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**Table 1** Clinical and other parameters in the 45 patients with herpes simplex virus encephalitis included in the present study

| Patient no. | (1) Sex | (2) Age* | (3) Days† | (4) GCS score‡ | CSF examinations: (5) Initial leucocyte cell count¶ | CSF examinations: (6) Initial CSF protein§ | (7) Corticosteroid administration** | Neuroradiological findings: (8) Detection of lesion by initial CT†† | Neuroradiological findings: (9) Detection of lesion by initial MRI‡‡ | (10) Detection of PLEDs on EEG¶¶ | Outcome§§ |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | M | 45 | 6 | 3 | 65 | 120 | 1 | 1 | 1 | 1 | Poor (death) |
| 2 | F | 57 | 7 | 3 | 377 (452) | 133 (358) | 1 | 1 | 1 | 1 | Poor (death) |
| 3 | M | 74 | 2 | 6 | 52 | 33 | 0 | 1 | 1 | 1 | Poor (moderate sequelae) |
| 4 | M | 34 | 4 | 14 | 148 | 36 | 0 | 1 | 1 | 0 | Good (complete recovery) |
| 5 | F | 60 | 7 | 3 | 4 (6) | 66 | 1 | 1 | 1 | 0 | Poor (severe sequelae) |
| 6 | M | 19 | 10 | 7 | 149 | 120 | 0 | 1 | 1 | 1 | Good (complete recovery) |
| 7 | F | 27 | 13 | 3 | 4 | 77 | 0 | 1 | 1 | 0 | Good (mild sequelae) |
| 8 | F | 21 | 11 | 6 | 22 | 92 | 1 | 0 | 1 | 0 | Good (mild sequelae) |
| 9 | M | 30 | 11 | 10 | 16 | 88 | 0 | 0 | 0 | 1 | Good (complete recovery) |
| 10 | F | 20 | 3 | 12 | 9 (12) | 33 | 0 | 0 | 0 | 1 | Good (mild sequelae) |
| 11 | F | 24 | 4 | 12 | 362 | 40 | 1 | 0 | 0 | 0 | Good (complete recovery) |
| 12 | F | 34 | 5 | 13 | 820 | 398 | 1 | 0 | 0 | 0 | Good (complete recovery) |
| 13 | M | 17 | 4 | 7 | 139 | 104 | 0 | 1 | 1 | 1 | Good (mild sequelae) |
| 14 | M | 57 | 7 | 3 | 944 | 410 | 1 | 1 | 1 | 0 | Poor (death) |
| 15 | F | 28 | 5 | 7 | 4 (6) | 77 | 0 | 1 | 1 | 1 | Good (mild sequelae) |
| 16 | M | 20 | 10 | 10 | 22 | 92 | 1 | 0 | 1 | 1 | Good (complete recovery) |
| 17 | M | 30 | 10 | 10 | 16 | 88 | 0 | 0 | 1 | 0 | Good (complete recovery) |
| 18 | F | 53 | 7 | 6 | 458 | 189 (235) | 1 | 1 | 1 | 1 | Poor (moderate sequelae) |
| 19 | F | 53 | 1 | 3 | 45 | 72 | 0 | 1 | 1 | 1 | Good (mild sequelae) |
| 20 | F | 34 | 5 | 6 | 22 | 68 | 1 | 1 | 1 | 0 | Good (complete recovery) |
| 21 | M | 34 | 3 | 13 | 22 | 86 | 1 | 1 | 1 | 0 | Good (mild sequelae) |
| 22 | M | 58 | 11 | 7 | 132 | 103 | 1 | 1 | 1 | 0 | Poor (severe sequelae) |
| 23 | M | 29 | 6 | 6 | 1376 | 240 | 0 | 1 | 1 | 0 | Good (complete recovery) |
| 24 | M | 76 | 12 | 3 | 102 | 305 | 1 | 1 | 1 | 0 | Poor (severe sequelae) |
| 25 | F | 33 | 9 | 12 | 61 | 61 | 1 | 0 | 0 | 1 | Good (complete recovery) |
| 26 | M | 58 | 6 | 3 | 24 | 56 | 1 | 1 | 1 | 1 | Poor (death) |
| 27 | M | 56 | 2 | 8 | 503 | 37 | 0 | 0 | 1 | 1 | Good (mild sequelae) |
| 28 | M | 77 | 1 | 12 | 571 | 580 | 0 | 0 | 1 | 0 | Good (mild sequelae) |
| 29 | F | 49 | 5 | 6 | 377 | 134 | 0 | 1 | 1 | 0 | Poor (moderate sequelae) |
| 30 | F | 77 | 3 | 3 | 7 (63) | 88 (104) | 0 | 1 | 1 | 0 | Poor (moderate sequelae) |
| 31 | F | 68 | 5 | 10 | 41 | 173 | 0 | 0 | 1 | 0 | Good (mild sequelae) |
| 32 | M | 38 | 7 | 10 | 4 (8) | 55 | 1 | 1 | 1 | 1 | Good (complete recovery) |
| 33 | M | 39 | 7 | 3 | 70 | 72 | 0 | 1 | 1 | 0 | Poor (moderate sequelae) |
| 34 | F | 71 | 1 | 3 | 422 | 90 | 0 | 0 | 1 | 1 | Good (mild sequelae) |
| 35 | F | 72 | 10 | 12 | 128 | 85 | 1 | 0 | 1 | 0 | Poor (severe sequelae) |
| 36 | M | 32 | 4 | 8 | 22 | 40 | 0 | 0 | 1 | 1 | Poor (moderate sequelae) |
| 37 | M | 71 | 10 | 12 | 18 | 39 | 0 | 1 | 1 | 1 | Good (mild sequelae) |
| 38 | F | 66 | 12 | 11 | 95 | 74 | 1 | 1 | 1 | 1 | Poor (severe sequelae) |
| 39 | M | 41 | 5 | 14 | 190 | 159 | 1 | 0 | 0 | 0 | Good (complete recovery) |
| 40 | F | 56 | 10 | 4 | 150 | 72 | 1 | 0 | 0 | 1 | Poor (death) |
| 41 | M | 27 | 4 | 13 | 21 (42) | 45 (86) | 1 | 1 | 1 | 0 | Good (complete recovery) |
| 42 | M | 66 | 5 | 3 | 56 | 85 | 1 | 1 | 1 | 0 | Poor (severe sequelae) |
| 43 | M | 45 | 12 | 8 | 4 (8) | 28 (42) | 0 | 0 | 1 | 0 | Good (complete recovery) |
| 44 | M | 47 | 6 | 3 | 15 (20) | 240 | 1 | 1 | 1 | 1 | Poor (moderate sequelae) |
| 45 | M | 45 | 10 | 3 | 60 | 84 | 0 | 1 | 1 | 0 | Poor (moderate sequelae) |

*Age in years.
†Days after onset at initiation of aciclovir.
‡GCS score at initiation of aciclovir.
¶Initial leucocyte cell count(/μl) (maximum values in serial CSF samples when the initial and maximum values were different).
§Initial CSF protein (mg/dl) (maximum values in serial CSF samples when the initial and maximum values were different).
††Detection of lesion by initial computed tomography (CT): 0 = absent, 1 = present.
‡‡Detection of lesion by initial magnetic resonance imaging (MRI): 0 = absent, 1 = present. ¶¶Detection of periodic lateralized epileptiform discharges (PLEDs) on electroencephalogram (EEG): 0 = absent, 1 = present.
**Corticosteroid administration: 0 = given, 1 = not given.
§§Outcome at 3 months after completion of aciclovir treatment.
GCS, Glasgow Coma Scale; CSF, cerebrospinal fluid.

and multiple logistic regression analyses in relation to outcome are summarised in table 3. The independent variables that were significantly related to the outcome of HSVE in the multiple logistic regression analysis included patient age, GCS score at initiation of aciclovir, and corticosteroid administration in the acute stage (see table 3). The best set of predictors for the outcome of HSVE as estimated by stepwise logistic regression analysis of the 10 variables included patient age, GCS score at initiation of aciclovir, and corticosteroid administration ($R^2 = 0.594$, $p<0.0001$). No interactions were found between age and corticosteroid administration, or between age and GCS. A poor outcome was thus evident in patients with older age, lower GCS scores, and no corticosteroid administration in the acute stage (see table 3). Details of the manner of corticosteroid administration in the 22/45 patients (treated with corticosteroids) are presented in table 4. Corticosteroids were administered at the same time as the initiation of aciclovir treatment. Dexamethasone was given to 82% of patients and prednisolone to 18%. The initial dosage of corticosteroid, converted to the dosage of prednisolone, ranged from 40.0 mg/day to 96.0 mg/day (mean 64.6 mg/day; median, 64.0 mg/day). The duration of corticosteroid treatment ranged from 2 days to 6 weeks (mean 13.6 days; median 6.0 days), during which time the corticosteroid dosage was tapered off gradually when it had been administered for more than seven days. These differences between hospitals in the manner of corticosteroid administration were not statistically significant (table 4).

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**Table 2** Baseline clinical characteristics of the patient groups under aciclovir therapy with and without concomitant corticosteroid administration

| | Corticosteroid administration (n = 22) | No corticosteroid administration (n = 23) | Difference between the two groups* |
|---|---|---|---|
| (1) Male (%) | 64 | 52 | NS |
| (2) Age in years (minimum, mean, median, and maximum) | 17.0, 45.0, 42.0, 77.0 | 20.0, 46.8, 47.0, 76.0 | NS |
| (3) Days after onset at initiation of aciclovir (minimum, mean, median, and maximum) | 1.0, 5.9, 5.0, 13.0 | 3.0, 7.3, 7.0, 12.0 | NS |
| (4) GCS score at initiation of aciclovir (minimum, mean, median, and maximum) | 3.0, 7.3, 7.0, 14.0 | 3.0, 7.5, 6.0, 14.0 | NS |
| (5) Initial and <maximum> leucocyte cell count (/μl) in CSF (minimum, mean, median, and maximum) | 4, 184, 49, 1376 <4, 187, 56, 1376> | 4, 178, 65, 944 <6, 178, 65, 944> | NS |
| (6) Initial and <maximum> CSF protein (mg/dl) (minimum, mean, median, and maximum) | 28, 106, 81, 580 <33, 107, 81, 580> | 40, 132, 86, 410 <40, 145, 86, 410> | NS |
| (8) Detection of lesion by initial CT (%) | 59 | 65 | NS |
| (9) Detection of lesion by initial MRI (%) | 91 | 78 | NS |
| (10) Detection of PLEDs on EEG (%) | 50 | 43 | NS |

*Statistical differences between the two groups were evaluated by Fisher's exact probability test and the Mann–Whitney U test.
CSF, cerebrospinal fluid; CT, cranial computed tomography; EEG, electroencephalogram; GCS, Glasgow Coma Scale; MRI, magnetic resonance imaging; NS, not significant; PLEDs, periodic lateralized epileptiform discharges.

## DISCUSSION

Most of the previous results for predictors of outcome in HSVE have been based on between-group comparative analysis—for example, Wilcoxon's two sample test, $\chi^2$ analysis, or Fisher's exact two tailed test.[1-4] Cut-off values for each of the predictors were determined arbitrarily in these studies. An important problem thus remains: estimation of the associations among the predictors of the outcome of HSVE. One recent report based on multivariable analysis in 85 patients with HSVE indicated that a Simplified Acute Physiology Score II of 27 or more and a delay of more than two days between admission to hospital and initiation of aciclovir therapy were predictors of a poor outcome in HSVE.[6] Previously reported predictors such as patient age and GCS score at initiation of aciclovir were not identified as significant predictors. Although the number of patients was limited this previous study investigated numerous factors as predictors of outcome in HSVE. Furthermore, the time of

**Table 3** Results for the estimation of predictors of outcome in herpes simplex encephalitis by single and multiple logistic regression analyses

| Variable | Unit of increase | Spearman's rank correlation against outcome | Single logistic regression analysis: Odds ratio (95% CI) | Single logistic regression analysis: p value | Multiple logistic regression analysis: Odds ratio (95% CI) | Multiple logistic regression analysis: p value |
|---|---|---|---|---|---|---|
| (1) Sex | NA | 0.266 | 0.992 (0.30 to 3.29) | 0.989 | – | – |
| (2) Age | 1 year | 0.605 | 1.075 (1.03 to 1.12) | 0.001* | 1.088 (1.02 to 1.16) | 0.006* |
| (3) Days after onset at initiation of aciclovir | 1 day | 0.305 | 1.097 (0.92 to 1.31) | 0.144 | – | – |
| (4) Modified value of GCS score at initiation of aciclovir† | 1 score | 0.602 | 1.424 (1.14 to 1.77) | 0.002* | 1.452 (1.08 to 1.95) | 0.014* |
| (5) Initial leucocyte cell count in CSF‡ (maximum values in serial CSFs) | 0–10 | 0.245 (0.284) | 1.00 (reference) | 0.566 (0.719) | – | – |
| | 11–100 | | 0.200 (0.02 to 2.03) (0.275 (0.03 to 2.90)) | | | |
| | 101–300 | | 0.200 (0.02 to 2.58) (0.250 (0.02 to 3.34)) | | | |
| | ≥301/μl | | 0.300 (0.03 to 3.63) (0.375 (0.03 to 4.71)) | | | |
| (6) Initial CSF protein‡ (maximum values in serial CSFs) | ≤50 | 0.253 (0.246) | 1.00 (reference) | 0.441 (0.388) | – | – |
| | 51–100 | | 0.444 (0.07 to 2.74) (0.650 (0.10 to 4.18)) | | | |
| | ≥101 mg/dl | | 0.292 (0.04 to 1.94) (0.311 (0.05 to 2.11)) | | | |
| (7) Corticosteroid administration | 0 = given 1 = not given | 0.521 | 3.467 (0.99 to 12.09) | 0.041* | 8.964 (1.13 to 70.99) | 0.038* |
| (8) Detection of lesion by initial CT | 0 = absent 1 = present | 0.562 | 6.222 (1.45 to 26.65) | 0.044* | 3.690 (0.43 to 31.85) | 0.235 |
| (9) Detection of lesion by initial MRI | 0 = absent 1 = present | 0.567 | 5.400 (0.59 to 49.27) | 0.135 | – | – |
| (10) Detection of PLEDs on EEG | 0 = absent 1 = present | 0.269 | 1.050 (0.32 to 3.44) | 0.936 | – | – |

*Statistically significant ($p<0.05$).
†Subtraction GCS score from 16.
‡The values of (5) and (6) were arbitrarily categorised as above based on our clinical experience.
CSF, cerebrospinal fluid; CT, cranial computed tomography; EEG, electroencephalogram; GCS, Glasgow Coma Scale; MRI, magnetic resonance imaging; NA, not applicable; PLEDs, periodic lateralised epileptiform discharges.

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**Table 4** Details of corticosteroid treatments given along with aciclovir treatment in the acute stage of herpes simplex virus encephalitis

| | | Difference between (A) and (B)* |
|---|---|---|
| Patients given corticosteroids (n/N (%)) | 22/45 (49)<br>(A)† 15/30 (50)<br>(B)‡ 7/15 (47) | NS |
| Time of initiation of corticosteroid treatment | All patients were administered corticosteroid at the same time as the initiation of aciclovir treatment | |
| Type of corticosteroid (n/N (%)) | | |
| Dexamethasone | 18/22 (82)<br>(A) 12/15 (80)<br>(B) 6/7 (86) | NS |
| Prednisolone | 4/22 (18)<br>(A) 3/15 (20)<br>(B) 1/7 (14) | NS |
| Initial dosage of corticosteroid converted to dosage of prednisolone (mean, median, distribution) | 64.6, 64.0 mg/day, 40.0–96.0<br>(A) 67.9, 64.0, 48.0–96.0<br>(B) 61.7, 64.0, 40.0–96.0 | NS |
| Duration of corticosteroid treatment (mean, median, distribution) | 13.6, 6.0 days, 2.0–42.0<br>(A) 13.9, 3.0, 3.0–42.0<br>(B) 12.0, 8.0, 2.0–35.0 | NS |

*Statistical differences between the two different hospital groups were evaluated by Fisher's exact probability test or the Mann-Whitney U test.
†(A) Nihon University Itabashi Hospital and two affiliated hospitals.
‡(B) Tohoku University Hospital and two affiliated hospitals.
NS, not significant.

initiation of aciclovir after admission, and both the duration and the dosage of aciclovir in each patient were variable.[6] In our study, the time of initiation of aciclovir after admission, and both the duration and the dosage of aciclovir in each patient with HSVE were uniform and comparable. Significant predictors become difficult to detect as the number of variable factors increases, particularly when the numbers of patients are limited. We therefore limited our analysis to 10 variable factors which included previously reported predictors[1–4] based on between-group comparative analysis. Our results, based on multiple logistic regression analysis showed that patient age, GCS score at initiation of aciclovir, and corticosteroid administration in the acute stage of HSVE were significant predictors of outcome in 45 adult patients with HSVE. There were no obvious interactions between age and corticosteroid administration, or between age and GCS. The duration from onset of HSVE to the initiation of aciclovir treatment, and the detection of focal lesions by cranial CT at the initiation of therapy were not significant predictors as estimated by the multiple logistic regression analysis. The possible reasons for the lack of significance for these two factors may be, firstly, the number of patients in our study might not have been sufficient to allow detection as significant predictors, and secondly, the two factors were dependent on other predictors such as the degree of consciousness disturbance indicated by the GCS score at the initiation of aciclovir; therefore the two factors were not independent predictors of outcome in HSVE by themselves.

A notable finding of our study is that corticosteroid administration in the acute stage of HSVE represented one of the significant independent predictors of outcome in HSVE. The exact effectiveness of such combination therapy in HSVE has not yet been established. However, corticosteroids have been administered empirically with aciclovir treatment in some patients to improve brain oedema. There have been recent reports about the effects of corticosteroid administration with aciclovir treatment in an animal model of HSVE.[10 11] The data indicate that the HSV viral load of the brain tissue of animals treated with both aciclovir and corticosteroid was similar to that of the brain tissue of the animals which were treated with aciclovir alone. These studies also revealed that corticosteroids do not inhibit the antiviral action of aciclovir and may decrease the extent of HSVE infection.[10 11] Furthermore, it has been suggested that a host immune response associated with HSVE, such as that involving cytokines,[12] could play some role in the outcome of HSVE based on the findings of an in vitro experimental study on infection with HSV.

The pharmacological mechanism of corticosteroid treatment in the acute stage of HSVE may thus involve not only improvement of brain oedema but also regulation of the host immune response associated with the acute stage of HSVE. The present investigation is the first to provide statistical evidence that combination therapy using both aciclovir and corticosteroid can achieve a better outcome in adult patients with HSVE. Our study revealed three independent variables based on a multivariable regression analysis of the variables which had been found to be significant in the single variable analysis. However, multivariable regression analysis using all 10 variables is needed to achieve greater reliability, which will be possible when the number of recruited patients is sufficiently increased. Further investigations including clinical prospective randomised controlled studies are also required to determine the optimal type, dosage, and duration of corticosteroid administration.

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### Authors' affiliations

**S Kamei, H Shiota, T Mizutani, T Takasu,** Division of Neurology, Department of Medicine, Nihon University School of Medicine, Tokyo, Japan

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**T Sekizawa,** Department of Health Science, Yamagata Prefectural University of Health Science,Yamagata-shi, Japan
**Y Itoyama,** Department of Neurology, Tohoku University School of Medicine, Seiryo-cho, Aoba-ku, Sendai-shi, Japan
**T Morishima,** Department of Health Science, Nagoya University School of Medicine, Higashi-ku, Nagoya-shi, Japan
**K Hirayanagi,** Department of Hygiene and Public Health, Nihon University of Physical Education, Tokyo, Japan

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# *N*-METHYL-D-ASPARTATE RECEPTORS PLAY IMPORTANT ROLES IN ACQUISITION AND EXPRESSION OF THE EYEBLINK CONDITIONED RESPONSE IN GLUTAMATE RECEPTOR SUBUNIT δ2 MUTANT MICE

**Y. KATO,[a] K. TAKATSUKI,[a] S. KAWAHARA,[a*] S. FUKUNAGA,[a] H. MORI,[b] M. MISHINA[b] AND Y. KIRINO[a]**

[a]*Laboratory of Neurobiophysics, School of Pharmaceutical Sciences, The University of Tokyo, and Core Research for Evolution Science and Technology, Japan Science and Technology Agency, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan*

[b]*Department of Molecular Neurobiology and Pharmacology, Graduate School of Medicine, The University of Tokyo, and Solution Oriented Research for Science and Technology, Japan Science and Technology Agency, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan*

**Abstract—Classical eyeblink conditioning has been known to depend critically on the cerebellum. Apparently consistent with this, glutamate receptor subunit δ2 null mutant mice, which have serious morphological and functional deficiencies in the cerebellar cortex, are severely impaired in delay paradigm. However, these mutant mice successfully learn in trace paradigm, even in '0-trace paradigm,' in which the unconditioned stimulus starts just after the conditioned stimulus terminates. Our previous studies revealed that the hippocampus and the muscarinic acetylcholine receptors play crucial roles in 0-trace paradigm in glutamate receptor subunit δ2 null mutant mice unlike in wild-type mice, suggesting a large contribution of the forebrain to 0-trace conditioning in this type of mutant mice. In the present study, we investigated the role of *N*-methyl-D-aspartate receptors in 0-trace eyeblink conditioning in glutamate receptor subunit δ2 null mutant mice. Mice were injected intraperitoneally with the noncompetitive *N*-methyl-D-aspartate receptor antagonist (+)MK-801 (0.1 mg/kg) or saline, and conditioned with 350-ms tone conditioned stimulus followed by 100-ms periorbital shock unconditioned stimulus. Glutamate receptor subunit δ2 null mutant mice that received (+)MK-801 injection exhibited a severe impairment in acquisition of the conditioned response, compared with the saline-injected glutamate receptor subunit δ2 null mutant mice. In contrast, wild-type mice were not impaired in acquisition of 0-trace conditioned response by (+)MK-801 injection. After the injection solution was changed from (+)MK-801 to saline, glutamate receptor subunit δ2 null mutant mice showed a rapid and partial recovery of performance of the conditioned response. On the other hand, when the injection solution was changed from saline to (+)MK-801, glutamate receptor subunit δ2 null mutant mice showed a marked impairment in expression of the pre-acquired conditioned response, whereas impairment of the expression was small in wild-type mice. Injection of (+)MK-801 had no significant effects on spontaneous eyeblink frequency or startle eyeblink frequency to the tone conditioned stimulus in either glutamate receptor subunit δ2 null mutant mice or wild-type mice. These results suggest that *N*-methyl-D-aspartate receptors play critical roles both in acquisition and expression of the conditioned response in 0-trace eyeblink conditioning in glutamate receptor subunit δ2 null mutant mice.** **© 2005 Published by Elsevier Ltd on behalf of IBRO.**

**Key words: classical conditioning, learning, hippocampus, cerebellum, MK-801.**

Classical conditioning of the eyeblink response is a type of motor learning that depends critically on the cerebellum in rabbits (McCormick et al., 1982) and mice (Chen et al., 1996). Using a standard delay paradigm, in which the unconditioned stimulus (US) is delayed, overlaps and coterminates with the conditioned stimulus (CS), the essential neural circuit that resides in the cerebellum and brainstem has been extensively studied in rabbits. Thus this learning task has become a model system that is suitable for analysis of the neural substrates of learning and memory (Thompson et al., 1997). In addition to these essential brain regions, the forebrain including the hippocampus and the medial prefrontal cortex plays an important role in trace paradigm, in which a long trace-interval (a stimulus-free period of time) intervenes between the CS and US (Solomon et al., 1986; Kronforst-Collins and Disterhoft, 1998; McLaughlin et al., 2002; Takehara et al., 2002, 2003). Although this important basic framework for learning mechanism in classical eyeblink conditioning has been established in rabbit, mouse is becoming another important model because of its current progress in gene-manipulating techniques.

We have studied the eyeblink conditioning of mutant mice that lack glutamate receptor subunit δ2 (GluRδ2) (Kishimoto et al., 2001b,d; Takatsuki et al., 2002, 2003). GluRδ2 mRNA is expressed predominantly in Purkinje cells of the cerebellum whereas its expression in the forebrain is hardly detectable (Araki et al., 1993; Lomeli et al., 1993). Within Purkinje cells, GluRδ2 proteins are localized exclusively at parallel fiber-Purkinje cell synapses (Takayama et al., 1996; Landsend et al., 1997). GluRδ2 null mutant mice (GluRδ2$^{-/-}$ mice) have several morphological and functional impairments in the cerebellar cortex such as a deficit in long-term depression (LTD) at parallel fiber–Purkinje cell synapses, a 50% reduction in the number of these synapses, persistent multiple innervation of Purkinje cells by climbing fibers, and abnormal oscillating Purkinje cell activity (Kashiwabuchi et al., 1995; Kurihara

*Corresponding author. Tel: +81-3-5841-4801; fax: +81-3-5841-4805.
E-mail address: kawahara@mol.f.u-tokyo.ac.jp (S. Kawahara).
*Abbreviations:* APV, 2-amino-5-phosphonovaleric acid; CR, conditioned response; CS, conditioned stimulus; EMG, electromyogram; GluRδ2, glutamate receptor subunit δ2; LTD, long-term depression; NMDA, *N*-methyl-D-aspartate; US, unconditioned stimulus.

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et al., 1997; Yoshida et al., 2004). Impairments in motor coordination (Kashiwabuchi et al., 1995) and vestibular compensation (Funabiki et al., 1995) were also found in GluRδ2$^{-/-}$ mice.

Apparently consistent with the hypothesis that the cerebellum and synaptic plasticity in the cerebellar cortex play a crucial role in classical eyeblink conditioning (Ito, 1989; Thompson and Krupa, 1994), GluRδ2$^{-/-}$ mice exhibited a severe impairment in a delay paradigm with a 252-ms interstimulus interval between the 352-ms CS and 100-ms US (Kishimoto et al., 2001d), as did other cerebellar LTD-deficient mice (Aiba et al., 1994; Shibuki et al., 1996; Miyata et al., 2001; Kishimoto et al., 2001a; Koekkoek et al., 2003). However, in trace paradigm with a 500-ms trace interval between the CS end and US onset, GluRδ2$^{-/-}$ mice successfully acquired the eyeblink conditioned response (CR) (Kishimoto et al., 2001d). Further analysis using various temporal intervals between the CS and US revealed that GluRδ2$^{-/-}$ mice learned as successfully as wild-type mice in trace paradigms with a 50-, 100-, and 250-ms trace interval, and even in the '0-trace paradigm' in which the US starts just after termination of the CS (Kishimoto et al., 2001b). However, in a delay paradigm with a 352-ms interstimulus interval that is equal to that of the 0-trace paradigm with a 352-ms CS, GluRδ2$^{-/-}$ mice exhibited a severe learning impairment. These results suggest that another learning mechanism, one that does not require the cerebellar LTD, underlies the ability of GluRδ2$^{-/-}$ mice to learn in eyeblink conditioning paradigms in which the US does not overlap with the CS. Eyeblink conditioning in another cerebellar LTD-deficient mouse lacking the phospholipase Cβ4 subunit also showed similar results (Kishimoto et al., 2001a).

In the course of discovering this cerebellar LTD-independent learning mechanism, we found that ablation of the hippocampus (Takatsuki et al., 2003) or blockade of muscarinic acetylcholine receptors by systemic administration of scopolamine (Takatsuki et al., 2002) severely impaired acquisition of the CR during 0-trace conditioning in GluRδ2$^{-/-}$ mice. These results suggest that the forebrain including the hippocampus contributes to the successful learning of GluRδ2$^{-/-}$ mice in 0-trace conditioning. In wild-type animals, the hippocampus has an ability to modulate acquisition during a standard delay conditioning (Salafia et al., 1979; Prokasy et al., 1983; Solomon et al., 1983) and is required in more difficult paradigms such as long-trace paradigm (Solomon et al., 1986; Moyer et al., 1990; Weiss et al., 1999; Tseng et al., 2004) and discrimination reversal paradigm (Berger and Orr, 1983). The medial prefrontal cortex also plays an important role in acquisition (Kronforst-Collins and Disterhoft, 1998; Weible et al., 2000; McLaughlin et al., 2002) and retention (Takehara et al., 2003) in long-trace conditioning paradigm. Therefore, contribution of the forebrain seems to increase when the task demand on the animal is raised. Based on this consideration, we have hypothesized that contribution of the forebrain might also increase in GluRδ2$^{-/-}$ mice because the several deficiencies in the cerebellar cortex will make it difficult for the mutant mice to learn the short-trace eyeblink conditioning.

*N*-methyl-D-aspartate (NMDA) receptors are abundantly expressed in the forebrain (Watanabe et al., 1993) and many studies using NMDA receptor antagonists or gene-targeted mutant mice have revealed that NMDA receptors play important roles in several kinds of learning that depend on the forebrain. In eyeblink conditioning, Thompson and Disterhoft (1997) have demonstrated that the noncompetitive NMDA receptor antagonist (+)MK-801 slows the rate of acquisition in delay paradigm and completely blocks acquisition in 500-ms trace paradigm in rabbits. This drug is also effective in mice, in which the impairment with (+)MK-801 administration is greater in trace paradigm with a longer trace interval (Takatsuki et al., 2001). In addition, acute injections of the competitive NMDA receptor antagonist 2-amino-5-phosphonovaleric acid (APV) into the dorsal hippocampus severely impaired acquisition during 500-ms trace conditioning in mice (Sakamoto et al., 2005). Consistent with the effects of pharmacological blockade, those mutant mice that lack the NMDA receptor subunit ε1 (NR2A) exhibit a mild impairment in delay paradigm and a severe impairment in long-trace paradigm (Kishimoto et al., 1997, 2001c). These results suggest that blockade of NMDA receptors greatly affects the role of the forebrain in eyeblink conditioning in mice.

In the present study, we investigated the effect of (+)MK-801 on 0-trace eyeblink conditioning in GluRδ2$^{-/-}$ mice. As described above, these mutant mice critically depend on the hippocampus to learn the 0-trace eyeblink conditioning. Therefore, blockade of NMDA receptors would result in impairment of learning as reported in long-trace eyeblink conditioning in wild-type animals.

## EXPERIMENTAL PROCEDURES

### Animals

Previously-developed GluRδ2-mutant mice were repeatedly backcrossed with C57BL/6 mice to yield heterozygous GluRδ2$^{+/-}$ mice with a more than 99.99% C57BL/6 genetic background (Kashiwabuchi et al., 1995; Kishimoto et al., 2001d). These heterozygous mice with highly homogeneous genetic background were crossed with each other to obtain homozygous GluRδ2$^{-/-}$ mice. Both male and female mice were used and randomly assigned to saline-injected control group and (+)MK-801-injected group. No differences were detected between the results obtained from male and female mutant mice. We also investigated the effect of (+)MK-801 in wild-type C57BL/6 male mice obtained from a breeder (Japan SLC, Hamamatsu, Shizuoka, Japan). These animals were kept on a 12-h light/dark cycle with *ad libitum* access to food and water and weighed 18–27 g at the time of surgery. All experiments were performed in accordance with the guidelines established by the Institutional Animal Investigation Committee at the University of Tokyo and the United States National Institutes of Health Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize the use of animals and to optimize their comfort.

### Surgery

Surgical procedures were the same as those described previously (Takatsuki et al., 2001). Under anesthesia with ketamine (80 mg/kg, i.p.; Sankyo, Tokyo, Japan) and xylazine (20 mg/kg, i.p.; Bayer, Tokyo, Japan), four Teflon-coated stainless steel wires

(No. 7910, A-M Systems, Carlsborg, WA, USA) were implanted under the left eyelid. Two of these wires were used to record electromyograms (EMG) and the remaining two to deliver the US. These wires were soldered to connector pins, which were secured to the skull with dental acrylic resin and stainless steel screws. After surgery, the animals were returned to their home cages and their recovery was monitored.

### Drug treatments

(+)MK-801 (0.1 mg/kg, Tocris Cookson, St. Louis, MO, USA) or saline was administered by i.p. injection 10 min before the second adaptation session to the experimental apparatus (spontaneous recording day 2) and the subsequent daily conditioning sessions. (+)MK-801 was diluted in saline and injected in a volume of 5 ml/kg body weight. Control mice were injected with saline (5 ml/kg). The dose and the timing of injection used in the present study is sufficient to cause a severe impairment in a hippocampus-dependent long-trace eyeblink conditioning in wild-type mice as revealed in our previous study (Takatsuki et al., 2001), in which we also found higher dose (0.2 mg/kg, i.p.) reduced the startle eyeblink responses to the tone CS.

### Conditioning procedure

On a day (spontaneous recording day 1) three to five days after the surgery, spontaneous eyeblink frequency was recorded for approximately 50 min without the US, CS, or drug injection. On the next day (spontaneous recording day 2), spontaneous eyeblink frequency was recorded in the same way as the day before except that (+)MK-801 or saline was injected 10 min before the start of spontaneous eyeblink recording. The eyeblink conditioning began the next day. A daily conditioning session consisted of 100 trials divided into 10 blocks, which had nine CS-US paired trials followed by a CS-alone trial. Trials were separated by a variable intertrial interval, randomized between 20 and 40 s. The CS was a 350-ms tone (1 kHz, 85 dB) with a 5-ms rise and a 5-ms fall time. The US was a 100-ms periorbital shock (100 Hz square pulses) and was adjusted daily to elicit an eyeblink/head-turn response in each animal. We used '0-trace paradigm,' in which the US starts just after the CS terminates. (+)MK-801 or saline was injected 10 min before the daily conditioning. All experiments were carried out during the light phase of the light/dark cycle.

### Data analysis

EMG activity was band-pass filtered between 0.15 and 1.0 kHz and fed into a computer with a sampling rate of 10 kHz. These data were analyzed off-line. The maximum amplitude of the EMG signals during the time period of $t \pm 1$ ms was calculated and designated the "EMG amplitude" at $t$. Then, the mean+S.D. of the EMG amplitude data for 300 ms before the CS onset in 100 trials was defined as the threshold, which was then used in the analysis below. In each trial, the average values for the EMG amplitude above threshold were calculated for a period of 300 ms before CS onset (pre-value), 30 ms after CS onset (startle-value), and 200 ms before the US onset (CR-value). When both the pre-value and startle-value were less than 10% of threshold, the trial was considered a valid trial. Among the valid trials, a trial was assumed to contain the CR if the CR value was larger than 1% of threshold and exceeded two times the pre-value. For the CS-alone trials, the period for CR-value calculation was extended to the presumptive US termination. The frequency of CRs in the valid trials (CR%) was expressed as mean±S.E.M. To evaluate the effects on the startle eyeblink response to the tone CS, the number of trials whose startle value exceeded 10% of threshold during the acquisition session was computed and its ratio to the number of trials was calculated (after removal of those trials whose pre-value exceeded 10% of threshold). Statistical significance was determined by an ANOVA or $t$-test. $P<0.05$ was considered significant.

## RESULTS

### Effect of (+)MK-801 on acquisition of the CR

Using the noncompetitive NMDA receptor antagonist (+)MK-801, we investigated the role of NMDA receptors in 0-trace conditioning paradigm in GluRδ2$^{-/-}$ mice. Our previous study revealed that these mutant mice require an intact hippocampus to acquire the eyeblink CR during 0-trace conditioning, while wild-type mice do not (Takatsuki et al., 2003). GluRδ2$^{-/-}$ mice and wild-type mice received an i.p. injection of (+)MK-801 (0.1 mg/kg) or saline 10 min before daily conditioning for 7 days. Fig. 1A shows the CR% for the saline-injected control group ($n=8$) and the (+)MK-801-injected group ($n=8$) of wild-type mice. Both of them readily acquired the CR. A two-way repeated-measures ANOVA revealed that the drug had no significant effect ($F_{1,14}=2.35$, $P>0.1$). In GluRδ2$^{-/-}$ mice, the CR% of the saline-injected control group ($n=12$) gradually increased to around 80% by the 7th day of conditioning (Fig. 1B). In contrast, the (+)MK-801-treated GluRδ2$^{-/-}$ mice ($n=12$) exhibited a severe impairment in their acquisition of the CR. Their CR% reached only 30% after 7 days of conditioning. A two-way repeated-measures ANOVA confirmed a significant interaction between sessions and the drug ($F_{6,132}=10.54$, $P<0.001$). These results indicate that (+)MK-801 at this dose greatly impairs learning during 0-trace eyeblink conditioning in GluRδ2$^{-/-}$ mice, but not in wild-type mice.

### Effect of (+)MK-801 on spontaneous eyeblink frequency and startle eyeblink frequency

To confirm that the severe impairment of learning in GluRδ2$^{-/-}$ mice was not due to deficits in basic sensory or motor performance, the effects on spontaneous eyeblink frequency measured during adaptation sessions and on startle eyeblink frequency to the tone CS were assessed (Fig. 2). Statistical analysis using a two-way repeated-measures ANOVA indicated no significant differences in spontaneous eyeblink frequency between the saline-injected control group and the (+)MK-801-injected group in wild-type mice ($F_{1,14}=4.12$, $P>0.05$) and GluRδ2$^{-/-}$ mice ($F_{1,22}=2.12$, $P>0.1$). The frequency of startle eyeblink to the tone CS during conditioning also did not differ significantly between control group and MK-801 group in wild-type mice ($t$-test, $P>0.1$) and GluRδ2$^{-/-}$ mice ($t$-test, $P>0.1$).

### Effect of (+)MK-801 on expression of the pre-acquired CR

Because the impairment in the mutant mice was so severe during the acquisition sessions, we also checked the possibility that expression of the CR might be being suppressed by the (+)MK-801 treatment during acquisition. Among the mice that received a saline injection during the 7 days of acquisition sessions with 0-trace conditioning (Fig. 1), we selected those mice whose average CR% over the last three days of conditioning exceeded 60%. All the wild-type mice ($n=8$) and nine of 12 GluRδ2$^{-/-}$ mice met

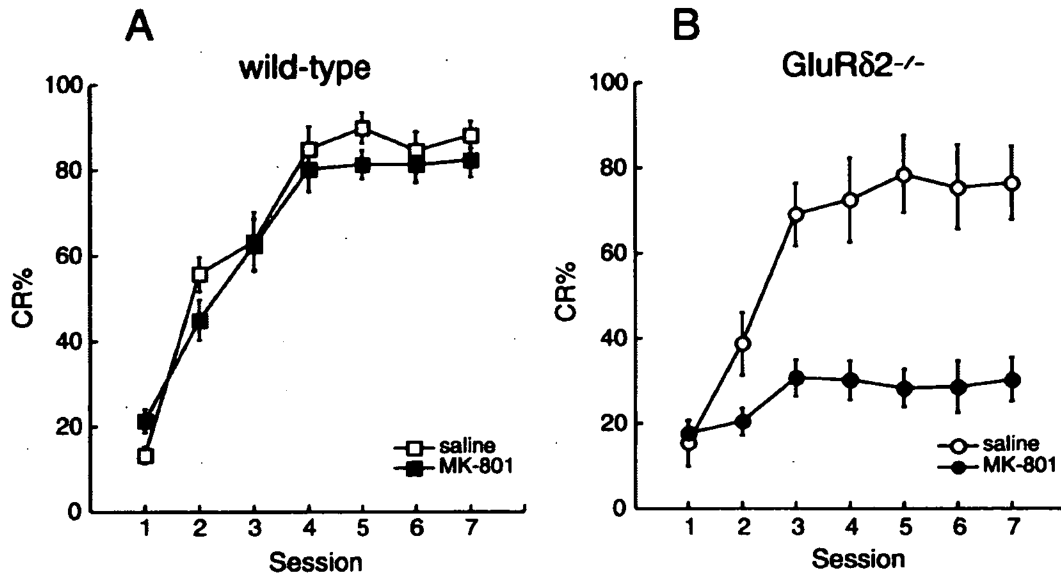


**Fig. 1.** Effects of (+)MK-801 on acquisition of the CR during 7 days of 0-trace conditioning in wild-type mice (A) and GluRδ2$^{-/-}$ mice (B). Saline or (+)MK-801 (0.1 mg/kg) was intraperitoneally injected 10 min before daily conditioning in wild-type mice ($n$=8 for saline and MK-801 group each) and GluRδ2$^{-/-}$ mice ($n$=12 for each group). The error bar indicates the standard error of the mean.

this criterion, and they received three additional days of conditioning (the expression sessions) with (+)MK-801 injections (0.1 mg/kg, i.p.). When the injection solution was switched from saline to (+)MK-801, the CR% of the GluRδ2$^{-/-}$ mice decreased markedly, while that of the wild-type mice decreased only marginally (Fig. 3A). A paired $t$-test revealed a significant difference between the average CR% over the last three days with saline injections and the CR% on the first day with (+)MK-801 injection both in GluRδ2$^{-/-}$ mice ($P$<0.001) and wild-type mice ($P$<0.01).

To further confirm the inhibition of CR expression by (+)MK-801, we looked at the recovery in CR expression after changing the injection solution from (+)MK-801 to saline. The mice that received injections of (+)MK-801 during the 7 days of acquisition sessions (eight wild-type

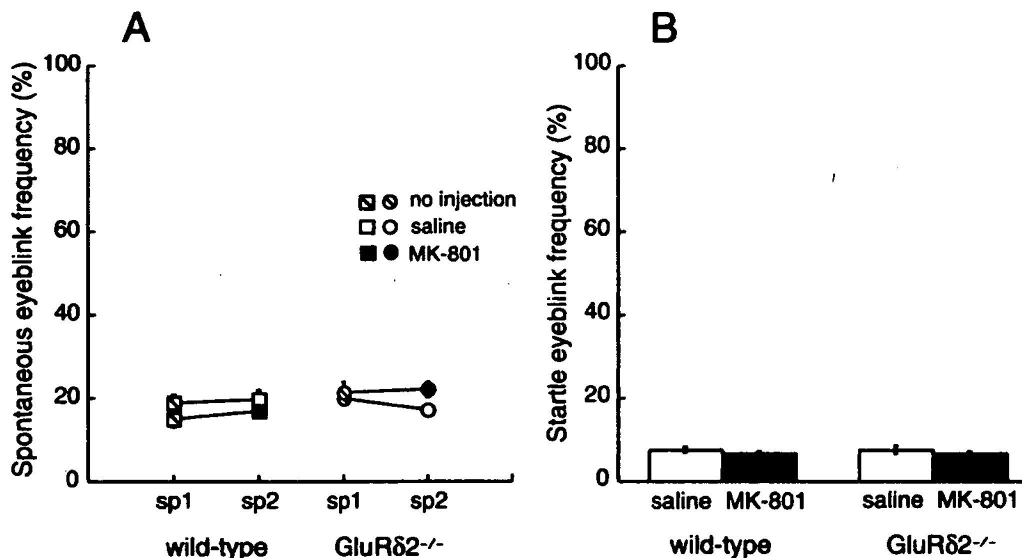


**Fig. 2.** Effects of (+)MK-801 on the spontaneous eyeblink and the startle eyeblink to the tone CS. (A) Effect on spontaneous eyeblink frequency during adaptation sessions, which were performed before the 7-day acquisition sessions. Eyeblink frequency was recorded using the same protocol as that used for the acquisition sessions except that no stimuli were presented. On the first day (sp1), spontaneous eyeblink frequency was recorded without drug injection. On the second day (sp2), saline or (+)MK-801 (0.1 mg/kg) was intraperitoneally injected 10 min before the adaptation session. (B) Effect on startle eyeblink frequency to the tone CS during conditioning. The data were collected from 7 days of conditioning. The error bar indicates the standard error of the mean.

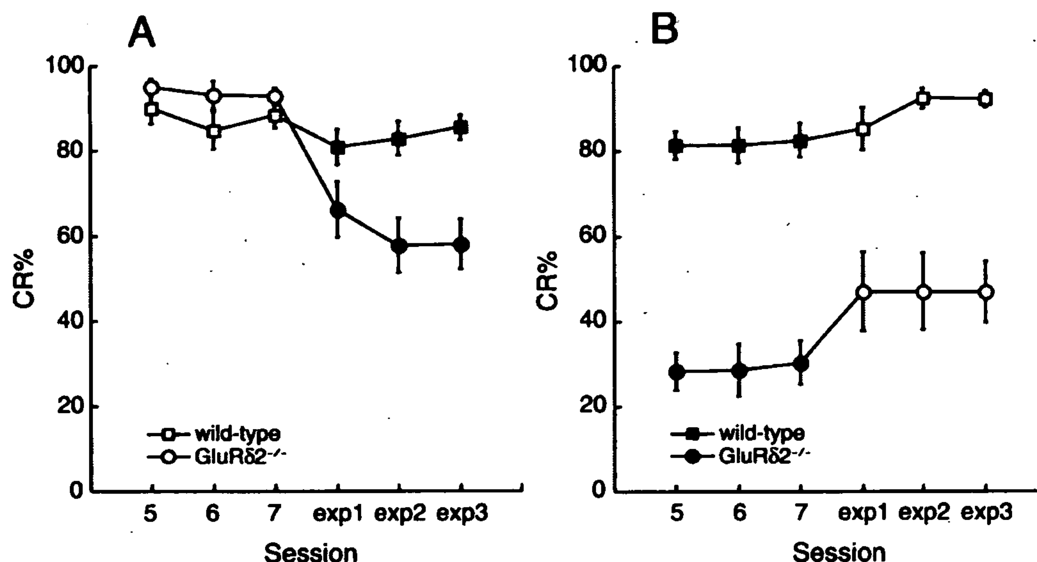


**Fig. 3.** Effects of (+)MK-801 on expression of the CR. After 7 days of conditioning (shown in Fig. 1), the injection solution was switched from saline to (+)MK-801 (A) or from (+)MK-801 to saline (B). The data during the last three days of conditioning (5th–7th days of conditioning) and the subsequent three days of expression sessions (exp1–3) are shown. (A) Inhibitory effect on expression of the pre-acquired CR. The wild-type mice ($n$=8) and the GluRδ2$^{-/-}$ mice ($n$=9), whose average CR% over the last three days with saline injection had exceeded 60%, received additional three days of conditioning with (+)MK-801 injection. (B) Recovery from inhibition by (+)MK-801. The wild-type mice ($n$=8) and the GluRδ2$^{-/-}$ mice ($n$=12), which were conditioned with (+)MK-801 injection for 7 days, received additional three days of conditioning with saline injection. Empty and filled symbols indicate saline and (+)MK-801 injection groups, respectively. The error bar indicates the standard error of the mean.

mice and 12 GluRδ2$^{-/-}$ mice used in the experiment shown in Fig. 1) received an additional three days of conditioning with saline injections. When the injection solution was switched from (+)MK-801 to saline, the CR% of the GluRδ2$^{-/-}$ mice rapidly increased, although the CR% did not fully recover during these additional days of conditioning (Fig. 3B). In contrast, the CR% of the wild-type mice showed almost no change on the first day. A paired $t$-test between the average CR% over the last three days with an (+)MK-801 injection and the CR% on the first day with a saline injection confirmed that there was a significant difference in GluRδ2$^{-/-}$ mice ($P<0.05$), whereas there were no significant differences in wild-type mice ($P>0.1$).

## DISCUSSION

In the present study, we investigated the effect of the noncompetitive NMDA receptors antagonist (+)MK-801 on the learning ability of GluRδ2$^{-/-}$ mice, which have a deficient cerebellar cortex, in '0-trace' eyeblink conditioning. We found that systemic application of (+)MK-801 (0.1 mg/kg, i.p.) severely impaired acquisition of the CR in GluRδ2$^{-/-}$ mice, while it did not in wild-type mice. Expression of the pre-acquired CR was also markedly impaired in GluRδ2$^{-/-}$ mice, whereas only a marginal impairment of CR expression was observed in wild-type mice. These results indicate that 0-trace conditioning in GluRδ2$^{-/-}$ mice depends largely on NMDA receptors both for acquisition and expression of the CR.

NMDA receptors are extensively expressed over the forebrain, including the prefrontal cortex and the hippocampus (Watanabe et al., 1993), which play critical roles in acquisition during long-trace eyeblink conditioning (Solomon et al., 1986; Kronforst-Collins and Disterhoft, 1998; McLaughlin et al., 2002). In addition to these areas, NMDA receptors are also expressed in the cerebellum (Watanabe et al., 1994a) and brainstem (Watanabe et al., 1994b). Consistent with this, microinfusion of the competitive NMDA receptor antagonist APV into the deep cerebellar nuclei severely impairs acquisition during delay eyeblink conditioning in rabbits (Chen and Steinmetz, 2000), suggesting that some of the effect of (+)MK-801 might be attributable to its effect on the cerebellum. However, in the present study, acquisition was not significantly impaired during 0-trace conditioning in wild-type mice (Fig. 1A) and the impairment of CR expression was minimal (Fig. 3). These results suggest that the effect of (+)MK-801 on the role of the cerebellum and brainstem in mouse 0-trace conditioning must be relatively weak under the present condition.

In contrast to the small effect in wild-type mice, (+)MK-801 severely impaired acquisition in GluRδ2$^{-/-}$ mice (Fig. 1). These results are consistent with our previous work that 0-trace conditioning depends largely on the hippocampus in GluRδ2$^{-/-}$ mice but not in wild-type mice (Takatsuki et al., 2003). Similar effects of (+)MK-801 have been observed in the hippocampus-dependent long-trace eyeblink conditioning in rabbits (Thompson and Disterhoft, 1997) and mice (Takatsuki et al., 2001). Therefore, GluRδ2$^{-/-}$ mice might learn 0-trace eyeblink conditioning using a similar mechanism to that used in long-trace conditioning in wild-type animals. However, in addition to the severe impairment during acquisition sessions, (+)MK-801 also partially inhibited expression of the pre-acquired CR in

GluRδ2$^{-/-}$ mice (Fig. 3A). Thus, a part of the severe impairment in GluRδ2$^{-/-}$ mice during acquisition sessions is attributable to inhibition of the CR expression. In contrast to the large effect in GluRδ2$^{-/-}$ mice, (+)MK-801 had only a little effect on CR expression in wild-type mice (Fig. 3A). Similarly, CR expression in long-trace conditioning was not significantly inhibited by (+)MK-801 in wild-type mice, while its acquisition was severely impaired (Takatsuki et al., 2001). Therefore, (+)MK-801 differently affects 0-trace conditioning in GluRδ2$^{-/-}$ mice and long-trace conditioning in wild-type mice, though both heavily depend on the hippocampus for successful learning.

This impairment of CR expression by (+)MK-801 after sufficient learning was unexpected, because hippocampal lesions made after sufficient learning in 0-trace conditioning paradigm did not affect CR expression in GluRδ2$^{-/-}$ mice, whereas a lesion made before the training did severely impair learning (Takatsuki et al., 2003). Therefore, the site of action of (+)MK-801 to inhibit the CR expression might be outside the hippocampus. At present, there are three candidates for this site. One is the cerebellum. Chen and Steinmetz (2000) have reported that some rabbits that were given APV injection to the deep cerebellar nuclei after sufficient learning showed impairment of CR expression, although most of the animals they examined did not show such impairment. Therefore, it is possible that GluRδ2$^{-/-}$ mice, which have several deficiencies in the cerebellar cortex, preferentially use the expression mechanism that depends on the NMDA receptors in the cerebellar deep nuclei, while wild-type mice use an NMDA receptor-independent mechanism for CR expression. The second candidate site is the medial prefrontal cortex, which plays an important role together with the hippocampus in the long-trace conditioning paradigm (Kronforst-Collins and Disterhoft, 1998; Weible et al., 2000; Takehara et al., 2003) and which also has an abundance of NMDA receptors (Watanabe et al., 1993). If GluRδ2$^{-/-}$ mice depend much more than wild-type mice on the medial prefrontal cortex for CR expression after sufficient conditioning, then blockade of the NMDA receptors in this region might result in an impairment of CR expression. The medial prefrontal cortex plays a critical role when the hippocampus becomes inessential, which occurs one month after completion of long-trace conditioning in rats (Takehara et al., 2003). Therefore, it is plausible that the medial prefrontal cortex also plays an important role in GluRδ2$^{-/-}$ mice, in which the hippocampus is no longer required after 7 days of conditioning (Takatsuki et al., 2003). Finally, the hippocampus cannot be excluded as a candidate, because it is possible that an abnormal hippocampus (caused by blockade of NMDA receptors in our study) could be much more detrimental than its absence (Solomon et al., 1983).

Impairment of CR expression by (+)MK-801 also has been reported in rabbits that received an i.v. injection of (+)MK-801 (Cox et al., 1994). The impairment of CR expression (as well as acquisition) in their study was much more profound than in other reports that adopted s.c. (Thompson and Disterhoft, 1997) or i.p. (Takatsuki et al., 2001) injection of (+)MK-801, suggesting that the i.v. administration may have selectively affected an area that participates in CR expression and that is susceptible to blockade of NMDA receptors in rabbits. Therefore, it is likely that wild-type mice also have a mechanism for CR expression that largely depends on the normal functioning of NMDA receptors.

The present results suggest that GluRδ2$^{-/-}$ mice may utilize an additional mechanism for expression of the CR and possibly for its acquisition as well to overcome the deficiencies in the cerebellar cortex. This mechanism may be present intrinsically but is not dominant in wild-type mice. Several characteristics of 0-trace conditioning in GluRδ2$^{-/-}$ mice (such as the dependence on the hippocampus, muscarinic acetylcholine receptors and on NMDA receptors) are more similar to the features of long-trace conditioning than to those of delay conditioning in wild-type animals (Solomon et al., 1986; Kaneko and Thompson, 1997; Thompson and Disterhoft, 1997). Consistent with the effects of hippocampal lesion in various classical conditioning paradigms, the case of GluRδ2$^{-/-}$ mice suggests that the forebrain may be more likely to be recruited if the task is more difficult for the cerebellum to perform because of deficiencies in the cerebellar cortex. Future study with microinfusion of APV into the cerebellum, the hippocampus and into the medial prefrontal cortex should provide more information about the mechanism underlying hippocampus-dependent eyeblink conditioning in GluRδ2$^{-/-}$ mice and in wild-type mice as well.

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ORIGINAL INVESTIGATION

Hiroshi Kimura · Masaru Ihira · Yoshihiro Enomoto
Jun-ichi Kawada · Yoshinori Ito · Tsuneo Morishima
Tetsushi Yoshikawa · Yoshizo Asano

# Rapid detection of herpes simplex virus DNA in cerebrospinal fluid: comparison between loop-mediated isothermal amplification and real-time PCR

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**Abstract** Loop-mediated isothermal amplification (LAMP) is a novel nucleic acid amplification method that amplifies DNA with high specificity, efficiency, and speed under isothermal conditions. To evaluate the usefulness of LAMP for diagnosing central nervous system infection with herpes simplex virus (HSV), we compared the LAMP method with real-time PCR, using samples that were previously tested by nested PCR. We examined 69 cerebrospinal fluid (CSF) samples from patients suspected of having HSV infection of the central nervous system. The results of the real-time PCR analysis and nested PCR assay were in complete accord. When nested PCR was regarded as standard, the sensitivity of LAMP was 81%, the specificity was 100%, the positive predictive value was 100%, and the negative predictive value was 90%. Although further improvement is necessary for the wide spread use, the LAMP method might be applicable to diagnosis of HSV infection of the central nervous system.

**Keywords** HSV · LAMP · Real-time PCR · CSF · Encephalitis

H. Kimura (✉) · J. Kawada · Y. Ito
Department of Pediatrics,
Nagoya University Graduate School of Medicine,
65 Tsuruma-cho, Showa-ku Nagoya, 466-8550, Japan
E-mail: hkimura@med.nagoya-u.ac.jp
Tel.: +81-52-7442295
Fax: +81-52-7442974

M. Ihira
Department of Medical Information Technology,
Fujita Health University College, Toyoake, Japan

Y. Enomoto · T. Yoshikawa · Y. Asano
Department of Pediatrics, Fujita Health University College,
Toyoake, Japan

T. Morishima
Department of Pediatrics,
Graduate school of Medicine and Dentistry,
Okayama University, Okayama, Japan

## Introduction

Herpes simplex viruses (HSV) are neurotropic viruses comprising type 1 (HSV-1) and type 2 (HSV-2) [23]. Both HSV-1 and HSV-2 cause central nervous system diseases in humans. Detection of HSV DNA in the cerebrospinal fluid (CSF), most commonly by PCR, is the gold standard for diagnosis of central nervous system infection by HSV [1, 3, 11–13, 17, 18, 23].

Recently, Notomi et al. [21] reported a novel nucleic acid amplification method, termed loop-mediated isothermal amplification (LAMP). The LAMP reaction requires a DNA polymerase with strand displacement activity and a set of four specially designed primers, termed inner and outer primers, which improve specificity. As a first step, a stem-loop DNA structure, in which the sequences of both DNA ends are derived from the inner primer, is constructed as the starting material. Subsequently, one inner primer hybridizes to the loop on the product in the LAMP cycle and initiates strand displacement DNA synthesis, yielding the original stem-loop and new stem-loop DNA with a stem twice as long. The final products are stem-loop DNAs with several inverted repeats of the target DNA and cauliflower-like structures with multiple loops, amplifying the amount to $10^9$ copies of the target [21]. The most significant advantage of LAMP is its ability to amplify specific sequences of DNA at 63–65°C, without thermocycling. Therefore, the technique requires relatively simple and cost-effective equipment, making it amenable for use in hospital laboratories.

This method has been used for the rapid diagnosis of various infectious diseases, including HSV [4–7, 9, 22, 24]. In this study, we examined the use of a type-specific HSV LAMP method for the diagnosis of HSV infection of the central nervous system. To evaluate the usefulness of LAMP, we tested CSF samples from patients with HSV encephalitis using the LAMP method, and compared the results with those obtained using the real-time PCR assay.

## Methods

### Samples

A total of 69 CSF samples obtained from 50 patients were analyzed. Clinically, all the patients were suspected of having HSV infection of the central nervous system. There were 43 samples from adults and 23 from neonates. In some patients, samples were obtained sequentially after the initiation of acyclovir therapy. We had previously performed nested PCR to detect HSV DNA in these samples; HSV DNA was detected in 26 of the 69 CSF samples from suspect cases using the nested PCR assay [10]. Restriction fragment length polymorphism of amplified products indicated that 11 samples contained HSV-1 DNA and 15 samples contained HSV-2 DNA [2, 15].

### Real-time PCR

The real-time quantitative PCR assay was carried out using a TaqMan PCR kit (Applied Biosystems, Foster City, USA), as described previously [8, 10, 14, 15], using the primers and fluorogenic probe based on the *UL30* gene sequence [8]. DNA was extracted from 200 μl of CSF, using a QIAamp Blood kit (QIAGEN, Hilden, Germany), and eluted in 50 μl of distilled water. Five microliters of the DNA extracted from each sample were used for the real-time PCR assay, which can detect both HSV-1 and HSV-2 with almost equal efficiency. Following 5 cycles of 20 s at 97°C and 1 min at 58°C, 45 cycles of 20 s at 96°C and 1 min at 58°C were carried out using a Model 7700 Sequence Detector (Applied Biosystems). Real-time fluorescence measurements were made, and threshold cycle value for each sample was calculated by determining the point at which the fluorescence exceeded a threshold limit (10 times the standard deviation for the base line). Standard curves were constructed using the values obtained from serially diluted positive control plasmids that contained *UL30* gene. The values from clinical samples were plotted on the standard curve, and the copy number was calculated by automatically using Sequence Detector v1.6 (Applied Biosystems). To determine a detection limit, a serially diluted plasmid control containing either HSV-1 or HSV-2 was used. A minimum of five copies of either HSV-1 or HSV-2 DNA per assay can be detected [10].

All experiments were done in duplicate. For borderline samples that were estimated as low copies or discordant with the results of LAMP, tests were repeated, and all the repeated results were in accord with the original ones. Sometimes, estimated copy numbers were lower than the detection limit of the real-time PCR assay. The reason was unclear, but inhibitors, which could not be eliminated from clinical samples by the DNA extraction, may influence on PCR reaction and underestimate the copy number [16].

### LAMP method

The LAMP reactions were conducted as described previously [4]. The LAMP method requires a set of four primers (B3, F3, BIP, FIP) that recognize a total of six distinct sequences within the target DNA [21]. Primers for the HSV-1- and HSV-2-specific LAMP assays were designed based on the HSV-1 gG and HSV-2 gG gene sequences, respectively. Primer BIP for the gG genes of HSV-1 and HSV-2 contained the B1 direct sequence and B2 complementary sequence, each specific for the respective strains. Primer FIP for the gG genes of HSV-1 and HSV-2 contained the F1 complementary sequence and the F2 direct sequence. Primers B3 and F3 for the gG genes of HSV-1 and HSV-2 were located outside the F2-B2 regions. As additional loop primers increase the amplification efficiency [20], loop primers specific for the HSV-1 gG and HSV-2 gG genes were also used. The primer sequences and binding locations have been described elsewhere [4]. The LAMP reaction was performed using a Loopamp DNA amplification kit (Eiken Chemical, Tochigi, Japan). The 25-μl reaction mixtures contained 1.6 μM each of the FIP and BIP primers, 0.8 μM of each outer primer (F3 and B3), 0.8 μM of each loop primer, 12.5 μl of 2X reaction mix, 1 μl of *Bst* DNA polymerase, and 5 μl of the extracted DNA solution. The mixture was incubated at 63°C for 45 min. The turbidity derived from magnesium pyrophosphate formation, due to release of pyrophosphate during polymerization, was detected on a TERAMECS LA200 real-time turbidimeter (Teramecs, Kyoto, Japan) and used as an indicator of the formation of amplified products. The turbidity of five negative control samples was $0.01 \pm 0.02$; we thus defined 0.1 as the cut-off value for discrimination between positive and negative samples. To determine a detection limit, a serially diluted plasmid control containing either HSV-1 or HSV-2 was used. A minimum of 500 copies of HSV-1 or 1,000 copies of HSV-2 DNA per assay was detected by the HSV type-specific LAMP method [4]. To avoid contamination between samples, different rooms were used for DNA extraction and LAMP set up, using filter-containing pipette tips for aerosol protection.

## Results

We used both real-time PCR and a type-specific HSV LAMP method to detect HSV DNA in the 69 previously tested CSF samples. The results of real-time PCR analysis were completely in accordance with those of the previously conducted nested PCR assay. On the other hand, 5 samples that were positive by nested PCR were negative by LAMP, although there were no false positive results (Table 1). When nested PCR was regarded as standard, the sensitivity of LAMP was 81%, the specificity was 100%, the positive predictive value was 100%, and the negative predictive value was 90%.

**Table 1** Comparison between nested PCR and loop-mediated isothermal amplification (LAMP) techniques for detection of herpes simplex virus DNA in cerebrospinal fluid

| LAMP | Nested PCR | | |
|---|---|---|---|
| | Positive | Negative | Total |
| Positive | 21 | 0 | 21 |
| Negative | 5 | 43 | 48 |
| Total | 26 | 43 | 69 |

The samples that were positive by any test are listed in order of the number of copies of HSV DNA detected (Table 2). The copy number measured by the real-time PCR assay ranged from 13 to 3,100,000 copies/ml of CSF. All 5 LAMP-negative samples had low HSV DNA copy numbers (23 to 190 copies/ml of CSF), as determined by real-time PCR. Three of the five samples were obtained during acyclovir therapy. None of the samples that had more than 200 copies/ml of HSV DNA were negative by LAMP. The differentiation of HSV types by LAMP was in complete accordance with those of restriction fragment length polymorphism. Although 4 out of 5 LAMP-negative samples were HSV type 1, there was no statistical difference of sensitivity between type 1 and type 2. A recent study indicates that the time required for the turbidity to exceed a cut-off value is dependent of initial template DNA [19]. The time required for the turbidity in each sample is shown in Table 2. There was no correlation between the copy number measured by real-time PCR and the required time by LAMP.

## Discussion

LAMP is a novel technique for amplification of specific DNA sequences, and has several advantages over PCR [20, 21]. First, the specificity of LAMP is high because the LAMP method uses multiple primers, recognizing six distinct sequences in the target DNA. Second, the method is both rapid and simple; only 45 min are needed to amplify the target sequences. Third, the cost of the equipment is inexpensive compared with PCR; equipment cost remains one of the major reasons why PCR diagnostics have not been more widely utilized. With these advantages, LAMP has the potential to become adopted for widespread use in hospital laboratories.

Recently, we established a LAMP-based HSV type-specific DNA amplification method and applied it to swab samples collected from patients with vesicular skin lesions and gingivostomatitis [4]. We showed that the HSV type-specific LAMP is sensitive enough for the detection of viral DNA in swab samples, which contain large quantities of HSV DNA. In this study, we applied the type-specific HSV LAMP method to detect HSV

**Table 2** Characteristics of cerebrospinal fluid samples that were positive for herpes simplex virus (HSV) DNA

| No. | Age | Sex | Diagnosis | Acyclovir therapy | HSV type | Real-time PCR (copies/ml) | LAMP | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | Type 1 | Type 2 | Time required* (min) |
| 1 | < 1 month | F | Neonatal herpes | No | 2 | 13 | – | + | 30 |
| **2** | **61 year** | **M** | **Encephalitis** | **No** | **1** | **23** | – | – | **45<** |
| 3 | 66 year | M | Encephalitis | No | 1 | 40 | + | – | 28 |
| **4** | **<1 month** | **M** | **Neonatal herpes** | **Yes** | **1** | **96** | – | – | **45<** |
| **5** | **<1 month** | **F** | **Neonatal herpes** | **Yes** | **2** | **120** | – | – | **45<** |
| **6** | **1 year** | **F** | **Encephalitis** | **Yes** | **1** | **160** | – | – | **45<** |
| **7** | **34 year** | **M** | **Encephalitis** | **No** | **1** | **190** | – | – | **45<** |
| 8 | <1 month | F | Neonatal herpes | Yes | 1 | 230 | + | – | 33 |
| 9 | 71 year | M | Encephalitis | No | 1 | 250 | + | – | 38 |
| 10 | 10 year | F | Meningitis | No | 2 | 500 | – | + | 37 |
| 11 | 15 year | M | Encephalitis | Yes | 1 | 630 | + | – | 30 |
| 12 | 15 year | M | Encephalitis | No | 1 | 830 | + | – | 26 |
| 13 | <1 month | F | Neonatal herpes | No | 2 | 1200 | – | + | 34 |
| 14 | <1 month | F | Neonatal herpes | Yes | 2 | 2100 | – | + | 33 |
| 15 | <1 month | M | Neonatal herpes | Yes | 2 | 2200 | – | + | 28 |
| 16 | <1 month | F | Neonatal herpes | Yes | 2 | 4500 | – | + | 32 |
| 17 | <1 month | F | Neonatal herpes | Yes | 2 | 5500 | – | + | 30 |
| 18 | 10 year | F | Meningitis | No | 2 | 5900 | – | + | 37 |
| 19 | 1 year | F | Encephalitis | No | 1 | 6500 | + | – | 29 |
| 20 | 34 year | M | Encephalitis | Yes | 1 | 11000 | + | – | 24 |
| 21 | <1 month | F | Neonatal herpes | No | 2 | 12000 | – | + | 36 |
| 22 | <1 month | F | Neonatal herpes | Yes | 2 | 130000 | – | + | 24 |
| 23 | <1 month | F | Neonatal herpes | No | 2 | 180000 | – | + | 27 |
| 24 | <1 month | M | Neonatal herpes | No | 2 | 240000 | – | + | 25 |
| 25 | <1 month | M | Neonatal herpes | No | 2 | 690000 | – | + | 36 |
| 26 | <1 month | M | Neonatal herpes | No | 2 | 3100000 | – | + | 30 |

Samples are listed in order of the number of copies of HSV DNA. Samples in bold print had discordant results in real-time PCR and LAMP

*LAMP* loop-mediated isothermal amplification

*Time required for the turbidity to exceed the cut-off value

DNA in CSF. The specificity and positive predictive value were very high, indicating that this method is highly reliable. HSV typing, which is important in the diagnosis of neonatal HSV infection [14, 15], was accurate with this method. However, the sensitivity of LAMP was 81%, whereas that of real-time PCR was 100%, indicating that the LAMP method is not as sensitive as real-time or nested PCR. This is probably due to the difference of sensitivity between the two methods. The real-time PCR can detect a minimum of five copies of either HSV-1 or HSV-2 DNA, while the HSV type-specific LAMP method detect a minimum of 500 copies of HSV-1 or 1000 copies of HSV-2 DNA per assay.

This study includes a considerable number of samples from neonates. We had shown previously that the viral loads in CSF were significantly higher in neonates than in older patients with HSV encephalitis [2, 10]. In neonatal HSV infection, HSV is frequently detected from the mucous membrane, even if there are no apparent vesicle lesions [23]. Swabs from throat, eye, or vesicles are widely used for the diagnosis of neonatal HSV infection. As shown in our previous experiments, the HSV type-specific LAMP is sensitive enough to detect viral DNA in swab samples [4]. Furthermore, direct amplification from swab samples is possible, which can omit DNA extraction step [4]. The type-specific HSV LAMP method may be potentially useful for the bedside diagnosis of neonatal HSV infection. Although further improvement in sensitivity is necessary for the wide spread use, this system might be applicable to diagnosis of HSV infection of the central nervous system.

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## Case Report

# A common variable immunodeficient patient who developed acute disseminated encephalomyelitis followed by the Lennox-Gastaut syndrome

Kondo M, Fukao T, Teramoto T, Kaneko H, Takahashi Y, Okamoto H, Kondo N. A common variable immunodeficient patient who developed acute disseminated encephalomyelitis followed by the Lennox-Gastaut syndrome.
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Common variable immunodeficiency (CVID) is a primary disorder characterized by impaired antibody production. CVID patients may develop recurrent infections, autoimmune disorders, and malignant lymphomas, but to our knowledge, there is no report on CVID patients who develop acute disseminated encephalomyelitis (ADEM) or the Lennox-Gastaut syndrome. We describe a 1-yr-old female CVID patient with ADEM who evolutionally manifested the Lennox-Gastaut syndrome. She was admitted with convulsions and T2-weighted magnetic resonance imaging (MRI) revealed high-intensity areas in the right temporal lobe and the left fronto-parietal region but she became conscious soon. Her serum findings showed severe hypogammaglobulinemia and a follow up MRI revealed that these areas had diminished. Consequently, she was diagnosed as having CVID with ADEM. After 5 months, she fell to having tonic and absence seizures and we diagnosed her as having the Lennox-Gastaut syndrome from electroencephalograms (EEG) and the seizure pattern. She is now 7 yr old and her tonic seizures are controlled with valproic acid, clobazam, and immunoglobulin replacement therapy which is administrated every 2 wk. It is well known that the immune and neurologic systems have a close relationship. We suspect that a genetic defect in the immune system of our patient might also be associated with the neurologic disorders of ADEM and the Lennox-Gastaut syndrome.

**Masashi Kondo[1], Toshiyuki Fukao[1], Takahide Teramoto[1], Hideo Kaneko[1], Yukitoshi Takahashi[1], Hiroyuki Okamoto[2] and Naomi Kondo[1]**
[1]Department of Pediatrics, Graduate School of Medicine, Gifu University, Gifu, [2]Department of Pediatrics, Kizawa Memorial Hospital, Minokamo, Gifu, Japan

Key words: common variable immunodeficiency; hypogammaglobulinemia; acute disseminated encephalomyelitis; magnetic resonance imaging; Lennox-Gastaut syndrome; experimental allergic encephalomyelitis; autoimmune disease; CD4; CD8

Masashi Kondo, Department of Pediatrics, Graduate School of Medicine, Gifu University, Yanagido 1-1, Gifu 501-1194, Japan
Tel.: +81-58-230-6386
Fax: +81-58-230-6387
E-mail: g2104012@guedu.cc.gifu-u.ac.jp

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Common variable immunodeficiency syndrome (CVID) is the most common symptomatic primary antibody-deficient syndrome, characterized by hypogammaglobulinemia, and most patients have recurrent pyogenic infections predominantly in the upper and lower respiratory and gastrointestinal tracts (1). CVID patients are prone to malignancies such as gastric carcinoma and lymphoma, and to autoimmune and granulomatous diseases (1). Acute disseminated encephalomyelitis (ADEM) is an inflammatory demyelinating disorder related to autoimmunity (2), the typical clinical features of which are characterized by the acute onset of multifocal neurologic signs and may include impaired consciousness, convulsions, psychotic symptoms, or behavioral change (3). This disorder is usually associated with preceding infections or vaccinations (4). Only nine patients with CVID-associated encephalomyelitis have been reported in the literature (1, 5, 6). Virus infections such as an enterovirus have been identified as the cause of encephalomyelitis in some cases, but an autoimmune central nervous system disease has also been considered in other cases (5).

To our knowledge there is no report on CVID patients who develop ADEM. In this report, we present a CVID patient with ADEM, who evolutionally manifested the Lennox-Gastaut syndrome.

## Case report

A 1-yr-old female was hospitalized in May 1998 with a fever, impaired consciousness, and right hemi-convulsions. Two weeks before admission, she suffered from otitis media with effusion and had taken antibiotics for 2 wk. There was no family history of neurologic disorders or immunodeficiency, and her developmental milestones were within the normal range until her hospitalization. On neurologic examination in the post-convulsive state, upward nystagmus and intention tremors were observed. Hematologic examination showed mild anemia [hemoglobin (Hb) 9.7 g/dl] and lymphocytosis [white blood cell (WBC) counts 11,000/mm$^3$; lymphocytes 70.8%] and biochemical analyses were not significant except for a mild elevation of the C-reactive protein (CRP; 0.68 mg/dl; control range: 0.00–0.50). The cerebrospinal fluid (CSF) was clear, with normal pressure, 10 leukocytes/mm$^3$ (mononuclear cells 8/mm$^3$; neutrophils 2/mm$^3$), glucose 88 mg/dl, and protein 24 mg/dl. Bacterial culture was negative. An electroencephalogram (EEG) showed diffuse high-voltage slow waves at the left hemisphere and spindles mainly at the right frontal region. Brain magnetic resonance imaging (MRI) depicted areas of increased signal intensity in subcortical white matter in the right temporal lobe and the left fronto-parietal region on T2-weighted images (Fig. 1). This patient became conscious soon and developed no further symptoms. A follow up EEG showed the disappearance of diffuse high-voltage slow waves on the left hemisphere. She did not receive methylprednisolone pulse therapy or high-dose immunoglobulin therapy. She was prescribed carbamazepine (CBZ), 50 mg/day, and discharged on the 11 hospital day. One month later, a follow up MRI revealed that areas of high intensity had diminished on the T2-weighted images. Retrospectively, she was diagnosed as having ADEM.

She was again admitted with a cough and a low-grade fever in July 1998 to this hospital. Hematologic and serum laboratory tests showed a WBC count of 3800/mm$^3$ with 25.0% neutrophils, 47.7% lymphocytes, 22.6% monocytes, and 3.6% eosinophils; red blood cell (RBC) count 515 × 10$^4$/mm$^3$; Hb 8.7/dl; Ht 25.4%; platelet count 170 × 10$^3$/mm$^3$; aspartate aminotransferase (AST) 17 IU/l; alanine aminotransferase (ALT) 6 IU/l; CRP 6.81 mg/dl; immunoglobulin G (IgG) 14 mg/dl (control range: 516–990); IgA 2 mg/dl (21–140); IgM 22 mg/dl (42–159); IgE, 1 IU/ml (5–30). On examination, she had mild wheezing and her cervical lymph nodes were palpable normally. She was diagnosed as having bronchitis with hypogammaglobulinemia. Retrospective examination of frozen sera from the first admission also revealed hypogammaglobulinemia (IgG 79 mg/dl; IgA 5 mg/dl; IgM 16 mg/dl). Lymphocyte surface marker data were as follows: CD3+, 78% (62–69); CD4+, 72.9% (30–40); CD8+, 5.9% (25–32); CD19+, 11% (21–28); surface IgM+, 8% (3–12); surface IgG+, 0% (1–3); surface IgA+, 0% (1–3); surface IgD+, 10% (1–10; $\kappa$+, 5% (3–8); $\lambda$+, 4% (1–5). Adenosine deaminase activity was 33.6 U/l (9–19). Lymphocyte proliferation was normal by stimulation with phytohemagglutinin (PHA; mitotic index: 261) and concanavalin A (mitotic index: 235).

She was referred to Gifu University Hospital for further immunologic examination. We analyzed the ability of IgM and IgG secretion in peripheral blood mononuclear cells (PBMCs) according to the method reported by Bryant et al. (7). However, the PBMCs derived from the patient did not secrete detectable levels of IgM and IgG (data not shown), so this patient was revealed to belong to group A CVID. Thereafter, she has received immunoglobulin replacement therapy once a month from September 1998 and has never developed a severe bacterial infection thus far.

In October 1998, she fell into a 'drowsy' state and started to have tonic seizures, upon which she was again admitted to our hospital. A brain MRI revealed no high-intensity areas on T2-weighted images and EEG recordings demonstrated a diffuse rapid rhythm in the sleeping state. She had myoclonic, tonic and absence seizures and was diagnosed as having the Lennox-Gastaut syndrome. Valproic acid (VPA) was added to CBZ, but the seizures occurred frequently and developmental delay became more evident.. Several combinations of antiepileptic drugs (zonisamide, phenytoin), methylprednisolone pulse therapy, and high-dose immunoglobulin therapy (400 mg/kg/day) could not control her seizures. From July 2000, a new combination of immunoglobulin replacement therapy (every 2 wk), VPA and clobazam, reduced the seizure frequency. She is now 7 yr old and her tonic seizures are under control, but her psychomotor developmental age is about 3 yr old.

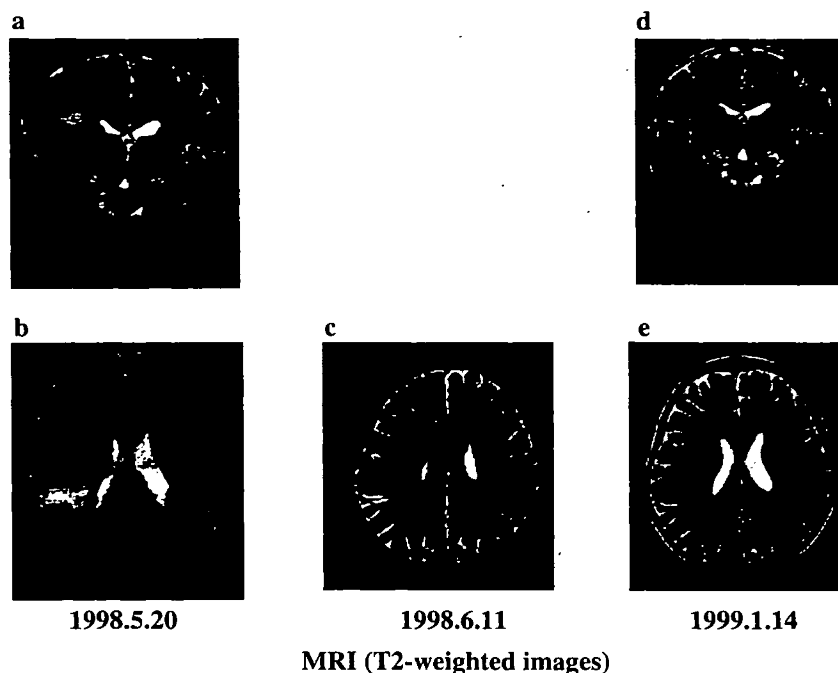


*Fig. 1.* (a and b) Non-contrast axial and coronal T2-weighted magnetic resonance imaging (MRI), obtained after first convulsions, demonstrates high intensity in subcortical white matter in the right temporal lobe and left fronto-parietal region. (c) Axial T2-weighted MRI at 4 wk after admission; high-intensity areas had almost disappeared. (d and e) Axial and coronal T2-weighted MRI at 6 months after first admission, when diagnosed as having the Lennox-Gastaut syndrome; abnormal areas had improved.

## Discussion

This CVID patient developed ADEM followed by the Lennox-Gastaut syndrome. CVID is the most common symptomatic primary antibody-deficient syndrome and has a heterogeneous etiology (8). In most cases, the gene defects responsible for CVID are unknown. The characteristic immunologic features in this patient are (i) decreased serum IgM, IgG, and IgA, (ii) normal number of surface IgM- and IgD-positive B cells, (iii) no IgG and IgM secretion from B cells stimulated with CD40 and interleukin (IL)-4, (iv) decreased number of CD8+ lymphocytes, and (v) normal natural killer (NK) cell activity. We have been investigating the defects in this patient at the molecular level but have failed to identify the cause thus far.

It is known that approximately 20% of patients with CVID develop one or more autoimmune diseases such as autoimmune hemolytic anemia, autoimmune thrombocytopenia, rheumatoid arthritis, and pernicious anemia (1), indicating that CVID is a disease of abnormal immunity regulation as well as an immunodeficiency. This patient developed ADEM, which is an inflammatory demyelinating disorder related to autoimmunity (4), so it is possible that CVID patients develop ADEM. However, to our knowledge, there is no report on ADEM in CVID patients. ADEM is believed to be the clinical counterpart of experimental allergic encephalomyelitis (EAE) (9). CD4+ myelin-specific T cells induced EAE (10). The role of CD8+ T lymphocytes has been described as having both effector and suppressor functions in the pathogenesis of EAE (11). This CVID patient has the peculiar feature of an increased ratio of CD4/CD8 and a reduced number of CD8+ T lymphocytes. This unbalance of the T-cell subpopulation may be associated with the development of ADEM in this patient.

The diagnosis of ADEM in our patient depended on MRI (2). Although the clinical symptoms disappeared without steroid or immunoglobulin therapy, asymmetrical and multiple T2 high lesions in subcortical white matter were noted when she developed the first convulsive state and then subsequently disappeared 4 wk later. This finding highly supports the diagnosis of ADEM.

The Lennox-Gastaut syndrome is characterized by tonic seizures associated with myoclonic seizures, atypical absence seizures, rapid rhythm in the sleeping state, and cognitive impairment (12). The etiologies of this syndrome have been divided into symptomatic and cryptogenic types, the former being attributed to brain disorders such as meningitis, encephalitis, head injury, and cerebrovascular diseases (13). ADEM might be etiologically related to the Lennox-Gastaut syndrome in our patient, as ADEM sometimes involves not only white matter but also gray matter (2). However, to our knowledge, the Lennox-Gastaut syndrome has not been reported as a sequela of ADEM.

The CVID is a syndrome caused by heterogeneous genetic defects. It is well known that the immune and neurologic systems have a close relationship. We suppose that the genetic defect in our patient might also be associated with the neurologic disorders of ADEM and the Lennox-Gastaut syndrome. It is of interest to identify the gene defect in this patient.

## References


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