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Reversible loss of dendritic spines and altered excitability after chronic epilepsy in hippocampal slice cultures

(neurodegeneration/plasticity)

MICHAEL MÜLLER, BEAT H. GÄHWILER, LOTTY RIETSCHIN, AND SCOTT M. THOMPSON

Brain Research Institute, University of Zurich, August Forel-Strasse 1, CH-8029 Zurich, Switzerland

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ABSTRACT **The morphological and functional consequences of epileptic activity were investigated by applying the convulsants bicuculline and/or picrotoxin to mature rat hippocampal slice cultures. After 3 days, some cells in all hippocampal subfields showed signs of degeneration, including swollen somata, vacuolation, and dendritic deformities, whereas others displayed only a massive reduction in the number of their dendritic spines. Intracellular recordings from CA3 pyramidal cells revealed a decrease in the amplitude of evoked excitatory synaptic potentials. γ-Aminobutyric acid-releasing interneurons and inhibitory synaptic potentials were unaffected. Seven days after withdrawal of convulsants, remaining cells possessed a normal number of dendritic spines, thus demonstrating a considerable capacity for recovery. The pathological changes induced by convulsants are similar to those found in the hippocampi of human epileptics, suggesting that they are a consequence, rather than a cause, of epilepsy.**

Temporal lobe epilepsy and status epilepticus are associated with a characteristic pattern of cell damage in the hippocampus. There is usually a prominent loss of pyramidal and/or granule cells (1, 2). In addition, numerous deformities are observed in surviving neurons, including swollen and "beaded" dendrites, as well as a complete or partial loss of dendritic spines, as revealed in Golgi-stained preparations (3, 4). Whether this neuropathology is the primary cause of the seizure disorder or rather a consequence of epileptic seizures resulting from some other etiology is not yet resolved. We have attempted to distinguish between these two possibilities by examining the effects of epileptic activity of known origin—namely, that induced by application of convulsant drugs—on neurons in hippocampal slice cultures maintained *in vitro*.

Synchronized, epileptiform burst discharge can be elicited in hippocampal slices and slice cultures by blocking inhibitory postsynaptic potentials (IPSPs) mediated by γ-aminobutyric acid (GABA) with either bicuculline methochloride (BMC) or picrotoxin (5, 6). We report here that after several days of such a treatment, morphological and physiological changes occur in pyramidal cells that are similar to those reported in tissue from human epileptics. Furthermore, the loss of dendritic spines can be reversed by returning treated cultures to normal medium.

MATERIALS AND METHODS

Preparation and Treatment of Cultures. Hippocampal slice cultures were prepared as described (7). In brief, transverse hippocampal slices were prepared from neonatal rat pups, attached to glass coverslips, and placed in sealed test tubes containing semi-synthetic medium. Test tubes were then inserted in a roller drum in an incubator. Within 12 days *in vitro*, the pyramidal and granule cells have developed their characteristic dendritic architecture and have elaborated their axons (8), which remain stable for an additional 2–3 weeks.

After 12–20 days *in vitro*, 100 μM BMC and/or 500 μM picrotoxin was added daily for 3 days in the fresh chemically defined serum-free medium, 75% Eagle's basal medium/25% Hanks' balanced salt ion/27.8 mM glucose/1 mM L-glutamine. Electrophysiological experiments showed that under these conditions, pyramidal cells spontaneously exhibit paroxysmal depolarization shifts of 30–50 mV in amplitude and 600–800 ms in duration (5, 6) at ≈0.02 Hz and will continue to do so over many hours. At the same time, sister cultures from the same series received fresh serum-free medium daily for 3 days and served as controls.

Histochemistry. Living CA3 pyramidal cells were impaled under visual control on an inverted microscope with microelectrodes containing 4% (wt/vol) horseradish peroxidase. After 1- to 3-hr postinjection survival, to allow for diffusion of horseradish peroxidase throughout the dendritic tree, cultures were fixed in glutaraldehyde and processed using diaminobenzidine as the chromagen, followed by a toluidine blue counterstain for Nissl substance (8). In other experiments, the anti-GABA monoclonal antibody 3A12 was used to detect glutaraldehyde-linked GABA–protein conjugates in fixed, trypsinized cultures, as described (6). Untreated sister cultures were processed simultaneously to ensure equivalent staining.

Intracellular Recording. Intracellular recording of membrane potential was obtained as described (9) by using methyl sulfate-filled sharp microelectrodes. Depolarizing or hyperpolarizing current was injected through the recording electrode via an active bridge circuit to change the membrane potential.

Isolated excitatory postsynaptic potentials (EPSPs). EPSPs were pharmacologically isolated as described (9). Synaptic potentials were elicited with monopolar stimuli (100-μs duration) delivered via a micropipette placed within the dentate gyrus. EPSPs were recorded at ≈−75 mV in a solution containing D-2-amino-5-phosphonovalerate (AP5, 20 μM), BMC (10 μM), and CGP 35348 (500 μM) to block *N*-methyl-D-aspartate, GABA A-type, and GABA B-type receptors, respectively, as well as 10 μM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) to partially block non-*N*-methyl-D-aspartate receptors. The EPSP remaining under these conditions can be reduced by further increasing concentration of the competitive antagonist CNQX, has a linear dependence on membrane potential, and follows high-frequency stimulation, indicating that it is mediated by glutamate release, probably from the

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Abbreviations: AP5, D-2-amino-5-phosphonovalerate; BMC, bicuculline methochloride; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; EPSP, excitatory postsynaptic potential; GABA, γ-aminobutyric acid; IPSP, inhibitory postsynaptic potential.

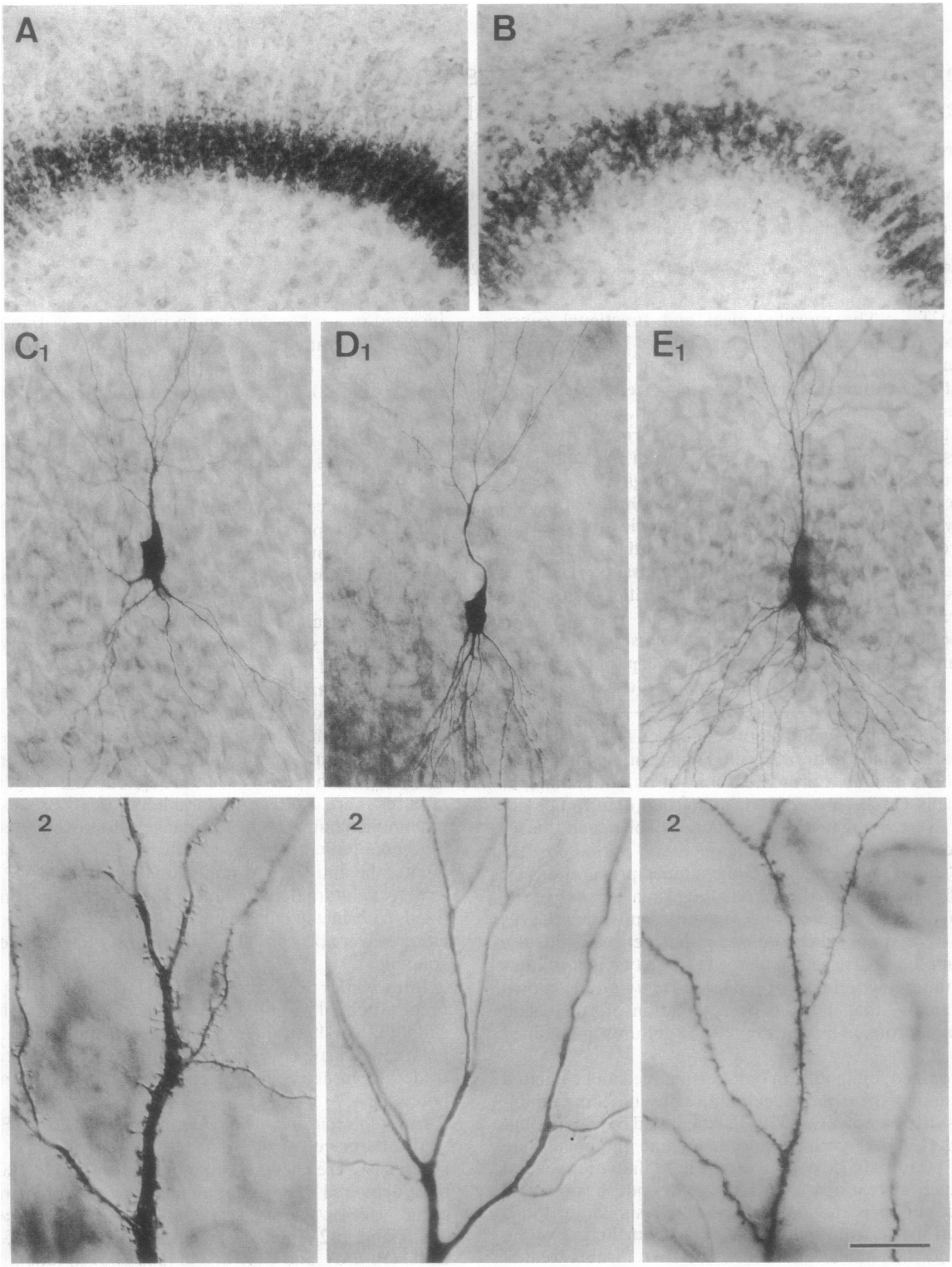

FIG. 1. Morphological consequences of chronic epilepsy. (*A*) Area CA3 of a control hippocampal culture. (*B*) Area CA3 of a sister culture after treatment for 3 days with 100 μM BMC, in which many swollen, degenerating cells can be seen. (*C–E*) CA3 pyramidal cells injected with horseradish peroxidase at low (*1*) and high (*2*) magnification. (*C*) Control cell displaying the normal dendritic structure and complement of dendritic spines. (*D*) CA3 cell (from sister culture to cell in *C*) after 3 days of treatment with 500 μM picrotoxin, exhibiting an almost total loss of dendritic spines. (*E*) CA3 cell in a culture treated with 500 μM picrotoxin for 3 days and then 1 week in control medium, showing clear evidence of recovery: no vacuolation was apparent, and spines were present on all dendrites. [Bar = 100 μm (*A* and *B*), 50 μm (C_1–E_1), and 10 μm (C_2–E_2).

axons of dentate granule cells, that acts on non-*N*-methyl-D-aspartate receptors.

IPSPs. Monosynaptic IPSPs were elicited with monopolar stimulation at the border between stratum pyramidale and stratum radiatum within 300 μm of the recorded cell in the presence of CNQX and AP5 (20 μM each) (9, 10).

Confocal Microscopy. Laser confocal microscopy, combined with digital image processing, was used to obtain high-resolution images of dendritic spines without out-of-focus regions. The two-dimensional images illustrated were prepared by acquiring a series of seven to eight images at focal planes 0.2 μm apart in the non-confocal transmitted-

light mode (Bio-Rad mounted on a Zeiss microscope). Each image was recorded digitally with a resolution of 768 × 512 pixels (pixel size, 0.066 × 0.066 μm^2). Postacquisition processing was done by using the SEMPER image-processing system (Synoptics, Cambridge, U.K.) running on a Sun4 workstation. The images of the desired slices were first added together. The result was then filtered by local averaging, and a directional differencing operator was applied to simulate a differential interference-contrast mode. Finally, the contrast of the image was further enhanced by stretching the intensities selected from a central region of this image.

RESULTS

Cellular degeneration was first seen after 1–2 days of treatment and appeared maximal after 3 days in the presence of convulsants. Cultures stained for Nissl substance contained considerable numbers of swollen and vacuolated cells after 3 days of treatment (n = 87 of 107 cultures) (Fig. 1 *A* and *B*). Such cells could typically be found throughout all pyramidal cell regions and in the dentate gyrus, although the pyramidal cell region at the border between areas CA1 and CA3 often appeared to contain fewer degenerating cells.

Individual pyramidal cells in treated cultures that did not appear grossly swollen were injected with horseradish peroxidase (Fig. 1). These cells exhibited a characteristically disturbed dendritic morphology. Dendritic shafts were thinner than in control cells and often contained pronounced nodules and swellings in distal processes and at branch points (i.e., "string-of-beads" deformities). Most strikingly, the number of spines on both apical and basal dendrites was considerably reduced (Figs. 1 and 2). Occasionally, cells lacked dendritic spines altogether. Reductions in the number of dendritic spines were observed in cells showing considerable somatic vacuolation, as well as in cells with apparently healthy somata. Within any one culture, individual cells could exhibit a greater or lesser loss of spines. Likewise, different dendrites on any single cell could show various amounts of spine loss. In general, the paucity of spines was greatest in the cultures showing the largest number of swollen, vacuolated cells.

Identical pathological changes were seen as a result of either picrotoxin or BCM treatment, whereas both together produced greater degeneration. Furthermore, simultaneous application of convulsants together with tetrodotoxin (2 μM) prevented all degenerative changes (n = 14 cultures). This characteristic pathology must, therefore, result from the epileptiform activity *per se* and not result from a direct toxic action of the convulsants themselves.

The physiological consequences of these pathological changes were examined with intracellular microelectrode recording from CA3 cells after 3 days of treatment with convulsants. The resting membrane potential and input resistance of these cells were not different from values in untreated cells. Stimulation of mossy fiber afferents elicited an EPSP followed by a biphasic IPSP, as in control cultures. Neither epileptiform bursting nor hyperexcitability was ever observed in control saline (n = 26 cultures). The amplitude of pharmacologically isolated EPSPs, obtained by stimulating within the dentate gyrus after completely blocking GABA receptors and incompletely blocking excitatory amino acid receptors (9) was determined as a function of stimulation intensity. The amplitude of evoked EPSPs at a given stimulation intensity was found to be much smaller in treated cultures than in control sister cultures (Fig. 3). These results demonstrate that chronic epileptic activity can lead to a decrease in synaptic excitation and does not necessarily produce self-sustaining hyperexcitability.

In some models of chronic epilepsy, the number of GABAergic inhibitory synapses has been found to be greatly reduced (11–14), and evoked IPSPs have been shown to be depressed (14–18). Given that blockers of GABAergic IPSPs produce epileptic discharge (5), such a loss of inhibition

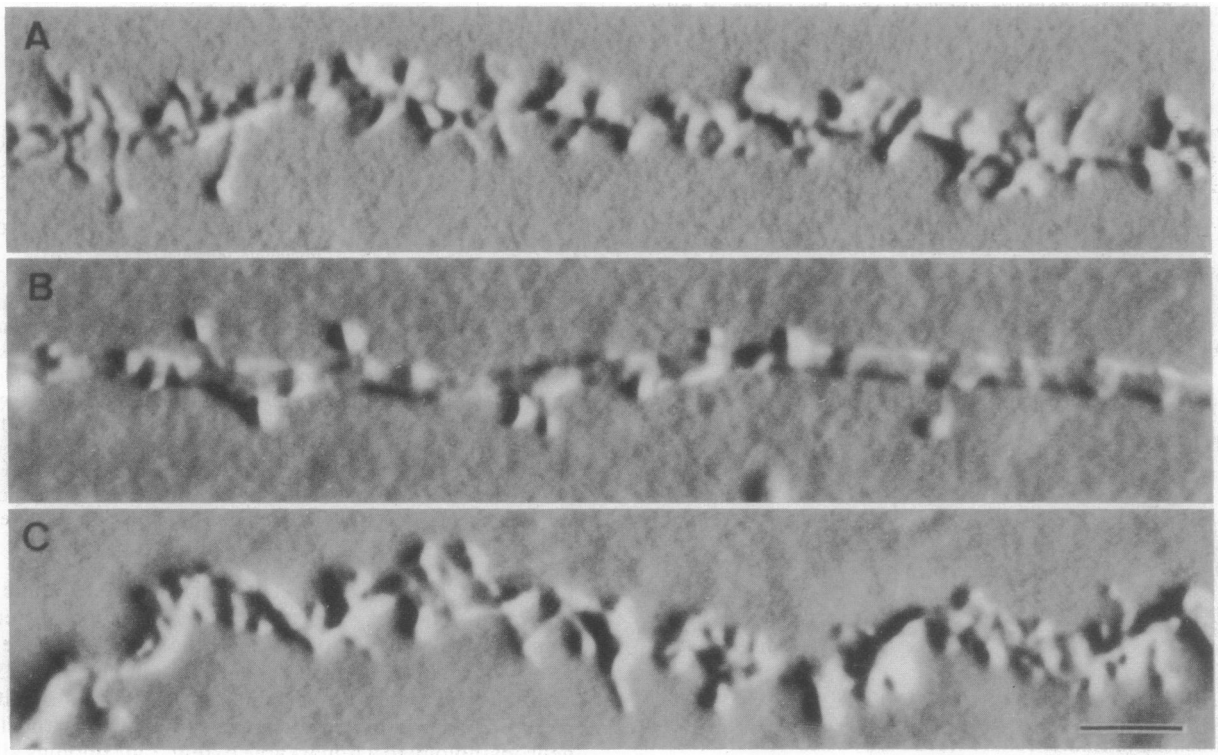

FIG. 2. Dendritic spines in CA3 pyramidal cells (same cells as Fig. 1 *C–E*). Control culture (*A*), sister culture treated with 500 μM picrotoxin for 3 days (*B*), and cell after 3 days of picrotoxin treatment followed by 7-day recovery in control medium (*C*). Images were obtained on a scanning laser microscope with transmitted light mode. A series of three images at different focal planes have been superimposed and differentiated to enhance contrast. (Bar = 4 μm.)

would be an important functional consequence of epileptic activity. We therefore used GABA immunohistochemistry to examine the effects of epileptic activity on GABAergic interneurons. After application of convulsants for 3 days, however, the number and morphology of GABA-immunoreactive neurons were identical to untreated sister cultures (Fig. 4 *A* and *B*). The mean (±SD) number of immunopositive cells in treated cultures was 799 ± 120 cells per culture (n = 5), not significantly different than in untreated sister cultures (718 ± 48 cells per culture, n = 5). Monosynaptic IPSPs, obtained in the presence of antagonists of excitatory amino acids (9, 10), were compared with intracellular recording from CA3 cells in control and treated cultures. The amplitude and reversal potential of monosynaptic IPSPs in treated cultures were not different than those in untreated sister cultures (Fig. 4*C*). These results suggest that damage to GABAergic interneurons is not an obligatory concomitant of epileptic activity.

The permanence of these pathological changes was assessed by returning cultures to normal medium after 3 days of treatment with convulsants. After 1 week of recovery, stratum pyramidale of Nissl-stained convulsant-treated cultures apparently contained fewer neurons than control sister cultures at the same age, suggesting that cell death had occurred. The remaining cells, however, had a healthy cell morphology and possessed a relatively normal complement of dendritic spines (Figs. 1*E* and 2*C*). Physiologically, the amplitude of evoked EPSPs (assessed with input/output curves as in Fig. 3) remained slightly depressed relative to untreated sister cultures at the same age but were nonetheless larger than those obtained immediately after treatment (data not shown). Thus, hippocampal cells and their dendritic spines apparently have the innate capacity to recover significantly after intense epileptic activity.

DISCUSSION

The hippocampi of human epileptics typically exhibit considerable sclerotic damage characterized by a loss of pyramidal cells and (to a lesser extent) dentate granule cells, deformed dendrites, and a decreased number of dendritic spines (1–4). A depletion of dendritic spines has been suggested in other *in vivo* models of chronic epilepsy (19, 20). Chronic epilepsy in hippocampal slice cultures results in a pattern of morphological changes that mimics the human neuropathology to a remarkable extent. Our findings allow us to conclude that such changes are a consequence and not necessarily a direct cause of epilepsy. Furthermore, our anatomical and physiological observations of intact GABAergic inhibition in treated cultures suggest that loss of

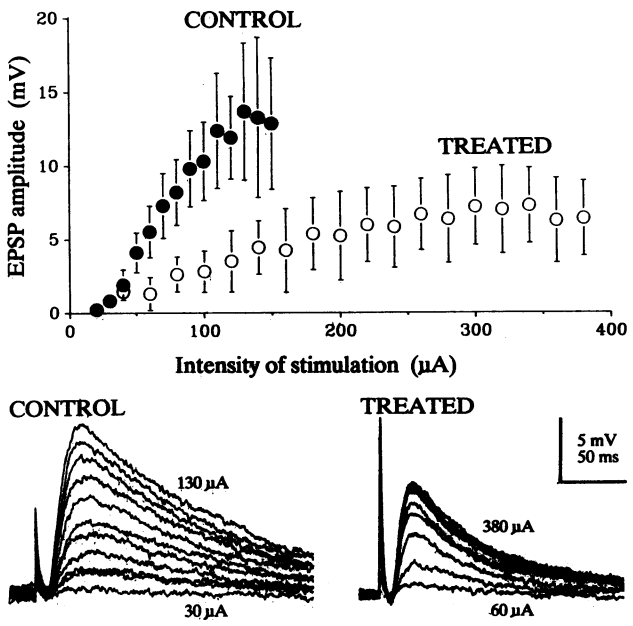

FIG. 3. EPSPs were depressed after chronic epilepsy. Amplitude of isolated EPSPs (means ± SEM) is plotted as a function of stimulation intensity in control cultures (●, 10 cells in four cultures) and in cultures treated with BMC (100 μM) for 3 days (○, 9 cells in four cultures). Representative data from one control and one treated cell are shown below. Chronic epilepsy resulted in a decrease in EPSP amplitude at any given stimulation intensity. Further increases in stimulation intensity were unable to compensate for this depression.

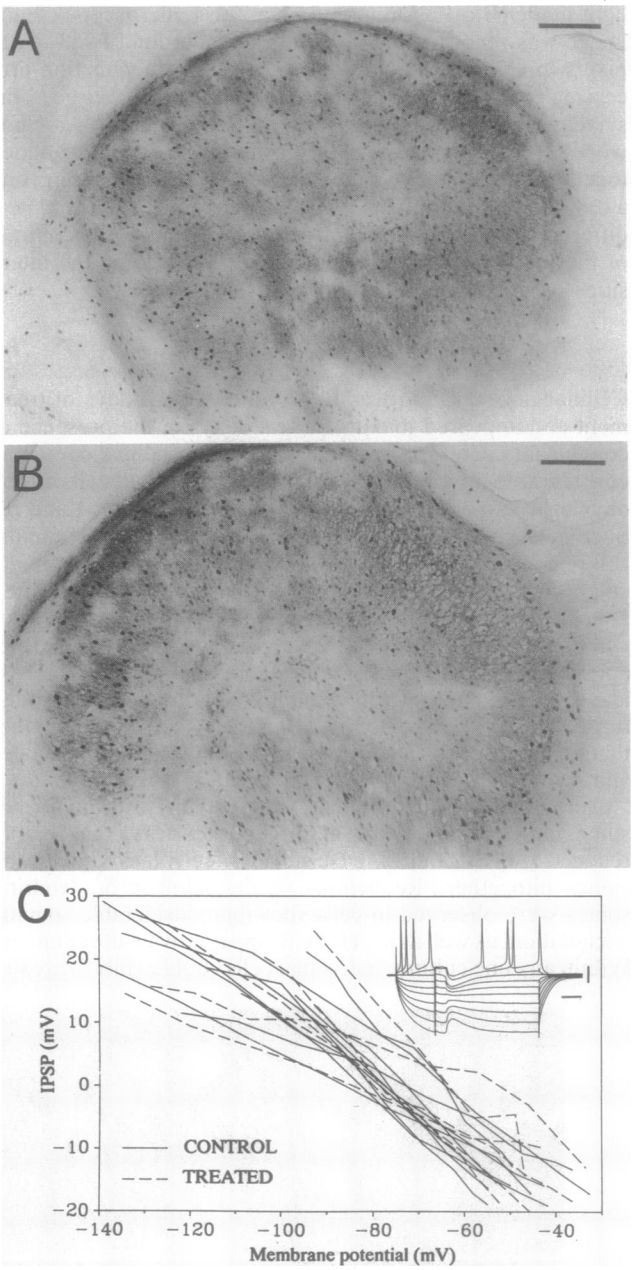

FIG. 4. GABAergic inhibition was unaffected by chronic epilepsy. Immunohistochemical localization of anti-GABA monoclonal antibody 3A12 in a control culture (*A*) and after 3 days of exposure to 100 μM BMC (*B*). The treated culture showed clear evidence of degeneration; yet the number of immunoreactive neurons was not altered. (*C*) The amplitude of the monosynaptic IPSP is plotted as a function of membrane potential for several CA3 cells in control cultures (——) and in cultures treated with 100 μM BMC for 3 days (----). No differences in either reversal potential or conductance were apparent. (*Inset*) Monosynaptic IPSPs from a representative BMC-treated cell. (Bar = 20 mV, 20 ms.)

inhibition is not always a consequence of epileptic activity. Indeed, evidence suggests that GABA immunoreactivity is also preserved in hippocampi from human epileptics (21). Nonetheless, our observation of depressed EPSPs is compatible with the recent suggestion of Sloviter (18) that decreased excitation of inhibitory interneurons could contribute to hyperexcitability in epilepsy.

Loss of dendritic spines has been reported after sensory deprivation or lesion of synaptic afferents (22); however, these experimental manipulations create a decrease in the level of activity at the affected synapses. In addition, high-frequency stimulation of excitatory afferents during induction of long-term potentiation reportedly leads to an increase in the size of dendritic spines (23–25). In our experiments, in contrast, dendritic spines were apparently destroyed as a result of strong synaptic activation, perhaps due to the excessive release of glutamate and its associated Ca^{2+} influx (26). The subsequent depression of excitatory synaptic transmission may represent a self-protective mechanism that allowed these cells to survive excitotoxic cell death (27, 28).

This model of chronic epilepsy offers the opportunity to examine the mechanisms underlying an intrinsically produced neuropathology under carefully controlled conditions *in vitro*. It will be particularly useful for examining the role of excitatory amino acid receptors in producing a pattern of damage resembling the human neuropathology in great detail. Finally, it is to be hoped that use of this *in vitro* method will minimize the number of animals currently needed for *in vivo* screening of anticonvulsant and neuroprotective substances.

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