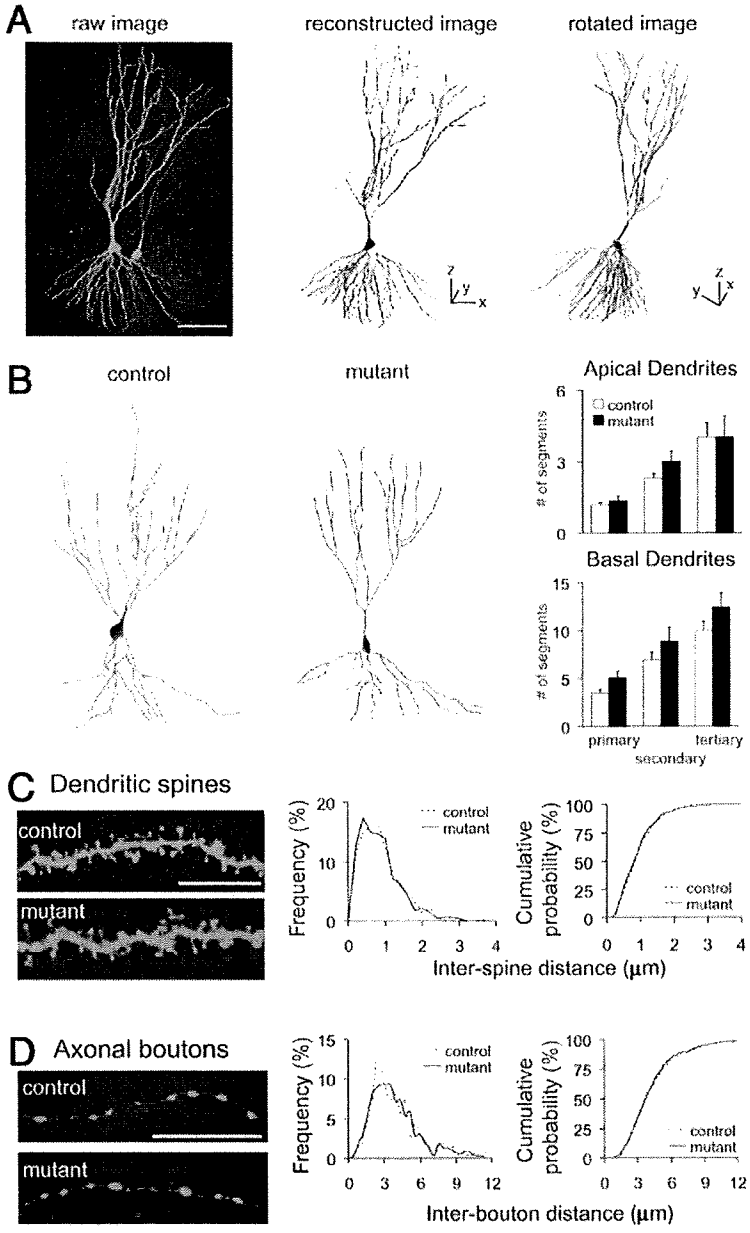

Figure 5. Dendritic branching and distribution of postsynaptic spines and presynaptic boutons in CA3 pyramidal neurons of control and mutant mice. A, Examples of three-dimensional reconstruction using IMARIS and FilamentTracer software. **B**, Three-dimensional reconstruction of AAV-EGFP-infected CA3 pyramidal neurons. Graphs represent the numbers of primary, secondary and tertiary dendrites of CA3 pyramidal neurons in control (open boxes, $n = 10–12$) and mutant mice (filled boxes, $n = 6–7$). There were no significant differences between control and mutant mice in the numbers of primary (apical, $P = 0.58$; basal, $P = 0.06$; t-test), secondary ($P = 0.13$, $P = 0.21$) and tertiary dendrites ($P = 1.0$ $P = 0.16$). **C**, Tertiary dendritic segments in control (left, top) and mutant (left, bottom) mice. Normalized distribution of inter-spine distances (middle, bin size, 0.1 μm). Cumulative distribution of inter-spine distances (right, same data set). There were no significant differences in inter-spine intervals of CA3 pyramidal neurons between two genotypes (control $n = 428$ from 10 dendrites of 4 mice; mutant, $n = 459$ from 9 dendrites of 4 mice; $P = 0.74$, Kolmogorov-Smirnov test). **D**, Boutons on the axon in the CA3 stratum radiatum of control (left, top) and mutant (left, bottom) mice. Normalized distribution of inter-bouton distances (middle, bin size, 0.4 μm). Cumulative distribution of inter-bouton distances (right, same data set). There were no significant differences in inter-bouton intervals of CA3 pyramidal neurons between two genotypes (control $n = 262$ from 18 axons of 4 mice; mutant, $n = 322$ from 24 dendrites of 4 mice; $P = 0.90$, Kolmogorov-Smirnov test).
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tional/commissural fibers and inhibitory interneurons (and their dendrites and axons), and then $GABA_A$-IPSCs were measured with the same stimulus strength at 0 mV in the presence of both the non-NMDA receptor antagonist CNQX and the NMDA receptor antagonist D-APV. The ratio of $GABA_A$-IPSCs to AMPA-EPSCs was indistinguishable between the two genotypes

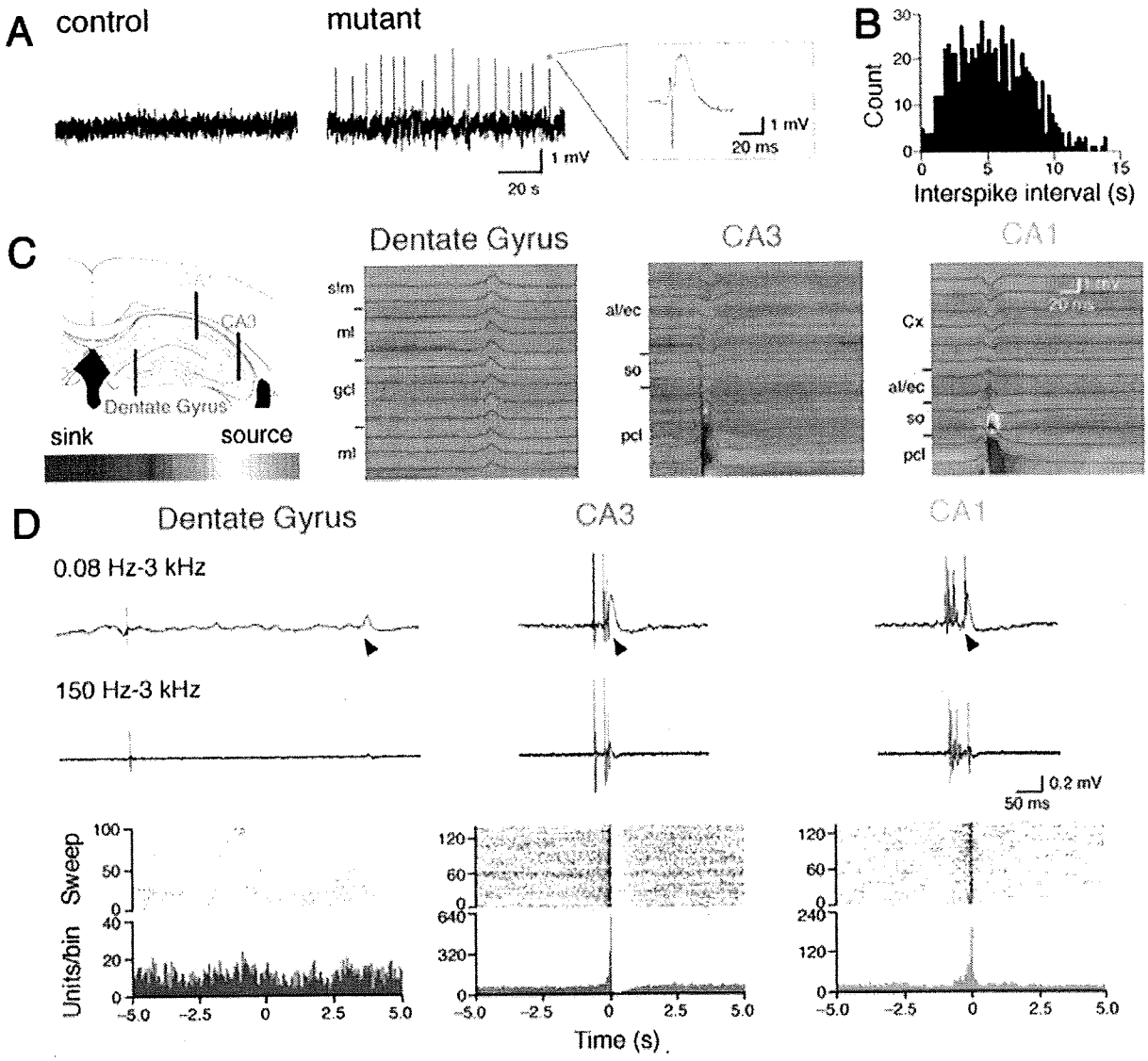

Figure 6. Characteristic large EEG spikes in the hippocampal CA3 region of mutant mice. **A**, Representative local field potential recordings from the CA3 region. **B**, Histogram of interspike intervals (bin, 0.25 s). **C**, Laminar profiles of field potentials and CSD analysis. Recording positions are illustrated on the left. Sinks and sources are indicated by cold and warm colors, respectively. Abbreviations: al/ec, alveus and external capsule; Cx, cortex; gcl, granule cell layer; ml, molecular layer; pcl, pyramidal cell layer; slm, stratum lacunosum-moleculare; so, stratum oriens. **D**, Wide-band recordings of extracellular activities (top), filtered MUA (middle) and raster plots and peri-event time histograms between MUA (bin, 200 ms) and EEG spikes in the dentate gyrus (left), CA3 (center) and CA1 regions (right). Arrowheads indicate the onset of spikes. MUA were aligned to the onset of spikes (time 0).
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(control, 0.52±0.09; mutant, 0.61±0.16; $n=12$ each; t-test, $P=0.65$) (Fig. 7B). Thus, there was no significant electrophysiological imbalance between AMPA receptor-mediated excitatory and $GABA_A$ receptor-mediated inhibitory synaptic transmission in the hippocampal CA3 region.

High-frequency stimulation failed to induce slow hyperpolarizing currents in hippocampal CA3 pyramidal neurons of mutant mice

In hippocampal CA1 pyramidal neurons, synaptic excitation is followed by an early GABA-mediated hyperpolarization and late AHP mediated by Ca^{2+}-dependent K^+ channels [40]. We thus examined the effect of NMDA receptor ablation on Ca^{2+}-dependent K^+ channels in hippocampal CA3 neurons. At a holding potential of −20 mV, high-frequency stimulation, which should activate both AMPA receptors and NMDA receptors in normal mice, induced slowly decaying outward currents in the pyramidal cells of control mice (Fig. 7C; peak amplitude, 46.1±5.4 pA, $n=12$). In contrast, such slow outward currents were hardly evoked by the same high-frequency stimulation in mutant mice (Fig. 7C; 0.5±2.1 pA, $n=12$, $P<0.001$). The outward currents in control mice were abolished by D-APV (Fig. 7D; control, 46.13±5.36 pA, $n=13$; D-APV, 0.38±2.41 pA, $n=12$,

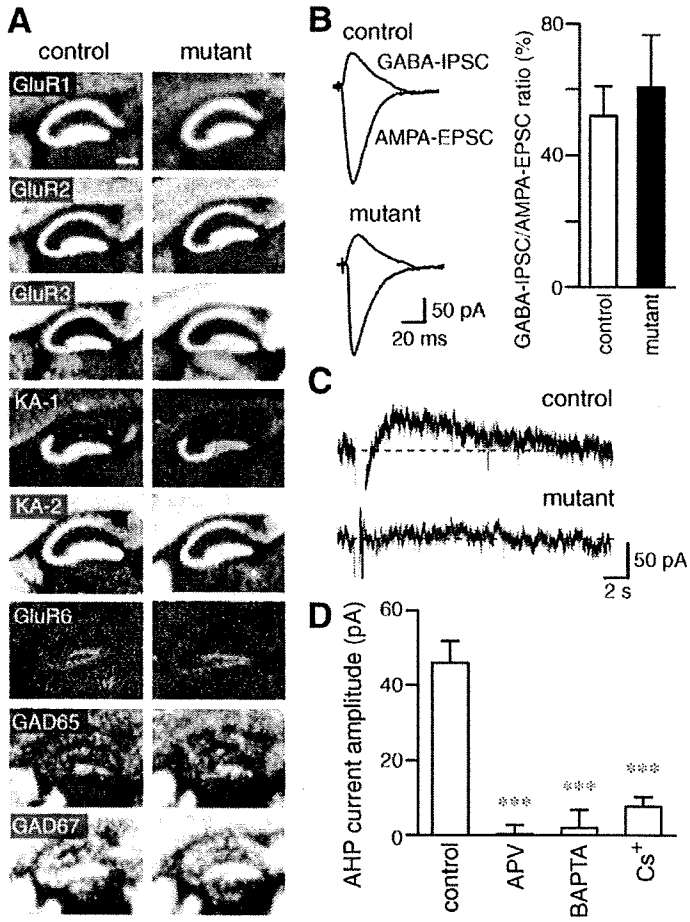

Figure 7. High-frequency stimulation failed to induce slow hyperpolarizing currents in hippocampal CA3 pyramidal neurons of mutant mice. **A**, X-ray film autoradiography for mRNAs of AMPA receptors, kainate receptors, and GADs. Scale bar, 200 μm. **B**, Representative traces of AMPA-EPSCs and $GABA_A$-IPSCs in the CA3 pyramidal cells. Graph shows the ratio of $GABA_A$-IPSCs to AMPA-EPSCs. **C**, Representative traces of slow hyperpolarizing currents. **D**, Peak amplitudes of the slow hyperpolarizing currents of the control mice in the absence (control) or presence of D-APV. Those recorded with a BAPTA-containing (BAPTA) or Cs^+-based internal solution (Cs^+) are also shown. ***, $P<0.001$, *t*-test.
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Table 1. Ratios of hybridization signal densities of GluR and GAD mRNAs in the CA3 region to those in the CA1 region.

mRNA	Control	Mutant
GluRζ1/NR1	0.86±0.02 (*n*=10)	0.04±0.01 (*n*=5)
GluRα1/GluR1	0.98±0.02 (*n*=10)	0.95±0.01 (*n*=12)
GluRα2/GluR2	0.91±0.03 (*n*=8)	0.89±0.03 (*n*=11)
GluRα3/GluR3	0.88±0.02 (*n*=10)	0.86±0.02 (*n*=12)
GluRγ2/KA-2	1.16±0.03 (*n*=9)	1.17±0.02 (*n*=10)
GluRβ2/GluR6	1.11±0.09 (*n*=8)	1.19±0.04 (*n*=8)
GAD65	1.12±0.05 (*n*=10)	0.99±0.05 (*n*=11)
GAD67	1.11±0.02 (*n*=10)	0.94±0.03 (*n*=12)

Slices were prepared from 3 mice of both genotypes.
Hybridization signal densities of the *GluRγ1/KA-1* mRNA in the CA3 region were 51.5±0.8 (*n*=10) in control mice and 32.3±0.5 (*n*=12) in mutant mice.
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$P<0.001$), suggesting that NMDA receptors are required for the response. NMDA receptor activation results in influx of Ca^{2+} into postsynaptic cells, which would activate Ca^{2+}-dependent K^+ channels. In fact, inclusion of the Ca^{2+} chelator BAPTA in the internal solution of patch pipettes diminished the outward currents (Fig. 7D; BAPTA, 1.99±4.76 pA, $n=7$, $P<0.001$). The outward currents were also diminished when recorded with a Cs^+-based internal solution (Fig. 7D; Cs^+, 7.74±2.35 pA, $n=4$, $P<0.001$), suggesting that the currents were mediated by postsynaptic K^+ channels. Taken together, the slow kinetics and sensitivities to D-APV, BAPTA and Cs^+ of the outward hyperpolarizing currents suggest that the high-frequency stimulation evokes slow AHP currents [41,42] mediated by Ca^{2+}-activated K^+ channels, which are activated by Ca^{2+} influx through NMDA receptor channels. These results suggest that the NMDA receptor-slow AHP coupling is diminished in the hippocampal CA3 pyramidal neurons of mutant mice, which may result in enhanced excitability of the CA3 recurrent network as a whole. The coupling between NMDA receptors and AHP currents is found in various regions [34,43–45]. However, the durations of AHP currents observed in our

experiment were much longer than those observed in previous studies.

These results with hippocampal CA3-specific NMDA receptor mutant mice raise an intriguing possibility that MDA receptors suppress the excitability of the CA3 recurrent network as a whole by restricting synchronous firing of CA3 neurons, although the possibility cannot be excluded that the enhanced excitability of the mutant mice might be due to subtle cytoarchitectural abnormalities of CA3 pyramidal neurons. To test the possibility, we then examined the effect of NMDA receptor ablation in the CA3 region of the adult brain on hippocampal network oscillations by employing a virus-mediated gene knockout technique [22,23].

Ablation of CA3 NMDA receptors in the mature brain also generated characteristic EEG spikes with large amplitudes

An adeno-associated viral expression vector for Cre recombinase (AAV-Cre, titer of $5–8\times10^{10}$) was streotaxically microinjected to the hippocampal CA3 region of *GluRζ1*$^{flox/flox}$ mice at 8–9 weeks old. Immunohistochemical analysis revealed that the infection of AAV-Cre was limited to the hippocampal CA3 region and spread within 40–70% of the region (Fig. 8A–C). Immunoreactivity for GluRζ1 was diminished in the well-demarcated infected CA3 region by 2 weeks after infection (Fig. 8C). Age-matched *GluRζ1*$^{+/+}$ mice microinjected with AAV-Cre served as controls.

Local field potential recording from the CA3 region showed characteristic EEG spikes with large amplitudes in *GluRζ1*$^{flox/flox}$ mice 2–3 weeks after AAV-Cre infection ($n=5$ out of 9 mice) (Fig. 8D). The frequency of large EEG spikes was variable among subjects, which may be related to the variance of AAV-infected regions among animals. No such spike activity was detected in EEG records from the CA3 region of AAV-Cre-infected *GluRζ1*$^{+/+}$ mice ($n=7$ out of 7 mice, $P=0.02$, Fisher's exact probability test) (Fig. 8D). CSD analysis revealed the sink in the pyramidal cell layer of the CA3 region and the sources in neighboring stratum oriens (Fig. 8E, $n=8$ spikes). Thus, the ablation of CA3 NMDA receptors induced by AAV-Cre infection in the adult brain also resulted in the generation of characteristic EEG spikes.

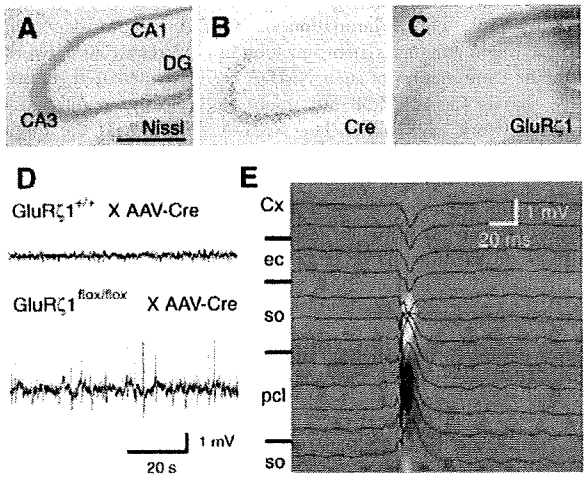

Figure 8. Hippocampal CA3 NMDA receptor ablation in the adult brain also generated characteristic EEG spikes with large amplitudes. A–C, AAV-Cre-mediated ablation of NMDA receptors in the hippocampal CA3 region. Nissl staining (**A**) and imunohistochemistry for Cre (**B**) and GluRζ1 (**C**). Scale bar, 0.5 mm. **D**, Representative local field potential recordings from the CA3 region. **E**, Laminar profiles of field potentials and CSD analysis. Recording positions are illustrated on the left. Sinks and sources are indicated by cold and warm colors, respectively. Cx, cortex; ec, external capsule; pcl, pyramidal cell layer; so, stratum oriens.
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Pharmacological blockade of CA3 NMDA receptors enhanced the susceptibility to kainate-induced seizure

We finally examined the seizure susceptibility of wild-type mice by focal injection of a competitive NMDA receptor antagonist, APV. We bilaterally injected 30 mM APV or aCSF into the hippocampal CA3 region of C57BL/6N mice at postnatal 8–10 weeks. About 20–30 minutes later, the animals were intraperitoneally administrated with the convulsive dose of kainate (30 mg/kg) [46]. Kainate-induced tonic-clonic seizures with loss of the postural tone appeared within 1 h in both groups of mice ($n=8$ each; $P=0.23$, Fisher's exact probability test) (Fig. 9). However, the latency to the onset of seizures was significantly shorter in mice injected with APV ($n=8$; $P=0.0044$, Log-rank test). Thus, the focal blockage of CA3 NMDA receptors also enhanced the susceptibility to kainate-induced seizure.

Discussion

Here, we generated hippocampal CA3 pyramidal neuron-specific NMDA receptor mutant mice on the C57BL/6N genetic background. The expression of the *GluRζ1* mRNA was comparable between mutant and control mice at P1 but strongly decreased in mutant mice at P7. The significant expression of GluRζ1 protein, though reduced, was found in the CA3 region at P7 but diminished to a negligible level by P14. We found that the mutant mice lacking NMDA receptors in the hippocampal CA3 pyramidal neurons showed enhanced susceptibility to kainate-induced seizures. This observation was rather unexpected since NMDA receptor-mediated LTP was implied to contribute to the generation of synchronous network activity by *in vitro* studies [14,15]. We found that characteristic EEG spikes with large amplitude were generated by the ablation of NMDA receptors in CA3 pyramidal neurons. Strong association of MUA with the characteristic EEG spikes in the CA3 pyramidal cell layer of mutant mice suggests that the CA3 NMDA receptor ablation increases the synchronous network activity possibly by affecting the firing pattern of CA3 neurons. In contrast, CA1 region-specific ablation of NMDA receptors appeared to hardly affect EEG *in vivo* [47]. NMDA receptor antagonists have minimal effects on basal synaptic transmission but completely block the generation of long-term potentiation in the CA1 region *in vitro* [48–50]. Hence, NMDA receptors in the CA1 region are not considered to be involved in spontaneous network activity. The difference in the neural wiring pattern such as the abundance of recurrent networks may underlie the different effects of NMDA receptor ablation in the hippocampal CA1 and CA3 regions on network activity. Our results raise an intriguing possibility that NMDA receptors may suppress the excitability of the CA3 network as a whole *in vivo*.

It is possible that the ablation of NMDA receptors may disturb the neural wiring of the hippocampal CA3 region, leading to abnormal excitability of the network. It is well known that the NMDA receptor plays a role in the activity-dependent refinement of synaptic connections and neural pattern formation [51–54]. Chronic blockade of NMDA receptors in hippocampal slice cultures during the first two weeks of postnatal development leads to a substantial increase in synapse number and results in a more complex dendritic arborization of CA1 pyramidal cells [31]. The activity blockade in hippocampus during postnatal 2–3 weeks by

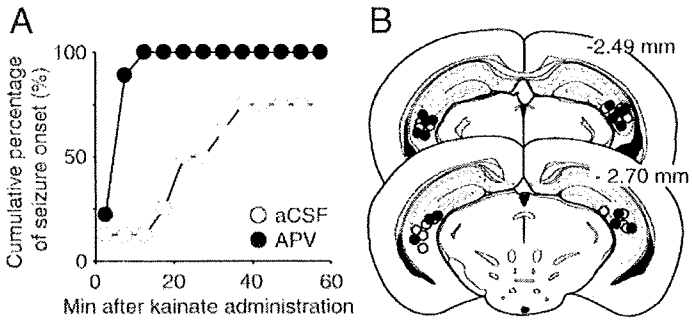

Figure 9. The pharmacological blockade of CA3 NMDA receptors increased the susceptibility to kainate-induced seizures. A, Cumulative curves for the onset of seizure. **B,** Illustration of the injection sites of APV (filled) and aCSF (open). Numbers represent distance (mm) of the section relative to the bregma landmark.
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tetrodotoxin infusion produced both behavioral and electrographic seizures 2 weeks after the infusion [55] and the increase in the density of axonal varicosities and postsynaptic AMPA receptor GluR1 and NMDA receptors [56]. Thus, reduced neuronal activity during development might potentially enhance the excitability. However, the cytoarchitecture was indistinguishable between control and mutant mice at P21–23. There were no detectable differences in the dendritic branching and the density of axonal boutons and dendritic spines between control and mutant mice at P21–23. The sustained expression of NMDA receptor proteins at least by P7 in mutant mice may support the development of CA3 pyramidal neuron cytoarchitectures. An alternative possibility is that the excitability of the CA3 network may be suppressed by NMDA receptor-mediated signaling. No significant differences were detectable in the basic membrane properties and balance between excitatory and inhibitory synaptic transmission between control and mutant mice. At synapses, activation of NMDA receptors evokes excitatory postsynaptic potential on the CA3 pyramidal neurons *in vitro* [57]. However, the enhancement of the kainate-induced seizure susceptibility and the emergence of characteristic EEG spikes associated with MUA in the mutant mice can be hardly explained if major roles of NMDA receptors would be simply mediating and strengthening the excitatory transmission at the commissural/associational synapses. Besides excitatory transmission and its enhancement, NMDA receptors may mediate diverse suppressive signals including spike-timing dependent long-term depression [58], LTP of slow GABA-IPSCs [59], the increase in I_h currents [60], and coupling with K^+ channels [34,43–45]. Thus, it is possible that NMDA receptor signaling may suppress the excitability of the CA3 network *in vivo*, although the possibility cannot be excluded that the enhanced excitability of the mutant mice might be due to subtle developmental abnormalities of CA3 pyramidal neurons.

We thus examined whether the excitability of the CA3 network is enhanced by ablation of NMDA receptors in the adult brain with a virus-mediated gene knockout technique [22,23]. We found that EEG spikes with large amplitude were generated by focal ablation of NMDA receptors in the CA3 region of adult mice by AAV-Cre infection. The frequency of large EEG spikes was variable among subjects, which may be related to the variance of AAV-infected regions among animals. Furthermore, the blockade of NMDA receptors by focal injection of APV into the hippocampal CA3 region enhanced the susceptibility to kainate-induced seizures. These results suggest that NMDA receptors control negatively the excitability of the hippocampal CA3 recurrent network as a whole *in vivo* by restricting synchronous firing of CA3 neurons, although the mechanism remains to be solved. Since slow AHP currents are involved in accommodation of action potential discharge of CA1 pyramidal neurons [40], it is possible that the frequency of action potentials may increase in a mutant CA3 pyramidal neuron where NMDA receptor-AHP coupling is eliminated. Prolonged discharges of CA3 pyramidal neurons might increase the chance of their synchronous firing, leading to the enhancement of the excitability of the CA3 network as a whole. Interestingly, Colgin et al. reported that blockade of NMDA receptors enhanced spontaneous sharp waves in rat hippocampal slices [61], supporting the idea that activation of NMDA receptors can serve to dampen the excitation of sharp waves. On the other hand, studies through computational models showed that when recurrent networks with conductance delays exhibit population bursts, spike-timing-dependent plasticity (STDP) rules exert a strong decoupling force that desynchronizes activity [58]. Thus, elimination of NMDA receptor-dependent STDP might enhance synchronization in CA3 recurrent networks. One or combination of such NMDA receptor-mediated suppressive signals [34,43–45,58–60] might underlie the regulation of CA3 network excitability. The NMDA receptors in the hippocampal CA3 region are implied in rapid acquisition and recall of associative memory as well as paired associate learning [11–13]. These functions may be mediated not only by the plasticity at synapses but also by the NMDA receptor-mediated neural network oscillation.

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Author Contributions

Conceived and designed the experiments: FF KN MW TM MM. Performed the experiments: FF KN TS MF MW. Analyzed the data: FF KN TS MF MW. Contributed reagents/materials/analysis tools: SiM KS HK HM. Wrote the paper: FF KN MW TM MM.

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Inflammation Research

Enhancement of activated β_1-integrin expression by prostaglandin E_2 via EP receptors in isolated human coronary arterial endothelial cells: implication for the treatment of Kawasaki disease

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Abstract. *Objective:* Plasma prostaglandin E_2 (PGE_2) levels are markedly elevated in acute Kawasaki disease (KD). We evaluated the function of the EP receptors in the expression of activated β_1-integrin stimulated by PGE_2 in human coronary arterial endothelial cells (HCAEC).
Methods: We determined the mRNA expression of the PGE_2 receptors, EP receptors ($EP_{1\text{-}4}$) in HCAEC by RT-PCR and protein expression by Western blotting. We evaluated the function of the EP receptors in the expression of activated β_1-integrin stimulated by PGE_2 in HCAEC, using antagonists and agonists of the EP receptors, by flow cytometry.
Results: RT-PCR revealed mRNAs for all four EP receptors in HCAEC. Western blotting demonstrated EP_1, EP_2 and EP_3 expression in HCAEC. The EP_2 and EP_3 agonists enhanced the expression of activated β_1-integrin in HCAEC. The potency of the EP_2 agonist was significantly greater than that of the EP_3 agonist. Pretreatment with the EP_1, EP_2 and EP_3 antagonists inhibited the expression of activated β_1-integrin induced by PGE_2 in HCAEC. The potency of the EP_2 antagonist was significantly greater than that of the EP_1 and EP_3 antagonists.
Conclusions: Our results suggest that PGE_2 mainly induces the activation of β_1-integrins via the EP_2 receptor in HCAEC. Our results further suggest that the EP_2 antagonist modulates the inflammatory response during KD vasculitis.

Key words: β_1-integrin – Coronary artery endothelial cells – Prostaglandin E_2 – Prostaglandin E receptor

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Introduction

During inflammation leukocytes interact with extracellular matrix proteins after migration through the vascular endothelium to the site of tissue injury or infection. These interactions are mediated through integrins, which exist as heterodimers of noncovalently associated α and β subunits [1]. At least thirteen different integrins have been reported to be expressed in the vascular system during developmental or postnatal angiogenesis: $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_4\beta_1$, $\alpha_5\beta_1$, $\alpha_6\beta_1$, $\alpha_6\beta_4$, $\alpha_8\beta_1$, $\alpha_9\beta_1$, $\alpha V\beta_1$, $\alpha V\beta_3$, $\alpha V\beta_5$ and $\alpha V\beta_8$ [2]. Recent studies have shown that endothelial cells and vascular smooth muscle express β_1-integrin, which is involved in leukocyte adhesion and angiogenesis [3–5]. It is believed that β_1-integrins play a role in atherosclerosis and myocardial infarction by promoting the adhesion and migration of vascular smooth muscle and endothelial cells, modulating matrix synthesis, and remodeling in tissue repair [6, 7].

Kawasaki disease (KD) is an acute illness of early childhood that is characterized by prolonged fever, diffuse mucosal inflammation, indurative edema of the hands and feet, a polymorphous skin rash and non-suppurative lymphadenopathy [8]. The histopathological findings in KD comprise panvasculitis with endothelial necrosis, and infiltration of mononuclear cells into small and medium-sized blood vessels [9]. A coronary artery lesion is the most important complication of KD and may cause significant coronary stenosis resulting in ischemic heart disease [10]. We previously reported that plasma prostaglandin E_2 (PGE_2) levels were markedly elevated during the acute stage of KD [11]. PGE_2 contributes to dilation of coronary arteries and increased vascular permeability, and acts via four receptor subtypes (EP_1, EP_2, EP_3 and EP_4) [12] in a complex manner: its action varies with different receptor subtypes in the same cell type and different cells with the same subtypes of the receptor [12, 13].

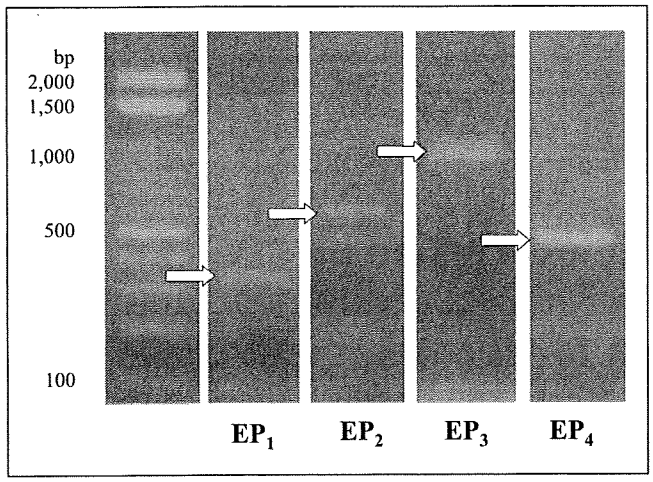

Fig. 1. mRNA expression of the EP subtypes measured by RT-PCR. mRNA expression of all EP receptors was observed in HCAEC. Representative data are shown. Samples were collected in 4 independent experiments. Similar results were obtained in 4 independent experiments.

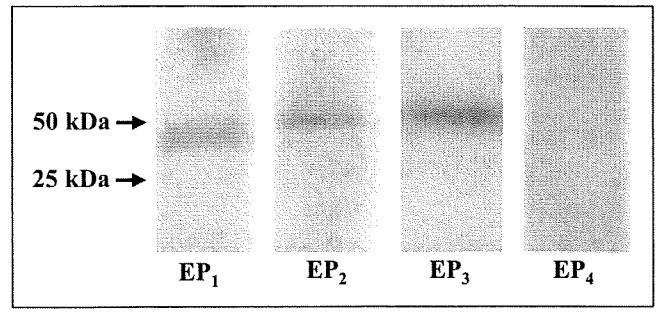

Fig. 2. Expression of the EP subtypes measured by Western blot analysis in HCAEC. EP_1, EP_2 and EP_3, but not EP_4 receptors, were expressed in HCAEC. Representative data are shown. Samples were collected in 2 independent experiments. Similar results were obtained in 2 independent experiments.

Since PGE_2 is an important factor in activating β_1-integrins, we examined the expression of EP receptors in human coronary arterial endothelial cells (HCAEC) and investigated the function of the EP receptors for expression of activated β_1-integrin stimulated by PGE_2 in HCAEC.

Materials and methods

Cell culture and stimulation conditions

HCAEC were obtained from BioWhittaker (Walkersville, MD) and maintained at 37 °C under humidified 5 % CO_2 in a stationary culture. HCAEC were grown using the EGM-2 BulletKit (BioWhittaker).

The cells were exposed to 1 mM Mn^{2+} (Sigma Aldrich, St. Louis, MO), and 10 ng/ml (30 nM) of PGE_2 (Sigma Aldrich), 10 µM of receptor agonists of EP_1 (ONO-DI-004, (17S)-2,5-ethano-6-oxo-17, 20-dimethylPGE$_1$), EP_2 (ONO-AE1-259-01, (16S)-9-Deoxy-9b-chloro-15-deoxy-16-hydroxy-17,17-trimethylene-19,20-didehydroPGF$_2$ sodium salt), EP_3 (ONO-AE-248, 11,15-O-DimethylPGE$_2$) or EP_4 (ONO-AE1-329, 16-(3-Methoxymethyl)phenyl-w-tetranor-3,7-dithiaPGE$_1$), provided by ONO Pharmaceutical Co. (Osaka, Japan). Some samples were pretreated with 10 µM of receptor antagonists against EP_1 (ONO-8713, 4-[2-[N-isobutyl-N-(2-furylsulfonyl)amino]-5-trifluoromethylphenoxy-methyl]cinnamic acid), EP_2 (AH 6809, 6-isopropoxy-9-oxoxanthene-2-carboxylic acid) (Cayman Chemical, Ann Arbor, MI), EP_3 (ONO-AE3-240, chemical structure not exhibited) or EP_4 (ONO-AE3-208, 4-{4-Cyano-2-[2-(4-fluoronaphthalen-1-yl)propionylamino]phenyl}butyric acid). The antagonists of ONO were provided by ONO Pharmaceutical Co. The cells were exposed for 30 minutes before addition of PGE_2. Each agonist is able to selectively bind and stimulate each receptor subtype. Each antagonist is able to selectively bind and block each receptor from stimulation with PGE_2 [14].

RNA isolation and reverse transcription-PCR

Total RNA was prepared from each cell type using TRIzol reagent (Invitrogen, Leek, The Netherlands). Reverse transcription (RT)-PCR was performed with Gene Amp and an oligo dT primer (Applied Biosystems, Foster City, CA) for RT, and Taq polymerase (Roche Diagnostics GmbH, Mannheim, Germany) for PCR. The primers specific for each EP were designed as described previously [15–17]. The sequences of the primers were as follows: EP_1 (322 bp), 5'-CTTGTCGGTATCATGGTGGTGTC-3'(forward) and 5'-GGTTGTGCTTAGAAGTGGCTGAGG-3'(reverse); EP_2 (654 bp), 5'-GCCACGATGCTCATGCTCTTCGCC-3'(forward) and 5'-CTTGTGTTCTTAATGAAATCCGAC-3'(reverse); EP_3 (837 bp), 5'- CGCCTCAACCACTCCTACACA -3'(forward) and 5'- GCAGACCGACAGCACGCACAT -3'(reverse); and EP_4 (435 bp), 5'-TGGTATGTGGGCTGGCTG-3'(forward) and 5'-GAGGACGGTGGCGAGAAT-3'(reverse). Quantitation of the bands was conducted with a Kodak Digital Science 1D Image Analysis Software (Eastman Kodak Company, New Haven, CT). All experiments were performed five times.

Western blot analysis

Whole cell lysates were obtained by incubation of cell samples in ice-cold lysis buffer (1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride) with protease inhibitors (1 µM leupeptin and 1 µM pepstatin) and centrifugation to remove debris (12,000 g for 10 min at 4 °C). The protein concentrations of the samples were determined with the Bio-Rad (Hercules, CA) protein concentration reagent. Samples containing 20 µg of protein were separated in denaturing 10 % polyacrylamide gels and then transferred to polyvinylidene difluoride membranes. After three washes in TBST (Tris buffer saline with Tween 20; 40 mM Tris-HCl, p7.6, 300 mM NaCl and 0.5 % Tween 20), the membranes were incubated with 1:200,1:100,1:500,and 1:500 dilutions of rabbit polyclonal anti-EP_1, EP_2, EP_3 and EP_4 receptor antibodies (Cayman Chemical), respectively, in TBST containing 5 % nonfat dry milk at room temperature for 1hr. After three washes in TBST, the membranes were incubated with a 1:2,000 dilution of horseradish peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad) for 1hr at room temperature. Immunoreactive proteins were detected using enhanced chemiluminescence (Amersham, Arlington Heights, IL) and analyzed by radiography. All experiments were performed three times.

Determination of activated β_1-integrin expression

The expression of activated and total β_1-integrins was determined by flow cytometric analysis. The cells were labeled with 10 µl of phycoerythrin labeled anti-human CD29 (clone name, HUTS-21) (BD Pharmingen, San Jose, CA) as the surface antigen for activated β_1-integrin [18], or with 10 µl of phycoerythrin labeled anti-human CD29 (clone name, MAR4) (BD Pharmingen) as the surface antigen for total (activated and non-activated) β_1-integrin. Immunofluorescence staining was analyzed using a FACScan flow cytometer equipped with CellQuest software (BD PharMingen). Five thousand cells were analyzed for each sample in the flow cytometric studies.

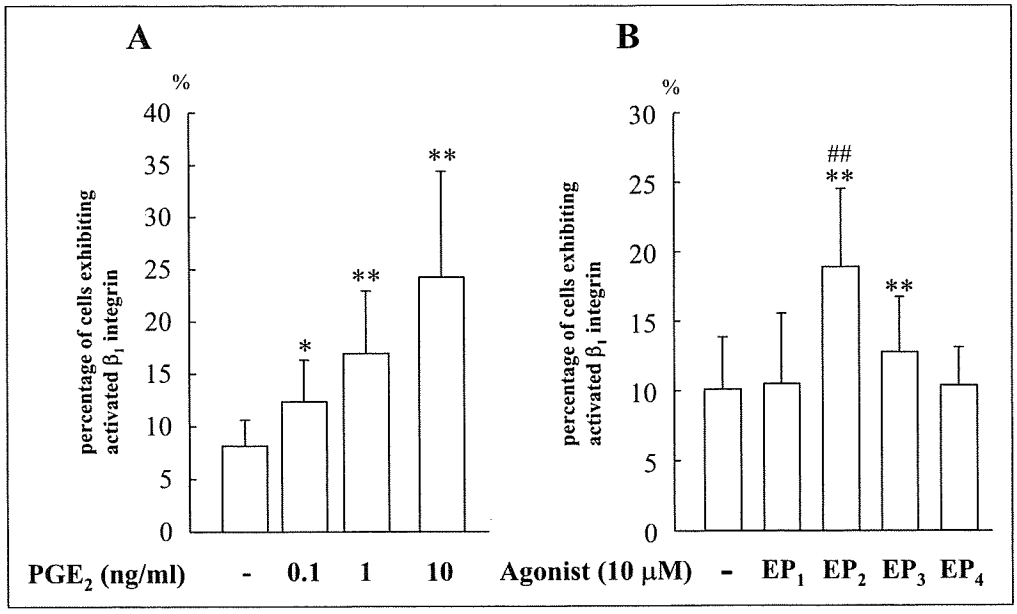

Fig. 3. The percentage of cells exhibiting activated β_1-integrin by PGE_2 (A) and EP_{1-4} agonists (B) in HCAEC determined by flow cytometric analysis. A; Cells were stimulated with 0.1, 1 and 10 ng/ml PGE_2 for 4 hrs. PGE_2 induced the expression of activated β_1-integrin in a dose-dependent manner. Data (n = 10) are presented as the means + 1 SD. B; Cells were stimulated with 10 μM EP_1, EP_2, EP_3 and EP_4 agonists for 4 hrs. EP_2 and EP_3 agonists induced the expression of activated β_1-integrin. Data (n = 14) are presented as the means + 1 SD. ** $p < 0.01$ and * $p < 0.05$, compared with cells without treatment. ## $p < 0.01$ compared with cells treated with EP_3 agonist.

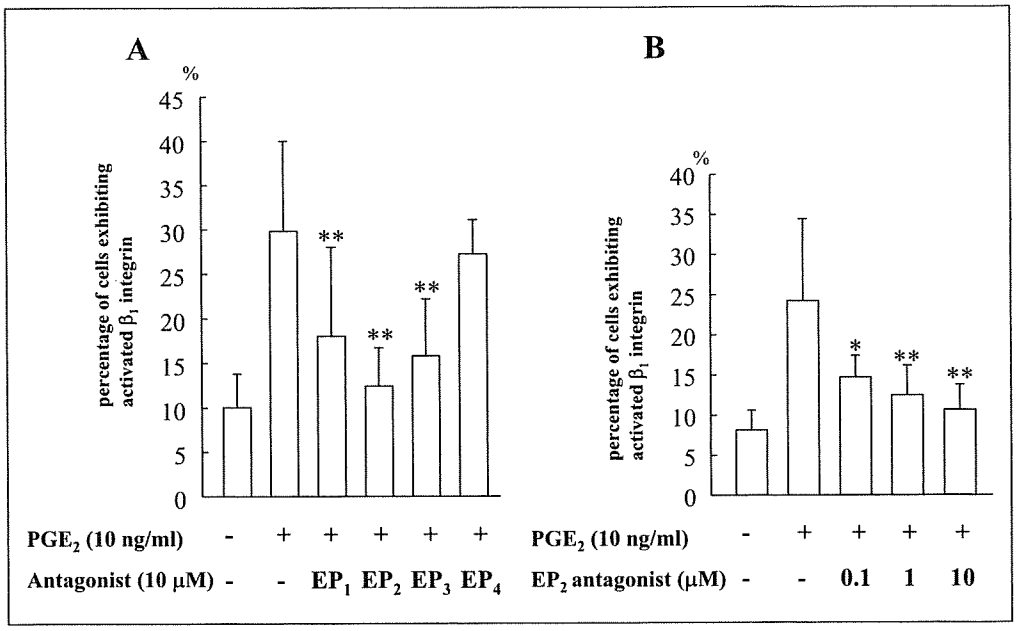

Fig. 4. The inhibitory effects of EP_{1-4} antagonists on activated β_1-integrin expression induced by PGE_2 in HCAEC determined by flow cytometric analysis. A; Cells were pretreated with 10 μM EP_1, EP_2, EP_3 or EP_4 antagonists for 30 min and stimulated with 10 ng/ml PGE_2 for 4 hrs. EP_1, EP_2 and EP_3 antagonists significantly inhibited the expression of activated β_1-integrin. Data (n = 14) are presented as the means + 1 SD. B; Cells were pretreated with 0.1, 1 and 10 μM of the EP_2 antagonists for 30 min and were stimulated with 10 ng/ml PGE_2 for 4 hrs. EP_2 antagonists inhibited the expression of activated β_1-integrin in a dose-dependent matter. Data (n = 10) are presented as the means + 1 SD. ** $p < 0.01$ and * $p < 0.05$, compared with the cells stimulated with PGE_2 only.

Statistical analysis

All values are presented as the mean ± SD. Differences in the results were analyzed by means of the Wilcoxon matched paired test, with a p-value less than 0.05 being considered significant.

Results

Expression of the EP receptors in HCAEC

Reverse transcription-PCR of HCAEC showed expression of all four EP receptor subtypes (Fig. 1). Western blotting analysis of HCAEC revealed the expression of EP_1 (42 kDa), EP_2 (52 kDa) and EP_3 (53 kDa) receptors, but not of the EP_4 receptor (65 kDa) (Fig. 2).

Expression of activated β_1-integrin

Flow cytometric analysis revealed that the expression of total (activated and non-activated) β_1-integrin was 100% before and after addition of PGE_2 in HCAEC (data not shown). Figure 3A demonstrates that 0.1, 1, and 10 ng/ml (0.3, 3, and

30 nM, respectively) PGE_2 significantly induced expression of activated β_1-integrin in HCAEC at each concentration (0.1 ng/ml, $p < 0.05$; 1 and 10 ng/ml, $p < 0.01$). Expression of activated β_1-integrin by PGE_2 occurred in a dose-dependent manner. Figure 3B presents the effect of the $EP_{1\text{-}4}$ agonists on the expression of activated β_1-integrin in HCAEC. EP_2 and EP_3 agonists significantly induced expression of activated β_1-integrin (both $p < 0.01$), but EP_1 or EP_4 agonists did not. The EP_2 agonist significantly induced the expression of activated β_1-integrin compared with that for EP_3 ($p < 0.01$).

Figure 4A presents the inhibitory effects of the $EP_{1\text{-}4}$ antagonists on the expression of activated β_1-integrin induced by PGE_2. Pretreatment with EP_1, EP_2 and EP_3 antagonists significantly inhibited the expression of activated β_1-integrin induced by PGE_2 (all $p < 0.01$), but the EP_4 antagonist did not. The EP_2 antagonist significantly inhibited the expression of activated β_1-integrin, compared with the EP_1 and EP_3 antagonists (both $p < 0.05$). Pretreatment with 0.1, 1 and 10 µM of the EP_2 antagonist significantly inhibited the expression of activated β_1-integrin (0.1 ng/ml, $p < 0.05$; 1 and 10 ng/ml, $p < 0.01$) (Fig.4B). The effect of the EP_2 antagonist was dose-dependent. The time courses of expression of activated β_1-integrin induced by PGE_2 and the inhibitory effects of pretreatment with 10 µM EP_2 antagonist were examined in HCAEC over 24 hrs. PGE_2 significantly induced the expression of activated β_1-integrin at 4, 8, and 24 hrs (all $p < 0.01$). The EP_2 antagonist attenuated the expression of activated β_1-integrin at 4, 8, and 24 hrs (all $p < 0.01$) (data not shown).

Discussion

Our present study indicated that HCAEC expressed mRNA for all four EP receptors, and expressed the proteins for the EP_1, EP_2 and EP_3 receptors. We suggested that the protein for EP_4 could not be synthesized, despite the fact that the mRNA of EP_4 was produced in HCAEC.

Flow cytometric analysis showed that the EP_2 and EP_3 agonists significantly increased the expression of activated β_1-integrin, and that the potency of the EP_2 agonist was greater than that of the EP_3 agonist. The EP_1, EP_2 and EP_3 antagonists significantly inhibited the expression of activated β_1-integrin induced by PGE_2, and the potency of the EP_2 antagonist was greater than that of the EP_1 or EP_3 antagonists. These results suggested that PGE_2 induced the expression of activated β_1-integrin mainly via EP_2 receptors, and in part via EP_3 receptors. The EP_1 agonist did not increase the expression of activated β_1-integrin, while the expression was significantly inhibited by the EP_1 antagonist. The K_i values of the EP_1 antagonist are 3.0 µM and 1.0 µM for the EP_2 and EP_3 receptors, while the EP_1 agonist is a selective ligand for the EP_1 receptor with a K_i value of 0.3 nM [19]. In our present study, cells were pretreated with 10 µM of each antagonist. Therefore, it is possible that the inhibition of activating β_1-integrin by the EP_1 antagonist might be due to non-specific inhibition with excess dose (30 times higher) of this antagonist. The concentrations of PGE_2 used in this study are similar to the plasma concentrations of PGE_2 in patients during acute KD [11].

High levels of PGE_2 are involved in the pathogenesis of KD [11]. PGE_2 is mainly produced by monocytes/macrophages, and exhibits pyrogenic and vascular permeability-increasing actions [20, 21]. Macrophage lineage cell infiltration in the dermis, necrotic changes of endothelial cells, and dilation of capillary blood vessels have been observed in coronary arterial tissue and skin biopsy specimens in KD [9, 22–24]. We previously demonstrated that nuclear factor-κB, a transcription factor for genes that encode various inflammatory mediators, was markedly activated in peripheral blood monocytes/macrophages of children with acute KD [25]. These findings suggest that there is ample evidence supporting a central role of monocytes/macrophages during acute KD. It has been reported that activated $\alpha_4\beta_1$-integrin on the surface of endothelial cells binds fibronectin, which binds to $\alpha_4\beta_1$-integrin on the surface of monocytes and enables firm adhesion of monocytes to endothelial cells [26]. A previous study reported that plasma fibronectin concentrations were decreased significantly in the early days of acute KD, increased gradually thereafter, and reached significantly higher concentrations by the fourth week of the disease, suggesting massive consumption of fibronectin in vasculitis lesions [27]. Taking these reports into consideration, it is likely that the adhesion of monocytes to endothelial cells by activated β_1-integrin on endothelial cells stimulated with PGE_2 may occur in KD vasculitis. If so, alternative therapy, such as the use of an EP_2 antagonist may be effective during acute KD, through modulation of the β_1-integrin system. It will be necessary to further examine the effects of PGE_2 on monocyte binding to HCAEC and the inhibitory effects of the EP_2 antagonist in the future.

Aspirin is widely administered as an anti-thrombotic and anti-inflammatory agent for acute KD [28]. PG metabolites exhibit various activities, and their pro- but also anti-inflammatory activities have been reported [29]. PGI_2 [30–32] and PGD_2 [33, 34] exhibit anti-inflammatory effects. For example, PGI_2 exhibits anti-platelet and anti-thrombotic effects [35, 36], and an anti-inflammatory effect on permeability through endothelial cells in vivo [31]. Favorable effects of a stable analogue of PGI_2 were reported in various patients with vasculitis, including thromboangiitis obliterans and Raynaud's phenomenon [32]. PGD_2 is a potent inhibitor of platelet aggregation. Endogenous PGD_2 decreases vascular cell adhesion molecule-1 (VCAM-1) expression and VCAM-1 mRNA expression in human umbilical vein endothelial cells [34]. Clinical doses of aspirin for acute KD (30–50 mg/kg/day) are able to sufficiently inhibit production of PGI_2 and PGD_2 [37, 38]. Regarding the PG cascade, it is unlikely that aspirin is an adequate treatment for acute KD because PGI_2 and PGD_2 could act to attenuate vasculitis, and impairment of their production by aspirin would be disadvantageous. Therefore, it is likely that an EP_2 antagonist, in addition to being an anti-thrombotic agent, is better as a medicine for KD than aspirin. Further in vivo studies are needed to further elucidate this hypothesis.

In conclusion, HCAEC express EP_1, EP_2 and EP_3 receptors. Activated β_1-integrin is produced by PGE_2 mainly via EP_2 receptors in HCAEC. Among the $EP_{1\text{-}4}$ antagonists, the EP_2 antagonist most significantly inhibited the expression of activated β_1-integrin induced by PGE_2 in HCAEC.

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Serial cerebrospinal fluid neurofilament concentrations in bacterial meningitis

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ABSTRACT

Neurofilament (NF) is one of the major cytoskeleton proteins of neurons. We investigated the concentrations of the heavy subunit of NF (NF-H) in cerebrospinal fluid (CSF) as biomarkers of neuronal injury in bacterial meningitis. Concentrations of NF-H in CSF of 26 children with bacterial meningitis and in 16 control subjects were measured by ELISA. The CSF NF-H levels were elevated in 22 of the 26 children (85%) with bacterial meningitis. The peak CSF NF-H level occurred at a median period of 10.5 days after onset of illness (range, 1 to 35 days). The peak CSF NF-H levels of the patients with neurological sequelae ($n=4$) were significantly higher than those without sequelae ($n=22$) (7.06 vs. 2.46 ng/mL as median, $p=0.048$). There was no significant difference in CSF NF-H levels between patients with and without severe neurological sequelae up to day 14 of illness, but the CSF NF-H levels in patients with sequelae were significantly higher than in those without sequelae after day 14 of illness (2.04 vs. 1.19 ng/mL as median, $p=0.024$). We suggest that neuronal injury occurs in bacterial meningitis regardless of the presence or absence of neurological sequelae.

1. Introduction

Neurofilament (NF) is a major structural element of neurons and is composed of three subunits: the light (NF-L), medium (NF-M) and heavy (NF-H) subunits [1]. NF is a specific biomarker for axonal injury, degeneration and neuronal loss, and detection of NF in cerebrospinal fluid (CSF) provides information on the degree of neuronal injury [1]. The phosphorylated forms of NF-H are resistant to proteases and are particularly concentrated in larger diameter axons [1]. It has been reported that NF-L or NF-H in CSF is increased in neurological diseases, including multiple sclerosis, hydrocephalus, subarachnoid hemorrhage, brain damage after cardiac arrest, AIDS dementia complex, Parkinsonian syndromes, amyotrophic lateral sclerosis, and Guillain–Barré syndrome [2–11].

Bacterial meningitis remains a serious and life-threatening disease. Antibiotics and adjunctive dexamethasone therapy improve the prognosis, but the condition can result in both severe neurodisability and milder motor and psychometric impairment. In this study, we determined serial CSF NF-H concentrations to evaluate neuronal injury in pediatric patients with bacterial meningitis.

2. Materials and methods

Informed consent was obtained from the parents of the patients and controls in the study. The protocol was approved by the Institutional Review Board of Yamaguchi University Hospital.

2.1. Bacterial meningitis

Ninety-six CSF samples were obtained from 26 children (13 females and 13 males, mean age: 1.1 years old, range: 2 days to 4 years old) with bacterial meningitis on admission to Yamaguchi University Hospital from August, 1990 to August, 2007 (Table 1). The day of onset of fever was considered to be day 1 of illness. Serial CSF samples were obtained from patients (mean: 3.7 times, range: 2 to 9 times), with the initial CSF sample obtained during days 1 to 15 (median: 1.0 days) of illness. Samples were stored at −80 °C until assay. CSF cultures from patients with bacterial meningitis yielded *Haemophilus influenzae* ($n=17$), *Streptococcus pneumoniae* ($n=3$), *Escherichia coli* ($n=3$), Group B *Streptococcus* ($n=2$), and methicillin-resistant *Staphylococcus aureus* ($n=1$). The patients were treated with multiple antibiotics that were effective against these bacteria, and adjunctive dexamethasone therapy was performed in 16 of the 26 patients according to the standard method (0.6 mg/kg/day in four intravenous doses) [12]. Administration of dexamethasone was started before the first administration of antibiotics. Four patients had severe neurological sequelae, including motor paresis ($n=2$), mental retardation ($n=1$), and sensorineural hearing impairment ($n=1$). The relationships between

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CSF NF-H levels and CSF cell counts and total protein levels were investigated at the time of specimen collection.

2.2. Control subjects

The control subjects were 16 afebrile and noninfectious children (7 females and 9 males, age: 2 days to 4 years old, median: 1.7 years old), including 10 patients with epilepsy, 3 with gait disturbance, 2 with psychomotor delay, and 1 with clubfoot (Table 1). CSF samples were obtained for routine analysis and all the controls had normal CSF cell counts. There was no significant difference in age or gender between patients with bacterial meningitis and controls by Mann–Whitney U test or χ^2 test.

2.3. Determination of CSF NF-H concentrations

The CSF concentrations of NF-H were measured with a phosphorylated NF-H ELISA kit (EnCor Biotechnology Inc., Gainesville, FL, USA). An anti-NF-H monoclonal coating antibody was adsorbed onto polystyrene microwells. NF-H in the samples or standard bound to the adsorbed antibodies and the NF-H/antibody complex was detected with an alkaline phosphatase-conjugated secondary antibody. The amount of captured NF-H was measured using an ELISA plate reader based on a color reaction. The detection limit was 0.10 ng/mL.

2.4. Statistical analysis

Values lower than the detection limit were taken to be 0.05 ng/mL (half of the detection limit). Differences were analyzed using a Mann–Whitney U test and correlations were determined by Spearman's correlation coefficient test. P-values less than 0.05 were taken to be significant. Calculations were performed using SPSS v. 12.0 (SPSS, Inc., Chicago, IL, USA).

3. Results

The median CSF NF-H concentration in controls was 0.05 ng/mL (range:<0.1–0.84 ng/mL) and all values in controls were lower than 0.94 ng/mL, the normal upper limit [13,14]. There was no significant correlation of the CSF NF-H concentration with age in our controls. Serial CSF NF-H concentrations in the 26 patients with bacterial meningitis are shown in Fig. 1. The peak CSF NF-H levels in the patients were significantly higher than those in controls ($p<0.001$) and the peak level occurred at a median of 10.5 days after onset of illness (range: 1 to 35 days). The peak CSF NF-H levels of patients with severe neurological sequelae were significantly higher than for those without sequelae (median, range; 7.06, 5.16–13.89 vs. 2.46,<0.10–26.67 ng/mL, $p=0.048$). There was no significant difference in CSF NF-H levels between patients with and without severe neurological sequelae up to day 14 of illness (median, range; 1.38, 0.37–1.84 vs. 0.88,<0.10–26.67 ng/mL, $p=0.822$), but the CSF NF-H levels in patients with sequelae were significantly higher than in those without sequelae after day 14 of illness (median, range; 2.04, 0.75–13.89 vs. 1.19, 0.23–4.82 ng/mL, $p=0.024$). There were no significant differences in peak CSF NF-H concentrations between patients who did ($n=16$) and did not ($n=10$) receive adjunctive dexamethasone therapy (median, range; 2.45,<0.10–7.74 vs. 6.98, 0.86–26.67 ng/mL, $p=0.077$), or between patients who tested positive ($n=17$) and negative ($n=9$) for *H. influenzae* in the CSF culture (median, range; 2.56,<0.10–8.80 vs. 6.46, 0.86–26.67 ng/mL, $p=0.220$). The CSF NF-H concentrations showed no correlation with CSF cell counts or total protein levels in patients with bacterial meningitis.

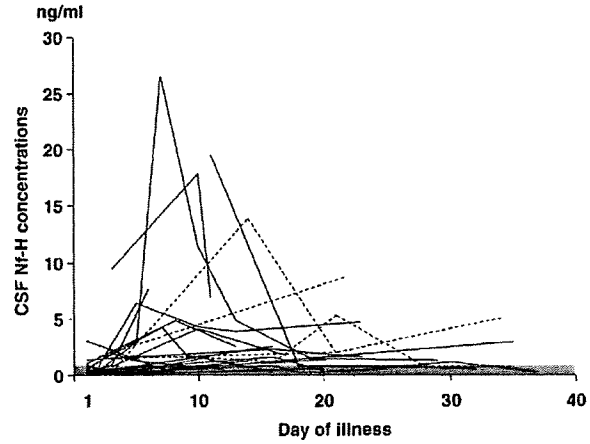

Fig. 1. Serial CSF NF-H concentrations in 26 children with bacterial meningitis. Dot lines indicate patients with severe neurological sequelae. The shaded area shows the normal value (<0.94 ng/mL).

Table 1
Clinical characteristics of the children with bacterial meningitis and controls.

	Bacterial meningitis $N=26$	Controls $N=16$
Age (median, range)	8 months, 2 days–4 yr	1.7 yr, 2 days–4 yr
Sex (female: male)	13: 13	7: 9
Primary causative bacteria	*Haemophilus influenzae*	17
	Streptococcus pneumoniae	3
	Escherichia coli	3
	GBS	2
	MRSA	1
Outcome	Normal	22
	Motor paresis	2
	Mental retardation	1
	Hearing impairment	1
CSF NF-H concentrations (ng/mL; median, ranges)	3.08, <0.1–26.67	0.05, <0.1–0.84

GBS, Group B *Streptococcus*; MRSA, methicillin-resistant *Staphylococcus aureus*.

4. Discussion

Measurement of NF is useful for confirmation of neuronal injury, evaluation of therapeutic effect, prediction of prognosis, and differential diagnosis [2–11]. However, there are only a few reports of NF in pediatric disorders, including perinatal asphyxia, cerebral white matter abnormalities and subacute sclerosing panencephalitis [15–17]. Here, we provide the first report of CSF NF levels in bacterial meningitis. Our data show that these levels were elevated in most patients regardless of the presence or absence of neurological sequelae. However, the peak CSF NF-H levels of patients with severe neurological sequelae were significantly higher than those without sequelae. These findings suggest that CSF NF-H levels may reflect the severity of neuronal damage in bacterial meningitis, in which such damage is common.

NF-H levels tend to increase several days after onset of acute disease. A significant increase in these levels is typically seen 7 days after subarachnoid hemorrhage [18], and peak levels have been observed 3 days after experimental spinal cord injury and 2 days after experimental traumatic brain injury [13]. Our results showed peak CSF NF-H levels at a median of 10.5 days after onset of bacterial meningitis. The CSF NF-H levels in patients with severe neurological sequelae were significantly higher than in those without sequelae after day 14 of illness, but up to day 14 there was no significant difference in CSF NF-H levels between patients with and without sequelae. These findings suggest that the late rise in CSF NF-H levels might be related to secondary brain damage as a complication of meningitis. In addition, the peak CSF NF-H levels were not related to adjunctive dexamethasone therapy. This therapy suppresses acute inflammation, whereas CSF NF-H levels reflect neuronal damage followed by acute inflammation. These results suggest that dexamethasone therapy

during the early phase did not affect the late peak of CSF NF-H levels. There was also no significant difference in CSF NF-H levels based on the type of causative bacteria in our patients, but a further large-scale study is necessary to clarify the relationship between CSF NF-H levels and causative bacteria in bacterial meningitis.

Bacterial meningitis can have severe neurological sequelae in 12 to 29% of survivors, and milder impairment of neurological function occurs in another 15 to 38% [19]. Based on previous reports and our present data, most patients with bacterial meningitis have neuronal damage and may develop severe neurological sequelae and milder impairment of neurological function. Therefore, careful long-term clinical follow-up and comprehensive developmental assessments are necessary for patients with bacterial meningitis to evaluate sequelae. We also conclude that CSF NF-H concentrations are elevated in most patients with bacterial meningitis.

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Clinical Characteristics of Benign Convulsions With Rotavirus Gastroenteritis

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Convulsions sometimes occur in infants and toddlers with mild gastroenteritis. We retrospectively investigated the hospital records of 106 patients admitted to our hospital who had rotavirus gastroenteritis from February 2002 to April 2008. There were 23 patients with convulsions, including 13 with benign convulsions, 9 with febrile seizures, and 1 with epilepsy. Gastroenteritis in patients with benign convulsions was mild from the viewpoint of body weights and serum creatinine concentrations on admission and the duration of admission. Serum Na^+ and Cl^- concentrations of patients with benign convulsions were relatively lower than those without convulsions on admission ($P = .006$, and $P = .008$, respectively). Twelve of thirteen patients had no other seizures after oral administration of 5 mg/kg of carbamazepine, while 1 patient had 1 convulsion 15 minutes after the therapy. In conclusion, carbamazepine therapy was effective for benign convulsions with rotavirus gastroenteritis.

Keywords: carbamazepine; convulsion; rotavirus gastroenteritis

Convulsions associated with mild gastroenteritis sometimes occur in infants and young children.[1-6] The condition is characterized by (1) previously healthy infants and young children aged 6 months to 3 years having afebrile, brief, mostly generalized tonic-clonic convulsions between the first and fifth days of mild gastroenteritis; (2) mild dehydration (less than 5%); (3) seizures tending to occur repetitively over several days; (4) an interictal electroencephalogram (EEG) showing no epileptic discharges; (5) normal other laboratory examinations, including cerebrospinal fluid, serum electrolytes, and blood glucose; and (6) good prognosis without sequelae.[4] The causative agent for gastroenteritis has been known to be a rotavirus, and norovirus also induces such convulsions.[5] This report also suggested that other agents that cause gastroenteritis might also induce such convulsions. The convulsions frequently recur after the administration of diazepam or phenobarbital.[5,7]

In this study, we retrospectively investigated the clinical aspects of 106 children with rotavirus gastroenteritis in the past 6 years, particularly regarding the clinical characteristics of benign convulsions, compared with children without convulsions. We report that oral administration of 5 mg/kg carbamazepine once per day was dramatically effective for such convulsions.

Patients and Methods

Patients

One hundred and six children aged 15 days to 9 years who were admitted to Yamaguchi University Hospital from February 2002 to April 2008 were enrolled in our study. The diagnosis of rotavirus infection was based on virus antigen detection in the feces using the latex agglutination test. There were 23 patients with convulsions and 83 without convulsions. We excluded 9 patients who had seizures with fever, which was defined as a temperature of $\geq$38.0°C, as having febrile seizures, and 1 patient with epilepsy who was being treated with anticonvulsants. Hence, there were 13 patients in all who had benign convulsions associated with gastroenteritis (Tables 1 and 2; Figure 1). Lumbar punctures were performed in 10 of 13 patients, and cerebrospinal fluid cell counts and protein concentrations were normal in 10 patients. There were 5 boys and 8 girls, aged from 9 to 24 months (mean, 1.9 years). The day of onset of gastroenteritis was considered as the first day of illness. The convulsions occurred from day 1 to

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Table 1. Clinical Data of Patients With Rotavirus Gastroenteritis[a]

	Patients With Benign Convulsions; n = 13	Patients Without Benign Convulsions; n = 83	*P*
Age (years)	1.9 ± 0.6	1.7 ± 1.7	.053
Male	5	54	.226
Female	8	29	
Day of admission[b]	2.8 ± 1.0	3.0 ± 1.9	.780
Body weight on admission (kg)	10.9 ± 1.7	9.6 ± 4.2	.029
Duration of admission (days)	3.9 ± 1.5	6.4 ± 3.9	.011

a. Results are expressed as means ± 1 SD.
b. Onset day of clinical symptom of rotavirus gastroenteritis is the first day.

4 (mean, day 2.8). All patients were previously healthy. Examination excluded acute encephalitis/encephalopathy, meningitis, hypoglycemia, cyclic vomiting, and metabolic disorders.

Clinical data

The relationships between patients who had rotavirus gastroenteritis with and without benign convulsions and clinical data including leukocyte counts, serum concentrations of Na^+, K^+, Cl^-, C-reactive protein, blood urea nitrogen, creatinine, aspartate transaminase, and aspartate aminotransferase on admission were investigated.

Protocol of Carbamazepine Therapy

This protocol was approved by the Institutional Review Board of Yamaguchi University Hospital. The patients were immediately given 5 mg/kg carbamazepine orally after a definitive diagnosis of benign convulsions associated with rotavirus gastroenteritis. The patients were given carbamazepine as soon as possible because the convulsions often occur in clusters. The patients were given carbamazepine through a nasogastric tube when they could not take carbamazepine orally because they were sleeping due to the postictal state or administration of diazepam. The patients were given 5 mg/kg of carbamazepine once per day until the diarrhea had stopped.

Statistical analysis

The differences between groups were analyzed using the Mann-Whitney *U* test or the χ^2 test. *P* values less than .05 were considered significant. Analyses and calculations were performed using SPSS-12.0 (SPSS, Inc, Chicago, Ill).

Results

Table 1 shows clinical profiles of patients with/without benign convulsions. The body weights on admission of patients with benign convulsions were significantly higher than those without benign convulsions ($P = .029$). Duration of admission of patients with benign convulsions was significantly shorter than those without benign convulsions ($P = .011$). There were no significant differences of age, gender, or day of admission from the onset of rotavirus gastroenteritis.

Serum Na^+, Cl^-, and creatinine concentrations of patients with benign convulsions were significantly lower than those without convulsions on admission, as shown in Table 3 ($P = .006$, $P = .008$, and $P = .018$, respectively). There were no significant differences in leukocyte counts or serum concentrations of K^+, C-reactive protein, blood urea nitrogen, aspartate transaminase, or aspartate aminotransferase between the patients with benign convulsions and those without convulsions (Table 3).

The clinical courses of the 13 patients with benign convulsions are shown in Figure 1. Eleven of the thirteen patients were subjected to intravenous and/or suppository administration of diazepam (0.3-0.5 mg/kg/time) before the administration of carbamazepine (Table 2; Figure 1). In all patients who were given diazepam, the convulsions recurred after the administration of diazepam. The convulsions occurred 1 to 5 times (mean, 3.3 times) before the administration of carbamazepine. Twelve of the thirteen patients had no seizures after the administration of carbamazepine. One patient (No 11) had 1 convulsion 15 minutes after the administration of carbamazepine. All patients were treated with 5 mg/kg of carbamazepine once per day until the diarrhea had stopped for 1 to 9 days (mean, 4.4 days). No definite side effects were seen in any patients. None had neurological sequelae or abnormal follow-up EEG findings.

Discussion

Benign convulsions with mild gastroenteritis may be frequent in infants and young children in Eastern Asia, including Japan, Korea, and Taiwan, because such convulsions have been mostly reported in these areas.[1,4,5,7-10] Seizures occurred in 31 of 1200 Japanese patients with rotavirus gastroenteritis.[5] Afebrile convulsions have been reported in 9 of 77 Korean children with rotavirus gastroenteritis in 1 clinical center.[11] However, such convulsions have also been reported in the United States and United Kingdom.[3,12,13]

In this study, body weights on admission of patients with benign convulsions were significantly higher than those without the convulsions, serum creatinine levels of those with the convulsions were significantly lower than those without the convulsions, and duration of admission of those with the convulsions was significantly shorter than that of those without the convulsions, suggesting the severity of gastroenteritis, including dehydration, of inpatients with benign convulsions was relatively more mild than those without the convulsions.

Table 2. Data for 13 Patients Who Had Benign Convulsions With Rotavirus Gastroenteritis

Patient Number	Age	Gender	Day of Onset of Convulsions	Number of Convulsions	Administration of Diazepam Before Carbamazepine
1	1 year 1 month	Female	1	5	+
2	2 year 3 months	Female	3	3	+
3	2 year 9 months	Female	4	5	+
4	1 year 7 months	Male	2	3	+
5	1 year 10 months	Male	4	1	−
6	1 year 7 months	Female	3	4	+
7	1 year	Female	1	1	−
8	1 year 9 months	Female	4	4	+
9	1 year 11 months	Female	3	4	+
10	1 year 6 months	Female	3	5	+
11	1 year 9 months	Male	3	5	+
12	9 months	Male	2	2	+
13	2 years	Male	3	2	+

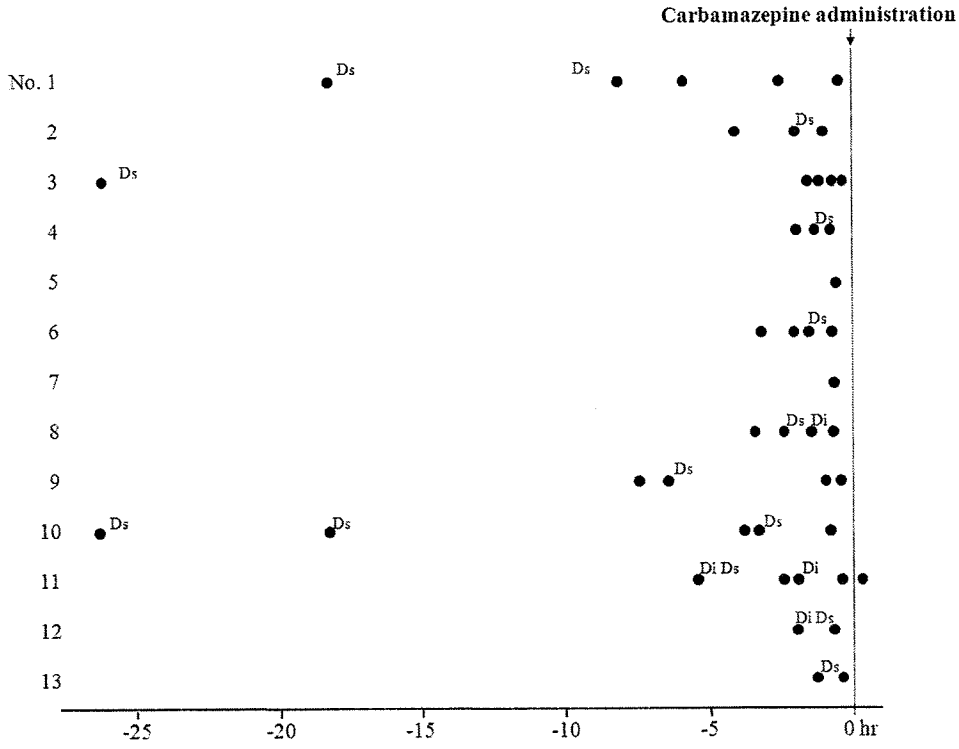

Figure 1. Clinical courses of 13 patients with benign convulsions with rotavirus gastroenteritis. The time of oral administration of carbamazepine is considered to be 0 hours. ● = convulsion; Di = intravenous administration of diazepam; Ds = suppository administration of diazepam.

Diazepam is generally and widely used for any convulsive condition, such as epilepsy, meningitis, encephalitis, and so on. Previous reports demonstrated that the elimination half-life of diazepam was 9 to 96 hours.[14,15] In addition, the minimum effective plasma level of diazepam was maintained for about 8 hours after single rectal administration of 0.5 mg/kg diazepam.[16] Therefore, we interpreted that diazepam was not effective for the benign convulsions based on the clinical courses of our patients. A previous study also revealed that diazepam or phenobarbital cannot suppress benign convulsions associated with mild gastroenteritis.[7] In this study, the low-dose carbamazepine therapy was effective for the convulsions of 12 patients. The reason that 1 patient (No 11) had 1 convulsion 15 minutes after the administration of carbamazepine may be that the medicine had not been absorbed yet. We

Table 3. Laboratory Findings for Patients With Rotavirus Gastroenteritis

	Patients With Benign Convulsions; n = 13	Patients Without Benign Convulsions; n = 83	*P*
Na^+ (mmol/L)	135 ± 2	138 ± 5	.006
K^+ (mmol/L)	4.5 ± 0.4	4.4 ± 0.6	.530
Cl^- (mmol/L)	100 ± 3	105 ± 6	.008
Leukocyte counts (per μL)	9646 ± 5728	11354 ± 4809	.163
C-reactive protein (mg/dL)	0.98 ± 1.33	1.20 ± 2.56	.458
Blood urea nitrogen (mg/dL)	14 ± 3	16 ± 11	.877
Creatinine (mg/dL)	0.22 ± 0.04	0.29 ± 0.14	.018
Aspartate Transaminase (U/L)	26 ± 6	37 ± 56	.996
Aspartate Aminotransferase (U/L)	49 ± 10	47 ± 22	.253

have previously published the first report on the usefulness of carbamazepine on benign convulsions associated with mild gastroenteritis.[17] We focused on benign convulsions associated with rotavirus gastroenteritis in this study and believe firmly in the usefulness of carbamazepine. Recently, a supportive study was reported.[18] The efficacy of lidocaine drip infusion on convulsions has been previously reported.[7] However, oral administration of carbamazepine is easier than drip infusion of lidocaine for infants and young children.

The reason that low-dose carbamazepine is effective for such convulsions, but diazepam or phenobarbital is not, is unclear. Carbamazepine and lidocaine inhibit Na^+ channels.[19,20] Benzodiazepine promotes binding of γ-aminobutyric acid to the γ-aminobutyric acid receptor and exhibits an anticonvulsive action.[21] Previous reports revealed that benign familial infantile convulsion, which has a characteristic seizure similar to convulsions associated with mild gastroenteritis, is associated with the Na^+ channel β1 subunit gene of chromosome 19q.[22,23] If benign convulsions with mild gastroenteritis are also associated with Na^+ channels, it can be conceived that this is a reason for the validity of carbamazepine. Although phenobarbital also blocks Na^+ channels as does carbamazepine,[24] carbamazepine may be quickly effective on the disorder because the absorption speed of carbamazepine is higher than that of phenobarbital. Our present data demonstrated that serum Na^+ and Cl^- levels in patients with benign convulsions were relatively lower than those without benign convulsions, while the changes of Na^+ and Cl^- levels were within normal ranges. These findings may be related to the above hypothesis. The results presented here provide an impetus for large-scale studies to explore the pathogenesis of the benign convulsions with mild gastroenteritis and the mechanism that accounts for the effectiveness of the low-dose carbamazepine therapy.

Although the prognosis of benign convulsions with mild gastroenteritis is good, repetitive convulsions over several days may not only make the family uneasy, but also lengthen the hospitalization period and increase cost. Therefore, we again recommend 5 mg/kg of carbamazepine once per day until the diarrhea has stopped as a cure for convulsions associated with mild gastroenteritis. In conclusion, benign convulsions with gastroenteritis induced by rotavirus were mild; serum Na^+ and Cl^- levels of patients with benign convulsions were relatively lower than those without the convulsions, and low-dose carbamazepine therapy was effective on the convulsions.

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