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L-TYPE Ca^{2+} CHANNEL BLOCKER INHIBITS MOSSY FIBER SPROUTING AND COGNITIVE DEFICITS FOLLOWING PILOCARPINE SEIZURES IN IMMATURE MICE

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Abstract—Behavioral and cognitive deficits are one of the most frequent sequelae of childhood epilepsy. Accumulating evidence indicates that epilepsy induces aberrant development of the mossy fibers in the hippocampus, the region that is commonly accepted to play a key role in learning and memory. We have therefore proposed that such abnormal maturation of the central nervous system may cause the adverse prognoses following epilepsy. Based on this hypothesis, using primary cultures of the dentate granule cells, we showed that the L-type Ca^{2+} channel blocker nicardipine was neuroprotective against excessive mossy fiber synaptogenesis induced by prolonged depolarization that was assumed to mimic epileptiform conditions. Therefore, we evaluated the *in vivo* effect of nicardipine on aversive sequelae following epileptiform seizures. We found aberrant sprouting of the mossy fibers and poor performance of spatial and contextual tasks in the mice that had received treatment with pilocarpine at their early postnatal age. Repetitive administration of nicardipine prevented the mossy fiber sprouting and ameliorated the cognitive deterioration, although it did not show anticonvulsant actions against pilocarpine seizures.

In the present study, we proposed two *in vitro* and *in vivo* models for evaluating epilepsy sequelae and noticed that L-type Ca^{2+} channel blocker nicardipine was effective in both models. L-type Ca^{2+} channel blocker may be a good candidate for a preventive for childhood epilepsy sequelae. Likewise, these useful systems will disclose additional candidates in future. © 2000 IBRO. Published by Elsevier Science Ltd. All rights reserved.

Key words: childhood epilepsy, hippocampus, dentate gyrus, L-type calcium channel, synaptogenesis, learning and memory.

Behavioral and cognitive deficits are a common consequence of epilepsy that occurs in infancy or early adolescence.[1,22,47,56,65] The sequelae are one of the most serious clinical issues because intractable seizures frequently appear early in life,[29] during which an increase in susceptibility to epilepsy is inevitably due to dynamic changes in excitatory and inhibitory neural contributions.[34] However, the cellular basis remains obscure, and the medical precautions or treatments have not been well established. Because even the etiology of epilepsy has not been fully clarified, the researchers have made efforts predominantly to elucidate the etiology or treatment for epilepsy, and less attention has been paid to the consequences of epilepsy. However, the present clinical therapy can remedy only half of epileptic patients.[13,20,43] Therefore, to develop the treatment for childhood epilepsy sequelae is a pressing need for clinical research and could benefit a number of medically refractory patients.

A discrepancy between experimental and clinical observations has also delayed progress in studies on childhood epilepsy sequelae so far.[28,30] While clinical data suggest that seizures beginning in early childhood are associated with a higher risk of intellectual impairment than those beginning in late adolescence or adult,[9,14,17,18,50] experimental studies of epileptic animals have demonstrated that adult animals are more vulnerable to seizures in terms of long-term sequelae.[59–61,70] Thus, chemically or electrically induced seizures in juvenile animals have been generally believed to produce no intellectual impairment. Indeed, we preliminarily examined single or repetitive administration to immature rodents (mouse or rat) with several convulsants (kainate, picrotoxin, pentylenetetrazol, pilocarpine, etc.) but only the muscarinic agent pilocarpine successfully produced cognitive impairment (Y. Ikegaya, N. Nishiyama and N. Matsuki, unpublished data). Actually, the result confirms a previous report indicating long-term behavioral deficits following pilocarpine seizures in immature rats.[38] Accordingly, pilocarpine seizure model is a unique and useful system to allow us to investigate the behavioral and morphological aberrations in premature epileptic animals.

Histological analysis in human epileptics or experimentally-induced animal models of epilepsy has revealed pathological alterations in the hippocampus[25,45,53] (the region that is thought to be involved in learning and memory), and hence such topographic alterations are assumed to cause cognitive deterioration.[67] Particularly, aberrant sprouting of the mossy fibers originating from the dentate granule cell has often been described in epileptic hippocampus.[16,19,60] Therefore, blockade of epilepsy-related alteration of the mossy fibers is supposed to prevent the sequelae. In the present study, we evaluated this possibility using a novel model of mossy fiber sprouting and pilocarpine seizure model, and found that L-type Ca^{2+} channel blockers, that prevented mossy fiber aberration both *in vivo* and *in vitro*, practically amended the cognitive deterioration. Indeed, Ca^{2+} channel blockers have been used as neuroprotective agents in epilepsy and stroke.[36,46] As for ischemic stroke, in particular, many *in vivo* and *in vitro*

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Abbreviations: ANOVA, analysis of variance; CaMK, Ca^{2+}/calmodulin-dependent kinase; DiV, days *in vitro*; EDTA, ethylenediaminetetra-acetate; EEG, electroencephalogram; fEPSP, field excitatory postsynaptic potential; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; LTP, long-term potentiation; P, postnatal day; PKC, Ca^{2+}-dependent protein kinase.

studies indicated that post-ischemic neuronal death is prevented by non-selective Ca^{2+} channel blockers,[5] L-type Ca^{2+} channel blockers,[73] N-type Ca^{2+} channel blockers,[52] or P/Q-type Ca^{2+} channel blockers.[41] None the less, the present study is the first indication that Ca^{2+} channel blockers inhibited epilepsy-related brain dysfunction including cognitive deficit. Thus, the results will provide valuable suggestions for clinical applications of Ca^{2+} channel blockers.

EXPERIMENTAL PROCEDURES

All efforts were made for the care and use of animals according to the National Institutes of Health guideline. The experimenter was blinded to the treatment the experimental group had received at the time of analysis.

Astrocyte-conditioned medium

Glial cells were cultured in Dulbecco's modified Eagle's medium (Nissui Pharmaceuticals, Tokyo, Japan) containing $NaHCO_3$ 1.89 mg/ml, sodium pyruvate 120 μg/ml and 1% fetal bovine serum (Sanko-Junyaku, Tokyo, Japan). Astrocyte-conditioned medium was prepared from cultures of cortical astrocytes. Postnatal day (P) 3 Wistar (SLC, Shizuoka, Japan) rat was deeply anesthetized with ether, and the cerebral cortex was dissected out and cut into pieces. Following incubation with 0.25% trypsin (Difco, Detroit, MI, U.S.A.) and 0.01% deoxyribonuclease I (DNase I) (Sigma, St Louis, MO, U.S.A.) at 37°C for 40 min, the tissue was centrifuged at 1200 r.p.m. for 5 min and the pellet was resuspended in Dulbecco's medium; single cells were mechanically dissociated by being passed through plastic tips with a 850-μm hole five to12 times. After passing through double nylon nets (25-μm mesh) to remove cell lumps, the suspension was diluted to the optimal concentration and cells were plated on 75 cm^2 culture flasks at a density of 1.5×10^5 cells/cm^2 and then cultivated at 37°C in a humidified 5% CO_2 and 95% air atmosphere. To purify astrocyte type I cells, cultures were shaken at 200 r.p.m. for 2 min twice after two days *in vitro* (DiV). Thereafter, the culture medium was changed once every three days. About 30 days after plating, the primary astrocyte culture which had become confluent was treated with Ca^{2+}-free trypsin solution containing EDTA 5 mM and re-plated at a density of 5.0×10^3 cells/cm^2. As the culture became confluent again, the medium was conditioned for three days, filtered through 0.22-μm pore membrane and was subsequently used for neuron culture as astrocyte-conditioned medium.

Neuron culture

Unless otherwise specified, neurons were cultivated in Neurobasal (Life Technologies, Gaithersburg, MD, U.S.A.) supplemented with L-glutamine 73 μg/ml and 2% (v/v) B-27 supplement (Life Technologies). P3 Wistar rat pups (SLC) were deeply anesthetized with ether, the hippocampi were immediately removed, and the dentate region was isolated with extreme care prior to dissociation so that cultures contained neurons predominantly from this part of the hippocampus. Briefly, following isolation of the hippocampal formation, the subicular complex was removed along the sulcus hippocampi, and then the remaining part of the hippocampal formation was divided into the two pieces, i.e. the dentate gyrus and Ammon's horn. They were separately cut into pieces and treated with trypsin and DNase I at 37°C for 30 min. The incubation was terminated by addition of heat-inactivated horse serum (Cell Culture, Cleveland, OH, U.S.A.). The tissue fragments were centrifuged at 1200 r.p.m. for 5 min, the supernatant was removed and the pellet was suspended in a mixture of 50% Neurobasal/B-27 and 50% astrocyte-conditioned medium. The suspension was gently triturated until visibly dispersed, followed by being filtered through nylon net. We were able to obtain about 5.0×10^5 granule cells from one pup. The cells were plated at a density of 4.0×10^4 cells/cm^2 onto 48-well plates (Costar, Cambridge, MA, U.S.A.) coated with poly-D-lysine (Sigma) and were cultivated at 37°C in a humidified 5% CO_2 and 95% air atmosphere. To prevent proliferation of glial cells, the culture medium was changed to the conditioned medium-free Neurobasal/B-27 medium supplemented with 2 μm cytosine β-D-arabino-furanoside (Sigma) 24 h after the plating. The culture medium was changed again at 4 DiV.

FM1-43 imaging

To visualize synaptic contacts, 7-DiV neurons were loaded with the fluorescent styryl membrane probe FM1-43 (Molecular Probe, Eugene, OR, U.S.A.).[6,57] Briefly, at least 1 h after the culture medium was changed to balanced salt solution 0.25 ml, the same volume of the solution containing FM1-43 20 μM and KCl 40 mM, which was prepared by iso-osmotic replacement of NaCl, was added to the culture (the final concentrations of FM1-43 and KCl were 10 μM and 20 mM, respectively). One minute later, the culture was gently washed twice with 0.25 ml of the balanced salt solution. The fluorescence images of FM1-43 were obtained with a confocal microscope. Tiny fluorescent punctae were identified as functional synapses because the fluorescence disappeared following exposure to 40 mM KCl-containing solution (data not shown). In several experiments, some of the granule cells received intracellular injection of 1% Lucifer Yellow CH ammonium salt (Molecular Probe) by applying negative current pulses (2 nA, 200-ms duration at 1 Hz) for 2 min so that they were able to be visualized.[64] The balanced salt solution was composed of NaCl 150 mM, KCl 5 mM, $MgCl_2$ 1 mM, $CaCl_2$ 2 mM, glucose 10 mM, HEPES 10 mM.

Confocal microscopy

Confocal imaging was conducted with a laser scanning confocal system MRC-600 (Biorad, Herculeus, CA, U.S.A.) equipped with an inverted microscope (Nikon, Tokyo, Japan), an argon ion laser and a host computer system. All image generation and processing operations were performed with COMOS Ver 6.01 (Biorad). For the measurements of FM1-43 and Lucifer Yellow CH, the cells were illuminated with the excitation wavelengths of 488 and 515 nm, and the fluorescence images were obtained through 515 and 550 nm long-pass filters, respectively. Fluorescent punctae of FM1-43 were counted in four areas (each 211×317 μm^2) per well, and at the end of the experiment neurons were enumerated in Nissl-stained cultures. The number of synaptic contacts per neuron was determined by dividing the total number of synapses by the number of surviving neurons.

Animals and drug injection

Male Std-ddY mice (SLC) were kept under temperature- and humidity-controlled conditions (22 ± 1°C, 55 ± 10%, respectively) and were housed five animals per cage after the weaning on P22. They had free access to food and water. We administered P14 mice with pilocarpine in order to induce seizures similar to child epilepsy, because <P12 mice did not show a sufficient cognitive deficiency (data not shown). The P14 mouse is thought to correspond roughly to P16–21 in rats and five to 10 years of age in humans. Forty mice on P14 were randomly divided into 4 groups consisting of 10 animals, i.e. the group that received injection of saline and vehicle (SV), pilocarpine and vehicle (PV), saline and nicardipine (SN), or pilocarpine and nicardipine (PN). Animals were intraperitoneally injected with saline (the group SV or SN) or pilocarpine hydrochloride 200 mg/kg (Sigma, St Louis, MO, U.S.A.) (the group PV or PN), preceded by 30 min with subcutaneous scopolamine methylbromide 1 mg/kg (Sigma) to reduce peripheral effects of pilocarpine. Additionally, at the same time as the injection of scopolamine, the animals were subcutaneously treated with vehicle (20% dimethyl sulfoxide) (the group SV or PV) or nicardipine hydrochloride 2 mg/kg (Sigma) (the group SN or PN). Subsequently, they received repetitive administration of the vehicle (the group SV or PV) or nicardipine 2 mg/kg (the group SN or PN) once a day from the following day (P15) to P38. Pilocarpine was not injected on P15–38. After P38, all behavioral, histological and electrophysiological experiments were performed in a blind fashion. On P38, all animals were numbered, randomized, and thereafter again numbered (ID). In the subsequent experiment, each animal was individually recognized by the ID number, not by the group that it belonged to.

Electroencephalogram

P13 mice were anesthetized with a combination of intramuscular ketamine hydrochloride 15 mg/kg and xylazine 1 mg/kg, and stainless bipolar electrodes were stereotaxically implanted in the left dorsal hippocampus (1.5 mm posterior to bregma, 1.3 mm lateral to midline, 1.5 mm ventral to dura). Body temperature was kept at 37°C during convalescence from the anesthesia. The surgery was well tolerated. All

animals recovered with a normal righting and placing reflex within 2 h. On the following day, each mouse was placed in a stainless-steel wire cage at least 1 h prior to electroencephalogram (EEG) recording. Baseline EEG was recorded for 30 min, and then the drugs were administered. EEG recording was conducted continuously with the mice unstrained, and their behavior was observed for 6 h following pilocarpine injection.

Locomotor activity test

Five minutes after a mouse was placed in a doughnut-shaped apparatus (320-mm outer diameter, 160-mm inner diameter, 130-mm wall height) (AT-320, Toyo Sangyo, Toyama, Japan), the numbers of rearing actions, turning actions and horizontal movement actions were automatically monitored for 30 min with 144 infrared sensors at scanning rate of 10 Hz. Rearing actions were detected by the sensors that were fixed 65 mm in height from the cage bottom.

Water maze test

A modified water maze was used to assess visuospatial learning and memory.[49] A circular tank (680-mm diameter) was filled with 18–19°C water to a depth of 270 mm. The pool was illuminated by room lights, and visual cues around the room were kept constant from day to day. Four points on the perimeter of the pool were designated north (N), east (E), south (S) and west (W), thus dividing the pool into four quadrants (NW, NE, SE and SW). On the day before the test, each mouse was placed into the pool for 60 s in order to become habituated to the training environment. On the first day (Day 0), a 100-mm diameter black platform, onto which the mouse could escape, was positioned 5 mm over the water surface in the center of the quadrant SW, and animals were placed into the pool at point E. On the trial test days (Days 1–7), a transparent platform was positioned 5 mm below the water surface in the same quadrant and each mouse was trained for four trials a day. The immersion points varied between N, S, E and W in a quasi-random order for each day so that the mouse would not be able to predict the platform location from the point at which it was placed into the pool. On mounting the platform, the mouse was allowed to remain there for a 60 s period. If a mouse failed to find the platform within 90 s, it was manually placed on the platform for 60 s. Latency from the immersion into the pool to escape onto the platform (escape latency) was automatically recorded for each trial using a behavior tracing analyser (BTA-2A, Muromachi Kikai, Tokyo, Japan). On Day 8, the platform was removed from the pool, and each mouse was placed at the point E and was allowed 90 s of free swimming (probe trial). The total duration that the mouse swam in the quadrant NE, NW, SE or SW was recorded.

Spontaneous alternation behavior test

The behavior experiment was conducted in a Y-shaped maze. The three trough-shaped arms (95 mm in width, 395 mm in length, 120 mm in depth) were separated by angles of 120°. A mouse was placed in one arm of the apparatus and was allowed to explore the maze for a period of 8 min. Arm choices were manually recorded during this time. Any three consecutive choices of three different arms were counted as an alternation. The percentage of alternation was determined by dividing the total number of alternations by the total number of choices minus two. The memory component in this task is that the mouse must remember which arm is more recently visited in order to alternate.

Step-through test

The apparatus (PA M1, O'Hara, Tokyo, Japan) consisted of two compartments separated by a black wall with a hole in the lower middle part so that a mouse could go through the wall. One chamber was illuminated by a fluorescent light, and the other was dark with opaque walls. The test was performed on two consecutive days at the same time of day. In the learning trial (Day 1), each mouse was placed in the bright compartment. Immediately after stepping through into the dark compartment, the animal received an electric shock to its paws (36 V, AC) through a stainless-steel grid floor. The time until each mouse entered the dark chamber (latency) was recorded. In the test trial (Day 2), the same test procedure was followed and the latency was measured with a ceiling score of 300 s.

Timm staining

Animals were deeply anesthetized with pentobarbital, and the brains were removed and immediately frozen at −15°C. Each brain was coronally sliced at 14-μm thickness with a cryostat (Cyrocut 1800, Finetec, Tokyo, Japan). For Timm stain, the sections were washed with 0.1 M phosphate buffer and then immersed in 0.37% sodium sulfide solution for 10 min, immediately followed by fixation with 10% (v/v) formaldehyde solution for 15 min. After being washed with 0.1 M phosphate buffer, the sections were dehydrated with 70 and 96% ethanol, and were dried. To perform the sulfide silver staining, they were incubated with the physical developer, which consisted of citrate-buffered 20% Arabic gum solution containing 1.7% $AgNO_3$ and 0.085% hydroquinone, in a dark room at 26°C for 50 min. The slices were washed with distilled water at the end of the reaction. As a counter stain, Nissl stain was performed with 0.1% Cresyl Fast Violet for 5 min.

Cell count

Animals were deeply anesthetized with pentobarbital, and the brains were removed and immediately frozen at −15°C. Each brain was coronally sliced at 14-μm thickness with a cryostat. The sections were stained with 0.1% Cresyl Fast Violet for 5 min. The numbers of Nissl-stained cells (N_c) in the stratum granulosum, the dentate hilus or the stratum pyramidale were measured on coded photomicrographs. The diameters of cell bodies (D_c μm) of neurons in each hippocampal area (A_h μm^2) were also measured and the values were averaged. The cell density (mm^{-3}) was defined as $10^9 \times N_c / (17.724 \times A_h \times \sqrt{1 + D_c/7})$ (simplified Gaya's method).

Electrophysiology

A mouse was deeply anesthetized with ether, and then the brain was quickly removed. The hippocampus was cut into 300-μm-thick slices in the ice-cold artificial cerebrospinal fluid, and the slices were then submerged in the artificial cerebrospinal fluid at 32°C for >1 h. The stratum granulosum was stimulated with a bipolar electrode and the evoked potential was extracellularly recorded from the stratum lucidum with a glass capillary microelectrode filled with NaCl 0.15 M. Test stimulation (100-μs duration) was applied at intervals of 20 s, and its stimulus intensity was adjusted in the range of 100–400 μA so that it produced about 50% of the maximal amplitude of field excitatory postsynaptic potential (fEPSP). To induce long-term potentiation (LTP), at >30 min after the basal response became stable, a tetanic stimulation (twice 100 Hz for 1 s at an interval of 20 s) was applied to the mossy fibers through the same electrode used for the test stimulation. Potentiation of evoked potentials following tetanic stimulation was evaluated by measuring changes in fEPSP slope that is defined as the maximal slope in rise phase of the negative field potential via a computational analysis (Wave-kun Ver 1.01, Gaya, Tokyo, Japan) of the analog-to-digital converted signals (10 bits/10 mV, 20 kHz). The artificial cerebrospinal fluid was composed of NaCl 127 mM, KCl 1.6 mM, $CaCl_2$ 2.4 mM, $MgSO_4$ 2.4 mM, KH_2PO_4 1.3 mM, $NaHCO_3$ 1.24 mM and glucose 10.0 mM, and was saturated with 95% O_2 and 5% CO_2.

RESULTS

Dentate granule cell culture

The granule cells prepared from the hippocampal dentate gyrus were cultured in serum-free defined medium. At 7 DiV, they were incubated with FM1-43 to visualize their functional synaptic contacts (Fig. 1A). These synapses are believed to preserve morphological and physiological properties of the mossy fiber synapses.[3,39,40,69] Because mossy fiber sprouting is performed by excessive synaptogenesis induced by prolonged depolarization during epileptic discharges, neurons were cultivated under high K^+ concentration (30 mM) that induced chronic depolarization of membrane potentials, i.e. quasi-epileptic conditions. The cultures exposed to high K^+ predictably displayed a dramatic increase in the number of synaptic contacts (Fig. 1B).

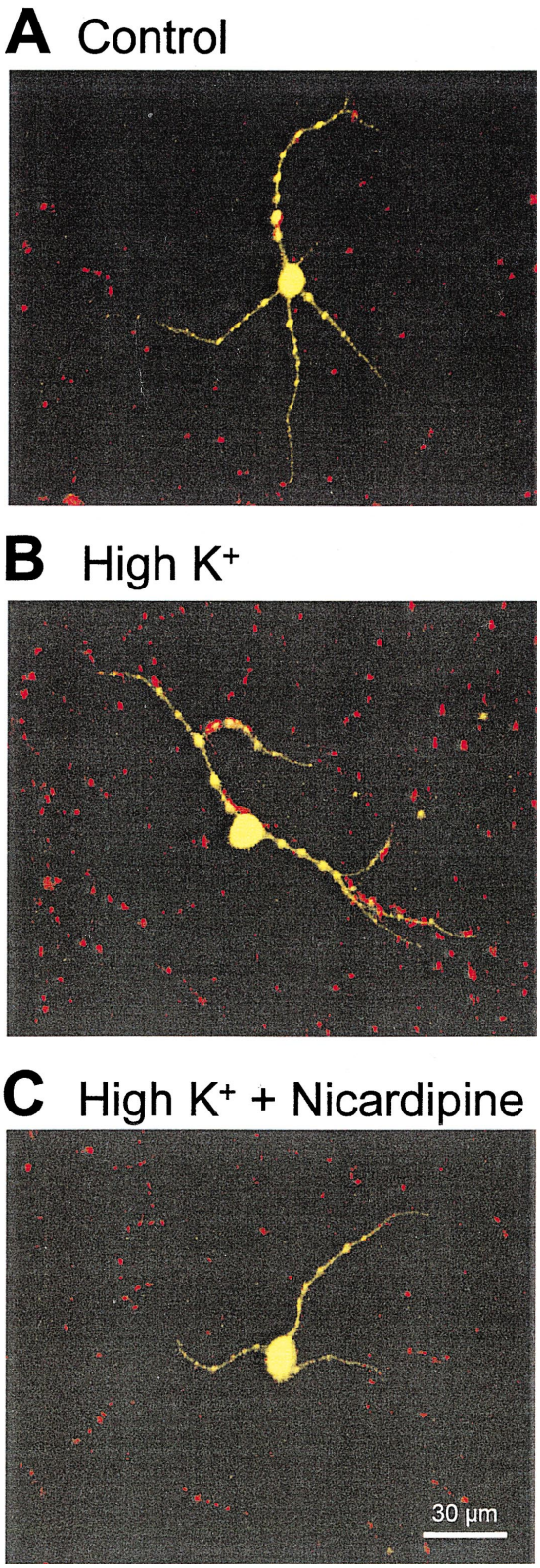

Fig. 1. Fluorescence images of neurons double-labeled with Lucifer Yellow CH (yellow) and FM1-43 (red). The granule cells were cultivated in normal culture medium (A), in the medium containing K^+ 30 mM (B) or in that containing a combination of K^+ 30 mM and nicardipine 2 μM (C), and confocal images were obtained at 7 DiV. The granule cells received intracellular injection of Lucifer Yellow CH and then the cultures were exposed to FM1-43 for 1 min. Red fluorescent punctae that represented functional synapse clusters increased in number by chronic treatment with high concentration of K^+, and the high K^+-driven synaptogenesis was canceled by an application of nicardipine.

Using this culture system, we made pharmacological investigations to clarify the molecular basis for the aberrant synaptogenesis (Table 1). The effects of Ca^{2+}-permeable channel blockers were first examined because a prolonged depolarization could allow Ca^{2+} influx through voltage-sensitive Ca^{2+} permeable channels. When drugs were applied during high K^+ (2–7 DiV), the aberrant synaptogenesis was prevented by the L-type Ca^{2+} channel blocker nicardipine 2 μM (Fig. 1C), but not by the T-type Ca^{2+} channel blocker $NiCl_2$ 30 μM, the N-type Ca^{2+} channel blocker ω-conotoxin GVIA 1 μM or the *N*-methyl-D-aspartate receptor blocker 2-amino-5-phosphonopentanoic acid 30 μM. Because intracellular Ca^{2+} serves as a mediator to stimulate Ca^{2+}/calmodulin-dependent kinase (CaMK),[10] Ca^{2+}-dependent protein kinase (PKC),[7] and certain types of tyrosine kinases,[24] we next examined the effects of the CaMKII inhibitor KN-93, the PKC inhibitor calphostin C and the tyrosine kinase inhibitor genistein, and found that high K^+-stimulated synaptogenesis was inhibited by KN-93 3 μM but not by calphostin C 30 nM or genistein 3 μM. Because the number of surviving neurons might have an influence on the number of synapses that they made, neuron survival was simultaneously monitored in the same series of experiments (Table 1). K^+ depolarization significantly increased the number of surviving neurons. Nicardipine or genistein blocked the promoted survival but the other drugs did not. Each drug employed here alone affected neither synaptogenesis nor neuronal survival in normal K^+ concentration.

The culture system, an assumed model of mossy fiber aberration in epilepsy, proposed nicardipine as a good candidate for a preventive for epilepsy sequelae. We hence attempted to estimate the *in vivo* effect of the drug in the following experiment, in which we developed a useful animal model for evaluating cognitive deterioration following childhood epilepsy.

Pilocarpine-induced seizures in immature mice

Injection of pilocarpine into P14 mice resulted in apparent behavioral alternations. About 10 min after the injection, animals began to show progressive body tremors and minor limbic seizures, and finally developed status epilepticus that continued for 3–6 h. The EEG changes correlated well with the behavior alternations. Although θ-rhythm regular spiking was constantly observed before pilocarpine injection (Fig. 2A), high-frequency spikes and then continuous ictal discharges appeared (Fig. 2B). Pretreatment of nicardipine did not affect pilocarpine-induced behavioral alternations. Nicardipine did not affect the number of myoclonic jerk masses 20 min, 2 or 6 h after pilocarpine injection [$F(1,54) = 0.445$, $P = 0.837$: repeated measure two-way analysis of variance (ANOVA)] (Fig. 2C). The duration of status epilepticus was also not changed by nicardipine: 230.7 ± 22.3 min in the control mice and 251.1 ± 36.9 min in the nicardipine-treated mice (means ± S.E.M., $n = 10$). EEG changes induced by pilocarpine were similar to those in nicardipine-pretreated group (Fig. 2B). Therefore, we concluded that nicardipine did not prevent pilocarpine-induced seizures. On P15–39, the mice were monitored for 1 h per diem, but spontaneous recurrent seizures were seen in neither PV nor PN mice.

Table 1. Effect of various drugs on high K^+-induced increase in synapses

Drug	*n*	No. of surviving neurons/cm^2	No. of synaptic contacts/neuron
In normal [K^+] medium			
Control	8	15021 ± 488	19.89 ± 1.77
Nicardipine	8	15704 ± 696	20.17 ± 1.06
$NiCl_2$	8	15343 ± 464	21.11 ± 1.82
ω-Conotoxin	8	14864 ± 883	22.13 ± 1.72
AP5	8	15202 ± 760	21.35 ± 2.05
KN-93	8	14469 ± 392	20.40 ± 1.82
Calphostin C	8	15560 ± 352	20.81 ± 1.72
Genistein	8	15125 ± 401	20.37 ± 2.05
In high [K^+] medium			
Control	8	18217 ± 760**	43.72 ± 4.64**
Nicardipine	8	15265 ± 272***	21.34 ± 2.35****
$NiCl_2$	8	17769 ± 504*	44.22 ± 3.98**
ω-Conotoxin	8	18440 ± 840**	43.34 ± 3.09**
AP5	8	17868 ± 424*	43.21 ± 4.08**
KN-93	8	17802 ± 356*	19.04 ± 1.20****
Calphostin C	8	19521 ± 880**	39.50 ± 3.73**
Genistein	7	15164 ± 402***	50.84 ± 3.75**

Drugs were added to the culture medium 24 h after plating. The densities of surviving neurons and the numbers of synaptic contacts were measured at 7 DiV. Concentrations of the drugs were as follows: high K^+ 30 mM, nicardipine 2 μM, $NiCl_2$ 30 μM, ω-conotoxin 1 μM, 2-amino-5-phosphonopentanoic acid (AP5) 30 μM, KN-93 3 μM, calphostin C 30 nM and genistein 3 μM. Data represent the means ± S.E.M. of *n* wells.
*$P < 0.05$.
**$P < 0.01$ vs Control in normal [K^+] medium.
***$P < 0.05$.
****$P < 0.01$ vs Control in high [K^+] medium: Tukey's test following one-way ANOVA.

Behavioral tests

The PV mice showed a delayed increase in body weight [$F(1,648) = 70.4842$, $P < 0.001$, SV vs PV: repeated measure two-way ANOVA], and repetitive administration of nicardipine did not affect the retarded growth [$F(1,648) = 0.663$, $P = 0.417$, PV vs PN] (Fig. 3). However, body weight of the groups PV and PN became comparable to that of the control mice by P35. Nicardipine alone had no effect on a change in body weights [$F(1,648) = 0.604$, $P = 0.438$, SV vs SN].

Locomotor activities of the mice were measured on P39 (Table 2). The number of rearing, turning and horizontal actions were recorded for 30 min but these parameters were not different among the groups [$F(3,108) = 0.826$, $P = 0.482$: repeated measure two-way ANOVA].

Water maze test was started on P40 to evaluate spatial memory (Fig. 4). On the first day (Day 1), mice were placed in the pool with the visual platform over the water surface; the latencies to get onto the platform were not different among the groups [$F(3,36) = 0.474$, $P = 0.702$: repeated measure one-way ANOVA]. For the following six days, the platform was hidden below the water surface. The mice acquired place learning of the platform and escape latencies were gradually shortened. However, the spatial learning in the group PV were significantly retarded [$F(1,108) = 5.817$, $P = 0.018$, SV vs PV: repeated measure two-way ANOVA]. The mice appeared to show learning disturbance on Days 1–3, but not on Days 4–6. The group PN showed a tendency to improve the impaired spatial learning of the PV mice [$F(1,108) = 2.413$, $P = 0.124$, PV vs PN]. Nicardipine alone did not affect the water maze performance [$F(1,108) = 0.116$, $P = 0.731$, SV vs SN]. The swimming time ratio or the swimming speed was not different among the groups. In the probe test, all groups displayed comparable preference for the quadrant SW where the platform had been located throughout the trial test, and there was no difference in ratio of the period of time during which the mice swam in the quadrant SW [$F(3,114) = 0.0462$, $P = 0.986$: repeated measure two-way ANOVA].

Spontaneous alternation behavior test was performed on P49 to examine spatial working memory (Table 3). Behaviors of the mice placed in the Y-shaped maze were observed for 8 min. No groups displayed either preference for a particular arm or variance in the number of arm choices. The SV mice showed a significant alternation ratio in arm choices, but alternation behavior in the group PV was almost collapsed [$Q(4,36) = 3.832$, $P < 0.05$, SV vs PV: Tukey's test following repeated measure one-way ANOVA]. The impaired performance was rescued in the group PN [$Q(4,36) = 3.793$, $P < 0.05$, PV vs PN]. Nicardipine alone did not affect spontaneous alternation behavior [$Q(4,36) = 0.451$, $P > 0.1$, SV vs SN].

Step-through test was conducted on P50–51 to assess passive avoidance performance (Table 4). On the first day (Day 1), all mice placed in the lightened compartment showed strong preference for the dark chamber and stepped through into the dark compartment within 60 s and subsequently received electrical foot shock. The latencies to enter the dark cell were not different among the groups [$F(3,36) = 1.227$, $P = 0.864$: repeated measure one-way ANOVA]. Although the same procedure was performed on the next day (Day 2), nine of 10 SV mice did not enter the dark compartment and halted in the bright room during 300 s. However, six of 10 PV mice got into the dark chamber. Accordingly, average latency to enter the dark compartment in the group PV was significantly shorter than that in the group SV [$Q(4,36) = 3.660$, $P < 0.05$, SV vs PV: a modified Dunnett's test following Kruskal–Wallis test]. In the group PN, no impaired performance was observed [$Q(4,36) = 4.097$, $P < 0.05$, PV vs PN]. The latency in the group SN was comparable to that in the group SV [$Q(4,36) = 0.325$, $P > 0.1$, SV vs SN].

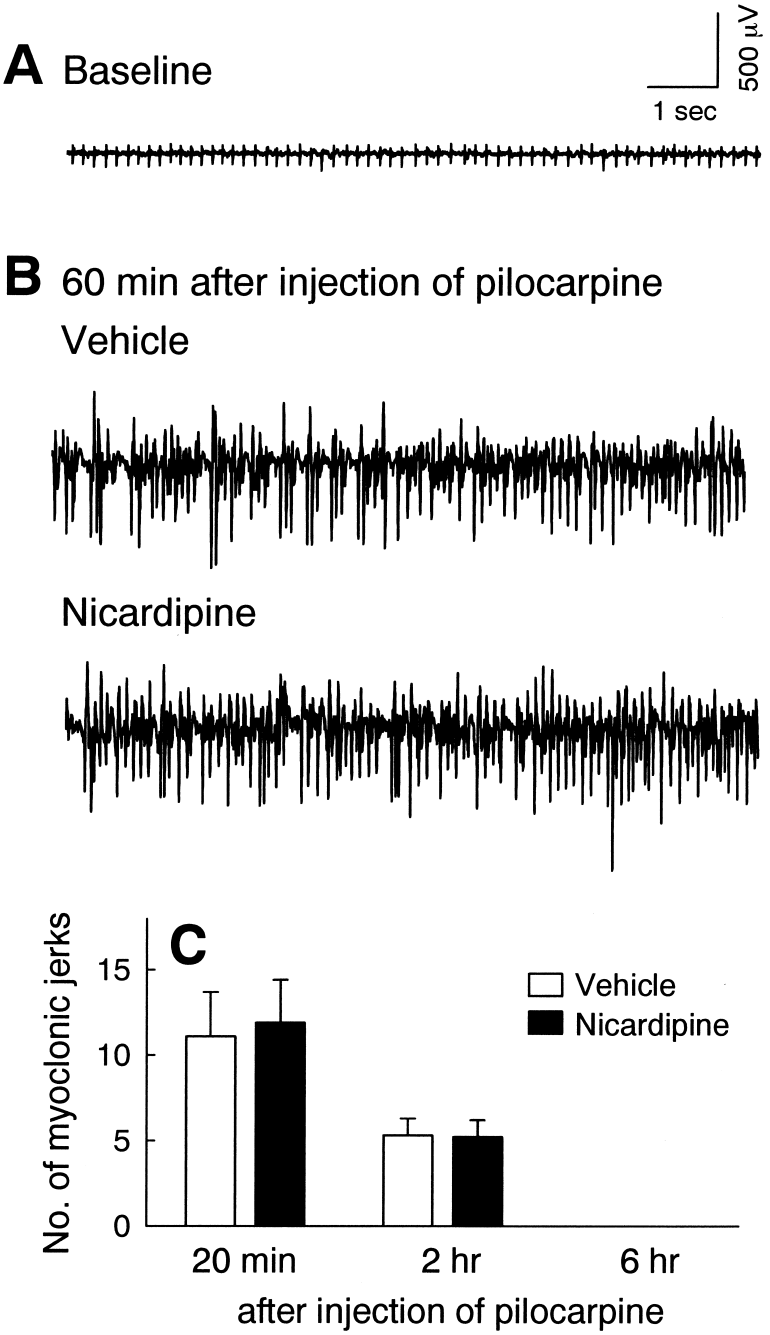

Fig. 2. Lack of effect of nicardipine on EEG changes and myoclonic jerks following pilocarpine injection into P14 mouse. Baseline EEG in A was recorded 30 min prior to pilocarpine injection, and EEGs in B were recorded 60 min after pilocarpine injection in the mouse pretreated with vehicle (upper) or nicardipine (lower). (C) The number of myoclonic jerk masses for 10 min were measured 20 min, 2 or 6 h after treatment with pilocarpine in the mice pretreated with vehicle (open columns) or nicardipine (white columns). Nicardipine did not show anticonvulsive actions against pilocarpine seizures. Data represent the means ± S.E.M. of 10 mice.

Histochemistry

Hippocampal sections were stained with Timm's method, a histochemical technique that selectively labeled synaptic terminals of the mossy fibers because of their high zinc content.[68] In the groups SV and SN, the dentate hilus (Fig. 5A, C) and the stratum lucidum (Fig. 5E, G) were Timm-positive areas. However, in the group PV, the mossy fiber terminals were detected in the molecular layer of the dentate gyrus (Fig. 5B) and the CA3 stratum oriens (Fig. 5F) as well. These ectopic synapse formations were not observed in the group PN (Fig. 5D, H).

The number of cells in the subregions of the hippocampus were recorded in Nissl-stained hippocampal sections but cell density in the stratum granulosum [$F(3,20) = 0.478$, $P = 0.701$: repeated measure one-way ANOVA] or the stratum pyramidale [CA3: $F(3,20) = 0.531$, $P = 0.666$; CA1: $F(3,20) = 0.544$, $P = 0.658$] was not different among the groups, except for a slight decrease in cell density in the dentate hilus of the group PV [$F(3,20) = 1.111$, $P = 0.215$] (Table 5).

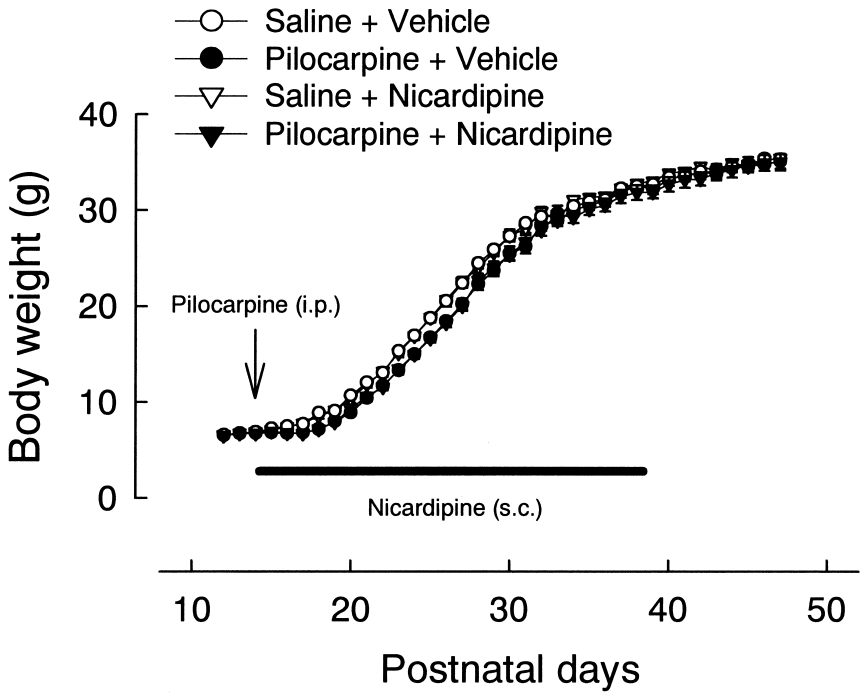

Fig. 3. Changes in body weights of the mice treated with pilocarpine or/and nicardipine. Animals received repetitive injection (once a day from P14 to P38) of vehicle (circles) or nicardipine (triangles) following the administration of saline (open symbols) or pilocarpine (closed symbols). Pilocarpine was injected on P14 (indicated by an arrow). Nicardipine was injected on P14–38 (indicated by a black bar). Data represent the means ± S.E.M. of 10 mice.

Long-term potentiation of mossy fiber–CA3 synapses

LTP in hippocampal synaptic transmission is believed to be a cellular basis of learning and memory.[42] fEPSP evoked in the mossy fiber–CA3 synapses was recorded to determine whether LTP was affected in our mice. The baseline fEPSPs before LTP induction were not uniform among the groups. The fEPSP slope was 0.52 ± 0.10 V/s in the group SV (mean ± S.E.M. of $n = 9$), 1.03 ± 0.11 V/s in the group PV ($n = 8$), 0.52 ± 0.07 V/s in the group SN ($n = 7$) and 0.70 ± 0.09 V/s in the group PN ($n = 7$). The fEPSP slope in the group PV was significantly larger [$Q(4,27) = 5.57$, $P < 0.01$, SV vs PV], and that in the group PN was also slightly enlarged. In the groups SV and SN, fEPSP slope was potentiated following tetanic stimulation, and robust LTP was produced (Fig. 6A, C). However, in the group PV or PN, LTP was not induced (Fig. 6B, D).

DISCUSSION

We have shown here that nicardipine, proposed as a neuroprotective candidate for epilepsy sequelae by our *in vitro* experiment in the present study as well as our previous reports,[32,33] actually prevented cognitive deficits and mossy fiber sprouting following pilocarpine-induced seizures in immature mice.

Pilocarpine-induced seizures

Although all mice that received treatment with pilocarpine displayed body tremors and severe myoclonic jerks and subsequently developed status epilepticus, they showed neither spontaneous recurrent behavioral seizures nor massive cell loss in the hippocampus. Pilocarpine is well known to produce these consequences.[15,44,71] Furthermore, mossy fiber sprouting was observed in the PV mice but the amount was slightly weak. These observations do not seem to corroborate prior works done with pilocarpine. One possible explanation for this discrepancy is that the dose of pilocarpine (200 mg/kg) employed here was lower than that in standard pilocarpine models using adult animals (320–380 mg/kg);[4,71] >200 mg/kg of pilocarpine caused high lethality (43.3%) in our premature mice. However, our preliminary experiments revealed that P14 mice surviving a high dose of pilocarpine (380 mg/kg) displayed neither spontaneous recurrent behavioral seizures nor hippocampal lesions. Therefore, it is our impression that

Table 2. Locomotor activities of the mice treated with pilocarpine or/and nicardipine

Treatment	Rearing actions	Turning actions	Horizontal movement
Saline + Vehicle	71.2 ± 8.1	83.1 ± 3.7	104.2 ± 5.0
Pilocarpine + Vehicle	67.8 ± 7.7	80.2 ± 9.8	98.8 ± 1.0
Saline + Nicardipine	62.9 ± 9.3	67.8 ± 9.7	104.8 ± 6.7
Pilocarpine + Nicardipine	53.2 ± 5.7	74.4 ± 6.3	106.6 ± 8.0

Locomotor activities of P39 mice were evaluated by the numbers of rearing actions, turning actions and horizontal movements. The animals had received injection of saline and sequentially repetitive treatment with vehicle (Saline + Vehicle), injection of pilocarpine and repetitive treatment with vehicle (Pilocarpine + Vehicle), injection of saline and repetitive treatment with nicardipine (Saline + Nicardipine) and injection of pilocarpine and repetitive treatment with nicardipine (Pilocarpine + Nicardipine). There was no difference among the groups. Data represent the means ± S.E.M. of 10 mice.

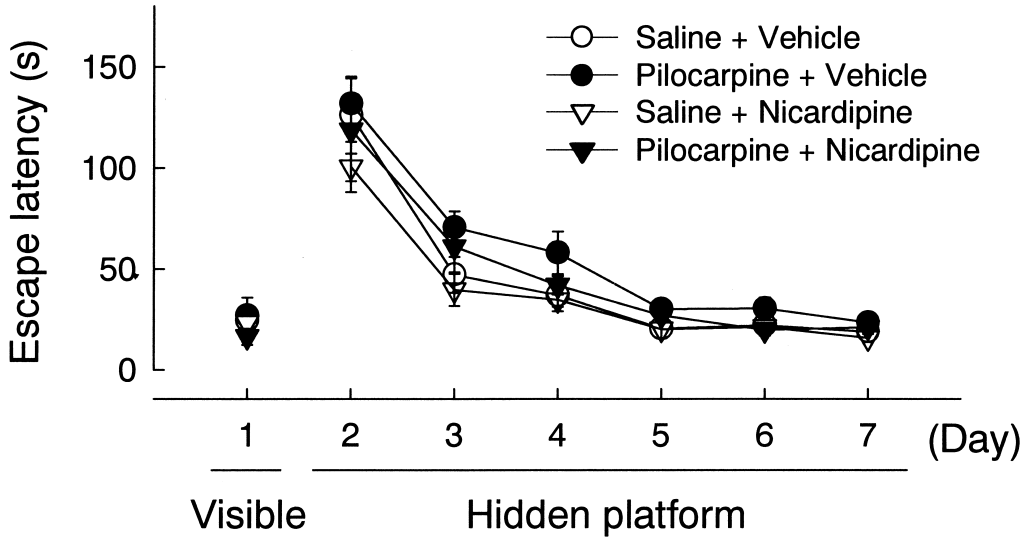

Fig. 4. Ameliorative effects of repetitive treatment of nicardipine on pilocarpine-induced cognitive deficits in water maze test. The ordinate indicates escape latencies to get onto the platform that was visible on Day 1 or hidden on Days 2–7. The symbols are the same as in Fig. 3. Nicardipine slightly prevented poor performance in spatial tasks in the pilocarpine-treated mice. Data represent the means ± S.E.M. of 10 mice.

less effectiveness of pilocarpine in the present study was not due to the low dose but rather due to the age of animals. Pilocarpine might be unable to produce a sufficient lesion in immature mice. Correspondingly, Liu *et al*. demonstrated that pilocarpine shows no spontaneous recurrent seizures in premature rats.[38]

Cognitive deficient in pilocarpine-treated mouse

The PV mice performed poorly in all learning tasks tested in this study, i.e. water maze test, spontaneous alternation behavior test and step-through test. First, it is important to determine which pilocarpine-induced seizures or its pharmacological effect induced the learning deficits. Several previous studies with adult animals indicated that pilocarpine-induced status epilepticus produces cognitive deterioration.[31,54] Furthermore, acute administration with muscarinic agonists including pilocarpine is generally known to rather improve learning abilities of animals.[8,21,23,35,55] Therefore, we concluded that the learning deficits were due not to the on-going effects of pilocarpine but to pilocarpine-induced seizures.

Although the water maze and spontaneous alternation behavior tests measure visual spatial learning tasks, the water maze performance depends on both working and reference memory but the spontaneous alternation behavior depends on working memory alone.[11,27,51] Accordingly, the poor performance in the latter task indicates working memory deficit of the PV mice. Furthermore, in the water maze test, the mice showed learning disturbance on Days 1–3, but not on Days 4–6 and the probe test. It is considered that animals perform this task using both working and reference memory in the early phase of sessions but using only reference memory in the late phase. Therefore, these results suggest that working memory deficit rather than reference memory deficit caused the poor achievement in these spatial tasks.

Of three kinds of tasks tested here, spontaneous alternation behavior test and step-through test revealed that nicardipine significantly prevented cognitive deficits induced by pilocarpine. The poor performance of PV mice in the water maze test also tended to be improved by repetitive treatment with nicardipine. The possibility that the ameliorative effect of nicardipine depended on changes in factors that affected the task performance can be ruled out for the following reasons. (1) Nicardipine alone did not affect the task performance. Any parameters recorded in the behavior tests were not different between the groups SV and SN. (2) General

Table 3. Collapsed spontaneous alternation behaviors were ameliorated by nicardipine

Treatment	% Alternation
Saline + Vehicle	66.82 ± 2.48
Pilocarpine + Vehicle	55.10 ± 1.47*
Saline + Nicardipine	68.20 ± 2.00
Pilocarpine + Nicardipine	66.70 ± 5.01**

Spontaneous alternation behavior in a Y-shaped maze was observed for 8 min. The column "Treatment" represents the same as in Table 2. The chance level of alternation ratio is ~50%. Data show the means ± S.E.M. of 10 animals. Nicardipine prevented the poor performance of spatial tasks in the pilocarpine-treated mice.
*$P < 0.05$ vs Saline + Vehicle.
**$P < 0.05$ vs Pilocarpine + Vehicle: Tukey's test following one-way ANOVA.

Table 4. Attenuated performance in passive-avoidance test were improved by nicardipine

Treatment	Latency (sec)	
	Day 1	Day 2
Saline + Vehicle	21.2 ± 3.8	276.1 ± 12.8
Pilocarpine + Vehicle	19.6 ± 1.8	194.0 ± 38.9*
Saline + Nicardipine	16.1 ± 2.7	283.4 ± 11.7
Pilocarpine + Nicardipine	26.3 ± 5.8	285.9 ± 14.1**

The values indicate latencies to enter the dark chamber in the learning trial (Day 1) or the testing trial (Day 2) of step-through test. The column "Treatment" represents the same as in Table 2. Nicardipine prevented the poor performance of contextual tasks in the pilocarpine-treated mice. Data show the means ± S.E.M. of 10 mice.
*$P < 0.05$ vs Saline + Vehicle.
**$P < 0.05$ vs Pilocarpine + Vehicle: modified Dunnett's test following Kruskal–Wallis test.

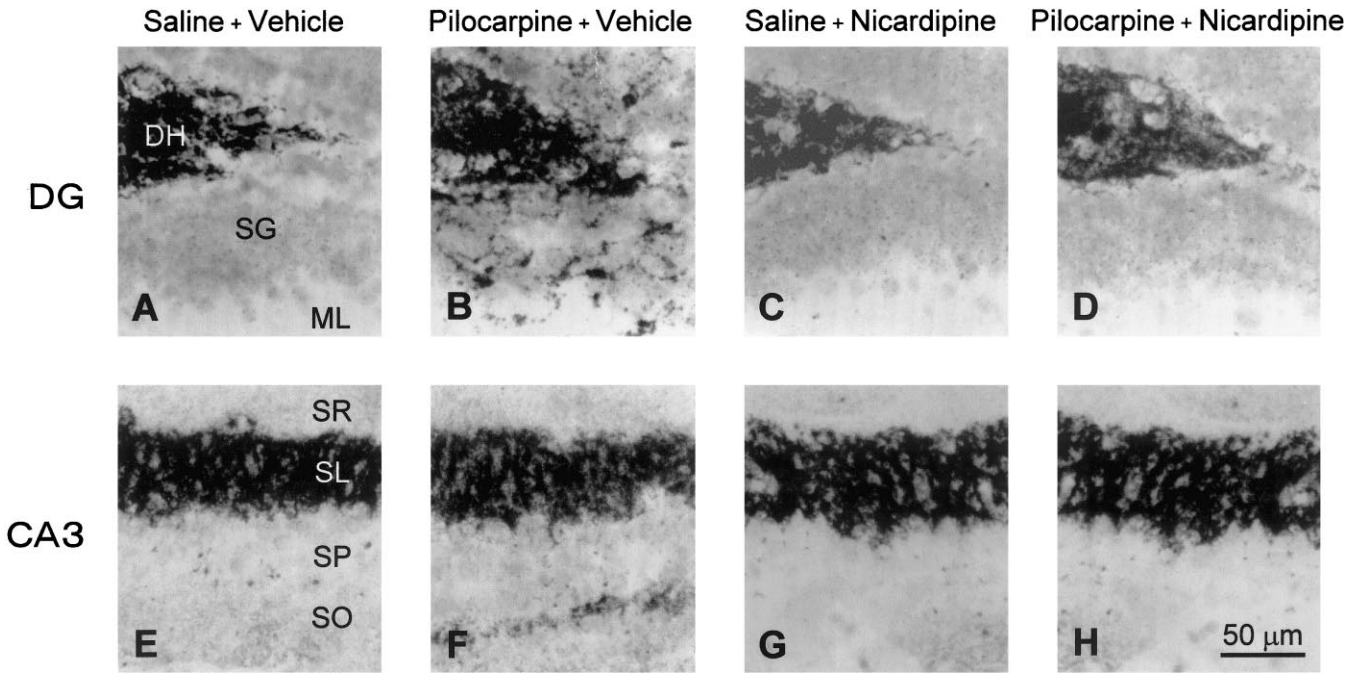

Fig. 5. Repetitive administration with nicardipine prevented aberrant sprouting of the mossy fibers induced by pilocarpine. The photographs A–D show light-phase microscopic images of the crest of the dentate gyrus that includes the dentate hilus (DH), the stratum granulosum (SG) and the molecular layer (ML) of Timm-stained hippocampal sections. E–H are photographs of the CA3 region containing the stratum radiatum (SR), the stratum lucidum (SL), the stratum pyramidale (SP) and the stratum oriens (SO). Nicardipine completely inhibited pilocarpine-induced mossy fiber sprouting.

behaviors that would affect task performance, i.e. the locomotor activity test, the visible platform test or the swimming speed in water maze test, and the number of arm choices in Y-shaped maze, showed no differences among the groups. These results indicate that motor abilities and behavioral habits of the mice were invariant among the groups. (3) The swimming time ratio in water maze test and the latency to enter the dark chamber on Day 1 in step-through test were not different among the groups. This suggests that emotional and motivational aspects were uniform among the groups. Based on these observations, we concluded that the effect of nicardipine depends on the improvement of learning disability in the epileptic mice.

Nicardipine blocked mossy fiber sprouting but did not ameliorate long-term potentiation attenuation

Consistent with our previous study indicating that nicardipine did not inhibit convulsant-elicited epileptiform discharges in the hippocampus *in vitro*,[32,33] it prevented neither pilocarpine-induced behavior alternations nor EEG changes. These suggest that nicardipine blocked pilocarpine seizure-induced structural or physiological changes that were associated with intellectual impairment. As candidates of such pilocarpine-induced alternations, we found mossy fiber sprouting and no LTP.

Because epileptiform neuron activities induce long-lasting enhancement of synaptic strength like LTP,[4] it is possible that LTP in the PV mice had been saturated by hyperexcitability of mossy fiber synapses.[48,62,63] The result that baseline fEPSP of the group PV was significantly enlarged does support the pre-existence of LTP. Therefore, no LTP in the group PV may account for the memory dysfunction. However, LTP behavior was not always in accord with cognitive abilities in the present study because repetitive treatment with nicardipine prevented the intellectual impairment but not the LTP abolishment. Rather, this suggests that nicardipine-induced improvement of cognitive deficit was not mediated by LTP changes. On the other hand, nicardipine prevented both mossy fiber sprouting and learning disability. Mossy fiber sprouting is accompanied by new circuitry formation as excitatory recurrent inputs and such abnormal circuit rearrangement possibly causes hippocampal dysfunction and thereby may

Table 5. Cell densities in the hippocampus of the mice treated with pilocarpine or/and nicardipine

Treatment	Cell density ($\times 10^5$ cells/mm^3)			
	SG	DH	SP–CA3	SP–CA1
Saline + Vehicle	5.48 ± 0.42	0.44 ± 0.05	0.89 ± 0.05	2.27 ± 0.16
Pilocarpine + Vehicle	5.55 ± 0.43	0.35 ± 0.03	0.95 ± 0.09	2.32 ± 0.09
Saline + Nicardipine	5.17 ± 0.41	0.47 ± 0.04	0.86 ± 0.07	2.23 ± 0.14
Pilocarpine + Nicardipine	5.33 ± 0.10	0.45 ± 0.04	0.79 ± 0.06	2.23 ± 0.14

Nissl-stained cells in the stratum granulosum (SG), the dentate hilus (DH) and the CA3 and CA1 stratum pyramidale (SP–CA3 and SP–CA1, respectively) of the mice treated with pilocarpine or/and nicardipine were counted. The column "Treatment" represents the same as in Table 3. Pilocarpine did not induce cell loss in any subregions of the hippocampus, except for a slight decrease in cell density of the dentate hilus. Data are the means ± S.E.M. of seven mice.

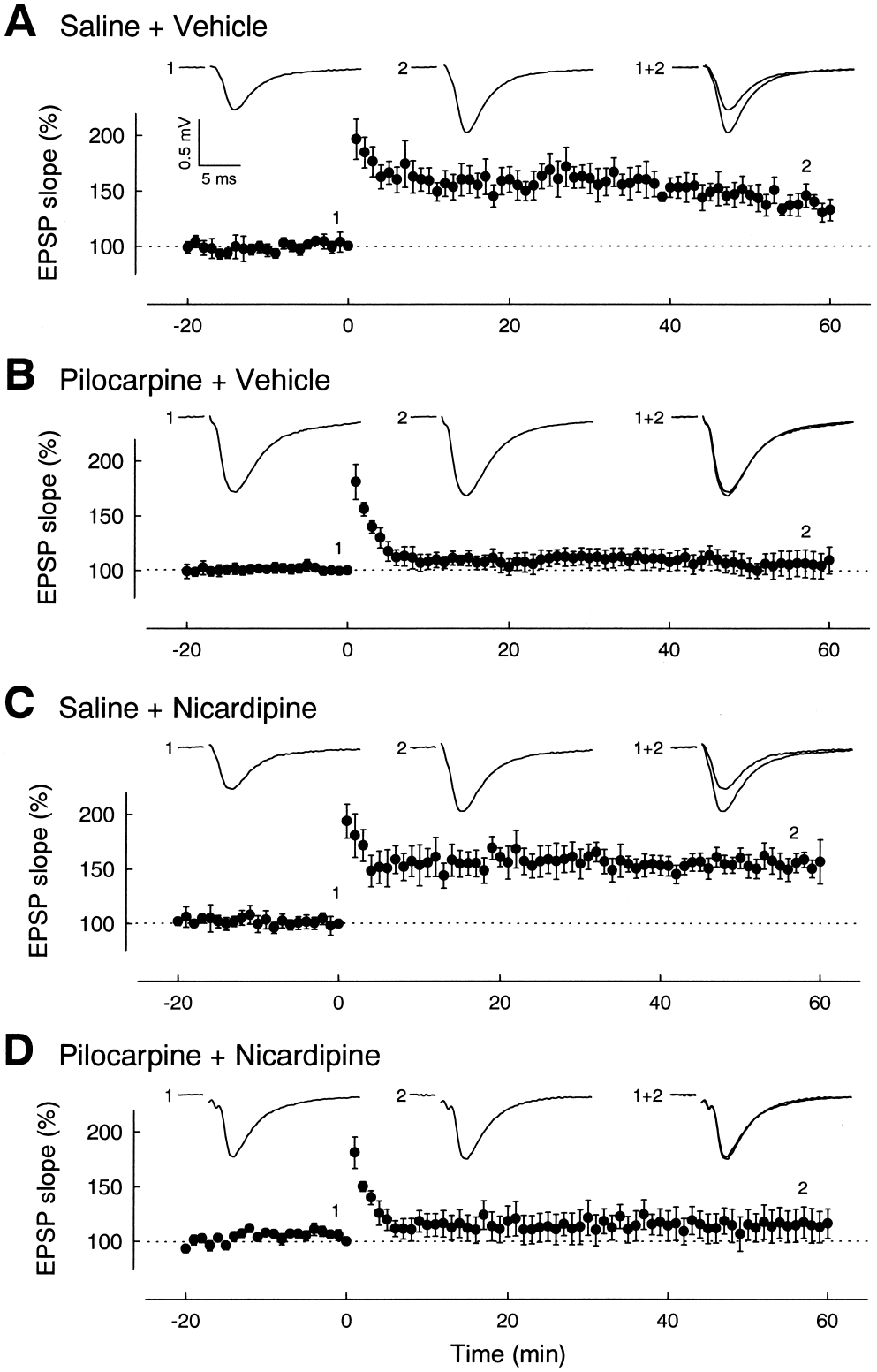

Fig. 6. Attenuation of LTP in mossy fiber–CA3 synaptic transmission in pilocarpine-treated mice. Each panel (A–D) contains representative traces recorded 1 min before (1) or 56 min after (2) tetanic stimulation and a superimposition of both traces (1 + 2), and a graph of the time-course of changes in fEPSP slope following tetanic stimulation applied at time 0. fEPSP slope was expressed as a percentage of the baseline value immediately before tetanic stimulation. Nicardipine did not rescue the attenuated LTP in pilocarpine-treated mice. Data are the means ± S.E.M. of seven slices prepared from three mice of each group.

be associated with cognitive deficits.[67] It is, therefore, plausible that the ameliorative effect of nicardipine on cognitive abilities is mediated by the inhibition of mossy fiber sprouting. Of course, there is much room for the affirmation. First, we examined only the mossy fiber LTP, based on numerous indications that the mossy fibers are most susceptible to structural or physiological alterations following epilepsy,[16,19,66] but LTP is found in many other regions such as

Schaffer collateral–CA1 synapses and perforant path–dentate gyrus synapses. Therefore, the possibility that nicardipine alters other types of LTP cannot be excluded. Secondly, although we found only mossy fiber sprouting and the collapse of LTP, there might presumably be unidentified aberrations in the animal treated with pilocarpine. Therefore, it still remained undetermined whether mossy fiber sprouting is involved in the memory disturbance. Now we consider that the results of LTP and the sprouting should not be too much associated with memory impairment following seizures. Further detailed investigation on epileptic brain would appraise our hypothesis. None the less, so far, our results do not apparently contradict the hypothesis that blockade of mossy fiber sprouting may correlate with prevention of the epilepsy sequelae.

L-type Ca^{2+} *channel and mossy fiber morphology*

The mechanism by which nicardipine affects the mossy fiber structures remains unclear. There are many reports indicating that intracellular free Ca^{2+} modulates neurite outgrowth and synaptogenesis.[2,12,37,43] Our study using the granule cell cultures indicated that high K^+-driven synaptogenesis was inhibited by pharmacological blockade of L-type Ca^{2+} channels but not other types of Ca^{2+}-permeable channels, strongly suggesting a pivotal role of L-type Ca^{2+} channels in mossy fiber synaptogenesis. This is consistent with our previous finding that L-type Ca^{2+} channel activation causes abnormal growth of the mossy fibers following epileptiform activities using rat hippocampal slice cultures.[32,33] In the present study, further pharmacological investigations revealed a possible contribution of CaMKII activities. Several reports indicate that CaMKII inhibitors prevent neurite outgrowth stimulated by K^+ depolarization or/and growth factors in PC12 cells[58] or 3T3 fibrobralst.[72] Additionally, Goshima *et al*. reported that overexpression of CaMKII α subunit in Neuro2a or NG108-15 neuroblastoma promoted neurite outgrowth.[26] Therefore, CaMKII activation following Ca^{2+} influx through L-type Ca^{2+} channels may be involved in mossy fiber sprouting. However, we were not able to determine yet which synaptogenesis or neurite outgrowth was promoted by Ca^{2+} through L-type Ca^{2+} channels. Because our culture had a high density of neurons, and thereby because their axon and dendrites were complicatedly intertangled, we were not able to measure the number of boutons per given length of dendrites. Therefore, it remains to be determined which is the main effect of Ca^{2+} on the length of the dendrites or on synaptogenesis in K^+-induced synapse increase.

Importantly, neither nicardipine nor KN-93 inhibited the background level of synaptogenesis, i.e. synapse formation in normal K^+ concentration. This result indicates that the drugs selectively blocked excessive synaptogenesis evoked by prolonged depolarization, and correlates well with the *in vivo* data, which denoted that nicardipine prevented pilocarpine-induced aberration of the mossy fibers without affecting the basal morphology. Therefore, L-type Ca^{2+} channels do not likely participate in normal mossy fiber morphology but their pathological activation may produce aberrant alterations in the structure.

The dentate granule cell culture provides a faithful and useful model of mossy fiber sprouting and allowed us to notice a novel potency of L-type Ca^{2+} channel blocker. To our knowledge, nicardipine is the first drug that prevents epilepsy prognosis without blocking seizures and hence may benefit intractable childhood epilepsy. In the present study, we pre-treated the animals with nicardipine in order to examine the effect on both pilocarpine seizures and the sequelae in the same series of experiments. However, our previous *in vitro* study indicated that nicardipine did show the same neuroprotective effects when it was applied four days after epileptiform activities were induced.[32] Therefore, to investigate the effect when nicardipine was administered following the seizures will provide more effective data from a clinical standpoint. The *in vitro* and *in vivo* system we established here will be able to disclose additional candidates for a preventive for epilepsy sequelae in future.

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