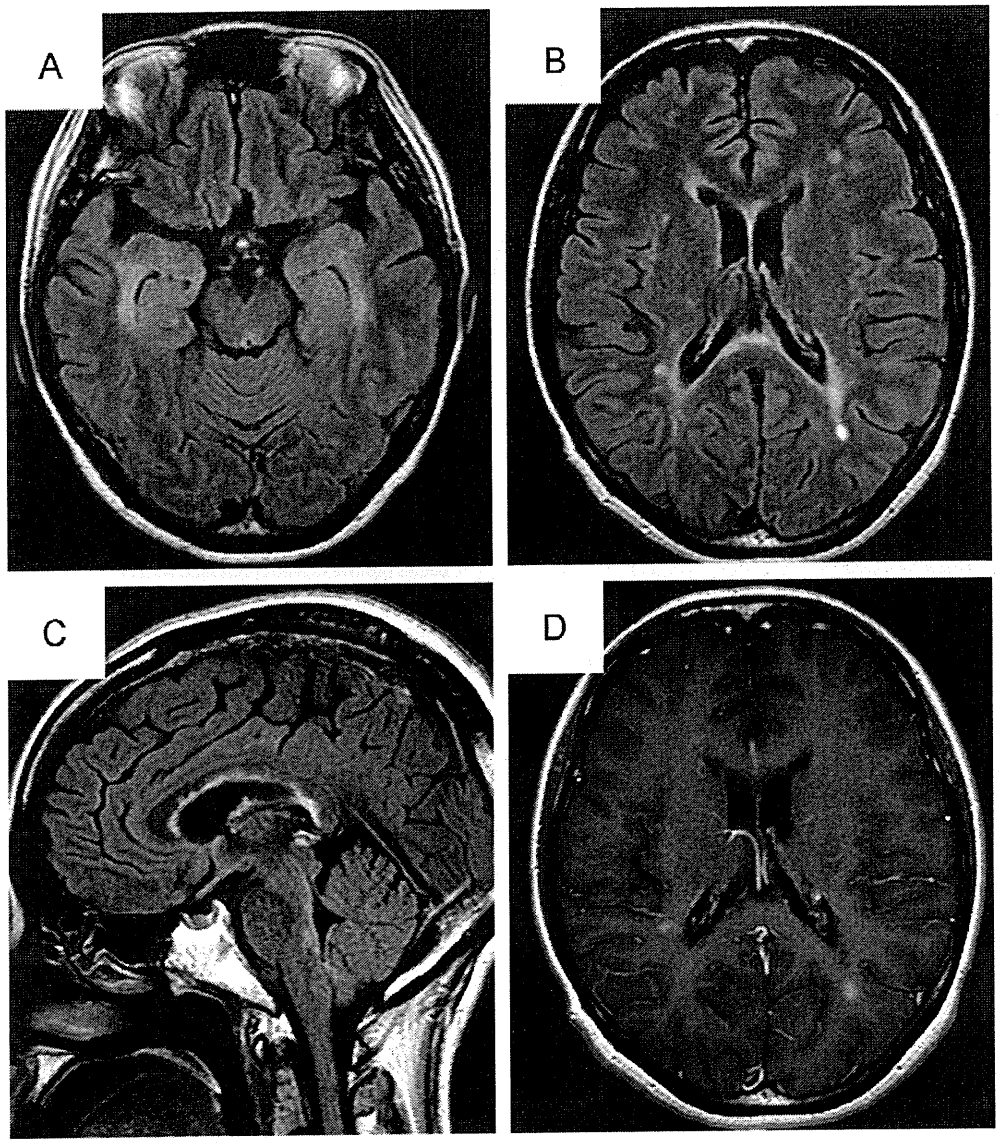

Fig. 1. Brain MRI performed on admission showing a high-intensity lesion on FLAIR images of the bilateral medial temporal lobes (A); some MS plaques (ovoid lesions) are noted around the lateral ventricle (B and C); an image obtained with gadolinium enhancement (D) is also shown.

3. Literature review

The coexistence of ON or neuromyelitis optica (NMO) with anti-NMDAR or anti-GluR antibody positive encephalitis has attracted attention recently. Four previously reported cases [2–5] and the present case are summarized in Table 1. Among these 5 patients, 2 were diagnosed with NMO, 2 with ON and 1 with MS. All 5 patients were female, and this predominance fits the clinical characteristics of their respective diseases. In all cases, anti-NMDAR or anti-GluR antibodies were detected in the CSF. The symptoms of the 3 patients with ON or MS preceded the development of anti-NMDAR encephalitis. Interestingly, no patient developed tumours and showed poor clinical prognoses.

4. Discussion

The early clinical course of this patient was typical of MS and the later course was compatible with anti-NMDAR encephalitis. The patient's fulminant neuropsychiatric manifestations and seizures, including the lesions that were present in the bilateral medial temporal lobes on MRI and positivity for the anti-GluRε2 antibody in the CSF, are atypical of MS, which encouraged the diagnosis of anti-NMDAR encephalitis with overlapping MS. GluRε2 (NR2B) is a subunit of NMDAR that is predominantly expressed in the hippocampus and forebrain and is involved in memory function. The mild cognitive impairment of the present case appears to have been caused by lesions in these areas.

Antibodies against NR1/NR2B heteromers are specific to NMDAR-associated encephalitis, with or without ovarian teratoma, whereas GluRε2 antibodies are also found in some other disorders, such as Rasmussen's encephalitis and progressive epilepsia partialis continua, and may lack syndrome specificity. Hence, the detection of anti-GluRε2 antibodies in the present patient's CSF may be related to her epilepsy or the destruction of her central nervous system. However, an activated autoimmune system in such patients may be related to the production of anti-NMDA antibodies; previous cases [4,5] and present case have manifested ON or

Table 1
Characteristics of patients with presenting with NMO, ON or MS with anti-NMDA or anti-GluR receptor antibody positive encephalitis.

Authors	Age/sex	Disease	Onset	Anti-NMDAR Ab in CSF	Epilepsy	NMO-IgG/ anti-AQP4 Ab	Spinal lesion	Optic nerve lesion	Tumour	Prognosis
Honda [2]	39 years/F	NMOSD	NA	Anti-GluRε2	Absent	Anti-AQP4 Ab (+)	+ (>3VL)	–	–	Good
Kruer [3]	15 years/F	NMO	NMDA → NMO	Anti-NMDAR	Present	NMO-IgG (–)	+ (>3VL)	+	–	Good
Motoyama [4]	10 years/F	ON	ON → NMDA	Anti-NMDAR	Present	Anti-AQP4 Ab (–)	–	+	NA	Good
Ishikawa [5]	12 years/F	ON	ON → NMDA	Anti-GluRε2	Present	NA	–	+	NA	Good
Our case	34 years/F	MS	MS → NMDA	Anti-GluRε2	Present	Anti-AQP4 Ab (–)	+ (<3VL)	+	–	Good

NMO: neuromyelitis optica; NMOSD: neuromyelitis optica spectrum disorder; MS: multiple sclerosis; ON: optic neuritis; NMDAR: *N*-methyl D-aspartate receptor; GluR: glutamate receptor; Ab: antibody; AQP4: aquaporin 4; NA: not available; VL: vertebral segments in length.

MS before the development of anti-NMDAR encephalitis. A previous review reported that 59% of anti-NMDAR encephalitis patients have tumours and 36% of patients without tumours showed severe deficits or died [1]. Most noteworthy were the facts that all reported cases [2–5] and present case who presented with ON, NMO or MS with anti-NMDAR encephalitis did not present with tumours, and that those cases demonstrated good recoveries. Positive outcomes may be possible following the use of intensive immune-modulating therapies.

5. Conclusion

We reported the first case of a patient who developed anti-NMDA glutamate receptor antibody-positive encephalitis with good recovery during the course of MS. There may be a possible linkage between these diseases, and concurrent autoimmune responses may be important for the development of autoimmune encephalitis. Anti-NMDAR encephalitis should be recognized as a rare manifestation that can occur in MS patients who develop psychiatric symptoms and seizures. However, further investigation of patients with related disorders and analysis is needed.

References

[1] Dalmau J, Gleichman AJ, Hughes EG, Rossi JE, Peng X, Lai M, et al. Anti-NMDA-receptor encephalitis: case series and analysis of the effects of antibodies. Lancet Neurol 2008;7:1091–8.
[2] Honda K, Yuasa T. A case of anti-aquaporin-4 and anti-glutamate receptor antibodies positive myelitis presented with modest clinical signs. Magn Reson Med Sci 2008;7:55–8.
[3] Kruer MC, Koch TK, Bourdette DN, Chabas D, Waubant E, Mueller S, et al. NMDA receptor encephalitis mimicking seronegative neuromyelitis optica. Neurology 2010;74:1473–5.
[4] Motoyama R, Shiraishi K, Tanaka K, Kinoshita M, Tanaka M. Anti-NMDA receptor antibody encephalitis with recurrent optic neuritis and epilepsy. Rinsho Shinkeigaku (in Japanese) 2010;50:585–8.
[5] Ishikawa N, Tajima G, Hyodo S, Takahashi Y, Kobayashi M. Detection of autoantibodies against NMDA-type glutamate receptor in a patient with recurrent optic neuritis and transient cerebral lesions. Neuropediatrics 2007;38:257–60.

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Case report

Anti-glutamate receptor δ2 antibody-positive migrating focal encephalitis

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1. Introduction

In recent years, increasing attention has been given to acute and subacute encephalitis related to glutamate receptors (GluR). GluRs are involved in excitatory neurotransmission on cell membrane surfaces of the spinous processes in dendrites of mammalian central nervous systems. Abnormal activity of GluR channels is thought to contribute to the neuronal death observed in acute and chronic encephalitis. GluRs have two types: ionotropic and metabotropic (Table 1). Ionotropic GluRs are classified pharmacologically as N-methyl-D-aspartate (NMDA)-type, non-NMDA-type (alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid {AMPA}-type, kainate-type), and δ-type with unknown ligands. NMDA-type GluR ε2 antibody is frequently found in acute limbic encephalitis or widespread encephalitis [1]. Moreover, NMDA receptor-related encephalitis is well known to be associated with ovarian teratoma. Much remains unknown regarding the pharmacological properties of the GluR δ2 subunit that is localized in the forebrain after birth, but it is known to be selectively expressed in cerebellar Purkinje cells.

Several recent reports [2–5] have revealed anti-GluR δ2 antibodies in the serum and cerebrospinal fluid of patients with autoimmune encephalitis.

We report herein a rare case involving a 42-year-old woman with anti-glutamate receptor δ2 antibody-positive encephalitis and migrating focal lesions mainly in the cerebral cortex.

2. Case report

A 42-year-old woman presented with loss of consciousness and convulsions after catching a cold. She was examined by a family physician the following day. No obvious abnormalities were apparent on brain computed tomography, but magnetic resonance imaging (MRI) with fluid-attenuated inversion recovery (FLAIR) showed high-intensity areas in bilateral frontal cortices, the left parietal cortex, and the cortex and subcortex of bilateral occipital lobes, and she was referred to our hospital for emergency hospitalization. On admission, body temperature was 37.4 °C and she was in a stupor. No cranial nerve or motor system abnormalities were identified, and nuchal rigidity was identified.

Blood tests showed a leukocyte count of 11,810/μl (neutrophils, 90.0%) and C-reactive protein level was 1.28 mg/dL. Thyroid function was normal, free T_3, 2.23 pg/ml, free T_4, 1.34 ng/dl, TSH, 097 μIU/ml. Anti-thyroglobulin antibody-RIA was ≤0.3 U/ml, Anti-thyroid receptor antibody <1.0 IU/ml, Antinuclear antibody <5 I.C., Anti-cardiolipin antibody IgG ≤8.0 U/ml, NSE-RIA 6.9 ng/ml, Anti-aquaporin 4 antibody, anti-Hu antibody, anti-Yo antibody were negative. Tumor markers did not show any abnormalities (CEA; 2.6 ng/ml, AFP 4.5 ng/ml, CA19-9 17.9 U/ml). Immune system findings showed an activated immune status. Immunoglobulin (Ig)M was mildly elevated to 275 mg/dl and among peripheral blood cells, the T lymphocyte subset of CD4/interleukin-2 receptor antibody-positive cells comprised

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Table 1
Subunit of glutamate receptor channel.

Subtype	Subfamily	Subunit
AMPA type	GluR1-4(GluRα)	GluR1(GluRA, GluRα1) GluR2(GluRB, GluRα2) GluR3(GluRC) GluR4(GluRD)
Kainic acid type	GluR5-7(GluRβ)	GluR5 GluR6(GluRβ2) GluR7
	KA1, 2	KI 1 KI 2(GluRγ2)
δ type	GluRδ	GluRδ1 GluRδ2
NMDA type	GluRε	GluRε1 GluRε2 GluRε3 GluRε4
	NR1(GluRζ1) GluRχ	NMDAR1(GluRζ1, NR1) GluRχ(NR3A) NR3B

AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate; KA, kainic acid, GluR, glutamate receptors, NMDA, N-methyl-D-aspartate.

23.1% (normal: 6.3–19.5%), CD4/CD29-positive cells comprised 27.3% (9.0–27.2%), and suppressor–effector cells comprised 1.02% (1.3–9.42%). Cerebrospinal fluid (CSF) examination showed elevated protein levels (61 mg/dl), and polynuclear cell-dominant cytosis (cell count, 54/μl; mononuclear cells, 12/μl; polynuclear cells, 42/μl), but glucose level was normal (72 mg/dl). Herpes simplex virus IgG 0.3 EIA, EB virus anti-EBNA IgG antibody <1 C.I, EB virus anti-VCA IgM antibody <1 C.I. Antibody titers against herpes simplex virus and Epstein–Barr virus in cerebrospinal fluid were negative. Oligoclonal bands was negative. All cultures of blood and cerebrospinal fluid likewise yielded negative results. Results for anti-GluR IgG-δ2 antibody were positive in serum, but negative results were obtained for IgG-ε2, IgM-ε2, and IgM-δ2 subtypes. Blood and CSF levels of lactate were normal. Whole body CT including enhanced did not show any abnormality.

Brain MRI findings on admission are shown in Fig. 1. High-intensity areas on T2-weighting image, FLAIR, and (apparent diffusion coefficient) ADC maps, iso- or hyperintense areas on diffusion-weighted imaging (DWI), and hypointense areas on T1-weighted imaging were seen in bilateral frontal and parietal lobes and the left occipital lobe. No gadolinium enhancement was seen. Meningoencephalitis was suspected at the time of admission, and

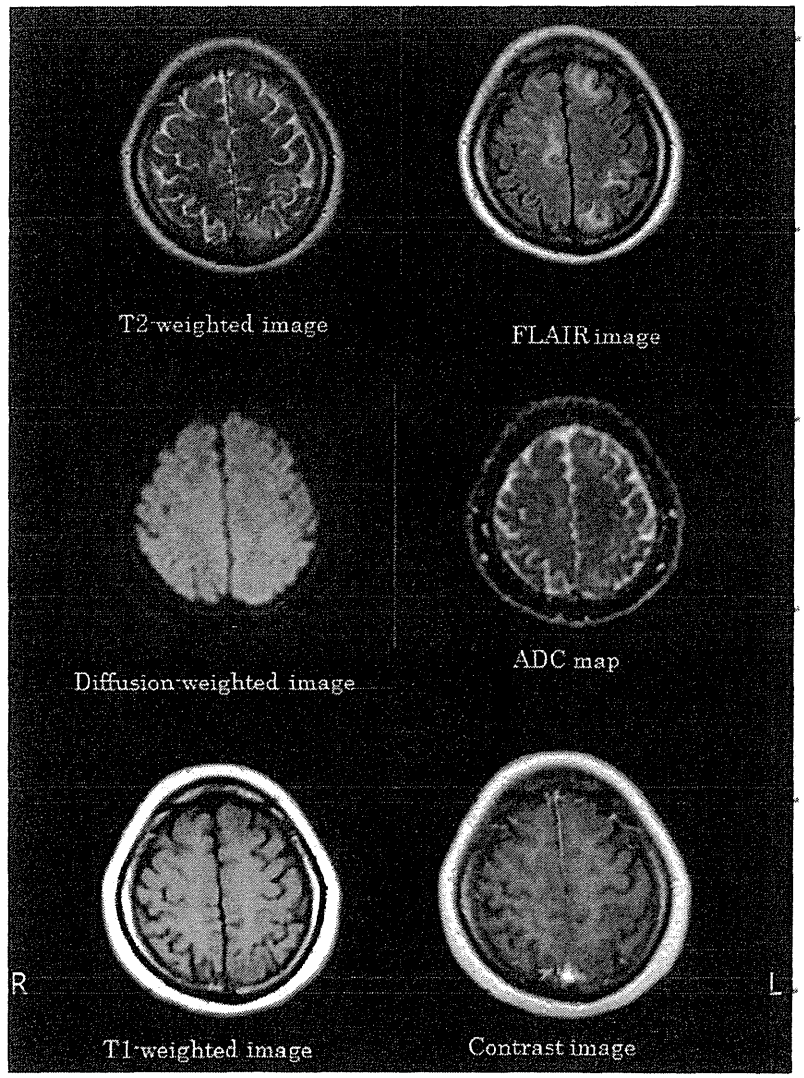

Fig. 1. Brain MRI on admission: bilateral cortex of the frontal, parietal and left occipital lobe showed high-intensity areas on T2-weighted, FLAIR and ADC images, iso- or hyperintense areas on DWI, but low-intensity areas o T1-weighted image. In contrast image, lesion was not enhanced.

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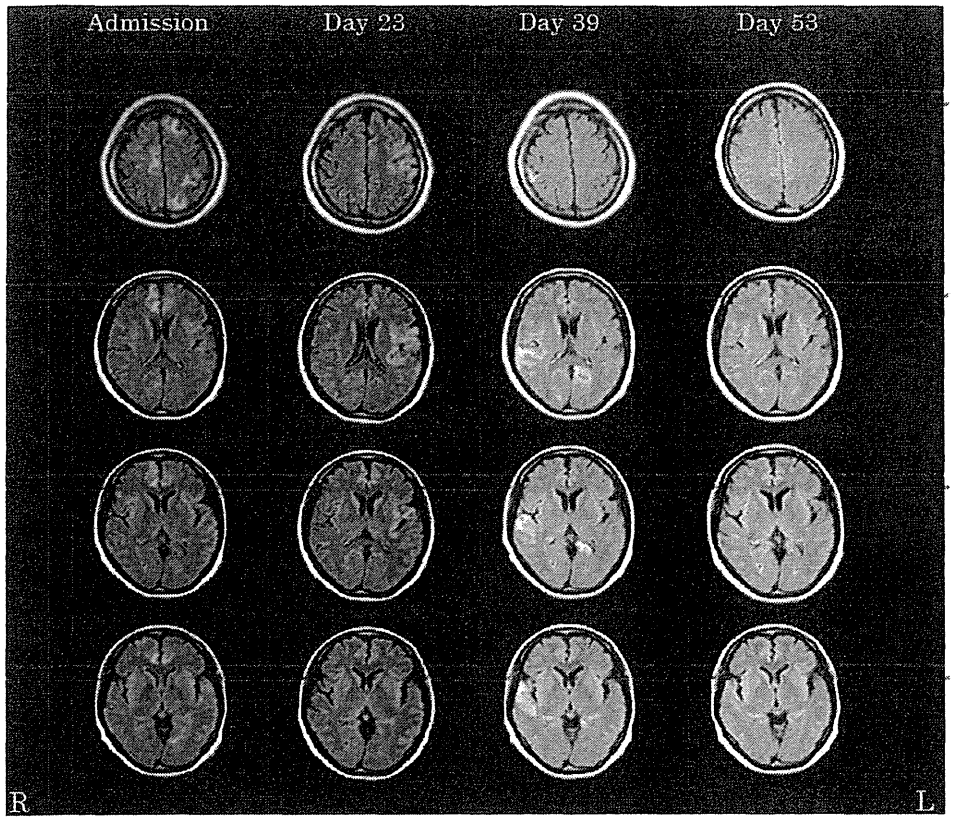

Fig. 2. Fluid-attenuated inversion recovery (FLAIR) MRI of the brain. High-intensity areas showed various changes with time after onset.

antibiotics (panipenem/betamiprom, 3 g/day), an antiviral agent (acyclovir, 1500 mg/day), and an antiedematous agent for cerebral edema (glycerol, 800 ml/day) were administered. Steroid pulse therapy (methylprednisolone 1000 mg/day) was performed for 5 days, followed by oral prednisolone at 40 mg/day. On day 2, the patient regained consciousnous. She was performed the neuropsychological test during day 7. Mini-mental state examination (MMSE) yielded a score of 27/30, digit span was 6 digits forward and 5 digits backward and Frontal Assessment Battery was 18/18. Cognitive behavior testing yielded a high score of 32 on the Frontal

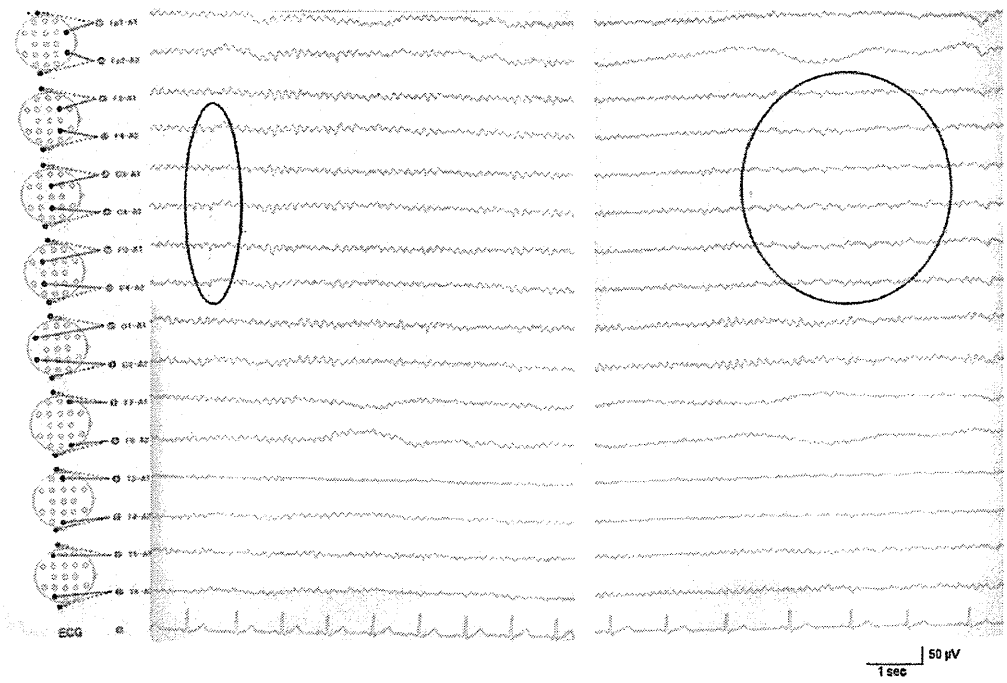

Fig. 3. Paroxysms and slow waves were seen with left dominance in the central and parietal regions.

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Behavioral Inventory. Chronological changes on MRI FLAIR images are shown in Fig. 2. Paroxysms and slow waves were seen with left dominance in the central and parietal regions on Day 8 (Fig. 3). Ovarian teratoma was not found by pelvic MRI.

On Day 23, when the dose of prednisolone was tapered to 20 mg/day, motor aphasia and right-sided hemiplegia occurred for about 30 min. MRI revealed new lesions showing hyperintense areas in the left temporal and frontoparietal lobes on FLAIR imaging. Prednisolone was therefore increased again to 40 mg/day. No neurological symptoms appeared in the subsequent course. On Day 32, those lesions had disappeared, and new lesions had appeared in the left occipital and frontal lobes. On Day 39, when the dose was 20 mg/day, new lesions in the right temporal and left occipital areas were seen on MRI, and the dose of prednisolone was again increased to 40 mg/day and tapered gradually. On Day 53, MRI findings had nearly normalized, and normal results were also obtained on magnetic resonance angiography and magnetic resonance venography following this increase in prednisolone dose. At 34 months later, no neurological symptoms have appeared and there have been no abnormal MRI findings.

3. Discussion

Characteristics of the present case were as follows: (1) clinical features of meningoencephalitis and CSF pleocytosis; (2) localized cortical lesions were frequently seen in various sites over time on MRI; (3) both clinical symptoms and MRI lesions responded well to steroid therapy; and (4) positive results were obtained for anti-GluR δ2 antibody in the serum.

From the perspective of clinical feature and MRI findings, differential diagnoses included multiple sclerosis, acute disseminated encephalomyelitis (ADEM), posterior reversible leucoencephalopathy syndrome (PRES), Hashimoto's encephalopathy, and mitochondrial encephalopathies. In the present case, the lesions were mainly in the cortex, in contrast to the white matter lesions seen in those disorders. Hashimoto's encephalopathy was also thought to be unlikely due to negative findings for anti-thyroid antibody. Mitochondrial encephalopathies were ruled out based on lactate levels.

Anti-GluR δ2 antibody in the serum and CSF has occasionally been detected in patients with acute cerebellitis or acute limbic encephalitis [2,4]. However, Tomioka et al. [4] and Hirano et al. [5] presented two cases of anti-GluR δ2 antibody-positive autoimmune encephalitis with cerebral cortical lesions. The case described by Tomioka et al. [4] involved a 16-year-old boy who showed meningitis-like symptoms and seizures. Brain MRI showed only mild hyperintensity in the parietal cortex on FLAIR and clinical symptoms were monophasic. The case described by Hirano et al. [5] involved a 17-year-old boy who presented with right hemiplegia, aphasia, and seizures. Brain MRI showed a hyperintense lesion in the left parietal cortex on T2-weighted imaging. That case responded to steroids and showed repeated episodes of encephalitis without new lesions on MRI. Our case resembles these in terms of cerebral cortical lesions. However, the migration of cerebral cortical lesions over time as verified by MRI represents a highly unusual finding.

Cortical lesions in the present case were considered to represent extracellular edema based on MRI findings. Although the role of anti-GluR δ2 antibody in the pathogenesis of this autoimmune migrating cortical-dominant localized encephalitis is unclear, cellular immunity that had been activated as a result of an antecedent infection was thought to have caused a cross-reaction in the central nervous system, giving rise to autoimmune encephalitis as a result of anti-GluR antibody production and cytokine release. It is unclear whether anti-GluR antibodies themselves were involved in the encephalitis or whether antibodies were produced as a result of cell destruction from encephalitis.

Conflict of interest

The authors have no conflicts of interest to report.

References

[1] Takahashi Y, Mori H, Mishina M, Watanabe M, Kondo N, Shimomura J, et al. Autoantibodies and cell-mediated autoimmunity to NMDA-type GluRepsilon2 in patients with Rasmussen's encephalitis and chronic progressive epilepsia partialis continua. Epilepsia 2005;46(Suppl. 5):152–8.
[2] Hayashi Y, Matsuyama Z, Takahashi Y, Wakida K, Hashizume T, Kimura A, et al. A case of non-herpetic acute encephalitis with autoantibodies for ionotropic glutamate receptor delta2 and epsilon2. Clinical Neurology 2005;45:657–62.
[3] Shimokaze T, Kato M, Yoshimura Y, Takahashi Y, Hayasaka K. A case of acute cerebellitis accompanied by autoantibodies against glutamate receptor delta2. Brain and Development 2007;29:224–6.
[4] Tomioka S, Shimono M, Kato A, Takano K, Shiota N, Takahashi Y. A 16-year-old boy with meningoencephalitis with auto-antibody against glutamate receptor. No To Hattatsu 2008;40:42–6 (in Japanese).
[5] Hirano K, Aiba H, Yano M, Watanabe S, Okumura Y, Takahashi Y. Effect of tacrolimus in a case of autoimmune encephalitis. No To Hattatsu 2007;39:436–9 (in Japanese).

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FULL-LENGTH ORIGINAL RESEARCH

Efficacy of stiripentol in hyperthermia-induced seizures in a mouse model of Dravet syndrome

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Summary

Purpose: We previously reported a mutant mouse carrying a severe myoclonic epilepsy in infancy (*SMEI*) mutation in *Scn1a*. In this study, we examined the susceptibility to hyperthermia-induced seizures of heterozygous *Scn1a* mutant mice ($Scn1a^{RX/+}$) and wild-type ($Scn1a^{+/+}$) mice. Then we assessed the efficacy of stiripentol (STP) monotherapy versus STP and clobazam (CLB) combination therapy to prevent hyperthermia-induced seizures in $Scn1a^{RX/+}$ mice.

Methods: The seizure-inducing body temperatures in $Scn1a^{RX/+}$ mice and age-matched $Scn1a^{+/+}$ mice were compared in three age groups (1 month, 3–5 months, > 6 months). Then STP, CLB, or STP + CLB was administered intraperitoneally to $Scn1a^{RX/+}$ mice of two age groups (p1M, aged 1 month; p5M, aged 5–10 months). The efficacy of medications was assessed by comparing the seizure-inducing body temperature and the duration of seizures.

Key Findings: The seizure-inducing body temperature was significantly lower in $Scn1a^{RX/+}$ than in $Scn1a^{+/+}$ mice for all age groups ($p < 0.01$). The seizure-inducing body temperature was significantly elevated after administration of STP in p1M ($p < 0.05$) but not in p5M ($p > 0.05$), and it was significantly elevated after administration of CLB in both age groups ($p < 0.05$). The seizure-inducing body temperature was significantly higher after administration of STP + CLB than after administration of CLB in p5M ($p < 0.05$).

Significance: $Scn1a^{RX/+}$ mice have increased susceptibility to hyperthermia-induced seizure in all age groups. STP monotherapy is effective in preventing hyperthermia-induced seizures in $Scn1a^{RX/+}$ mice aged 1 month, but not in those aged 5 months and older. When used in combination therapy with CLB, STP inhibits the metabolism of CLB and probably synergistically enhances the anticonvulsant effect in mice aged 1 month.

KEY WORDS: Stiripentol, Severe myoclonic epilepsy in infancy, Mouse model, Clobazam, Efficacy.

Severe myoclonic epilepsy in infancy (SMEI), or Dravet syndrome, is one of the most severe forms of epileptic encephalopathy. It is characterized by fever-sensitive and refractory generalized clonic or tonic–clonic seizures or unilateral seizures beginning in the first year of life. De novo mutation in the neuronal voltage-gated sodium-channel α subunit type 1 (*SCN1A*) gene is a major cause of this syndrome (Claes et al., 2003).

We succeeded to produce mice carrying an *Scn1a* gene mutation (Ogiwara et al., 2007). This knock-in mouse line carries a premature stop codon R1407X in exon 21 of mouse *Scn1a*, which is identical to the pathogenic mutation found in three unrelated patients with SMEI. Because SMEI patients and *Scn1a* knockout (KO) mice (Oakley et al., 2009) have fever-sensitive seizures, our first aim was to confirm the increased susceptibility to hyperthermia-induced seizures in this mouse model.

The seizures of Dravet syndrome are known to be resistant to therapy (Dravet et al., 2005). In general, conventional treatment is disappointing, and introduction of newer antiepileptic drugs (AEDs) including stiripentol (STP) has been recommended (Chiron et al., 2000; Ceulemans et al., 2004; Chiron, 2005). STP is an AED derived from phenyl-1-pentanol, which was first described in 1978 (Astoin et al., 1978). STP has been shown to efficiently reduce the metabolic degradation of several cytochrome P450 (CYP)–sensitive AEDs (Finnell et al., 1995; Levy et al., 1984, 1990). Accordingly, clinical studies have highlighted the usefulness of STP as adjunctive therapy in pediatric epilepsies, notably in Dravet syndrome (Perez et al., 1999; Chiron et al., 2000; Cazali et al., 2003). The striking efficacy of STP has raised the possibility that it may have a direct antiepileptic action. Some data recently showed that STP itself

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[1]These two authors contributed equally to this work.

enhances central γ-aminobutyric acid (GABA) transmission (Quilichini et al., 2006; Fisher, 2009). However, we are not able to perform clinical trials to verify the efficacy of STP monotherapy for Dravet syndrome because of limitation of licensed indication. In this animal study, we examined the efficacy of STP when given as monotherapy and in combination therapy in a mouse model.

Methods

All experiments were performed in accordance with the animal experiment committees of Shizuoka Institute of Epilepsy and Neurological Disorders and RIKEN Brain Science Institute.

Generation of the mice carrying an *Scn1a* gene mutation

A mutant mouse line carrying a nonsense mutation, R1407X, in *Scn1a* has been described previously (Ogiwara et al., 2007). This RX mutation is located within a loop between segments 5 and 6 of domain III and could lead to production of truncated mutant $Na_v1.1$ missing segment 6 of domain III, domain IV, and C-terminus. All experiments with heterozygous ($Scn1a^{RX/+}$) mice were done on a C57BL/6 genetic background. Study animals were generated by breeding $Scn1a^{RX/+}$ males with wild-type (WT) ($Scn1a^{+/+}$) females. The $Scn1a^{RX/+}$mice were distinguished by genotyping using a 4-oligonucleotide multiplex polymerase chain reaction (PCR) of genomic DNA samples isolated from mouse tails. PCR was performed with 1–100 ng of total genomic DNA, 0.2 mM deoxyribonucleotide triphosphates (dNTPs), and 2.5 U of Blend Taq-plus polymerase (Toyobo, Osaka, Japan) under the following conditions: 40 cycles of 94°C for 30 s and 60°C for 1 min. The nucleotide sequences of the 5′, RX, and WT primers were 5-ATGATTCCTAGGGGGATGTC-3, 5-TTTACTTTCACATTTTTCCATCA-3, and 5-CTTTCACATTTTTCCACCG-3, respectively.

$Scn1a^{RX/+}$ mice were maintained on a C57BL/6J background (Japan SLC, Hamamatsu, Japan). We used $Scn1a^{RX/+}$ mice (n = 28) and age-matched WT mice ($Scn1a^{+/+}$; n = 27) for the analysis. Mice were kept on a 12 h light/dark schedule with unlimited food and water.

Procedure of seizure induction and recording of electroencephalography

Procedures to provoke hyperthermia-induced seizures, modified from Oakley et al., 2009, were used. To elevate the body temperature, each mouse was put into a small box and heated by a hot plate from below and by electric light bulb from above. Rectal temperature was measured with a temperature probe (Nihon Kohden, Tokyo, Japan) at baseline and at seizure onset. We gradually elevated the body temperature by 0.1°C per 10 s. Each seizure was identified by EEG recording. When a seizure was induced, or when the rectal temperature reached 45°C, the heating bulb was turned off and the mouse was promptly put into another hyaline box with a room temperature environment. After the seizure stopped, the mouse was rescued by cooling with ice cubes. Video and electroencephalography (EEG) were recorded simultaneously when the heating procedure began. The body temperature threshold at which seizure was induced and the duration of seizure were recorded. EEG electrodes were implanted ≥24 h before first seizure was induced under isoflurane anesthesia with oxygen ventilation. Stainless-steel screws (1.1 mm in diameter) served as EEG electrodes, and were secured to the skull and dura over the right somatosensory cortex (1.5 mm lateral to midline, 1.0 mm posterior to bregma) and at the cerebellum (at midline, 2.0 mm posterior to lambda) as reference electrode.

Confirmation of the increased susceptibility to hyperthermia-induced seizures

We used $Scn1a^{RX/+}$ mice (n = 28) and age-matched $Scn1a^{+/+}$ mice (n = 27) for this analysis. We divided these mice into three groups according to age: 1, 3–5 months, and over 6 months. We induced seizure and recorded the body temperature threshold in each group.

To confirm the reproducibility of the hyperthermia-induced seizure of $Scn1a^{RX/+}$ mice, we induced seizure again after 24–48 h from the first induced seizure (n = 17, including all age groups). We compared each seizure-inducing body temperature (first seizure: BT_1; second seizure: BT_2) and duration of seizure (first seizure: D_1; second seizure: D_2).

Administration of STP and clobazam (CLB)

Two age groups, p1M (n = 18, aged 4 weeks) and p5M (n = 18, aged 5–10 months), of $Scn1a^{RX/+}$ mice were assigned in this experiment. Both groups were divided randomly into three subgroups (n = 6), and each subgroup was administered STP (300 mg/kg) alone, CLB (6.62 mg/kg) alone, or a combination of STP (p1M; 150 mg/kg, p5M; 300 mg/kg) and CLB (6.62 mg/kg). The dosages of STP and CLB were set according to previous studies (Shen et al., 1990; Trojnar et al., 2005; Nakajima, 2001). When STP (300 mg/kg) combined with CLB was administered to four $Scn1a^{RX/+}$ mice aged 1 month, all mice developed severe hypothermia (around 20°C) and died within 1 h. The reason could be that 1-month-old mice could not tolerate the high concentration of STP and CLB or that the intraperitoneal space was too narrow and the intraperitoneal contents compressed the thorax and obstructed breathing. Therefore, we reduced the dose of STP to 150 mg/kg in combination therapy in these younger mice. We induced seizures before administration of medication to determine the pretreatment seizure-inducing body temperature (BT_1) and duration of seizures (D_1) as baseline data. All drugs were administered by intraperitoneal injection (i.p.) after a 48-h recovery from baseline seizure study. STP was suspended in saline solution (0.9% NaCl) containing 1% Tween 80 (v/v) and CLB was

suspended in saline solution (0.9% NaCl). We induced seizure again at 1 h after administration of medication to determine the posttreatment seizure-inducing body temperature (BT_2) and duration of seizures (D_2). Blood samples were collected at 1 h and 20 min after administration of CLB or STP + CLB for measurement of plasma concentrations of CLB and *N*-desmethylclobazam (NCLB, a metabolite of CLB) (p1M; n = 5, p5M; n = 6, respectively). In p5M mice, blood samples were equally collected after administration of STP for measurement of plasma concentration of STP (n = 6).

Statistical analysis

Data are expressed as mean ± standard deviation (SD). Differences were considered statistically significant if p-value was <0.05 using paired or unpaired Student's *t*-test. The *t* value is noted when there is a statistically significant difference.

Results

Confirmation of increased and sustained susceptibility to hyperthermia-induced seizures in *Scn1a*$^{RX/+}$ mice

Scn1a$^{RX/+}$ mice manifested clonic tonic–clonic or clonic seizures as body temperature was elevated and all mice manifested seizures at body temperatures below 45°C, whereas approximately 20% (5/27) of *Scn1a*$^{+/+}$ mice did not manifest seizures even when body temperature was elevated to 45°C. The data of the mice that did not experience seizure were excluded from average value. The seizure-inducing body temperature of *Scn1a*$^{RX/+}$ mice was significantly lower than that of *Scn1a*$^{+/+}$ mice in each age group (Table 1).

Ictal EEG patterns of *Scn1a*$^{RX/+}$ mice and those of *Scn1a*$^{+/+}$ mice were similar, and composed of continuous spike-waves (Fig. 1). Seizure durations tended to be longer in *Scn1a*$^{RX/+}$ mice than in *Scn1a*$^{+/+}$ mice in all groups, but the differences did not reach statistical significance (Table 1). Approximately 90% (25 of 28) of *Scn1a*$^{RX/+}$ mice showed abnormal EEG patterns (spike, spike-waves, and/or, polyspike-waves) before seizure onset. On the other hand, no abnormal EEG patterns were observed in *Scn1a*$^{+/+}$ mice before seizure onset. In the heating process, we observed myoclonic seizures, which were confirmed by video recording accompanied by polyspike waves on EEG in approximately 60% (17 of 28) of *Scn1a*$^{RX/+}$ mice.

With regard to reproducibility of the hyperthermia-induced seizure, there was no significant difference between BT_1 (40.15 ± 0.90°C) and BT_2 (40.48 ± 0.78°C) and between D_1 (32.65 ± 14.87 s) and D_2 (37.01 ± 12.07 s).

Table 1. Body temperature at seizure onset and seizure duration in each age group

	Body temperature (°C)	Seizure duration (s)
Age 1 month		
Scn1a$^{RX/+}$ (n = 9)	40.19 ± 0.92*	31.32 ± 9.92
Scn1a$^{+/+}$	43.18 ± 0.45*	23.44 ± 9.93
Age 3–5 months		
Scn1a$^{RX/+}$ (n = 9)	40.00 ± 0.77*	36.03 ± 13.95
Scn1a$^{+/+}$	43.08 ± 0.62*	25.96 ± 11.95
Age over 6 months		
Scn1a$^{RX/+}$ (n = 10)	39.78 ± 1.07*	32.68 ± 14.89
Scn1a$^{+/+}$	43.04 ± 0.44*	32.43 ± 10.78

Values represent means ± SD.
*p < 0.05.

Assessment of efficacy of STP in monotherapy and combination therapy with CLB in *Scn1a*$^{RX/+}$ mice

p1M group

In mice treated with STP monotherapy, the difference between BT_1 (39.67 ± 1.09°C) and BT_2 (41.32 ± 1.05°C) reached statistical significance ($t = 3.097$, $p < 0.05$); In mice treated with CLB monotherapy, the difference between BT_1 (40.10 ± 1.35°C) and BT_2 (42.58 ± 0.55°C) also reached statistical significance ($t = 5.381$, $p < 0.01$) (see Table 2). The difference in BT_2 between STP monotherapy and CLB monotherapy was statistically significant ($t = 2.615$, $p < 0.05$). In mice treated with STP + CLB combination therapy, the difference between BT_1 (40.18 ± 0.58°C) and BT_2 (43.03 ± 0.49°C) reached statistical significance ($t = 10.44$, $p < 0.01$). The difference in BT_2 between CLB therapy and combination therapy did not reach statistical significance ($t = 1.49$, $p > 0.05$). The differences between D_1 and D_2 for all three treatment modalities were not statistically significant ($p > 0.05$).

p5M group

One *Scn1a*$^{RX/+}$ mouse did not develop hyperthermia-induced seizures after administration of combination therapy. In mice treated with STP monotherapy, the difference between BT_1 (40.82 ± 0.32°C) and BT_2 (40.98 ± 0.50°C) did not reach statistical significance ($t = 0.966$, $p > 0.05$). In mice treated with CLB monotherapy, the difference between BT_1 (40.67 ± 0.67°C) and BT_2 (42.85 ± 0.74°C) reached statistical significance ($t = 4.364$, $p < 0.01$). In mice treated with combination therapy, the difference between BT_1 (40.18 ± 0.56°C) and BT_2 (43.82 ± 0.75°C) was statistically significant ($t = 16.508$, $p < 0.01$). The difference in BT_2 between CLB monotherapy and combination therapy was statistically significant ($t = 2.241$, $p < 0.05$) (Table 3). The differences between D_1 and D_2 for all three treatment therapies did not reach statistical significance ($p > 0.05$).

We measured the plasma concentrations of STP and CLB after seizures (1 h 20 min after administration). The concentration of CLB was higher in combination therapy (p1M; 887.6 ± 211.60 ng/ml, p5M; 779.83 ± 262.53 ng/ml) than in CLB monotherapy (p1M; 54.00 ± 27.39 ng/ml, p5M;

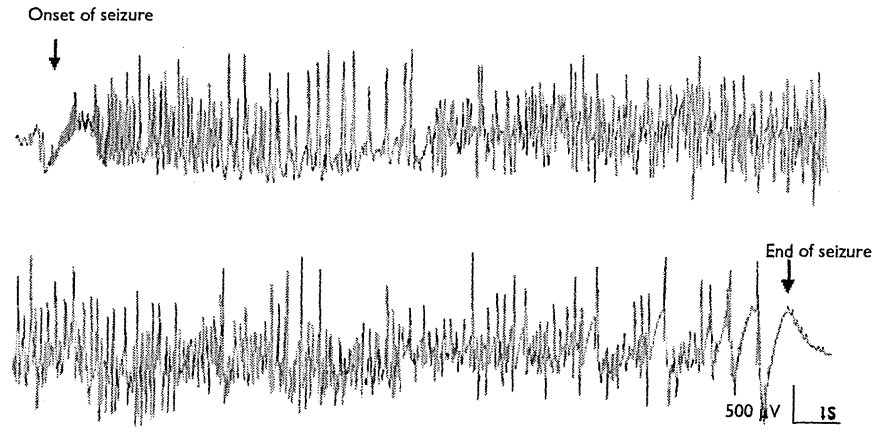

Figure 1.
EEG pattern. Ictal EEG pattern of Scn1a$^{RX/+}$ mice and that of wild-type mice were similar and composed of a crescendo and decrescendo frequency of spike-waves.
Epilepsia © ILAE

Table 2. The seizure-inducing body temperature and the duration of seizure in young age group (aged 4 weeks)

Group	Age (week)	BT_1 (°C)	D_1 (s)	BT_2 (°C)	D_2 (s)
STP (n = 6)	4	39.67 ± 1.09	39.75 ± 10.84	41.32 ± 1.05*	33.17 ± 6.86
CLB (n = 6)	4	40.10 ± 1.35	24.95 ± 11.06	42.58 ± 0.55*	36.29 ± 20.30
STP + CLB (n = 6)	4	40.18 ± 0.58	32.57 ± 6.12	43.03 ± 0.49*	23.51 ± 8.10

Values represent means ± SD.
*BT_2 is significantly higher than BT_1 ($p < 0.05$).

Table 3. The seizure-inducing body temperature and the duration of seizure on older age group (aged 5–10 months)

Group	Age (month)	BT_1 (°C)	D_1 (s)	BT_2 (°C)	D_2 (s)
STP (n = 6)	7.62 ± 1.74	40.82 ± 0.32	22.50 ± 6.29	40.98 ± 0.50	27.67 ± 6.71
CLB (n = 6)	7.53 ± 1.82	40.67 ± 0.67	28.00 ± 4.33	42.85 ± 0.74*	25.50 ± 4.51
STP + CLB (n = 6)	8.05 ± 1.74	40.18 ± 0.56	22.60 ± 4.62	43.82 ± 0.75*	30.90 ± 6.56

Values represent means ± SD.
*BT_2 is significantly higher than BT_1 ($p < 0.05$).

66.00 ± 29.17 ng/ml), with a significant difference (p1M; t = 8.74, p5M; t = 5.31, $p < 0.05$). The concentration of NCLB was lower in combination therapy (p1M; 710.00 ± 144.44 ng/ml, p5M; 526.33 ± 142.37 ng/ml) than in CLB monotherapy (p1M; 1504.4 ± 682.26 ng/ml, p5M; 1,371 ± 267.36 ng/ml), also with a significant difference (p1M; t = 2.54, p5M; t = 4.08, $p < 0.05$). The concentration of STP in STP monotherapy was 38.92 ± 13.41 mg/l in p5M mice.

Discussion

Increased and sustained susceptibility to hyperthermia-induced seizures in *Scn1a*$^{RX/+}$

Dravet syndrome is characterized by fever-sensitive seizures in infancy and childhood. We were interested in how susceptibility to hyperthermia-induced seizures changes with age. Recently, Oakley et al. (2009) reported the age-dependent susceptibility to hyperthermia-induced seizure in mice with heterozygous deletion of $Na_v1.1$, which showed hyperthermia-induced seizures at postnatal days (P) 20–22 and P30–46, but not at P17–18. But they did not mention the susceptibility at higher ages. In our study, we observed increased and sustained susceptibility to hyperthermia-induced seizure in *Scn1a*$^{RX/+}$ mice at various ages over 1 month. In patients with Dravet syndrome, febrile seizure was reported to ameliorate or disappear as the patients grew up (Ohtsuka et al., 1991), but this was not the case in *Scn1a*$^{RX/+}$ mice. We agree with Oguni et al. (2001) that fever sensitivity may last throughout the clinical course also in patients with Dravet syndrome, but due to the decreased incidence of fever with age as a result of maturation, medication, or safe-bathing, the frequency of fever-sensitive seizures may be reduced.

Scn1a$^{RX/+}$ mice had also spontaneous seizure (clonic or clonic tonic–clonic seizure, or myoclonic seizure)

from the age of day 16. Spontaneous seizures occur frequently before the third postnatal week, and tended to occur in a bursting manner. Many mice died immediately after seizure or died of starvation or whatever caused emaciated state during this period. After the third postnatal week, the frequency of spontaneous seizures decreases and seizures become irregular. Therefore the frequency of spontaneous seizure is not suitable as an index for evaluating the effects of drugs. On the other hand, hyperthermia-induced seizures can be recorded stably and the seizure-inducing body temperature is reproducible in the same mouse. Therefore, we chose the seizure-inducing body temperature as an index to evaluate drug efficacy.

Efficacy of STP monotherapy in *Scn1a*$^{RX/+}$ mice

Numerous animal experiments have revealed the anticonvulsant action of STP in different models of experimental seizures. The antiepileptic activity of STP was first demonstrated in the early 1970s. The drug was found to protect rats from seizures in the pentylenetetrazole (PTZ) and supramaximal electroshock models (Shen et al., 1990; Trojnar et al., 2005). In the experiments of PTZ-induced seizures in rats, a significant elevation of seizure threshold was observed after a single intraperitoneal injection of STP (300 mg/kg), which corresponded to plasma concentrations above 35 mg/L. In our study, the plasma concentration of STP was 38.92 ± 13.41 mg/L, which is close to that in the previous study (Shen et al., 1990). Quilichini et al. (2006) examined the interactions of STP with GABA transmission using patch-clamp methods in CA3 pyramidal neurons in neonatal rats. They showed that STP enhances $GABA_A$-receptor–mediated transmission in the immature hippocampus, because this drug increases the frequency and lengthens the decay time constant of miniature inhibitory postsynaptic currents (mIPSCs). STP enhances central GABA transmission through a barbiturate-like effect, suggesting that STP should possess an antiepileptic effect per se. Fisher (2009) reported that STP acts as a direct allosteric modulator of the $GABA_A$ receptor at a site distinct from many commonly used anticonvulsants, sedatives, and anxiolytic drugs. On the other hand, no clinical data are available to verify the efficacy of STP monotherapy in patients with Dravet syndrome. *Scn1a*$^{RX/+}$ mice provide a good model to examine the efficacy of STP when used as monotherapy. Our results indicate that STP has some protective effects against hyperthermia-induced seizures in *Scn1a*$^{RX/+}$ mice aged 1 month, but not in those aged 5 months or older. According to Fisher's (2009) investigation, STP is effective in the presence of the α3 subunit of $GABA_A$ receptor. In another study, Laurie et al. (1992) found that the subunit of $GABA_A$ receptor decreases in adult rodents. Therefore, we speculate that decreased expression of the α3 subunit of $GABA_A$ receptor may account for the lack of efficacy of STP in *Scn1a*$^{RX/+}$ mice aged 5 months or older.

Efficacy of STP in combination therapy to *Scn1a*$^{RX/+}$

STP is an anticonvulsant agent, and its clinical efficacy as an add-on treatment to CLB and valproate has been demonstrated in patients with Dravet syndrome (Chiron et al., 2000; Thanh et al., 2002; Inoue et al., 2009). Similarly, we confirmed that STP has definite efficacy when given with CLB as combination therapy in mice aged both 1 and 5 months or above.

STP is known to be an inhibitor of several CYPs (Tran et al., 1997). STP is a potent inhibitor of CYP2C19 both in vitro and in vivo (Giraud et al., 2006). It is known that CLB can be demethylated to NCLB or hydroxylated to 4-hydroxyclobazam (OH-CLB), and both NCLB and OH-CLB can be transformed to OH-NCLB (Volz et al., 1979). STP can inhibit the demethylation of CLB, and inhibit more potently the hydroxylation of NCLB (Giraud et al., 2006). Giraud et al. (2004) found that CYP3A4 and CYP2C19 are the main CYPs involved in the demethylation of CLB, whereas CYP2C19 is mainly involved in the hydroxylation of NCLB. Therefore, the patients' plasma concentrations of CLB and NCLB increase significantly when STP is used together with CLB in a continuous manner. The data for humans were obtained at steady state, that is, 4 weeks after STP and CLB were taken continuously, providing the time necessary for CLB to be demethylated to NCLB and NCLB hydroxylated to OH-NCLB. However, our study showed a significant increase in plasma concentrations of CLB and a decrease in NCLB following a single administration of STP together with CLB. We collected blood samples at 1 h and 20 min after the administration of STP and CLB. With this timing STP might have strongly inhibited the demethylation of CLB, whereas CLB was still not demethylated to NCLB.

When used as monotherapy, STP was effective in suppressing hyperthermia-induced seizures in 1-month-old mice, but not in mice aged 5 months or older. The prominent protective effect of STP was in combination therapy; STP probably synergistically enhanced the anticonvulsant effect of CLB and inhibited CYPs in 1-month-old mice, whereas STP mainly contributed by inhibiting the metabolism of CLB in mice aged 5 months or older.

In conclusion, our study demonstrates that mutant heterozygous mice carrying R1407X nonsense mutation have increased and sustained susceptibility to hyperthermia-induced seizures from 1 month of age. Thus the *Scn1a*$^{RX/+}$ mice have identical genotype and phenotype as Dravet syndrome, therefore serve as a useful tool to test the efficacy of medications. Whether STP per se has an anticonvulsant effect has been a clinical interest. Of course the present data were obtained from a mouse model, and there is limitation in the extrapolation to the clinical situation. However, the results of this study could be translated to this clinical question.

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Disclosure

None of the authors has any conflict of interest to disclose. We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

References

Astoin J, Mariwain A, Riveron A, Crucifix M, Laporte M, Torrens Y. (1978) Influence of novel alpha-ethylene alcohols on the central nervous system. *Eur J Med Chem* 13:41–47. [French.]

Cazali N, Tran A, Treluyer JM, Rey E, d'Athis P, Vincent J, Pons G. (2003) Inhibitory effect of stiripentol on carbamazepine and saquinavir metabolism in human. *Br J Clin Pharmacol* 56:526–536.

Ceulemans B, Boel M, Claes L, Dom L, Willekens H, Thiry P, Lagae L. (2004) Severe myoclonic epilepsy in infancy: toward an optimal treatment. *J Child Neurol* 19:516–521.

Chiron C. (2005) Stiripentol. *Expert Opin Investig Drugs* 14:905–911.

Chiron C, Marchand MC, Tran A, Rey E, d'Athis P, Vincent J, Dulac O, Pons G. (2000) Stiripentol in severe myoclonic epilepsy in infancy: a randomized placebo-controlled syndrome-dedicated trial. STICLO study group. *Lancet* 356:1638–1642.

Claes L, Ceulemans B, Audenaert D, Smets K, Löfgren A, Del-Favero J, Ala-Mello S, Basel-Vanagaite L, Plecko B, Raskin S, Thiry P, Wolf NI, Van Broeckhoven C, De Jonghe P. (2003) De novo SCN1A mutations are a major cause of severe myoclonic epilepsy of infancy. *Hum Mutat* 21:615–621.

Dravet C, Bureau M, Oguni H, Fukuyama Y, Cokar O. (2005) Severe myoclonic epilepsy in infancy (Dravet syndrome). In Roger J, Bureau M, Dravet Ch, Genton P, Tassinari CA, Wolf P (Eds) *Epileptic syndromes in infancy, childhood and adolescence*. 4th ed. John Libbey Eurotext, Montrouge, pp. 89–113.

Finnell RH, Bennett GD, Slattery JT, Amore BM, Bajpai M, Levy RH. (1995) Effect of treatment with phenobarbital and stiripentol on carbamazepine-induced teratogenicity and reactive metabolite formation. *Teratology* 52:324–332.

Fisher JL. (2009) The anti-convulsant stiripentol acts directly on the GABA(A) receptor as a positive allosteric modulator. *Neuropharmacology* 56:190–197.

Giraud C, Tran A, Rey E, Vincent J, Trluyer JM, Pons G. (2004) In vitro characterization of clobazam metabolism by recombinant cytochrome P450 enzymes: importance of CYP2C19. *Drug Metab Dispos* 32:1279–1286.

Giraud C, Treluyer JM, Rey E, Chiron C, Vincent J, Pons G, Tran A. (2006) In vitro and in vivo inhibitory effect of stiripentol on clobazam metabolism. *Drug Metab Dispos* 34:608–611.

Inoue Y, Ohtsuka Y, Oguni H, Tohyama J, Baba H, Fukushima K, Ohtani H, Takahashi Y, Ikeda S. (2009) Stiripentol open study in Japanese patients with Dravet syndrome. *Epilepsia* 50:2362–2368.

Laurie DJ, Wisden W, Seeburg PH. (1992) The distribution of thirteen GABA, receptor subunit mRNAs in the rat brain. III. Embryonic and postnatal development. *J Neurosci* 12:4151–4172.

Levy RH, Loiseau P, Guyot M, Blehaut HM, Tor J, Moreland TA. (1984) Stiripentol kinetics in epilepsy: nonlinearity and interactions. *Clin Pharmacol Ther* 36:661–669.

Levy RH, Rettenmeier AW, Anderson GD, Wilensky AJ, Friel PN, Baillie TA, Acheampong A, Tor J, Guyot M, Loiseau P. (1990) Effects of polytherapy with phenytoin, carbamazepine, and stiripentol on formation of 4-ene-valproate, a hepatotoxic metabolite of valproic acid. *Clin Pharmacol Ther* 48:225–235.

Nakajima H. (2001) A pharmacological profile of clobazam (Mystan), a new antiepileptic drug. *Nihon Yakurigaku Zasshi* 118:117–122. [Japanese.]

Oakley JC, Kalume F, Yu FH, Scheuer T, Catterall WA. (2009) Temperature- and age-dependent seizures in a mouse model of severe myoclonic epilepsy in infancy. *Proc Natl Acad Sci USA* 106:3994–3999.

Ogiwara I, Miyamoto H, Morita N, Atapour N, Mazaki E, Inoue Y, Takeuchi T, Itohara S, Yanagawa Y, Obata K, Furuichi T, Hensch TK, Yamakawa K. (2007) Nav1.1 localizes to axons of parvalbumin-positive inhibitory interneurons: a circuit basis for epileptic seizures in mice carrying an *Scn1a* gene mutation. *J Neurosci* 27:5903–5914.

Oguni H, Hayashi K, Awaya Y, Fukuyama Y, Osawa M. (2001) Severe myoclonic epilepsy in infants – a review based on the Tokyo Women's Medical University series of 84 cases. *Brain Dev* 23:736–748.

Ohtsuka Y, Maniwa S, Ogino T, Yamatogi Y, Ohtahara S. (1991) Severe myoclonic epilepsy in infancy: a long-term follow-up study. *Jpn J Psychiatry Neurol* 45:416–418.

Perez J, Chiron C, Musial C, Rey E, Blehaut H, d'Athis P, Vincent J, Dulac O. (1999) Stiripentol: efficacy and tolerability in children with epilepsy. *Epilepsia* 40:1618–1626.

Quilichini PP, Chiron C, Ben-Ari Y, Gozlan H. (2006) Stiripentol, a putative antiepileptic drug, enhances the duration of opening of GABA-A receptor channels. *Epilepsia* 47:704–716.

Shen DD, Levy RH, Moor MJ, Savitch JL. (1990) Efficacy of stiripentol in the intravenous pentylenetetrazol infusion seizure model in the rat. *Epilepsy Res* 7:40–48.

Thanh TN, Chiron C, Dellatolas G, Rey E, Pons G, Vincent J, Dulac O. (2002) Long-term efficacy and tolerance of stiripentol in severe myoclonic epilepsy of infancy (Dravet's syndrome). *Arch Pediatr* 9:1120–1127.

Tran A, Rey E, Pons G, Rousseau M, d'Athis P, Olive G, Mather GG, Bishop FE, Wurden CJ, Labroo R, Trager WF, Kunze KL, Thummel KE, Vincent JC, Gillardin JM, Lepage F, Levy RH. (1997) Influence of stiripentol on cytochrome P450-mediated metabolic pathways in humans: in vitro and in vivo comparison and calculation of in vivo inhibition constants. *Clin Pharmacol Ther* 62:490–504.

Trojnar MK, Wojtal K, Trojnar MP, Czuczwar SJ. (2005) Stiripentol: a novel antiepileptic drug. *Pharmacol Rep* 57:154–160.

Volz M, Christ O, Kellner HM, Kuch H, Fehlhaber HW, Gantz D, Hadju P, Cavagna F. (1979) Kinetics and metabolism of clobazam in animals and man. *Br J Clin Pharmacol* 7:41S–50S.

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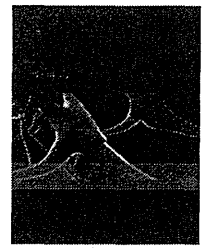

Risk factors for hyperammonemia associated with valproic acid therapy in adult epilepsy patients

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Epilepsy

Summary Hyperammonemia is one of the side effects of treatment with valproic acid (VPA), but the risk factors and mechanisms involved remain obscure. This study analyzed the risk factors for hyperammonemia associated with VPA therapy in adult epilepsy patients.

A retrospective analysis of 2724 Japanese patients (1217 males and 1507 females aged from 16 to 76 years) treated with VPA between January 2006 and December 2010 were analyzed.

The ammonia level increased markedly in a VPA dose-dependent manner, and was significantly elevated in patients who also used hepatic enzyme inducers such as phenytoin (PHT), phenobarbital (PB), carbamazepine (CBZ), and combinations of these drugs. When a blood ammonia level exceeding 200 μg/dl was defined as hyperammonemia, the risk factors for hyperammonemia according to multiple regression analysis were a VPA dose >20 mg/kg/day (odds ratio (OR): 4.1; 95% confidence interval (CI): 1.6–10.8) and concomitant use of PHT (OR: 11.0; 95% CI: 3.1–38.7), concomitant PB (OR: 4.3; 95% CI: 1.0–17.9), concomitant CBZ (OR: 2.8; 95% CI: 0.6–11.9), and concomitant topiramate (OR: 2.8; 95% CI: 1.2–6.5). Regimens containing multiple inducers were associated with an increased risk of hyperammonemia.

Identification of risk factors for hyperammonemia associated with VPA therapy can help to minimize side effects during its clinical use.

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Introduction

Valproic acid (VPA) is widely used for the treatment of seizure disorders, bipolar disorder, schizoaffective disorder, migraine, and neuropathic pain. However, VPA is known to cause an increase of the blood ammonia level and hyperammonemia can lead to vomiting, aggression, ataxia, and more frequent seizures. It is also rarely associated with disturbance of consciousness or fatal encephalopathy. Furthermore, hyperammonemia can damage the central nervous system, and is associated with an increased risk of cortical atrophy, ventricular enlargement, and demyelination (Cagnon and Braissant, 2007). Unfortunately, the mechanism by which VPA provokes hyperammonemia is poorly understood and the risk factors remain obscure.

Ammonia is normally converted to urea, which is eliminated via the kidneys. As the first step of the urea cycle, carbamoyl phosphate synthetase I (CPS-1) catalyzes the production of carbamoyl from ammonia and bicarbonate ion. CPS-1 activity is regulated by *N*-acetylglutamate (NAG), which is synthesized from acetyl-coenzyme A (acetyl-CoA) and glutamate by *N*-acetylglutamate synthetase (NAGS). Valpronyl-CoA and propionate, which are metabolites of VPA, reduce hepatocyte NAGS or CPS-1 activity, resulting in an increase of the blood ammonia level (Aires et al., 2011; Yagi et al., 2010; Verbiest et al., 1992). Another metabolite of VPA, 2-propyl-4-pentenoate (4-en-VPA) reduces the acetyl-CoA level, resulting in a decrease of NAG and an increase of ammonia (Kondo et al., 1992). Furthermore, carnitine is essential for the metabolism of VPA and it is consumed during long-term administration, resulting in an increased risk of blood ammonia elevation (Raskind and El-Chaar, 2000; Hamed and Abdella, 2009).

Previous studies have identified diverse risk factors for hyperammonemia associated with VPA therapy, including age (Altunbaşak et al., 1997), the VPA dose or blood concentration (Haidukewych et al., 1985; Verrotti et al., 1999), nutritional status (Laub, 1986; Warter et al., 1984), lupus erythematosus (Ichikawa et al., 1998), and concomitant drugs such as phenytoin (PHT), phenobarbital (PB), topiramate (TPM), and risperidone (Kugoh et al., 1986; Ratnaike et al., 1986; Hamer et al., 2000; Longin et al., 2002; Carlson et al., 2007; Knudsen et al., 2008; Deutsch et al., 2009). However, most of these previous investigations were case reports or prospective or cross-sectional studies of around 100 subjects, and the results were discordant. For example, Altunbaşak et al. (1997) reported that children younger than 2 years had a higher risk of hyperammonemia, but other authors have not found a relationship between age and the ammonia level. Similarly, only two studies have found that the VPA dose or blood concentration is positively correlated with the ammonia level (Haidukewych et al., 1985; Verrotti et al., 1999), while other studies have not identified any such association.

Accordingly, the aim of the present study was to identify clinical risk factors for hyperammonemia in adult Japanese patients with epilepsy receiving VPA therapy.

Methods

The study protocol was approved by the ethics committee of the National Epilepsy Center (Shizuoka, Japan) in March 2010. All patients treated at our hospital from January 2006 to December 2010 were eligible. There were 2724 adult Japanese patients with epilepsy who were on VPA therapy and underwent blood ammonia measurement. Patients who showed noncompliance with therapy, those with severe hepatic or renal dysfunction (aspartate aminotransferase or alanine aminotransferase >500 U/l and/or serum creatinine >3.0 mg/dl), those on a ketogenic diet, and those with intercurrent infection were excluded. The items investigated were the age, gender, body weight, blood ammonia level, liver function tests, concomitant anti-epilepsy drugs (AEDs), VPA dose, plasma concentration of VPA, and concentration to dose (CD) ratio of VPA (μg/ml per mg/kg).

Venous blood was collected into a heparinized tube, which was placed on ice as soon as possible. Then the blood ammonia level was rapidly measured by a bromophenol blue colorimetric test using a VITROS5600 autoanalyzer (Ortho Clinical Diagnostics, Tokyo, Japan). At the same time, another blood sample was obtained for measurement of the plasma VPA concentration. This sample was centrifuged at 3000 rpm, after which VPA was measured by a latex immunoagglutination assay using the same autoanalyzer.

If patients had multiple measurements during the study period, the highest blood ammonia level was used. Blood samples were obtained from the patients at 2 to 8 h after administration of VPA.

For analysis of the effect of concomitant AEDs, the other drugs being used at the time of obtaining the data were examined. Hepatic enzyme inducers (inducers) were defined as PHT, PB, and carbamazepine (CBZ). Primidone, which is converted to PB, was considered to be equivalent to PB. All other AEDs were defined as non-inducers. The VPA formulations used by the patients were Depaken (Kyowa Hakko Kirin, Tokyo, Japan) or Selenica (Kowa Pharmaceutical, Tokyo, Japan).

Statistical analysis

To assess the relationship between the blood ammonia level and the VPA dose, patients were classified into three VPA dose groups: <10 mg/kg, ≥10 and <20 mg/kg, and ≥20 mg/kg. These groups were compared by analysis of variance (ANOVA) with a post hoc Scheffe's multiple comparison test. Patients taking VPA with one or more inducers were analyzed in the same manner.

The relation between the blood ammonia level and various factors was examined by stepwise multiple regression analysis, with the partial regression coefficient (B) and standardized partial regression coefficient (β) being obtained. Multiple logistic regression analysis was performed to investigate risk factors for hyperammonemia, which was defined as a maximum blood ammonia level exceeding 150 or 200 μg/dl and was used as the dependent variable. The independent variables were factors that showed significance ($p < 0.05$) according to stepwise multiple regression analysis.

Results are expressed as the mean ± standard error. The level of significance was set at $p < 0.05$. Statistical analyses were conducted using SPSS Ver 19.0 (IBM, Tokyo, Japan).

Results

Patient characteristics

A total of 2724 adult patients (1217 males and 1507 females aged from 16 to 76 years) were analyzed (Table 1). Among them, 922 patients (33.8%) were receiving VPA monotherapy and 1402 patients (51.4%) were being treated with VPA plus inducers. Of these, 700 patients (49.9%) receiving VPA combined with both inducers and non-inducers. The mean blood ammonia level was 80.7 ± 1.0 (median: 69.0; range: 8–504) μg/dl (Fig. 1). Among the 2724 patients, there were

Table 1 Patient profile.

	Number of patients or mean ± SE
Number of patients	2724
Gender (female/male)	1507/1217
Age (years)	32.3 ± 0.24
Body weight (kg)	59.9 ± 0.28
Hepatocellular injury[a]	26
VPA therapy	
Dose (mg/kg)	15.4 ± 0.15
Concentration (μg/ml)	61.8 ± 0.49
Concomitant AEDs	
Phenytoin	700
Phenobarbital	535
Carbamazepine	611
Zonisamide	325
Clobazam	336
Clonazepam	226
Nitrazepam	71
Diazepam	76
Acetazolamide	62
Topiramate	77
Gabapentine	81
Lamotrigine	107
Other AEDs	60
Antipsychotics	189

Other AEDs were ethosuximide, bromide, ethotoin, and sultiame.
[a] Serum aspartate aminotransferase (AST) > 100 U/l or alanine aminotransferase (ALT) > 100 U/l.

81 patients (3.0%) who had blood ammonia levels exceeding 200 μg/dl, and 33 of these 81 patients (40.7%) treated for hyperammonemia with carnitine rescue, intravenous therapy, or VPA dose reduction. In contrast, 30 of these 81 patients (37.0%) suffered adverse effects such as lethargy, vomiting, anorexia, ataxia, and more frequent seizures.

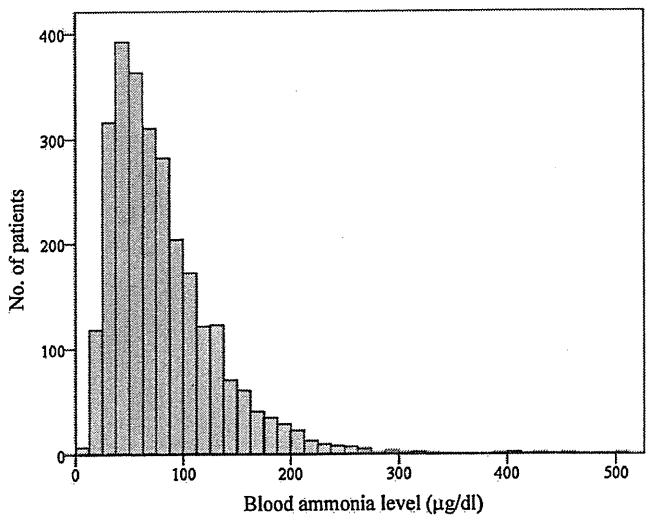

Figure 1 Ammonia levels in 2724 patients receiving VPA.

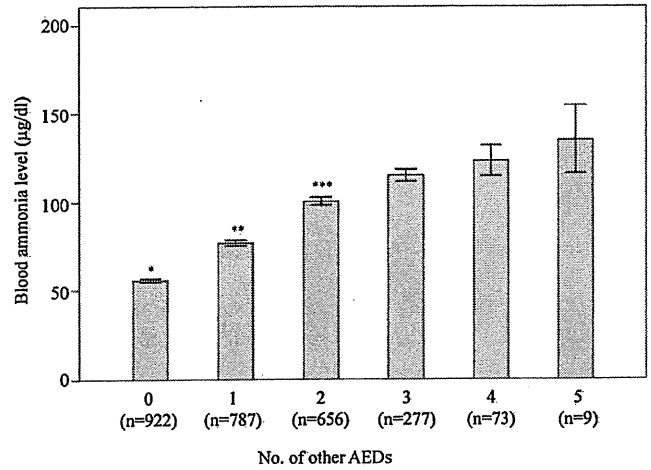

Figure 2 Blood ammonia level stratified by the number of combined AEDs. Significance was determined by ANOVA ($p < 0.001$) and Scheffe's multiple comparison test. *$p < 0.001$ versus 1, 2, 3, 4, and 5 AEDs. **$p < 0.001$ versus 2, 3, and 4 AEDs; $p < 0.05$ versus 5 AEDs. ***$p < 0.001$ versus 3 AEDs; $p < 0.01$ versus 4 AEDs.

Influence of concomitant AEDs and the VPA dose or plasma concentration

The relation between the blood ammonia level and the number of concomitant AEDs is shown in Fig. 2. The mean blood ammonia level was 55.7 ± 1.0 μg/dl in the patients on VPA monotherapy and the ammonia level showed a significant increase as the number of concomitant AEDs increased (ANOVA; $p < 0.001$). Post hoc analysis showed significant differences in relation to the concomitant use of 1, 2, 3, 4, and 5 AEDs compared with VPA monotherapy, but no difference among the concomitant use of 3, 4, and 5 AEDs.

Fig. 3 displays a stratified analysis of blood ammonia levels in the patients classified into three VPA dose groups. In all three groups, concomitant use of inducers

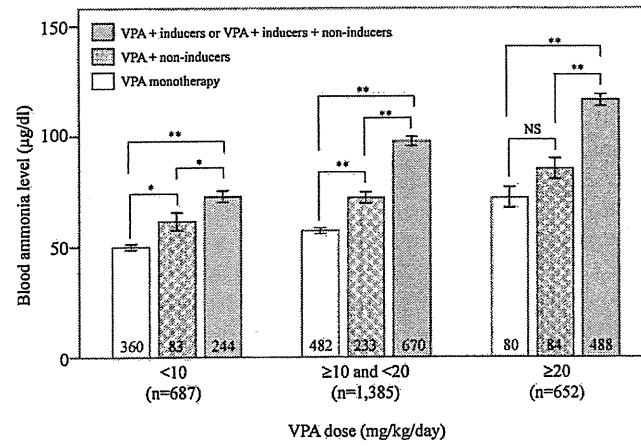

Figure 3 Blood ammonia level stratified by the VPA dose in patients on VPA monotherapy or combination therapy with inducers and/or non-inducers. In all three VPA dose groups, there were significant differences related to combined treatment with inducers or non-inducers (ANOVA; $p < 0.001$). Post hoc Scheffe's multiple comparison test. *$p < 0.05$, **$p < 0.001$.

significantly increased the blood ammonia level in comparison with monotherapy or use of non-inducers (Scheffe's test; $p < 0.001$). In the monotherapy group, the mean ammonia level of patients receiving ≥20 mg/kg of VPA was increased by 1.4-fold over that in patients receiving <10 mg/kg. There was a weak positive correlation between the blood ammonia level and the dose of VPA ($r = 0.23$, $p < 0.001$). In the non-inducer group, patients receiving <20 mg/kg of VPA had significantly higher blood ammonia levels in comparison with those on VPA monotherapy. In contrast, at VPA doses above 20 mg/kg, no significant difference of the ammonia level was observed. In summary, the blood ammonia level was markedly increased by concomitant treatment with inducers regardless of the VPA dose.

Table 2 shows the blood ammonia level, VPA dose, and CD ratio in patients receiving different inducers. Concomitant use of PHT was associated with a significantly higher blood ammonia level and VPA dose compared with use of PB or CBZ ($p < 0.001$). In contrast, use of PHT was associated with a lower mean CD ratio of VPA in comparison with PB and CBZ. All 4 poly-inducer regimens tended to be associated with higher blood ammonia levels than those seen with mono-inducer regimens, but there was no significant difference except for the VPA + PHT + PB regimen. When a blood ammonia level exceeding 150 or 200 μg/dl was defined as hyperammonemia, poly-inducer regimens were associated with a higher incidence of hyperammonemia in comparison with mono-inducer regimens.

Fig. 4 shows the correlations between the CD ratio for VPA and the plasma concentrations of PHT, PB, and CBZ. There were no significant correlation between the CD ratio for LTG and the concentrations of inducers. Meanwhile, there were weak, but significant, positive correlations between the blood ammonia level and the plasma concentrations of PHT, PB, and CBZ (Fig. 5).

Table 2 Relations between combinations of inducers and the blood ammonia level, VPA dose, and CD ratio.

Regimen	VPA mono (reference)	VPA + PHT	VPA + PB	VPA + CBZ	VPA + PHT + PB	VPA + PHT + CBZ	VPA + PHT + PB + CBZ	VPA + PB + CBZ	p-Value[a]
Number of patients	922	363	242	393	186	111	40	67	
Ammonia level (μg/dl)	55.7 ± 1.0	111.6 ± 2.7	89.1 ± 3.4*§	78.9 ± 2.3*§	126.6 ± 4.9§	105.2 ± 5.2	112.8 ± 13.5	111.6 ± 2.7	<0.001
Hyperammonemia (ammonia level)									
>150, n (%)	13 (1.4)	72 (19.8)	21 (8.7)	25 (6.4)	59 (31.7)	17 (15.3)	9 (22.5)	10 (14.9)	<0.001
>200, n (%)	3 (0.3)	21 (5.8)	5 (2.1)	5 (1.3)	27 (14.5)	5 (4.5)	5 (12.5)	4 (6.0)	<0.001
VPA dose (mg/kg)	12.0 ± 0.2	19.6 ± 0.5	17.1 ± 0.5†	15.9 ± 0.4*	18.7 ± 0.6	17.8 ± 0.8	15.6 ± 1.1	15.4 ± 0.8†	<0.001
CD ratio of VPA (μg/ml/mg/kg)	5.2 ± 0.06	3.7 ± 0.1	4.2 ± 0.1†	4.03 ± 0.08	3.6 ± 0.1‡	3.4 ± 0.12¶	3.7 ± 0.3	4.0 ± 0.2	<0.001

Post hoc Scheffe's multiple comparison test.
[a] Significance was determined by ANOVA or the χ^2 test.
* $p < 0.001$ versus VPA + PHT.
† $p < 0.05$ versus VPA + PHT.
§ $p < 0.001$ versus VPA + PHT + PB.
‡ $p < 0.05$ versus VPA + CBZ.
¶ $p < 0.005$ versus VPA + CBZ.

Risk factors for hyperammonemia

Stepwise multiple regression analysis was conducted by using the VPA dose, concomitant AEDs, antipsychotic drugs, age, and gender as independent variables to identify factors with an influence on the blood ammonia level. As shown in Table 3, the dose of VPA and concomitant use of PHT had the strongest positive correlations. In addition, gender and concomitant use of PB, CBZ, zonisamide, clobazam, TPM, acetazolamide, and antipsychotic drugs showed a significant correlation (these items were used as independent variables for multiple logistic regression analysis). No significant correlation was observed with age or concomitant use of clonazepam, diazepam, gabapentine, and lamotrigine, which were eliminated by the stepwise procedure.

Finally, multiple logistic regression analysis was performed. When a blood ammonia level >150 μg/dl was defined as hyperammonemia, concomitant use of PB, CBZ, clobazam, and acetazolamide were found to be significant risk factors, but no significant factors were observed in relation to an ammonia level >200 μg/dl. In contrast, the VPA dose and the concomitant use of PHT, multiple inducers, and TPM were significantly associated with hyperammonemia (>200 μg/dl). Concomitant PHT was the most important risk factor. The regimens VPA + PHT, VPA + PHT + PB,

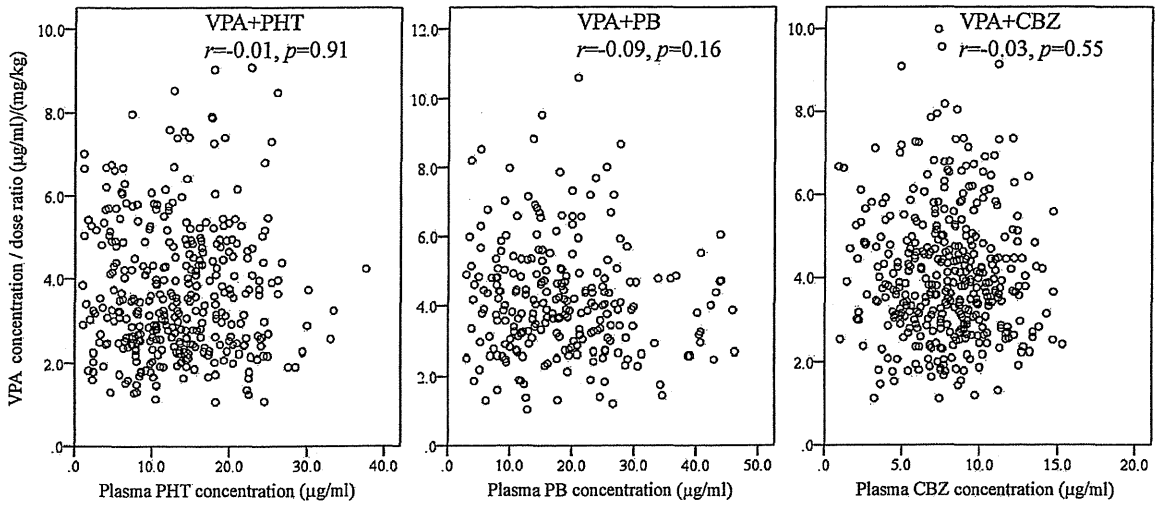

Figure 4 Correlation between the concentration to dose ratio for VPA and plasma concentrations of PHT, PB, and CBZ. *r*: Spearman's correlation.

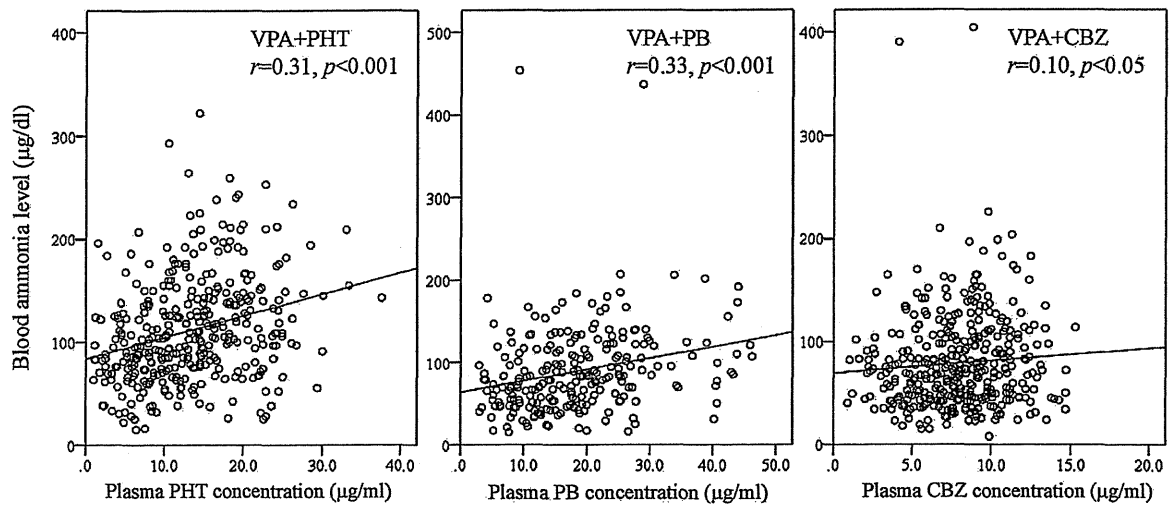

Figure 5 Correlation between the blood ammonia level and plasma concentrations of PHT, PB, and CBZ. *r*: Spearman's correlation.

Table 3 Factors influencing the ammonia level on multiple regression analysis.

Factors	β	*B* (95% CI)		*p*-Value
Gender (female = 1)	−0.04	−3.7	(−6.9, −0.4)	<0.05
VPA dose (mg/kg)	0.21	1.4	(1.2, 1.6)	<0.001
Concomitant drugs				
Phenytoin	0.27	31.5	(27.5, 35.4)	<0.001
Phenobarbital	0.16	20.2	(16.0, 24.3)	<0.001
Carbamazepine	0.06	7.4	(3.5, 11.3)	<0.001
Zonisamide	0.07	10.7	(5.6, 15.7)	<0.001
Clobazam	0.11	16.6	(11.6, 21.6)	<0.001
Topiramate	0.08	25.7	(15.9, 35.5)	<0.001
Acetazolamide	0.07	24.7	(13.8, 35.7)	<0.001
Antipsychotics	0.05	9.1	(2.7, 15.5)	<0.01

Stepwise forward selection method: $\geq F$ in = 0.05, $< F$ out = 1.0.
Multiple correlation coefficient: $R = 0.53$.
Multiple coefficient of determination: $R^2 = 0.28$.
β: standardized partial regression coefficient; *B*: partial regression coefficient; CI: confidence interval.

VPA + PHT + CBZ, and VPA + PHT + PB + CBZ increased the risk of hyperammonemia by 10.6, 32.9, 8.8, and 32.5-fold, respectively, in comparison with VPA alone or VPA plus non-inducers.

Discussion

In this study, we investigated a large cohort of 2724 epileptic adult patients receiving VPA to identify risk factors for hyperammonemia. Chicharro and Kanner (2007) reported that the mean prevalence of hyperammonemia caused by VPA therapy was 90.3% (range: 71–100%) and 47.4% (range: 16.2–100%) in prospective and cross-sectional studies, respectively. At our hospital, the normal range of blood ammonia is 16–76 μg/dl, and 1171 of the 2724 patients (43%) exceeded the upper limit of this range. Several studies have suggested that hyperammonemia causes few symptoms. Interestingly, Williams et al. (1984) reported that asymptomatic hyperammonemia was found in 58% of adult patients with mental retardation. In this study, symptoms were not investigated in all 2724 patients, but, 33 of the 81 patients (40.7%) with hyperammonemia exceeding 200 μg/dl had problems related to seizure control and additional costs because of treatment such as carnitine rescue, intravenous therapy, and VPA dose reduction. Lichter-Konecki (2008) reported that acute hyperammonemia can cause cerebral edema and severe brain damage, while chronic hyperammonemia in patients with congenital metabolic disorders is associated with developmental delay and intellectual disability. In general, long-term therapy is needed by epileptic patients using VPA. Therefore, it is important to identify risk factors for an increase of ammonia and to conduct routine measurement of ammonia irrespective of whether patients are asymptomatic or symptomatic.

According to multiple regression analysis, 28% of the blood ammonia level was explained by factors such as gender, the VPA dose concomitant AEDs, and antipsychotic drugs (Table 3). In this model, the β value indicates the magnitude of the influence on the blood ammonia level, so the VPA dose and concomitant use of PHT had a strong influence on elevation of ammonia. However, it was difficult to assess the influence of each factor accurately. When a blood ammonia level exceeding 200 μg/dl was defined as hyperammonemia, the risk factors for hyperammonemia were the VPA dosage, concomitant use of PHT, use of multiple inducers, and use of TPM (Table 4).

Several studies have not found any association between the VPA dose or blood concentration and the blood ammonia level. In contrast, we found that the increase of ammonia occurred in a VPA dose-dependent manner in adult epilepsy patients. Hamed and Abdella (2009) reported a negative correlation between the carnitine level and the VPA dosage ($r = -0.75$, $p < 0.001$), suggesting that a decrease of carnitine may be associated with an increased risk of hyperammonemia. Another possible explanation is that valpronyl-CoA may inhibit NAGS in a VPA dose-dependent manner, resulting in a decrease of CPS-1 activity.

Our study demonstrated that concomitant use of one or more inducers was an important risk factor for an increase of the blood ammonia level. Since the 1980s, several studies have showed that combination therapy with VPA + PHT and/or PB is associated with an increased blood ammonia level (Haidukewych et al., 1985; Verrotti et al., 1999), but the mechanisms involved remains to be identified. In general, inducers such as PHT, PB, and CBZ increase the activity of cytochrome P450 (CYP) enzymes, resulting in a decrease of the serum concentration of VPA. This drug interaction means that the dose of VPA has to be increased to maintain the optimum concentration, resulting in a higher blood level of ammonia (Table 2). However, we found that the blood ammonia level was significantly increased by combined treatment with inducers regardless of the VPA dose (i.e., in all three dose groups, ANOVA; $p < 0.001$) (Fig. 3). Another possible mechanism is that PHT, PB, and CBZ induce CYP 2A6, 2C9, 2C19, and 3A4 (Patsalos et al., 2002), resulting in more rapid metabolism of VPA to compounds such as propionate and 4-en-VPA, which inhibit CPS-1 activity and thus cause an increase of the blood ammonia level.

Among the three inducers, the blood ammonia level was increased in the order of PHT, PB, and CBZ. PHT also significantly reduced the CD ratio of VPA in comparison with PB and CBZ (Table 2; $p < 0.001$, 0.005). Therefore, PHT strongly induced VPA metabolism and thus increased the ammonia level.

Treatment regimens containing multiple inducers could be associated with enhanced interactions that increase the risk of hyperammonemia (Table 2). In particular, despite the lowest dosage of VPA, the incidence of hyperammonemia was the same in patients taking VPA + PB + CBZ as that in patients receiving VPA + PHT. Moreover, The plasma concentration of PHT, PB and CBZ caused a modest increase in blood ammonia level in a concentration-dependent manner, but dose not affect VPA metabolism (Figs. 4 and 5). It is possible that these inducers might have different mechanisms for increasing the ammonia level as well as induction of hepatic enzymes. Further studies will be necessary to evaluate ammonia levels in patients receiving one or more inducers without VPA.

Our study established the risk of hyperammonemia due to concomitant use of TPM (OR: 2.8; 95% confidence interval: 1.2–6.5). Several cases of hyperammonemic encephalopathy due to VPA and TPM therapy have been reported (Hamer et al., 2000; Carlson et al., 2007; Knudsen et al., 2008). Hamer et al. (2000) suggested that TPM inhibits carbonic anhydrase and thus blocks bicarbonate reuptake. Therefore, TPM-induced metabolic acidosis may lead to an increase of the blood ammonia level.

Among the non-inducers, we found that acetazolamide and clobazam had the potential to increase the ammonia level. Kim et al. (2007) have reported a patient with hyperammonemia due to acetazolamide monotherapy. Acetazolamide is a carbonic anhydrase inhibitor like TPM and the increase in blood ammonia would presumably be due to the same mechanism. An increase of the blood ammonia level due to clobazam has not been reported before and the mechanism of hyperammonemia is unknown. It is possible that clobazam influences the VPA concentration (Patsalos et al., 2008) or it might indirectly affect urea cycle enzymes in the liver, but further studies will be necessary to clarify the mechanism involved. Concomitant use of VPA and TPM and/or clobazam represents standard therapy for Dravet syndrome, but we suggest that special care might need to

Table 4 Multiple logistic regression analysis of risk factors for hyperammonemia.

Risk factors	Blood ammonia level			
	<150		<200	
	OR (95% CI)	*p*-Value	OR (95% CI)	*p*-Value
Gender (female = 1)	1.0 (0.7–1.3)	0.86	1.1 (0.7–1.7)	0.73
VPA dose (mg/kg)				
<10	1.0		1.0	
≥10 and <20	2.8 (1.6–4.9)	<0.001	2.7 (1.0–7.0)	<0.05
≥20	5.8 (3.3–10.3)	<0.001	4.1 (1.6–10.8)	<0.005
Anti-epilepsy regimen				
VPA alone or with non-inducers[a]	1.0		1.0	
VPA + PHT	7.9 (4.2–15.0)	<0.001	11.0 (3.1–38.7)	<0.001
VPA + PB	3.7 (1.8–7.7)	<0.001	4.3 (1.0–17.9)	0.06
VPA + CBZ	2.7 (1.3–5.5)	<0.01	2.8 (0.6–11.9)	0.18
VPA + PHT + PB	16.9 (8.8–32.4)	<0.001	32.7 (9.6–112.7)	<0.001
VPA + PHT + CBZ	6.4 (2.9–14.0)	<0.001	8.9 (2.0–38.8)	<0.005
VPA + PHT + PB + CBZ	12.4 (4.8–32.4)	<0.001	32.2 (7.2–144.6)	<0.001
VPA + PB + CBZ	8.3 (3.4–20.2)	<0.001	15.4 (3.3–71.8)	<0.001
Concomitant drugs				
Zonisamide	1.3 (0.9–1.9)	0.25	1.3 (0.7–2.5)	0.38
Clobazam	1.8 (1.3–2.6)	<0.001	1.2 (0.7–2.2)	0.47
Topiramate	2.4 (1.3–4.3)	<0.001	2.8 (1.2–6.5)	<0.05
Acetazolamide	2.9 (1.6–5.3)	<0.001	1.2 (0.4–3.7)	0.73
Antipsychotics	1.1 (0.7–1.9)	0.55	1.0 (0.4–2.0)	0.84

[a] Reference category. OR: odds ratio; CI: confidence interval.

be taken when a patient is treated with VPA in addition to TPM and/or clobazam.

The blood ammonia levels in patients with non-inducers were higher than those in patients with mono-therapy. Hung et al. (2011) reported that transient hyperammonemia in patients is significantly related to generalized tonic–clonic seizures and acidosis. Seizure control was better in patients on VPA monotherapy than in those on polytherapy, which may have contributed to higher ammonia levels in the latter. Also, TPM, zonisamide and acetazolamide can cause metabolic acidosis. However, seizure frequency and bicarbonate were not investigated in the present study. Further studies will be necessary to evaluate whether higher seizure frequency and acidosis are associated with one of the risk factors for hyperammonemia in patients using VPA.

This study had several limitations. First, genetic polymorphisms that are important for ammonia metabolism could not be confirmed because of its retrospective cross-sectional design. It has been reported that CPS-1 polymorphism is associated with an increase of the blood ammonia level in Japanese patients on VPA therapy (Yagi et al., 2010), although the VPA dose and use of inducers were not found to be significant risk factors for hyperammonemia in that study.

Furthermore, blood samples were not collected at a specific time. The blood ammonia level is affected by fasting, which may have contributed to the different results obtained in previous studies. The VPA concentration also fluctuates in the same patient depending on the blood sampling time and trough levels were not measured. However, despite the lack of trough levels, CD ratios for VPA showed significant differences among the combinations of inducers (Table 2).

Second, we showed that combining VPA with multiple inducers was linked to an increased risk of hyperammonemia, but the 95% confidence intervals were wide for regimens such as VPA + PHT + PB, VPA + PHT + PB + CBZ, or VPA + PB + CBZ (Table 4), probably because only 81 out of 2724 patients (3.0%) developed hyperammonemia.

In conclusion, our study identified some risk factors for hyperammonemia in a large cohort of Japanese patients with epilepsy. We suggest that the blood ammonia level should be monitored carefully when patients are receiving high-dose VPA, concomitant inducers, acetazolamide, clobazam, and TPM. Furthermore, regimens containing multiple inducers can result in enhanced interactions that increase the risk of hyperammonemia. Better identification of risk factors for hyperammonemia in patients on VPA therapy will help to minimize the side effects of this drug.

References

Altunbaşak, S., Baytok, V., Tasouji, M., Hergüner, O., Burgut, R., Kayrin, L., 1997. Asymptomatic hyperammonemia in children treated with valproic acid. J. Child Neurol. 12, 461–463.

Aires, C.C., van Cruchten, A., Ijlst, L., de Almeida, I.T., Duran, M., Wanders, R.J., Silva, M.F., 2011. New insights on the mechanisms of valproate-induced hyperammonemia: inhibition of hepatic *N*-acetylglutamate synthase activity by valproyl-CoA. J. Hepatol. 55, 426–434.

Cagnon, L., Braissant, O., 2007. Hyperammonemia-induced toxicity for the developing central nervous system. Brain Res. Rev. 56, 183–197.

Carlson, T., Reynolds, C.A., Caplan, R., 2007. Case report: valproic Acid and risperidone treatment leading to development of hyperammonemia and mania. J. Am. Acad. Child Adolesc. Psychiatry 46, 356–361.

Chicharro, A.V., Kanner, A.M., 2007. The measurement of ammonia blood levels in patients taking valproic acid: looking for problems where they do not exist? Epilepsy Behav. 11, 361–366.

Deutsch, S.I., Burket, J.A., Rosse, R.B., 2009. Valproate-induced hyperammonemic encephalopathy and normal liver functions: possible synergism with topiramate. Clin. Neuropharmacol. 32, 350–352.

Haidukewych, D., John, G., Zielinski, J.J., Rodin, E.A., 1985. Chronic valproic acid therapy and incidence of increases in venous plasma ammonia. Ther. Drug Monit. 7, 290–294.

Hamer, H.M., Knake, S., Schomburg, U., Rosenow, F., 2000. Valproate-induced hyperammonemic encephalopathy in the presence of topiramate. Neurology 54, 230–232.

Hamed, S.A., Abdella, M.M., 2009. The risk of asymptomatic hyperammonemia in children with idiopathic epilepsy treated with valproate: relationship to blood carnitine status. Epilepsy Res. 86, 32–41.

Hung, T.Y., Chen, C.C., Wang, T.L., Su, C.F., Wang, R.F., 2011. Transient hyperammonemia in seizures: a prospective study. Epilepsia 52, 2043–2049.

Ichikawa, H., Amano, T., Kawabata, K., Kushiro, M., Wada, J., Nagake, Y., Makino, H., 1998. Fatal hyperammonemia in a patient with systemic lupus erythematosus. Intern. Med. 37, 700–703.

Kim, J.M., Ryu, W.S., Hwang, Y.H., Kim, J.S., 2007. Aggravation of ataxia due to acetazolamide induced hyperammonaemia in episodic ataxia. J. Neurol. Neurosurg. Psychiatry 78, 771–772.

Knudsen, J.F., Sokol, G.H., Flowers, C.M., 2008. Adjunctive topiramate enhances the risk of hypothermia associated with valproic acid therapy. J. Clin. Pharm. Ther. 33, 513–519.

Kondo, T., Ishida, M., Kaneko, S., Hirano, T., Otani, K., Fukushima, Y., Muranaka, H., Koide, N., Yokoyama, M., Nakata, S., 1992. Is 2-propyl-4-pentenoic acid, a hepatotoxic metabolite of valproate, responsible for valproate-induced hyperammonemia? Epilepsia 33, 550–554.

Kugoh, T., Yamamoto, M., Hosokawa, K., 1986. Blood ammonia level during valproic acid therapy. Jpn. J. Psychiatry Neurol. 40, 663–668.

Laub, M.C., 1986. Nutritional influence on serum ammonia in young patients receiving sodium valproate. Epilepsia 27, 55–59.

Lichter-Konecki, U., 2008. Profiling of astrocyte properties in the hyperammonaemic brain: shedding new light on the pathophysiology of the brain damage in hyperammonaemia. J. Inherit. Metab. Dis. 31, 492–502.

Longin, E., Teich, M., Koelfen, W., König, S., 2002. Topiramate enhances the risk of valproate-associated side effects in three children. Epilepsia 43, 451–454.

Patsalos, P.N., Fröscher, W., Pisani, F., van Rijn, C.M., 2002. The importance of drug interactions in epilepsy therapy. Epilepsia 43, 365–385.

Patsalos, P.N., Berry, D.J., Bourgeois, B.F., Cloyd, J.C., Glauser, T.A., Johannessen, S.I., Leppik, I.E., Tomson, T., Perucca, E., 2008. Antiepileptic drugs – best practice guidelines for therapeutic drug monitoring: a position paper by the subcommission on therapeutic drug monitoring. ILAE Commission on Therapeutic Strategies. Epilepsia 49, 1239–1276.

Raskind, J.Y., El-Chaar, G.M., 2000. The role of carnitine supplementation during valproic acid therapy. Ann. Pharmacother. 34, 630–638.

Ratnaike, R.N., Schapel, G.J., Purdie, G., Rischbieth, R.H., Hoffmann, S., 1986. Hyperammonaemia and hepatotoxicity during chronic valproate therapy: enhancement by combination with other antiepileptic drugs. Br. J. Clin. Pharmacol. 22, 100–103.

Verbiest, H.B., Straver, J.S., Colombo, J.P., van der Vijver, J.C., van Woerkom, T.C., 1992. Carbamyl phosphate synthetase-1 deficiency discovered after valproic acid-induced coma. Acta Neurol. Scand. 86, 275–279.

Verrotti, A., Greco, R., Morgese, G., Chiarelli, F., 1999. Carnitine deficiency and hyperammonemia in children receiving valproic acid with and without other anticonvulsant drugs. Int. J. Clin. Lab. Res. 29, 36–40.

Warter, J.M., Marescaux, C., Chabrier, G., Rumbach, L., Micheletti, G., Reitzer, B., Hirsch, E., Imler, M., 1984. Increase of valproate-induced hyperammonemia in normal subjects by carbohydrate intake. Neurology 34, 1487–1489.

Williams, C.A., Tiefenbach, S., McReynolds, J.W., 1984. Valproic acid-induced hyperammonemia in mentally retarded adults. Neurology 34, 550–553.

Yagi, M., Nakamura, T., Okizuka, Y., Oyazato, Y., Kawasaki, Y., Tsuneishi, S., Sakaeda, T., Matsuo, M., Okumura, K., Okamura, N., 2010. Effect of CPS14217C>A genotype on valproic-acid-induced hyperammonemia. Pediatr. Int. 52, 744–748.