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Epileptic Activity Prevents Synapse Formation of Hippocampal Mossy Fibers via L-Type Calcium Channel Activation *In Vitro*

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ABSTRACT

Hippocampal slice from early postnatal rat was used to elucidate the influence of epileptic activity elicited by picrotoxin on synapse formation of mossy fibers. Neurite reelongation and synaptogenesis of mossy fibers transected at 8 days *in vitro* were confirmed by staining with DiI, a fluorescent membrane dye used as a neuronal tracer, and by recording field excitatory postsynaptic potentials (fEPSP) in the CA3 region evoked by stimulation of the dentate gyrus. Picrotoxin (50 μM), which evoked spontaneous epileptiform firing in the CA3 region that was occluded by tetrodotoxin (1 μM), hindered development of fEPSP amplitude after a lesion of mossy fibers. Furthermore, observations using a Timm method, a histochemical technique that preferentially labels synaptic terminals of mossy fibers, revealed that picrotoxin prevented synaptogenesis in the CA3 region. This inhibitory effect of picrotoxin was completely abolished by tetrodotoxin or nicardipine (10 μM), a L-type calcium channel blocker, but not by 2-amino-5-phosphonopentanoic acid (50 μM), a N-methyl-D-aspartate receptor antagonist, suggesting that influx of calcium ion via L-type calcium channels during epileptic bursts mediated the disturbance of appropriate synapse formation of mossy fibers.

Because ontogenetic maturation of several regions in the central nervous system extends until early postnatal period, certain forms of injury or disease during this critical stage are correlated with developmental disorders. It is well known that epilepsy, frequency of which is much higher in children than in adults, particularly in the first year of life, is associated with a broad spectrum of cognitive deficits when it occurs in this postnatal period (Alpherts and Aldenkamp, 1990; Mizrahi, 1994; Stafstrom, 1995). However, few previous reports identified characteristic changes in structure or function of the central nervous system of epileptic patients, which may underlie such cognitive deficits.

Hippocampal mossy fiber tract, axons projecting from the granule cells in the dentate gyrus mainly to the pyramidal cells in the CA3 region, is formed very late because the dentate granule cells generate postnatally (Stirling and Bliss, 1978; Amaral and Dent, 1981, Gaarskjær, 1986). This tract is believed to be involved in cognition and learning because its degeneration produces memory deficits (Conrad and Roy, 1993; Vaher *et al.*, 1994) and its synapses demonstrate a high degree of functional plasticity (Bradler and Barrionuevo, 1989; Mitsuno *et al.*, 1994; Malenka, 1995).

Although there are numerous reports concerning dynamic morphological plasticity of mossy fibers in epileptic seizure that often demonstrate aberrant sprouting of mossy fibers into the inner molecular layer of the dentate gyrus (Babb *et al.*, 1991, Mathern *et al.*, 1994) or massive reduction in the number of dendritic spines (Müller *et al.*, 1993), it has not been reported whether epilepsy has any influences on neurite outgrowth and synaptogenesis of mossy fibers during their developmental period. Fortunately, some recent reports showed that developmental and physiological properties of mossy fibers were retained in organotypic slice cultures of postnatal hippocampus (Dailey *et al.*, 1994; Robain *et al.*, 1994; Frotscher *et al.*, 1995). Therefore, we have asked whether epileptic activity disturbs normal neurite outgrowth and synaptogenesis of mossy fibers using hippocampal slice culture. As a result, we found severe suppression of synapse formation of mossy fibers by epileptic activity.

Methods

Preparation of organotypic slice cultures. For preparation of hippocampal slices, postnatal 8 day (P8) Wistar rats were decapitated and the brains were removed. The hippocampi were cut into 300-μm thick slices in cold glucose-enriched Gey's buffer and were then cultivated according to the method introduced by Stoppini *et al.* (1991). Briefly, selected sections were placed on moistened translucent membranes (0.4 μm Culture Plate Insert, 30 mm diameter, Millicell-CM, Millipore Corporation, Bedford, MA) that were inserted in six-well plates (35 mm in diameter) filled with 1 ml of medium (50% minimum essential medium, 25% Hanks' balanced

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ABBREVIATIONS: ACSF, artificial cerebrospinal fluid; AP5, 2-amino-5-phosphonopentanoic acid; DIV, day *in vitro*; fEPSP, field excitatory postsynaptic potential; GABA, γ-aminobutyric acid; NMDA, N-methyl-D-aspartate; DiI, 3,3′-dilinolenyloxacarbocyanin perchlorate.

salt solution, 25% heat inactivated horse serum). The cultures were kept at 36°C in a humidified, CO_2-enriched atmosphere. The culture medium was changed twice a week.

Lesioning of mossy fiber tract. In some slices, mossy fibers were transected at 8 DIV along the line linking the lips of the upper and lower blade of the granule cell layers (see fig. 1, C, E and G, fig. 5, B, C and D). The lesion was performed under an operating microscope using a manipulator with a razor blade.

DiI labeling. Cultured slices were fixed with 0.1 M phosphate buffer containing 4% paraformaldehyde 1 day after DiI crystal was placed on the dentate gyrus. After a 5-wk incubation in the fixative at room temperature, the DiI-labeled axons were observed using a fluorescent microscope (Honig and Hume, 1989).

Extracellular recordings. Cultured slices were submerged for 30 to 60 min in ACSF, which was composed of 124 mM NaCl, 5.0 mM KCl, 2.4 mM $CaCl_2$, 1.3 mM $MgSO_4$, 1.24 mM KH_2PO_4, 26.0 mM $NaHCO_3$ and 10.0 mM glucose and was saturated with 95% O_2-5% CO_2, and were transferred into a recording chamber filled with the same ACSF. The hilus of the upper blade of the dentate granule cell layer was stimulated with a bipolar electrode. The evoked potential was extracellularly recorded from the CA3 pyramidal cell layer with a glass capillary microelectrode filled with 0.9% NaCl. Positive field potential (see fig. 1, B, F and H) reflected fEPSP because it was blocked by 6-cyano-7-nitroquinoxaline-2,3-dione (10 μM), a non-NMDA 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 a lesion (Muller *et al.*, 1993; Stoppini *et al.*, 1993).

Timm staining. For Timm stain, cultures were washed with 0.1 M phosphate buffer and were then immersed for 10 min in 0.37% sodium sulfide solution, immediately followed by fixation for 15 min with 10% (v/v) formaldehyde solution. After washed with 0.1 M phosphate buffer, the cultures were dehydrated with 70 and 96% ethanol, and dried. To perform the sulfide silver staining, they were submerged in the physical developer according to the method of Sloviter (1982) and were then incubated in a dark room for 50 min at 26°C. The slices were washed with distilled water at the end of the reaction.

Drugs. In slice cultures, the drugs were applied in the culture medium on and after 8 DIV. For recording spontaneous activities, the drugs were dissolved in ACSF. All the drugs used were obtained from commercial sources; picrotoxin (Wako Pure Chemical Industry, Ltd., Osaka, Japan), a GABA receptor channel blocker; tetrodotoxin (Sigma Chemical Co., St. Louis, MO), a voltage-sensitive sodium channel blocker; nicardipine (Wako), a L-type calcium channel blocker; AP5 (Sigma), a NMDA receptor antagonist; 6-cyano-7-nitroquinoxaline-2,3-dione (Research Biochemical Incorporated, Natick, MA), a non-NMDA receptor antagonist.

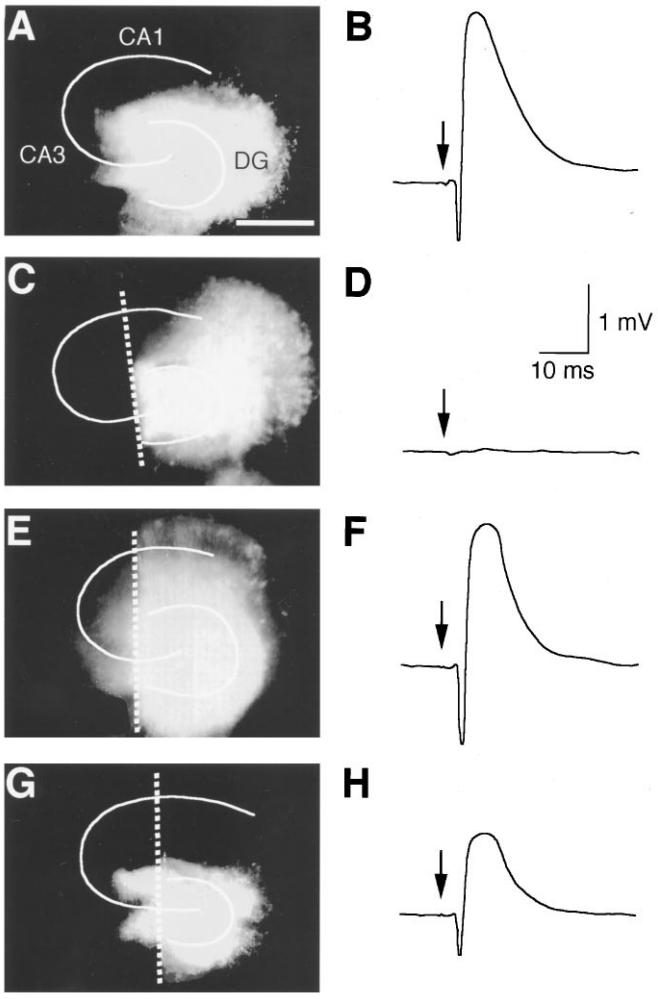

Fig. 1. Lesion-induced reorganization of mossy fibers. Fluorescent images of hippocampal slices stained with DiI were observed at 8 DIV (A), 0 day (C) or 7 days (E) after lesions at 8 DIV. White lines demonstrate the granule cell layer of the dentate gyrus (DG) and the pyramidal cell layer of the CA1-4 regions. Mossy fibers were transected along white broken lines. A scale bar represents 1 mm. Right traces were typical field potentials (average of four) in the CA3 region at 8 DIV (B), 0 day (D) or 7 days (F) after the lesion. The dentate gyrus was stimulated at the time indicated by arrows. DiI image in G and field potential in H were obtained from slices treated with picrotoxin (50 μM) for 7 days after lesions at 8 DIV.

Results

Mossy fiber growth and synapse formation after a lesion. In a series of these experiments, we investigated the effect of epileptic activity on reformation of synapses after a section of maturated mossy fibers because it was difficult to know exactly when mossy fiber formation starts *in vivo*. Many previous reports adopting this tissue lesion method indicated that organotypic characteristics, developmental processes and neuronal properties *in vivo* are well-preserved in hippocampal slice cultivated after the lesion (Gähwiler and Brown, 1985; Zimmer and Gähwiler, 1987, Heimrich and Frotscher, 1993; Li *et al.*, 1993; Stoppini, *et al.*, 1993; Dailey *et al.*, 1994; Frotscher *et al.*, 1995).

Mossy fibers were lesioned at 8 DIV because cultured slices were electrophysiologically stabilized by this time (fig. 2, open circle). First, we examined reelongation and synapto-

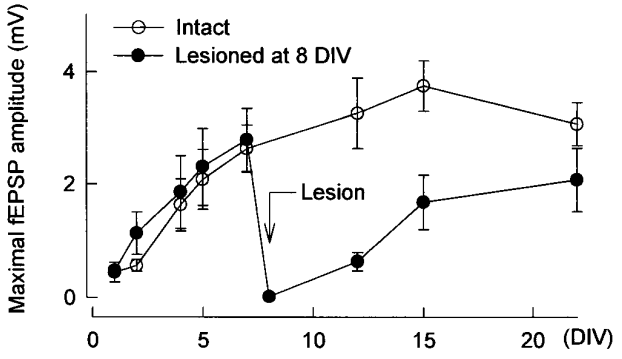

Fig. 2. Changes in amplitude of fEPSP recorded in the CA3 region elicited by supramaximal stimulation of the dentate gyrus in intact slices (n = 5–9) (open circle) or slices lesioned at 8 DIV (n = 6–9) (closed circle). Each point represents mean ± S.E.M.

genesis of transected mossy fibers under our culture conditions. Neurite outgrowth was observed by staining with DiI, which is a fluorescent membrane dye used as a neuronal tracer (fig. 1, A, C, E and G). Although mossy fibers were completely transected by the method adopted in this study (fig. 1C), they elongated close to the pyramidal cell layer of the CA3 region beyond the transection at 7 days after the section (fig. 1E), and formed functional excitatory synapses on the pyramidal cells, which was estimated by recording synaptic responses reflecting fEPSP in the CA3 region evoked by stimulation of the dentate gyrus (fig. 1, B, D and F). Because fEPSP in the CA3 region was not observed immediately after the lesion (n = 32) (fig. 1D), it was again confirmed that all mossy fibers were transected. At more than 4 days after the lesion, however, fEPSP appeared in all 82 slices tested. A change in the maximal size of evoked synaptic responses was shown in figure 2. For promoting comparisons, a change in fEPSP amplitude in nontransected slices was superimposed on the same figure. An extent of maximal response in slices at 14 days after the section was similar to that in DIV-matched intact slices.

Epileptic activity. Although epileptiform burst discharge can be elicited in acutely prepared hippocampus slices and cultured slices in a number of diverse ways, a simple procedure is to block inhibitory postsynaptic potentials mediated by GABA with its receptor antagonist (Dichter and Ayala, 1987; Thompson and Gähwiler, 1992). At 8 DIV, 42 of 43 slices (97.7%) exposed to picrotoxin (50 μM), a $GABA_A$ receptor channel blocker, showed spontaneous synchronized epileptiform bursts with a high regularity (2.05 ± 0.47 bursts/min; mean ± S.E.M. of eight slices) in the CA3 region, which individually consisted of 7.87 ± 0.85 (mean ± S.E.M. of eight slices) repetitive firings (fig. 3B), although epileptiform activity was not observed in normal ACSF (fig. 3A). Only 2 in 82 intact slices tested (2.4%) exhibited spontaneous activity that consisted of a single, but not repetitive, firing. The epileptic bursts induced by picrotoxin was blocked by application of tetrodotoxin (1 μM) (fig. 3C).

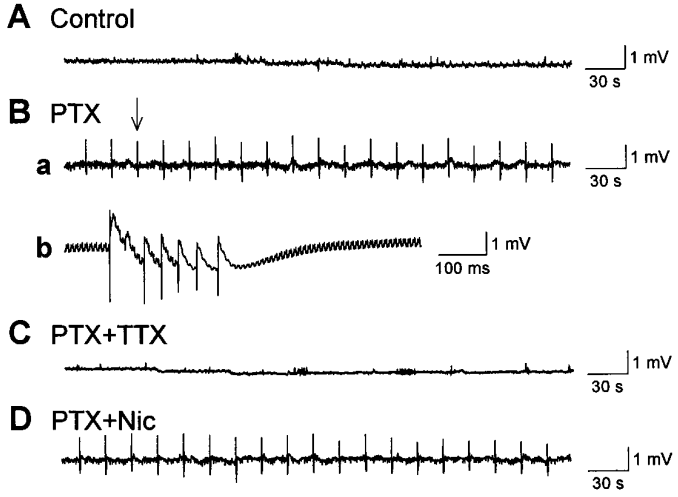

Fig. 3. Typical records of epileptic activity in the CA3 region of a hippocampal slice at 8 DIV. Field potentials were recorded in normal ACSF (A), in ACSF containing picrotoxin (PTX, 50 μM) (B), containing picrotoxin (50 μM) and tetrodotoxin (TTX, 1 μM) (C), or containing picrotoxin (50 μM) and nicardipine (Nic, 10 μM) (D). A burst indicated by an arrow in Ba was expanded in Bb.

Effect of picrotoxin on mossy fiber synapse formation. For evaluating the influence of epileptic activity on synapse formation of mossy fibers, picrotoxin was added to culture medium at a concentration of 50 μM immediately after the lesion. Development of fEPSP amplitude after the section of mossy fibers was prevented in slices cultivated in medium containing picrotoxin (figs. 1H and 4A). This inhibitory effect of picrotoxin was completely abolished by application of tetrodotoxin (1 μM) (fig. 4B). Picrotoxin did not reduce fEPSP amplitude in intact slices (maximal response amplitudes in nontransected slices cultivated for 7 days in medium containing picrotoxin was 2.52 ± 0.29 mV, and that in normal medium was 2.13 ± 0.42 mV; means ± S.E.M. of seven or six slices, respectively). To determine whether spontaneous activity in normal medium, which was rarely seen as above described, contributed to the recovery of fEPSP after the lesion, slices were cultivated in the medium containing tetrodotoxin for 7 days after the lesion. Tetrodotoxin (1 μM) did not affect fEPSP amplitude (maximal response amplitudes in slices cultivated in normal medium for 7 days was 2.37 ± 0.57 mV, and that in medium containing tetrodotoxin was 1.94 ± 0.58 mV; means ± S.E.M. of eight or nine slices, respectively). Inhibition of synaptogenesis by continuous epileptic activities was also confirmed with a Timm method, a

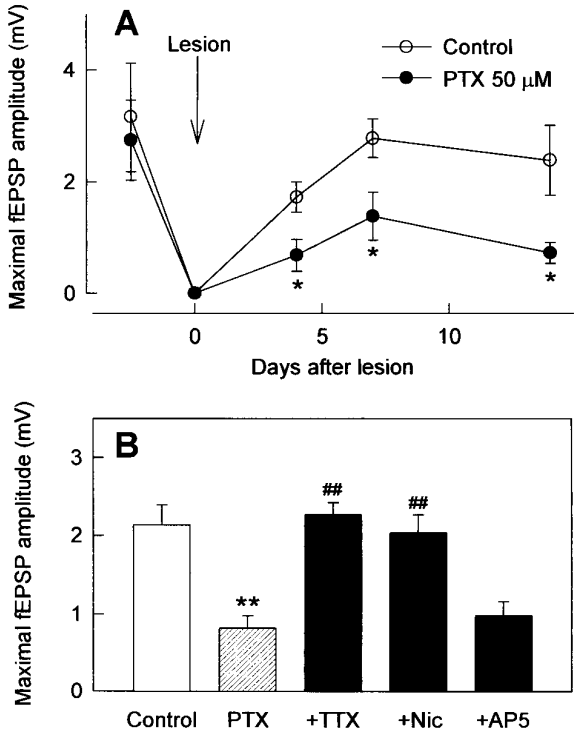

Fig. 4. Inhibitory effect of picrotoxin on mossy fiber synapse formation. A, Change in size of maximal fEPSP were observed in hippocampal slices cultivated in normal medium (n = 5–11) (open circle) or in medium containing picrotoxin (PTX, 50 μM) (n = 5–9) (closed circle). B, Field potentials were recorded at 7 days after the lesion in slices cultivated in normal medium (n = 12) (open column), in picrotoxin (50 μM, n = 6) (hatched column) or in coexistence of picrotoxin with tetrodotoxin (TTX, 1 μM, n = 6), nicardipine (Nic, 10 μM, n = 7) or 2-amino-5-phosphonopentanoic acid (AP5, 50 μM, n = 7) (closed column). Data are means ± S.E.M. of 6 to 12 cases. *P < .05, **P < .01 *vs.* control, ##P < .01 *vs.* PTX: Tukey's test after analysis of variance (ANOVA). Data in A and B were obtained from different series of experiments and were not pooled because deviation among experiments was large.

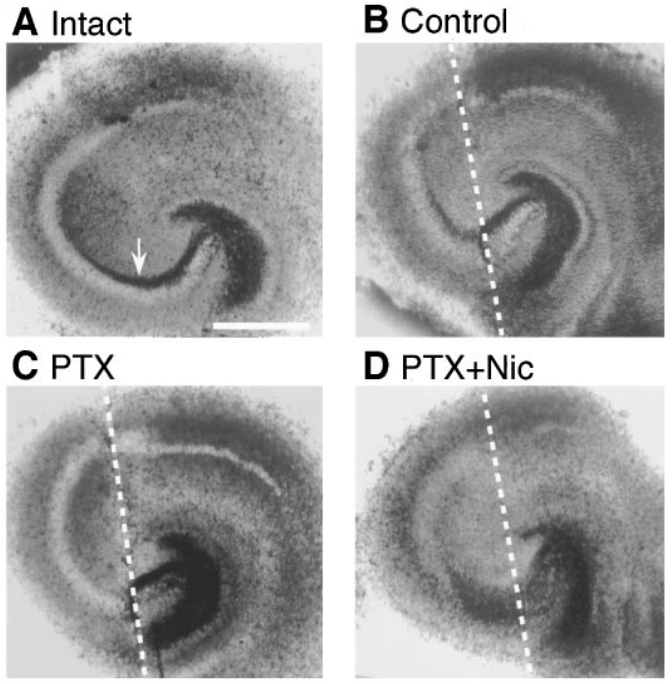

Fig. 5. Bright-field images of hippocampal slices stained with a Timm method were obtained from an intact slice at 15 DIV (A) or slices cultivated in normal medium (B), in picrotoxin (PIC, 50 μM) (C) or in picrotoxin (50 μM) and nicardipine (Nic, 10 μM) (D) for 7 days after lesions at 8 DIV. Mossy fibers were transected along white broken lines. The area indicated by an arrow is stratum lucidum, where terminals of mossy fiber tract form synapses on the CA3 pyramidal cells.

histochemical technique that labels synaptic terminals of mossy fibers because of their high zinc content (fig. 5). In extrahippocampal area, subiculum and entorhinal cortex were also stained, consistent with a previous report showing that synapse boutons in these regions contained zinc (Slomianka, 1992). In all 16 slices cultivated in normal medium, the stratum lucidum of the pyramidal cell layer in the CA3 region, which is indicated by an arrow in figure 5A, was stained across the transection (fig. 5B), but this was not observed in slices cultivated in picrotoxin in all 12 cases examined (fig. 5C). DiI labeling technique revealed that picrotoxin-treated mossy fibers grew past the lesion into the CA3 pyramidal cell layer at 7 days after the lesion (fig. 1G) in all nine slices tested. These results suggest that picrotoxin did not block outgrowth but inhibited synaptogenesis of mossy fibers. Another consistent feature in hippocampal slices treated with picrotoxin was aberrant sprouting of mossy fibers into the molecular layer of the dentate gyrus. In 3 of 15 slices cultivated in control medium after the lesions, this phenomenon was faintly observed (fig. 5B). This may be due to temporary loss of target produced by lesions because some reports showed that loss of hilus interneurons, one of the main postsynaptic targets of mossy fiber tract, caused such aberrant sprouting (Babb *et al.*, 1991).

Epileptic bursts elicit sustained depolarization shift of neuronal membrane potential that may allow influx of calcium ion via voltage-sensitive calcium channels or NMDA receptor channels. Finally, we tested the effects of nicardipine, a L-type calcium channel blocker, and AP5, a NMDA receptor antagonist, on picrotoxin-induced inhibition of synaptogenesis of mossy fibers. The inhibitory effect of picrotoxin on the recovery of fEPSP from the lesion was blocked by nicardipine (10 μM) but not by AP5 (50 μM) (fig. 4B). The ameliorative effect of nicardipine against picrotoxin was also confirmed morphologically by the Timm method (fig. 5D). We then examined if nicardipine altered the epileptiform activity induced by picrotoxin. Picrotoxin (50 μM) elicited the bursting even in nicardipine- (10 μM) containing ACSF in all eight patients tested (fig. 3D). This epileptiform activity showed high regularity and its frequency was 2.35 $\pm$ 0.59 min^{-1} (means $\pm$ S.E.M. of eight slices). Each burst was consisted of 6.82 $\pm$ 1.02 (means $\pm$ S.E.M. of eight slices) repetitive firings. These properties were very similar to those of bursts induced in normal ACSF. We concluded, therefore, that nicardipine did not change the character of picrotoxin-elicited bursts, consistent with a previous work reporting that dihydropyridine-type calcium channel blocker did not inhibit epileptic discharge (van Luijtelaar *et al.*, 1994). In addition, exposure of intact slices to nicardipine or AP5 in the absence of picrotoxin from 8 DIV to 15 DIV did not affect fEPSP amplitude evoked in the CA3 region (data not shown, n = 6–9). Taken together, it is suggested that calcium influx through L-type calcium channels during epileptic bursts mediated the disturbance of appropriate synapse formation of mossy fibers.

Discussion

Using hippocampal slice culture, we demonstrated that picrotoxin prevented reorganization of mossy fibers via L-type calcium channel activation.

Müller *et al.* (1993) found that the amplitude of evoked fEPSP was depressed after chronic application of $GABA_A$ receptor blockers. In our study, however, picrotoxin had no effect on fEPSP in intact slices. This apparent contradiction may come from the following: 1) Cultures prepared with the roller-tube method they used formed a monolayer explant and might be more delicate than slices cultivated with the static culture method we applied, which retained a few cell layers of thickness (Stoppini *et al.*, 1991). The difference in slice cultivation procedures may also account for the discrepancy in the extent of reinnervation in control cultures after the lesion. Indeed, both our cultures and those of Stoppini *et al.* (1993) showed 100% reinnervation, although in the previous studies by Zimmer and Gähwiler (1987) and Dailey *et al.* (1994) they could not produce such a high reinnervation rate in slices obtained by roller-tube method. 2) Concentration of picrotoxin Müller *et al.* (1993) applied was 500 μM that was 10 times higher than ours and might exert nonspecific or toxic effects.

Barbin *et al.* (1993) reported that blockade of $GABA_A$ receptors reduced neurite length of cultured hippocampal neurons and suggested the involvement of $GABA_A$ receptors in neurite outgrowth. Our result that picrotoxin inhibited synapse formation of mossy fibers can also be interpreted as a consequence of prevented reelongation of transected mossy fibers. However, this possibility is ruled out by an observation using DiI labeling technique that indicates that picrotoxin-treated mossy fibers extended close to the pyramidal cell layer of the CA3 region at 7 days after a lesion. Although we did not examine whether chronic application of picrotoxin produced epileptic activity in cultured slices, the inhibitory effect of picrotoxin on synapse formation was probably due to

epileptic activity *per se* because it was completely canceled by tetrodotoxin. In addition, aberrant sprouting of mossy fibers into the molecular layer of the dentate gyrus, that has been typically observed in epileptic hippocampus (Babb *et al.*, 1991, Mathern *et al.*, 1994), was confirmed in picrotoxin-treated slices by a Timm method. This also suggests that picrotoxin actually elicited epileptiform activity in cultured slices. Taken together, these data strongly suggest that epileptic activity hindered lesion-induced reorganization of mossy fibers.

Represa *et al.* (1989) found that high affinity binding sites for kainate increased in the CA3 region of childhood epileptics. Although their result seems to contradict our finding, it is known that the type of neuronal firings often determine the direction of plasticity. For example, the direction of the synaptic gain change depends on the membrane discharge of the postsynaptic cell in the hippocampus (Artola and Singer, 1993; Malenka, 1995). Thus, further detail examination on picrotoxin-induced bursts in cultured slice might elucidate the difference between the preceding report and our finding.

Recovery time course of maximal fEPSP amplitude after mossy-fiber lesions approximately matched to that of intrinsic formation of mossy fibers, which are completed during postnatal 1 to 2 wk (Stirling and Bliss, 1978; Amaral and Dent, 1981; Gaarskjær, 1986). Moreover, the maximal fEPSP amplitude recorded at 14 days after the section recovered to an extent comparable to that in DIV-matched intact slices. Additionally, synaptic terminals of regenerated mossy fibers were Timm-stain positive, that was one of the important characteristics of mossy fibers. These observations strongly support the idea proposed by several previous reports that developmental manner and organotypic nature *in vivo* are conserved in structures regenerated after the lesion (Gähwiler and Brown, 1985; Heimrich and Frotscher, 1993; Li *et al.*, 1993; Stoppini, *et al.*, 1993; Frotscher *et al.*, 1995). Accordingly, process and characteristics of reorganizing mossy fibers after a lesion in our study may correspond to those of developmentally programmed formation of mossy fibers (Zimmer and Gähwiler, 1987; Dailey *et al.*, 1994).

As mentioned above, mossy fibers are generated mainly in 1 to 2 wk after birth (Stirling and Bliss, 1978; Amaral and Dent, 1981; Gaarskjær, 1986). This postnatal period is hence a critical stage that is susceptible to injury or disease. Indeed, Represa *et al.* (1991) reported that neonatal irradiation selectively prevented the mossy fiber formation. Whereas childhood epilepsy is associated with a broad spectrum of cognitive deficits (Alpherts and Aldenkamp, 1990; Mizrahi, 1994; Stafstrom, 1995), no reports clarified characteristic changes in structure or function of the central nervous system that underlie cognitive deficits in childhood epilepsy. Although hippocampal plasticity in childhood epilepsy was reported (Represa *et al.*, 1989; Mathern *et al.*, 1994), it is unclear whether epilepsy is responsible for plasticity or plasticity contributes to epilepsy. Our result that picrotoxin prevents the recovery of lesioned mossy fiber might indicate that epilepsy disturbs hippocampal maturation. The hippocampus is thought to be involved in cognition and learning (Shen *et al.*, 1994; McClelland *et al.*, 1995), and both behavioral (Conrad and Roy, 1993; Vaher *et al.*, 1994) and physiological (Bradler and Barrionuevo, 1989; Mitsuno *et al.*, 1994; Malenka, 1995) analysis suggest that mossy fibers in this region participate in cognition and learning. Additionally, there are indications that reactive synaptogenesis may be involved in learning and memory (Greenough and Bailey, 1988; Moser *et al.*, 1994). Therefore, our results may account in part, for cognitive deficits elicited by childhood epilepsy, and further investigation using our method will provide further insights and understandings with respect to this syndrome.

Our results indicate that calcium ion influx through L-type calcium channels may mediate a disorder of synapse formation of mossy fibers, consistent with previous reports showing that calcium ion influx plays a major role in neuronal injury associated with epilepsy (Wasterlain *et al.*, 1993). However, many reports examining correlation between synaptogenesis and calcium ion movement implied contradictive information. Although most of these data demonstrate an essential role of calcium ion in synaptogenesis (Basarsky *et al.*, 1994), others suggest that synapse formation and increase in intracellular calcium ion is irrelevant (Verderio *et al.*, 1994). Our observation that blockade of calcium ion influx abolished epileptic activity-induced inhibition of synapse formation suggests a repressive role of calcium ion, which further complicated the discussion. One possible explanation is that excessive calcium concentration results in obstruction of synaptogenesis although intermediate degree of calcium ion level may be required for it. Despite ambiguous role of calcium ion in mossy fiber synaptogenesis, our results suggest a novel protective action of L-type calcium channel blockers against disturbance of normal synaptic maturation associated with epileptic seizure, besides its antiepileptic properties as have been proposed in various models of epilepsy (van Luijtelaar *et al.*, 1994;, Straub *et al.*, 1994). Further investigations on this finding may endow valuable information for applying calcium channel blockers as prophylactics against cognitive deficits induced by childhood epilepsy.

Finally, organotypic slice culture used in our study preserves *in vivo* nature to a high degree and renders a useful model for studying developmental cellular dynamics in a mammalian central nervous system.

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