecular layer. C–H: Immunoreactivity of synaptophysin in the dentate gyrus of pilocarpine-treated rats at 1 week (C,D), 2 weeks (E,F), and 16 weeks (G,H). In a pilocarpine-treated rat at 1 week, synaptophysin immunoreactivity was decreased in the inner one-third of the molecular layer (IML) of the DG, as compared with a control rat (cf. A with C and B with D). In pilocarpine-treated rats at 2 weeks and 16 weeks, synaptophysin immunoreactivity was increased in the inner one-third of the molecular layer (IML) of the DG, as compared with pilocarpine-treated animals at 1 week (cf. E and G with C and F and H with D). In control and pilocarpine-treated animals, synaptophysin immunolabeling was punctiform. Scale bars = 200  $\mu\text{m}$  in A,C,E,G; 50  $\mu\text{m}$  in B,D,F,H.

## DISCUSSION

### Acidic calponin is associated with neuroplastic changes observed in pilocarpine-treated rats

In the pilocarpine model of temporal lobe epilepsy, we report a striking increase in acidic calponin in the inner one-third molecular layer of the DG, the region of intensive network reorganization. These increased levels of acidic calponin occurred at 1 and 2 weeks after pilocarpine injection, when mossy fiber sprouting began to

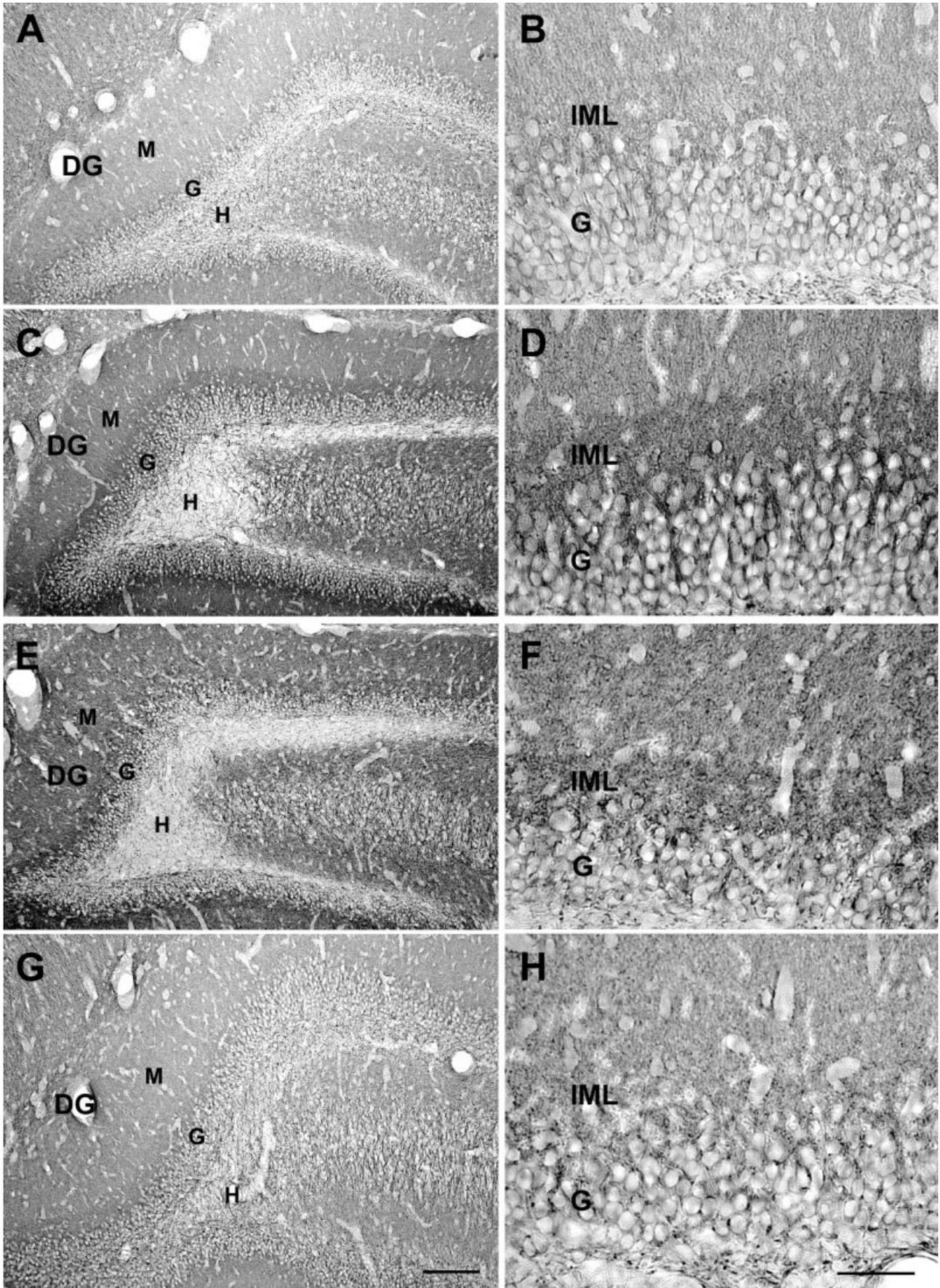


FIGURE 3.

appear in the supragranular layer of the DG (Mello et al., 1993). At the chronic stage, when the mossy fiber sprouting reached a plateau, the levels of acidic calponin in the IML were similar to those observed in control animals. These observations suggest that acidic calponin plays a role during the period of intense reorganization. In addition, our data strongly suggest that the transient increase in acidic calponin occurred within dendritic spines. Moreover, this increase was observed during the time of important remodeling of dendritic spines shape and density of dentate granule cells (Isokawa, 1998, 2000), suggesting that acidic calponin is involved in dendritic spine plasticity.

### Acidic Calponin May Be Involved in Spine Formation

In the rat pilocarpine model, dendrites of dentate granule cells displayed a generalized spine loss immediately after the status epilepticus induced by pilocarpine injection. However, this spine loss was transient and was followed by a recovery in spine density that started 3 days after status epilepticus and reached a plateau level 15 days later (Isokawa, 1998, 2000). The subsequent recovery of dendritic spine density probably reflects the formation of new spines on preexisting granule cell dendrites and/or development of spines on outgrowing dendrites of newly formed granule cells subsequent to epilepsy-induced neurogenesis (Parent et al., 1997). Since the increase in acidic calponin is observed during this period of recovery of spine density, we suggest that acidic calponin is involved in the formation of new dendritic spines. Our data show that the increase in acidic calponin levels does not occur in all dendritic spines because many puncta were labeled only for drebrin. Furthermore, this suggestion is strongly supported by our more recent data (Ferhat et al., manuscript in preparation) showing that overexpression of acidic calponin in hippocampal neurons induced a dramatic increase in spine density. This increase could be related to the properties of the calponin family, which is known to stimulate polymerization, bundling and stabilization of actin filaments

**FIGURE 3.** Microtubule-associated protein 2 (MAP2) immunoreactivity in coronal sections of dentate gyrus from control and pilocarpine-treated animals. A,B: In a control rat, immunoreactivity of MAP2 in the dentate gyrus (DG) is mainly observed in the cell body of granule cells (G) and all the dendritic layers, including the hilus (H) and the molecular layer (M). C-H: Immunoreactivity of MAP2 in the dentate gyrus of pilocarpine-treated rats at 1 week (C,D), 2 weeks (E,F) and 16 weeks (G,H). In pilocarpine-treated animals at 1 and 2 weeks, a clear increase in MAP2 immunoreactivity was observed in IML of the DG compared with control (A,B) and with pilocarpine-treated rats at 16 weeks (G,H). Scale bars = 200  $\mu\text{m}$  in A,C,E,G; 50  $\mu\text{m}$  in B,D,F,H.

**FIGURE 4.** Histograms comparing the mean intensities of immunolabeling for acidic calponin, microtubule-associated protein 2 (MAP2), and synaptophysin in the granule cell layer (G) and inner molecular layer (IML) of the dentate gyrus from control and pilocarpine-treated animals at 1, 2, and 16 weeks. Statistically significant differences in the mean estimated intensity of labeling are indicated (\* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ; ANOVA and Student's  $t$ -test). Error bars = SEM.

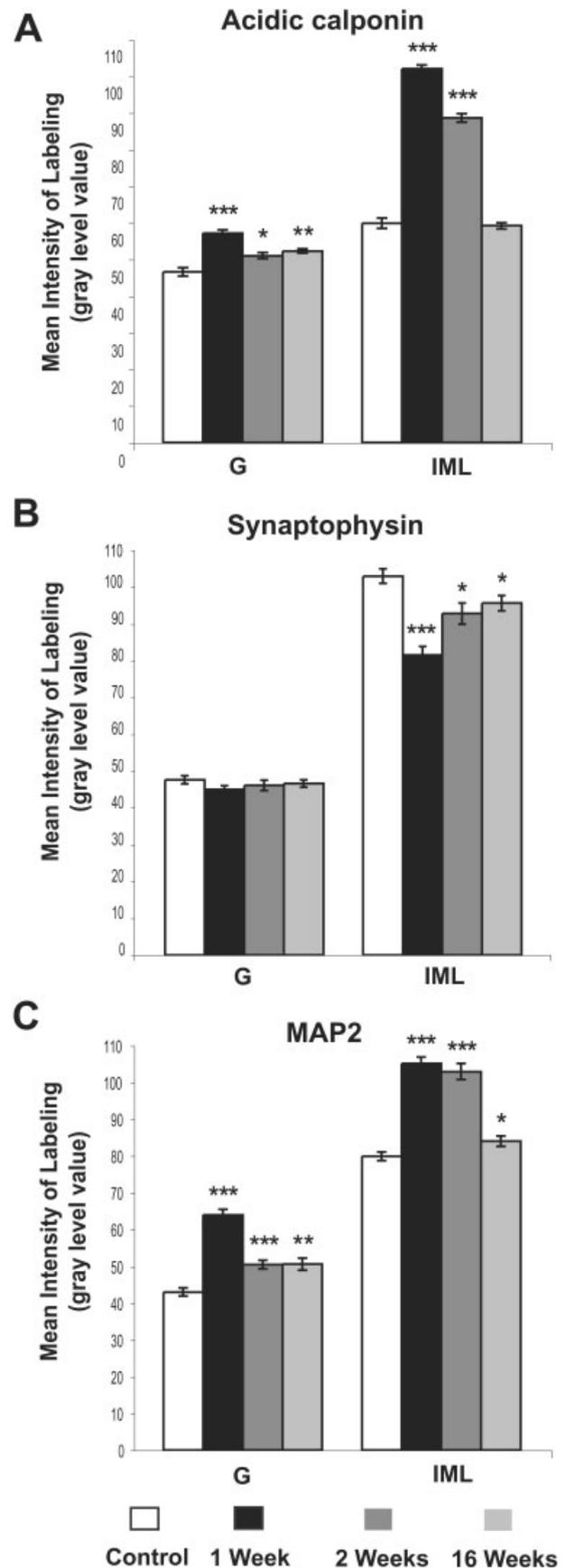


TABLE 1.

*Comparison of Mean Intensity of Labeling for Acidic Calponin, Synaptophysin, and MAP2, in Granule Cell and Inner Molecular Layers Between Control and Pilocarpine-Treated Animals at 1, 2 and 16 Weeks*

|                          | Mean intensity of labeling<br>in G $\pm$ SEM | <i>P</i> | Mean intensity of labeling in<br>IML $\pm$ SEM | <i>P</i> |
|--------------------------|--|----------|--|----------|
| <b>Acidic calponin</b>   |  |          |  |          |
| Control rats             | 46.53 $\pm$ 1.14                             |          | 59.88 $\pm$ 1.43                               |          |
| Pilocarpine-treated rats |  |          |  |          |
| 1 wk                     | 57.12 $\pm$ 0.89                             | <0.001   | 101.95 $\pm$ 1.05                              | <0.001   |
| 2 wk                     | 50.99 $\pm$ 0.86                             | <0.05    | 88.63 $\pm$ 1.18                               | <0.001   |
| 16 wk                    | 52.23 $\pm$ 0.58                             | <0.01    | 59.14 $\pm$ 0.84                               | NS       |
| <b>Synaptophysin</b>     |  |          |  |          |
| Control rats             | 47.43 $\pm$ 1.09                             |          | 102.60 $\pm$ 1.94                              |          |
| Pilocarpine-treated rats |  |          |  |          |
| 1 wk                     | 44.87 $\pm$ 1.03                             | NS       | 81.24 $\pm$ 2.33                               | <0.001   |
| 2 wk                     | 45.97 $\pm$ 1.41                             | NS       | 92.50 $\pm$ 2.87                               | <0.05    |
| 16 wk                    | 46.45 $\pm$ 1.05                             | NS       | 95.28 $\pm$ 2.05                               | <0.05    |
| <b>MAP2</b>              |  |          |  |          |
| Control rats             | 43.06 $\pm$ 1.07                             |          | 80.01 $\pm$ 1.18                               |          |
| Pilocarpine-treated rats |  |          |  |          |
| 1 wk                     | 50.58 $\pm$ 1.18                             | <0.001   | 103.10 $\pm$ 2.17                              | <0.001   |
| 2 wk                     | 64.02 $\pm$ 1.57                             | <0.001   | 105.33 $\pm$ 1.74                              | <0.001   |
| 16 wk                    | 50.66 $\pm$ 1.64                             | <0.01    | 84.15 $\pm$ 1.38                               | <0.05    |

MAP2, microtubule-associated protein; G, granule cell; IML, inner molecular layer; NS, not significant.

(Kake et al., 1995; Kolakowski et al., 1995; Danninger and Girona, 2000; Ferhat et al., 2001). As mentioned earlier, the period of new spine formation coincides with the period of mossy fiber sprouting (Mello et al., 1993; Okazaki et al., 1995), suggesting that these new spines will be involved in the formation of aberrant synapses with the newly formed mossy fiber terminals.

Under physiological conditions, granule cell dendrites in the IML are mainly innervated by hilar mossy cell axons (Amaral et al., 1990; Wenzel et al., 1997), whose main synaptic contacts with the dendritic spines in the IML are formed by commissural/associational projections (Buckmaster et al., 1996). It has been suggested that hilar mossy cells and their axon terminals degenerate in the pilocarpine model (Obenaus et al., 1993; Buckmaster et al., 2002). Thus, the loss of mossy cells and their terminals leads to a denervation of granule cell dendrites in the IML. These observations may explain the decrease in synaptophysin immunoreactivity that we observed in the IML 1 week after pilocarpine treatment. The subsequent recovery of synaptophysin immunostaining in the supragranular cell layer of the inner molecular layer 2 weeks after pilocarpine treatment is probably due to the development of aberrant sprouting of mossy fibers after status epilepticus (Mello et al., 1993; Okazaki et al., 1995), which is thought to be involved in the establishment of functional excitatory synaptic boutons on granule cell dendrites (Tauack and Nadler, 1985; Okazaki and Nadler, 2001). Similar observations have been reported in human temporal lobe epilepsy (Proper et al., 2000). However, we cannot com-

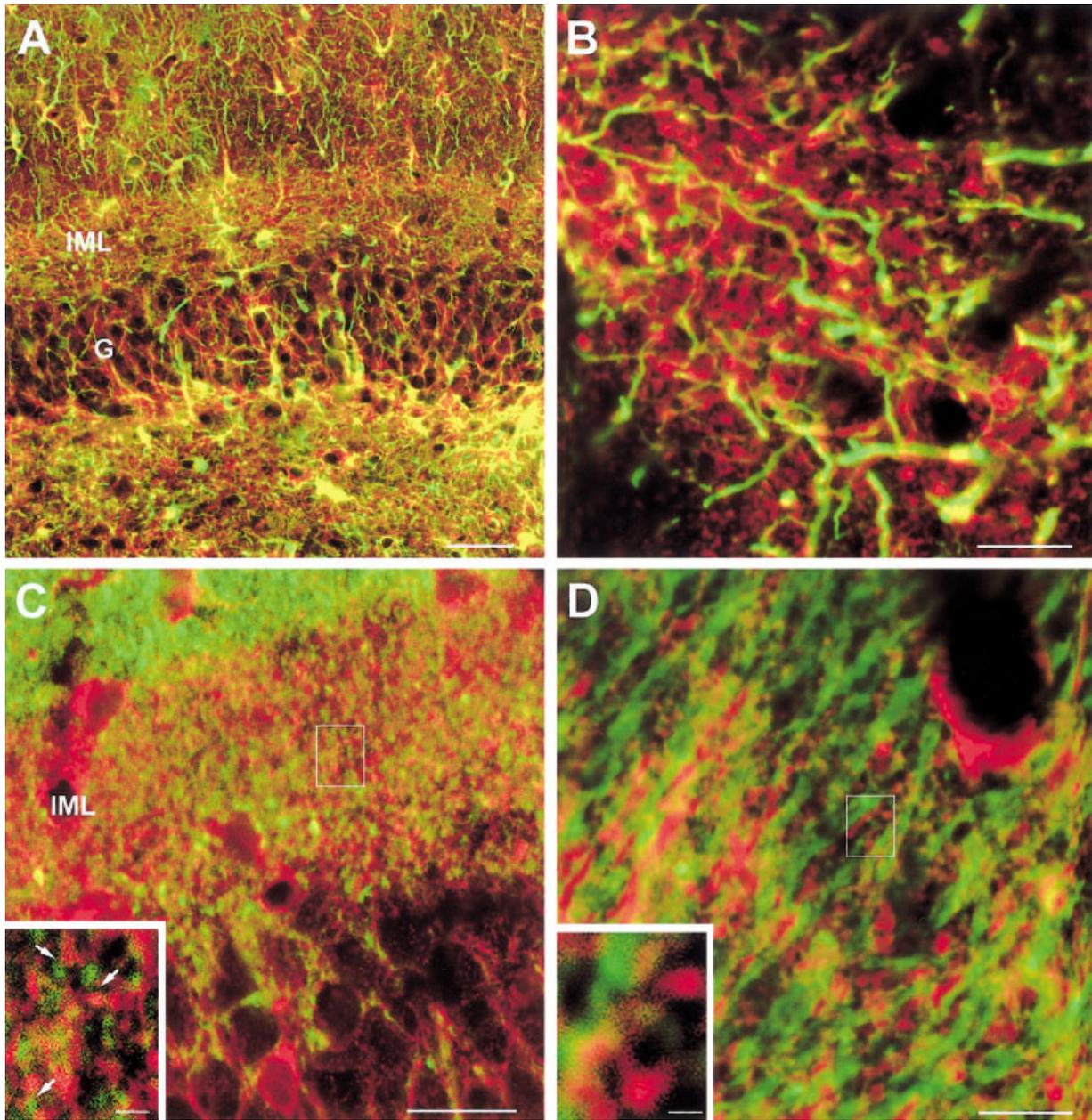
pletely exclude the possibility that these changes of synaptophysin immunoreactivity result from the regulation of the expression of synaptophysin in axon terminals.

If the increase in synaptophysin immunoreactivity indeed reflects an increased number of synaptic terminals due to mossy fiber sprouting, then we would expect an induction of acidic calponin in spines associated with newly formed synapses. Interestingly, our data clearly show an increase in acidic calponin associated with an increase in synaptophysin-containing terminals at 2 weeks after pilocarpine injection, further suggesting that the increase in acidic calponin levels occurs in dendritic spines that are associated with newly formed synapses.

### Acidic Calponin May Be Involved in Spine Shape Plasticity

In addition to a remodeling of spine density, changes in spine shape were reported in the pilocarpine (Isokawa, 1998, 2000) and kainate (Represa et al., 1993) models of temporal lobe epilepsy. Similar plastic changes in dendritic spines are observed during normal development (Smith, 1999; Van Rossum and Hanisch, 1999; Matus, 2000) as well as in adults after LTP (Fifkova and Morales, 1992).

Actin filament is the major cytoskeletal element of dendritic spines (Fifkova and Delay, 1982; Matus et al., 1982) and is thought to be important for determining spine shape and motility

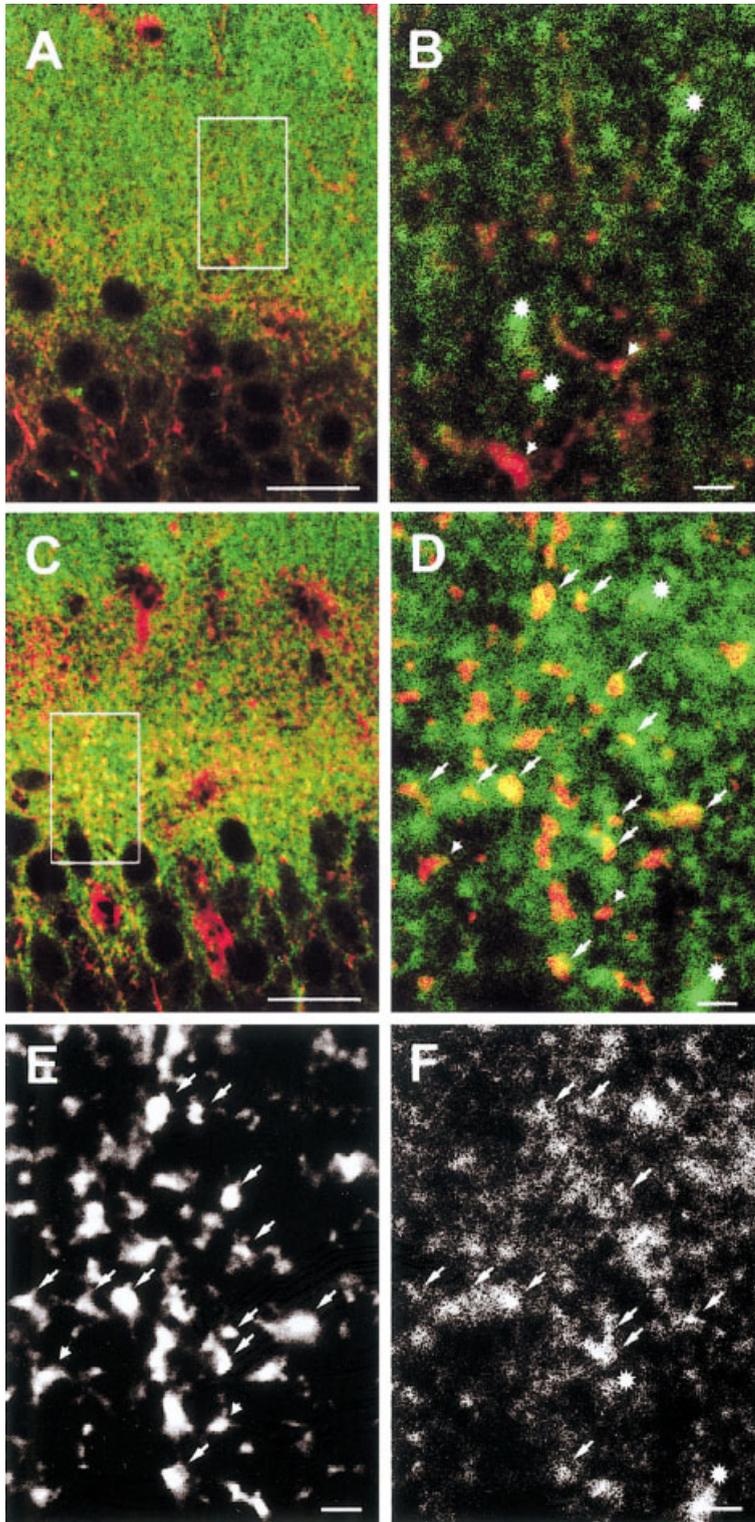


**FIGURE 5.** Double labeling for acidic calponin and either glial fibrillary acidic protein (GFAP), synaptophysin, or microtubule-associated protein 2 (MAP2) in coronal sections of dentate gyrus from pilocarpine-treated animals at 2 weeks. **A,B:** Simultaneous detection of acidic calponin (red) and GFAP (green) showed that many astrocytes that were immunoreactive for GFAP were labeled for the acidic calponin protein (yellow). **B:** High magnification of the double labeling for acidic calponin and GFAP in the inner molecular layer (IML). Many spots that were immunoreactive for acidic calponin were not labeled for GFAP. **C:** Simultaneous detection of acidic calponin (red)

and synaptophysin (green) showed no co-localization of the two proteins. (Inset) High magnification of the region outlined in the inner molecular layer (IML). The immunostaining for acidic calponin and synaptophysin appeared in adjacent spots. **D:** Simultaneous detection of acidic calponin (red) and MAP2 (green) showed no co-localization of the two proteins. (Inset) High magnification of the outlined region showed that acidic calponin immunoreactive spots appeared to be attached to the dendritic shafts labeled for MAP2. Scale bars = 50  $\mu\text{m}$  in **A**;  $\mu\text{m}$  in **B**–25; 2  $\mu\text{m}$  in **C,D** (inset).

(Fischer et al., 1998). In addition to actin filaments, myosin is also present at high concentrations in spines (Drenckhahn and Kaiser, 1983; Morales and Fifkova, 1989). Therefore, the actomyosin-based motility may be involved in the morphological changes in spines. However, little is known about the molecular mechanisms by which the motility of actin filaments is regulated in spines.

Because the increase in acidic calponin is observed during the period of important remodeling of dendritic spine shape in dentate granule cells, we postulate that this protein is one of the endogenous regulators of the motility of actin filaments during morphological changes of spines. Several lines of evidence reinforce this idea. First, in neurons acidic calponin is localized mainly in den-



**FIGURE 6.** Double labeling for acidic calponin and drebrin in coronal sections of dentate gyrus from control (A,B) and pilocarpine-treated animals at 2 weeks (C–F). **A:** In a control rat, many cellular elements immunolabeled for acidic calponin (red) are observed in the granule cell and molecular layers. The immunolabeling for drebrin (green) is mainly located in the molecular layer. No co-localization between the two proteins is detected. **B:** High magnification of the region outlined in A. Many dots (stars) probably corresponding to dendritic spines are labeled for drebrin only (green). Acidic calponin-labeled elements (arrowheads) are present and presumably correspond to glial processes. **C:** In a pilocarpine-treated animal, many cellular elements immunolabeled for acidic calponin only (red) are observed in the granule cell and molecular layers of the dentate gyrus (DG). Immunolabeling for drebrin only is mainly present in the molecular layer. In addition, many elements double-labeled (yellow) for acidic calponin and drebrin are observed in the inner one-third of the molecular layer (IML) in contrast to the control animal (A). No co-localization was observed in the outer two-thirds of the molecular layer. **D–F:** High magnification of the region outlined in C. **D** corresponds to the merge of acidic calponin (E) and drebrin (F). In the inner molecular layer of this pilocarpine-treated animal, some dots are double-labeled for the two proteins (D–F: white arrows; D: yellow), suggesting that acidic calponin is localized in some spines. Note also that, in the same region, some dots are immunoreactive for acidic calponin only (D,E: arrowheads) and many dots are only labeled for drebrin (D,F: stars). Scale bars = 25  $\mu\text{m}$  in A,C; 2  $\mu\text{m}$  in B,D–F.

dritic spines (Agassandian et al., 2000). Second, overexpression of acidic calponin in cultured HEK 293 cells induces major morphological changes through a reorganization of actin filaments (Ferhat et al., 2001). Third, we have recently shown that the overexpression of acidic calponin in hippocampal neurons is targeted to spines (Ferhat et al., in preparation). This overexpression of acidic calponin may affect the organization and the dynamic of actin

filaments in spines, leading to the alteration in the shape of dendritic spines. Finally, the main *in vitro* effect of the calponin family is to inhibit actomyosin activity (Gimona and Small, 1996; Winder and Walsh, 1996; Winder et al., 1998). This suggests that acidic calponin possibly modulates the motility of actin filaments that would alter the shape of dendritic spines. Indeed, consistent with this idea, overexpression of drebrin, another actin-binding

protein that affects the actomyosin machinery (Hayashi et al., 1996), has been shown to induce an elongation of dendritic spines in cortical neurons (Hayashi and Shirao, 1999). In addition to acidic calponin, other proteins may contribute to the remodeling of dendritic spines. In favor of this idea, more recent work of Roth et al. (2001) showed that the expression of synaptopodin, an actin-binding protein, also known to be enriched in dendritic spines, is associated with synaptic remodeling processes in the rat kainate model of epilepsy.

In conclusion, our data strongly support the notion that acidic calponin could contribute to the spine plasticity occurring in pilocarpine-treated rats. It is appealing to suggest that this protein may also be involved in the formation, growth and plasticity of spines during normal neuronal development. In brief, acidic calponin may constitute a novel molecular correlate of neuroplasticity.

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