Suppression of Synaptogenesis by Epileptiform Discharges in Hippocampal Slice Culture[1)]

Yuji IKEGAYA, Yuri IKEDA, Hiroshi SAITO, and Nobuyoshi NISHIYAMA*

Laboratory of Chemical Pharmacology, Graduate School of Pharmaceutical Sciences, The University of Tokyo, 7–3–1 Hongo, Bunkyo-ku, Tokyo 113, Japan. Received October 6, 1997; accepted November 19, 1997

Using an organotypic slice culture of the hippocampus, the effects of epileptic activities on synapse reorganization following axotomy were investigated. The maximal amplitude of field excitatory postsynaptic potentials that reflected the number of functional synaptic contacts were recorded 7 d after the mossy fibers or Schaffer collaterals were transected at 8 d *in vitro*. Fifty μM picrotoxin elicited epileptiform bursts, whose severity in the CA1 region was lower than that in the CA3 region. Synapse reformation of the mossy fibers was significantly prevented by picrotoxin, and that of Schaffer collaterals also tended to be attenuated. Ten μM bicuculline, 1 mM pentylenetetrazol or 2 mM 4-aminopyridine also induced epileptic activities in the CA3 region and significantly depressed synapse formation of the mossy fibers. Using cultures of dispersed neurons, we found that the prolonged depolarization of membrane potentials promoted neurite outgrowth. Taken together, we concluded that the preventing effects of epileptic activities on synapse reorganization following axotomy was due to the inhibition of the synaptogenesis process, not to a blockade of axon outgrowth.

Key words hippocampus; epilepsy; mossy fiber tract; synapse formation; neurite outgrowth; organotypic slice culture

Among the various structures of the brain, the hippocampal mossy fiber tract, axons originating the granule cells of the dentate gyrus, have received much attention in the study of synaptogenesis and synaptic or morphologic plasticity.[2,3)] Particularly, aberrant sprouting and notable changes in number of dendritic spines of the mossy fibers under epileptic conditions have been often described.[4—7)] Using an organotypic slice culture of the hippocampus receiving a transection of the mossy fibers, we previously demonstrated that picrotoxin, a γ-aminobutyric acid (GABA) receptor channel blocker, prevented its reorganization,[1)] and this finding may undergo *in vitro* evidence of the alternations in mossy fiber morphology following epilepsy. However, it remains unclear whether the effect of picrotoxin is restricted to the mossy fiber or whether it is due to epileptic activities elicited by this convulsant. In the present study, therefore, we further characterized effects of epileptic activities on synapse formation.

MATERIALS AND METHODS

Organotypic Slice Culture The hippocampi were prepared from postnatal 8-d-old Wistar rats and were cut into 300-μm thick slices. Sections were placed on polytetrafluoroethylene membranes which were inserted in 6-well plates filled with a medium consisting of 50% minimum essential medium (MEM) (Life Technologies, Gaithersburg, MD, U.S.A.), 25% Hanks' balanced salt solution and 25 % horse serum (Cell Culture Lab., Cleveland, OH, U.S.A.). The cultures were kept at 37 °C in a humidified and CO_2-enriched atmosphere. The culture medium was changed once every 3.5 d.

Lesion of Cultured Hippocampal Slice In some experiments, cultivated hippocampal slices were transected through the tips of the suprapyramidal and infrapyramidal blade of the stratum granulare at 8 d *in vitro* (DiV) (Fig. 3). The lesion was performed under an operating microscope using a manipulator with a razor blade.

Extracellular Recording Cultured slices were submerged in artificial cerebrospinal fluid (ACSF) at 32 °C for more than 1 h to withdraw the medium ingredients and convulsants. The stratum granulare or the CA1 stratum radiatum was stimulated with a bipolar electrode. The evoked potential was extracellularly recorded from the CA3 or CA1 stratum pyramidale with a glass capillary microelectrode filled with 0.15 M NaCl. Positive field potential (Fig. 2A) reflected field excitatory postsynaptic potential (fEPSP) because it was blocked by 10 μM 6-cyano-7-nitroquinoxaline-2,3-dione, a non-*N*-methyl-D-aspartate receptor antagonist (data not shown). The maximal size of fEPSP was used as an index of the number of functional synaptic contacts formed as a function of time after the lesion.[1,8,9)] All electrophysiological experiments were conducted in the ACSF composed of 127 mM NaCl, 1.6 mM KCl, 2.4 mM $CaCl_2$, 2.4 mM $MgSO_4$, 1.3 mM KH_2PO_4, 1.24 mM $NaHCO_3$ and 10.0 mM glucose, and was saturated with 95% O_2 and 5% CO_2.

Dispersed Neuron Culture The hippocampi from 18-d-old embryos of Wistar rats were dissected and were dissociated by incubation with 0.25% trypsin (Difco, Detroit, MI, U.S.A.) and 0.01% deoxyribonuclease I (Sigma, St. Louis, MO, U.S.A.) at 37 °C for 30 min. The dispersed neurons were plated on polylysine-coated culture dishes at a density of 2500 cells/mm^2 and were cultivated in Eagle's MEM (Nissui Pharmaceuticals, Tokyo, Japan) containing 10% fetal bovine serum (Sanko-Junyaku, Tokyo, Japan). After 24 h, the medium was changed to serum-free Eagle's MEM supplemented with 5 g/l transferrin (Sigma), 5 g/l insulin (Becton Dickinson, Bedford, MA, U.S.A.) and 20 nM progesterone (Sigma).

Measurement of Axon Length At 2 DiV, we selected neurons that did not come into contact with other cells and took photographs of them using an inverted microscope. Immediately after the recording, the culture medium was changed to normal or 30 mM KCl-containing MEM. The same neurons were photographed after 24 or 48 h, and their longest neurites were measured as axon lengths (Fig. 4).

* To whom correspondence should be addressed.

RESULTS

Synchronous Epileptiform Activities Although 8-DiV hippocampal slices did not show apparent spontaneous activities, synchronous epileptic bursts were recorded from the stratum pyramidale of both the CA3 and CA1 regions for at least 1 h when exposed to 50 μM picrotoxin in all 15 and 8 cases tested, respectively (Fig. 1A). Their severity in the CA1 region was lower than that in the CA3 region. Synchronous epileptic activities were also evoked in the CA3 region by 10 μM bicuculline or 1 mM pentylenetetrazol, GABA receptor blockers, or by 2 mM 4-aminopyridine, a voltage-sensitive potassium channel blocker, in all 5 slices tested for each convulsants (Fig. 1B).

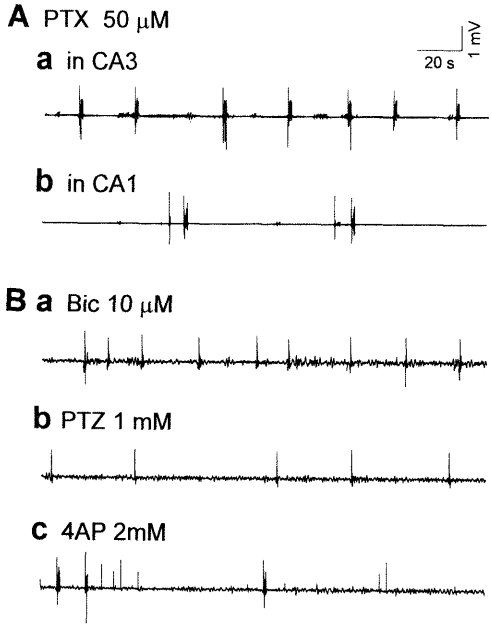

Fig. 1. Synchronous Epileptic Activities Elicited by Various Convulsants

Field potentials were extracellularly recorded from the CA3 or CA1 stratum pyramidale at 8 DiV. Fifty μM picrotoxin (PTX) evoked synchronous epileptiform bursts in both the CA3 (Aa) and CA1 (Ab) regions. Spontaneous activities were also elicited in the CA3 region by 10 μM bicuculline (Bic, Ba), 1 mM pentylenetetrazol (PTZ, Bb) or 2 mM 4-aminopyridine (4AP, Bc).

Synapse Formation No fEPSP was observed either in the CA3 or CA1 region immediately after lesion of the hippocampal slices at 8 DiV (data not shown, $n=13$), suggesting that the mossy fibers and Schaffer collaterals were completely transected using the method we employed here. At 7 d after the lesion, fEPSP in both the CA3 and CA1 regions appeared again (Fig. 2A). However, in slices exposed to 50 μM picrotoxin for 7 d after the lesion, the maximal amplitude of fEPSP evoked in the CA3 region was significantly small (Welch's test: $t(13.75)=3.744$, $p<0.001$, Fig. 2). The maximal CA1-fEPSP amplitude also tended to decrease in slices treated with picrotoxin (Student's t test; $t(14)=2.144$, $p=0.0502$, Fig. 2). Picrotoxin did not reduce the fEPSP amplitude in intact slices. CA3-fEPSP amplitude in naive slices cultivated in picrotoxin for 7 d was 112.7±8.9% of that in normal medium (Student's t test: $t(6)=0.9756$, $p=0.3671$, mean±S.E.M. of 4 slices.). CA1-fEPSP amplitude in picrotoxin was 91.6±8.6% ($t(6)=0.5721$, $p=0.5882$).

The hippocampal slices incubated with picrotoxin for 7 d after the lesion were stained with cresyl fast violet. Appear-

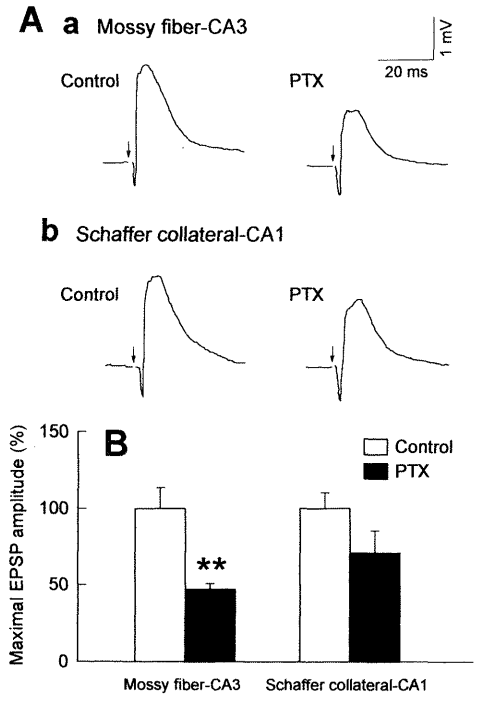

Fig. 2. The Effect of Picrotoxin on Reorganization of the Mossy Fiber-CA3 or Schaffer Collateral-CA1 Synapses after the Lesion

Panel A shows typical traces of fEPSP recorded from the CA3 (a) or CA1 (b) region evoked by stimulation of the stratum granulare (a) or the CA1 stratum radiatum (b) in the slices cultivated in normal medium (control) or in picrotoxin-containing medium (PTX) for 7 d after the axotomy. Data were summarized in panel B. Each value is expressed as a percentage of the control and represents a mean±S.E.M. ** $p<0.01$: Welch's test.

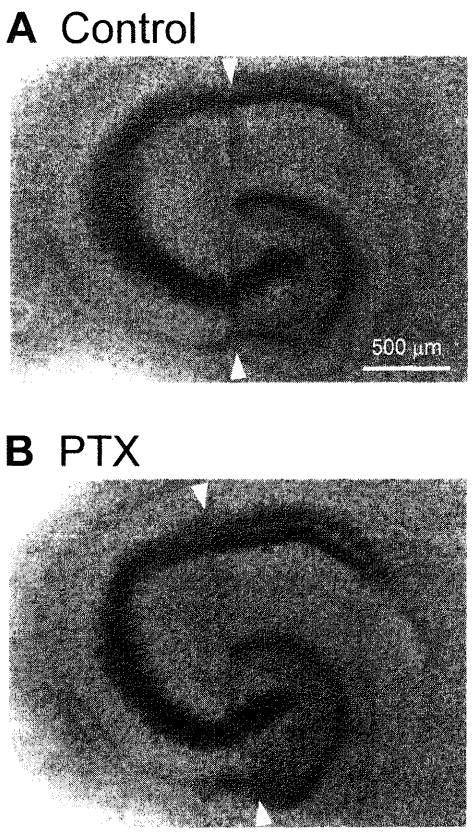

Fig. 3. Nissl-Stained Hippocampal Slice Cultures

Hippocampal slice cultivated in normal medium (A) or in 50 μM PTX-containing medium (B) for 7 d after lesion at 8 DiV was stained by cresyl fast violet. The slices in photographs A and B were obtained from the same rat pup. White arrowheads indicate the tips of the transection.

Table 1. The Effects of Various Drugs That Elicit Epileptic Activities on Synapse Formation of the Mossy Fibers

	n	Maximal amplitude of fEPSP (%)	*t* value	*p* value
Mossy-fiber lesioned				
Control	13	100.0±13.6		
Bic	7	28.2± 8.4	2.555	<0.001
Control	7	100.0±22.7		
PTZ	7	39.6±14.1	2.260	0.0435
Control	6	100.0±14.9		
4AP	8	44.5± 7.8	3.548	0.0043
Intact				
Control	4	100.0± 9.5		
Bic	4	98.3±21.6	0.072	0.9448
Control	4	100.0±24.6		
PTZ	4	94.3± 6.8	0.223	0.7738
Control	4	100.0±10.2		
4AP	4	128.9±54.4	0.522	0.4339

The mossy fibers of hippocampal slices were transected at 8 DiV (*mossy-fiber lesioned*). Immediately after the lesion, 10 μM bicuculline (Bic), 1 mM pentylenetetrazol (PTZ) or 2 mM 4-aminopyridine (4AP) was added to the culture medium. fEPSP evoked by stimulation of the stratum granulare was recorded from the CA3 stratum pyramidale 7 d after the lesion. In intact slices, field potentials were recorded at 15 DiV after exposure to drugs for 7 d (*intact*). The maximal size of fEPSP was used as an index of the number of functional synaptic contacts formed as a function of time after the lesion. Each value is expressed as a percentage of the control and represents a mean±S.E.M. *t*-value was calculated by Student's *t* test or Welch's test.

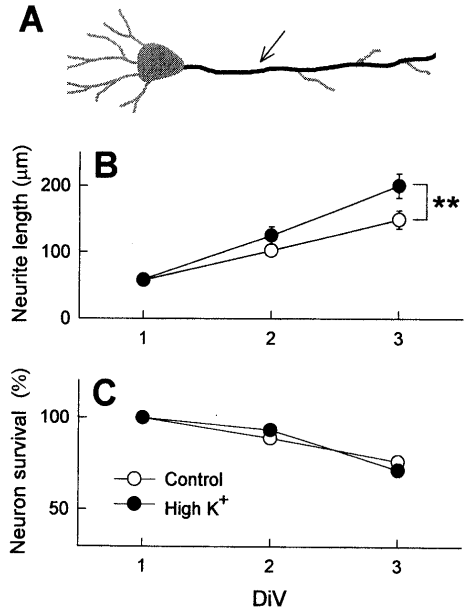

Fig. 4. The Effect of a High Concentration of Potassium on Neurite Outgrowth and Neuron Survival

Changes in the length of the longest neurite (a black line indicated by an arrow in A), *i.e.* axon length (B), and the number of alive neurons (C) were measured in dispersed hippocampal neuron culture. Neurons were cultivated in normal medium (open circles) or medium containing 30 mM K^+ (closed circles) from 3 to 5 DiV. ** $p<0.01$: two-way analysis of variance.

ances of the stratum pyramidale were not different between the control and the picrotoxin-treated slices in all 14 and 6 cases, respectively (Fig. 3).

Next, we examined the effect of 10 μM bicuculline, 1 mM pentylenetetrazol or 2 mM 4-aminopyridine on CA3-fEPSP recovery following the lesion. None of the tested convulsants had any influence on fEPSP in the intact slices, but they significantly attenuated fEPSP amplitude in the slices receiving axotomy (Table 1).

Axon Elongation The effect of continual membrane depolarization on neurite elongation was investigated using cultures of isolated neurons. Because it was difficult to evoke repetitive discharges in these cultured neurons by convulsants,[10,11] a chronic depolarization shift of membrane potentials was alternatively produced by a high concentration of KCl. Axon outgrowth was promoted by 30 mM KCl (two-way analysis of variance, $F(1,60)=7.1492$, $p<0.001$, Fig. 4B) which did not cause neuron loss (two-way analysis of variance, $F(1,2)=0.0002$, $p=0.9900$, Fig. 4C).

DISCUSSION

Picrotoxin, whcih evoked epileptiform discharges in the CA3 region, prevented the reorganization of transected mossy fibers. All other convulsants employed here, which used different pharmacological properties to exert their effects, also elicited epileptic activities in the CA3 pyramidal neurons and inhibited the mossy-fiber reformation. These results strongly suggest that it is epileptiform discharges evoked by these convulsants which suppress the recovery process of the mossy fibers following axotomy. On the other hand, picrotoxin evoked epileptic bursts in the CA1 region and also showed a strong tendency to prevent regeneration of lesioned Schaffer collaterals. The reason why the effect of this drug on the Schaffer collaterals was slightly weak may be that less epileptic activities were elicited in the CA1 region. We speculated, therefore, that the inhibitory effect of epileptiform discharges on synapse formation is not restricted to the mossy fibers but is a universal phenomenon in the nervous system.

Because excessive excitatory activities often cause a loss of neurons,[12—14] it is possible that reduced fEPSP in the slices treated with convulsants was due to a massive loss of the CA3 pyramidal neurons, a main target of the mossy fibers. Two kinds of experiments were thus designed to ascertain this possibility. Although the cell distribution of slices treated with picrotoxin was examined using the Nissl-staining method, there was no aberration in the appearance of the stratum pyramidale in picrotoxin-treated slices. Additionally, in the cultures of isolated neurons, a high concentration of KCl did not induce neuronal death. Based on these data, the target loss of the mossy fibers is not likely involved in the inhibition of synapse formation by picrotoxin.

It remains unclear which the elongation process and/or synaptogenesis of truncated mossy fibers was prevented under epileptic conditions. We evaluated whether prolonged depolarization inhibits axon outgrowth, using cultures of isolated neurons, which provided a less complicated and more fundamental system than organotypic slice culture, because the former included fewer glial cells and each neuron had no physical contact with the other cells. We found here that axon outgrowth was not inhibited, but rather promoted, by a continual depolarization shift, compatible with several previous reports.[15,16] Accordingly, depolarization evoked by epileptic discharges may not hinder the re-extension of amputated axons. Taken together, it is likely that the synaptogenesis process, the last step of reformation of the mossy fibers, was inhibited by epileptic activities. Further histological study is now underway in our laboratory to evaluate whether the mossy fibers re-extended close to their target in

picrotoxin-treated slices or whether the total number of synapses that the mossy fibers formed following reelongation was changed under epileptic conditions.

Our results suggest that the mossy fibers did not come into contact with accurate targets under epileptic circumstances. Requirement of appropriate firing patterns for precise neural circuit formation has been intensively argued.[17,18] Cesare *et al.*[19] observed a unique pattern of firings from the CA3 pyramidal cells during the second postnatal week. Because this week is known to be a critical period in which the mossy fibers are mainly formed,[2,20,21] it is probable that such a specific pattern of neuron activities is necessary for the mossy fibers to come close to their correct destination and to form synaptic contacts with it.[6,19] Therefore, it is plausible that epileptic activities evoked by convulsants applied exogenously had confused an appropriate pattern of activities, and thereby that the mossy fibers failed to form accurate synaptic contacts.

There is growing information available concerning abnormal morphological and physiological changes in the hippocampus of human epileptics or epileptic animals.[7,22] Particularly, the mossy fibers have provoked much interest because of their high plasticity, such as aberrant sprouting and notable changes in number of dendritic spines in the epileptic hippocampus.[4—7] Because the reorganization processes of the lesioned tissue in a slice culture system dependably retain its developmental manners and organotypic properties *in vivo*,[9,23—25] the irregularity of mossy-fiber reformation under epileptic conditions, as we showed here, may provide a simple and reliable model for the study of epilepsy induced morphological alternation in the hippocampus.[1]

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