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Ⅲ．研究成果の刊行物・別刷（主なもの）

Original article

Thermolabile CPT II variants and low blood ATP levels are closely related to severity of acute encephalopathy in Japanese children

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Abstract

Despite the decrease in Reye syndrome after the discontinuation of aspirin, acute encephalopathy (non-Reye syndrome type) has been continually reported in Japan. Recent studies suggested that the thermolabile phenotype of carnitine palmitoyltransferase II (CPT II) variation [F352C] was closely related to the pathomechanism of influenza-associated encephalopathy (IAE) in Japanese, causing mitochondrial ATP utilization failure during periods of high fever, resulting in brain edema. So, we analyzed CPT II polymorphism and peripheral blood ATP levels as a signal of "energy crisis" in 12 and 10 patients with acute encephalopathy, respectively. Out of the 12 patients with acute encephalopathy, six showed thermolabile CPT II variants [F352C], and of these six, two patients died in spite of intensive care. In contrast, the remaining six patients with no thermolabile CPT II variant [F352C] showed a relatively mild clinical course. Blood ATP levels of the 10 patients in the acute phase of encephalopathy were significantly lower than those during the convalescent phase and also those of patients with febrile seizure status. Our data suggest that the thermolabile F352C CPT II variant, found only in Japanese, might be one of the predisposing factors to trigger the pathomechanism of acute encephalopathy in the Japanese population, and that it is causally related to the severity of disease. The decreased blood ATP level seems to reflect systemic mitochondrial dysfunction including the blood brain barrier during the acute phase of encephalopathy.
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Keywords: Acute encephalopathy; Carnitine palmitoyltransferase II; Thermolabile variants; ATP; Mitochondrial dysfunction

1. Introduction

Acute encephalopathy in children is clinically characterized by high fever, prolonged consciousness disturbance associated with brain edema, and prolonged or multiple generalized seizures. Acute encephalopathy distinct from Reye syndrome is not rare in Japan. The precise pathogenesis of acute encephalopathy including influenza-associated encephalopathy (IAE) remains unclear. An epidemiological study revealed that aspirin use was closely related to the pathogenesis of Reye syndrome [1]. However, despite the decrease in Reye syndrome after the discontinuation of aspirin, acute encephalopathy (non-Reye syndrome type) has been continually reported in Japan [2].

Recently, acute encephalopathy was classified into several types according to magnetic resonance imaging (MRI) findings together with the clinical course, such

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as acute necrotizing encephalopathy [3], acute encephalopathy with biphasic seizures and late reduced diffusion [4], and hemorrhagic and shock encephalopathy [5,6]. Although the influenza virus and HHV-6 (human herpes virus-6) are the main causative agents of these acute encephalopathies, many other viruses are also considered to be responsible for the disease [3,4,7].

It is estimated that more than 100 children die of IAE every year in Japan [8,9]. According to the first nationwide clinical survey of IAE in Japan, in many patients with IAE, multiple-organ failure developed, and rates of mortality (31.8%) and disability (27.7%) were high [2]. Although clinical and neuropathological studies suggested that blood–brain barrier destruction and hypercytokinemia in cerebrospinal fluid were closely related to the pathogenesis of IAE, the pathophysiology and mechanisms of disease onset are still unclear [3,7,10,11].

Recently, Chen et al. [12] reported that the thermolabile phenotypes of carnitine palmitoyltransferase II (CPT II) variations, [1055T > G/F352C] alone, and [1055T > G/F352C] + [1102G > A/V368I] were closely related to the pathomechanisms of IAE. The CPT system is a pivotal component of ATP generation through mitochondrial fatty acid oxidation in mammals [13]. Yao et al. [14] further characterized the enzyme properties of the CPT II variants as follows: (1) dominant-negative effect, (2) reduced activities, (3) thermal instability, and (4) short half-lives compared with the wild-type. They demonstrated that the thermolabile CPT II variants might cause mitochondrial fuel utilization failure in various organs and endothelial cells during periods of high fever, and, thus, might play an important role in the pathogenesis of brain edema in IAE. In the present study, we analyzed the CPT II polymorphism and peripheral blood ATP levels as a signal of "energy crisis" in patients with acute encephalopathy with and without influenza virus infection, septic encephalopathy, and febrile delirium during influenza virus infection, and analyzed the relationships among these data, age, and clinical manifestations.

2. Patients and methods

2.1. Patient profile for the study of CPT II polymorphism

This investigation was approved by the Ethics Review Committee for human genome analysis of our institution. All participants' caregivers gave written informed consent. Fifteen patients were included in the study. The clinical details are summarized in Table 1. The diagnoses of the 15 patients were as follows: 12 patients with acute encephalopathy (7 IAE, one human herpes virus type 6 (HHV-6) associated, one varicella-associated, one septic encephalopathy associated with *Hemophilus influenzae* type b, two acute encephalopathy with an unknown pathogenesis, highly suspected of being of viral origin), and three febrile delirium associated with influenza virus infection. Two patients (Case 1, IAE, and Case 2, septic encephalopathy) died 30 and 3 days after admission, respectively, despite intensive care. All 12 patients with acute encephalopathy were diagnosed based on prolonged seizures with high fever and/or consciousness disturbance lasting longer than 12 h associated with brain CT or MRI abnormalities.

3. Representative case presentations

3.1. Case 1

This 4-year-old girl was admitted to our hospital because of feeding difficulty, a lethargic state, and high fever lasting longer than 12 h. A rapid test for influenza A virus antigen in the nasal discharge was positive. She has been followed at our outpatient clinic with a diagnosis of severe psychomotor delay and epilepsy due to chromosome abnormality (46, XX, dup(2)(q21.1q24.2)) since the age of 3 years. Her seizure disorder was well-controlled with phenobarbital. On admission, except for a lethargic tendency, she showed no neck stiffness, involuntary movement, or convulsion, and her respiratory and circulatory conditions were stable. She was also able to follow an object. Neurological examination revealed normal light and corneal reflexes and normal deep tendon reflexes. Pathological reflexes were not induced. Her consciousness level, however, deteriorated 12 h after admission. On laboratory tests, blood glucose, ammonia, the white blood cell count (WBC), hemoglobin (Hb), and platelet count (Plt) were within normal ranges, and cereblospinal fluid (CSF) findings were unremarkable. Blood and CSF cultures were negative. Because she also showed sudden respiratory insufficiency and reduced blood pressure, she was immediately resuscitated and intubated. After that, she could not move and all brainstem reflexes disappeared. On brain CT the next day, as shown in Fig. 1a, cisterns surrounding the brainstem and cerebellum were not identified and auditory brainstem responses (ABR) showed only bilateral wave I. Rapid consciousness deterioration as well as brain CT and ABR findings suggested cerebral herniation due to influenza-associated brainstem encephalopathy. On the second CT 3 weeks later, severe brain edema and subarachnoid hemorrhage were observed. Despite intensive care, she died on the 31st day of hospitalization. She had a thermolabile F352C CPT II variant.

3.2. Case 2

This previously healthy 2-year-old boy was admitted to our hospital because of consciousness disturbance, a brief seizure cluster, and high fever lasting 24 h. On admission, neurological examination revealed coma, the absence of light and corneal reflexes, dilated and anisocoric pupils, and flaccid extremities. Neck stiffness was

Table 1
Clinical summary of patients and CPT II polymorphism.

Case no.	Age at onset	Pathogen	Diagnosis	CPT II polymorphism	Duration of high fever	Duration of seizure (min)	Therapy	Outcome
1[c]	4 years 10 months	Flu A	IAE	F352C	24 h	(−)	Gly, IVIG, m-PSL, Venti	Died
2	2 years 2 months	H. influenza	Hib septic AE	F352C, V368I	2 days	3	Venti, Epin, DOA, CTX	Died
3[c]	1 year	Unknown	AEU	F352C, V368I	2 days	30	Mann, MDZ	Severe MR, MD, Epi
4[c]	1 year 7 months	Flu A	IAE	(−)	30 h	40	Gly, m-PSL, Venti	Moderate MR, MD, Epi
5[a]	4 years 5 months	Flu A	IAE	F352C, V368I	5 days	90	Gly, MDZ, Pen, IVIG, m-PSL	Moderate MR
6[a]	2 years 1 months	Varicella	Varicella AE	F352C, V368I	24 h	90	Mann, MDZ, m-PSL	Mild MR
7[c]	6 years	Unknown	AEU	F352C, V368I	2 days	30	Mann, MDZ, m-PSL, HT	Mild MR
8[a]	1 years 4 months	Flu A	IAE	V368I	5 days	60	Gly, MDZ, Pen, PB, m-PSL	Mild MR
9[a]	2 years	Flu A	IAE	V368I	5 days	60	Gly, MDZ, Pen, IVIG, m-PSL	Mild MR
10[a]	11 months	HHV-6	HHV-6 AE	V368I	36 h	100	Gly, MDZ, Pen, m-PSL	Good
11[b]	2 years 5 months	Flu A	IAE	V368I, M647 V	24 h	40	MDZ, PB, m-PSL, HT	Good
12[a]	3 years 11 months	Flu A	IAE	V368I	2 days	40	MDZ, PB, m-PSL, HT, Venti	Good
13	4 years 9 months	Flu A	FD	F352C, V368I	4 days	2	(−)	Good
14	9 years 5 months	Flu A	FD	(−)	3 days	(−)	(−)	Good
15	11 years	Flu A	FD	V368I, M647V	3 days	(−)	(−)	Good

IAE: Influenza-associated encephalopathy, AEU: acute encephalopathy of unknown pathogen, FD: febrile delirium, Flu A: influenza A, HHV-6: human herpes virus-6, MR; mental retardation, MD: motor delay, Epi: epilepsy, Mann: mannitol, MDZ: midazolam, m-PSL: methylpredonisolone, HT: hypothermia, Venti: artificial ventilator, Epin: epinephrine, DOA: dopamine, CTX: cefotaxim, PB: Phenobarbital, Pen: pentobarbital, IVIG: intravenous infusion of gamma-globlin, Gly: glycerole.

[a] AESD (acute encephalopathy with biphasic seizures and late reduced diffusion).
[b] This case partially resembles ANE (acute necrotizing encephalopathy).
[c] Unclassified acute encephalopathy.

not observed. A rapid test for influenza virus antigen in the nasal discharge was negative. His head CT demonstrated diffuse brain edema, as shown in Fig. 1b. On laboratory investigation, blood glucose and ammonia, as well as liver and renal functions were within normal limits. WBC was 18,000/µL, Hb 11.6 g/dL, Plt 3,60,000/µL, and prothrombin time 68.7 s. Blood culture identified H. influenzae type b. Spinal tap was not performed because of the risk of cerebral herniation. The blood ATP level was 0.58 mM on admission. The acylcarnitine ratio ((C16 + C18:1)/C2) was high, at 0.203, on admission, compared with the upper cutoff value of 0.048 [12]. We diagnosed him with septic encephalopathy. Despite intensive care including antibiotics, ventilator support, and catecholamine infusion, he died 2 days later. He had compound thermolabile CPT II variants [F352C + V368I].

3.3. Case 12

This previously healthy 3-year-old boy was admitted to our hospital because of a febrile seizure status and high fever lasting longer than 24 h. His generalized tonic clonic seizure was suppressed with pentobarbital infusion 40 min after the onset. A rapid test for influenza virus antigen in the nasal discharge was positive for flu A. Brain CT revealed mild brain edema. So, he was sedated and intubated. Methylpredonisolone (m-PSL) pulse and hypothermia therapies were immediately started based on the diagnosis of IAE. The blood ATP value was 0.77 mM on admission, and it increased to 1.35 mM 2 weeks later. On the 6th day of hospitalization, he developed brief right-sided clonic seizure. Brain MRI (diffusion-weighted images) showed an abnormal high intensity in the left hemisphere (Fig. 1e). The clinical course and MRI findings were compatible with acute encephalopathy with biphasic seizures and late reduced diffusion [4]. Additional m-PSL therapy was given and the hypothermia therapy gradually discontinued. His neurological condition subsequently showed a full recovery. No apparent mental, motor, and social skill impairment was noted during follow-up 1 year later. He had a V368I CPT II variant.
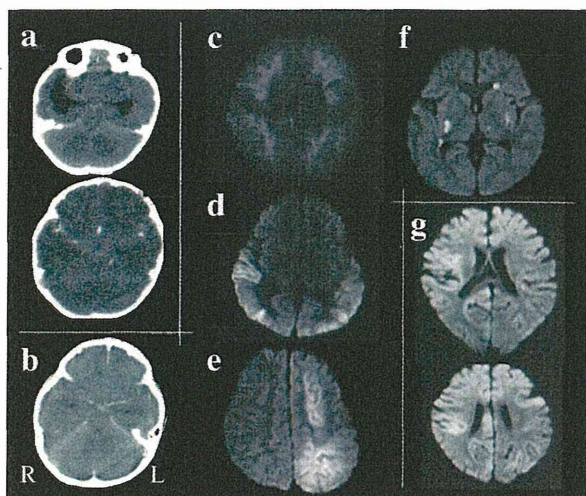
Fig. 1. (a) Brain CTs on 2nd and 21st day of hospitalization in Case 1 showing disappearance of cistern between brainstem and cerebellum (upper image) and severe brain edema (lower image). (b) Brain CT on admission showing severe brain edema in Case 2. (c–g) Brain MRIs showing abnormal high intensities in diffusion-weighted images in Cases 6,7,12,11 and 10, respectively.

3.4. Patient profile for the study of the blood ATP level

Twenty-five patients were included in this study. The diagnoses of the 25 patients were as follows: 10 patients with acute encephalopathy (mean age: 3 years and 11 months, age range: 7 months–10 years and 8 months, one IAE, one *Salmonella*-associated, one HHV-6-associated, three unknown virus-associated, one methylmalonic aciduria, one hepatic encephalopathy, one hemolytic uremic syndrome, and one septic encephalopathy (Case 2 in Table 1)), nine febrile seizure status (mean age: 1 year and 5 months, age range: 4 months–4 years 9 months), and six mitochondrial disease (mean age: 9 years and 8 months, age range: 2–25 years, two partial cytochrome c oxidase deficiency, three Leigh syndrome, and one chronic progressive external ophthalmoplegia). All 10 patients with acute encephalopathy were analyzed regarding the blood ATP levels in the acute phase (within 24 h of disease onset), and five of the 10 patients were also analyzed in the convalescent phase. Among the 15 patients who were analyzed for CPT II polymorphism, only Cases 2 and 12 were included in this study.

4. Methods

4.1. Analysis of CPT II polymorphism

Genomic DNA from whole blood was purified as previously described [15]. PCR of five exons of the CPT II gene was carried out with intron-based primers in genomic DNA. For haplotype analysis, the CPT II exon four region was cloned into the pCR® 2.1 vector (Invitrogen). The sequences of the PCR products and cloned CPT II gene were analyzed employing the ABI DyeDeoxy Terminator Cycle Sequencing Kit with an ABI-PRISM 3100 Genetic Analyzer (PE-Applied Biosystems). Each PCR product was sequenced at least twice independently.

4.2. Preparation of patients' lymphoblasts and culture

Blood samples (2 mL) were obtained from patients by venipuncture into a sterile EDTA blood collection tube. Lymphocytes were separated from peripheral blood, diluted (1:1, v/v) with sterile saline, by centrifugation ($800 \times g$, 20 min) over 2 mL of Lymphoprep (Nycomed). The lymphocyte layer was recovered and washed twice with PBS by centrifugation at $250 \times g$ for 10 min each, and then maintained in PRMI-1640 (GIBCO) supplemented with 12.5% FCS. Cells were incubated with 5% CO_2 at 37 °C for 7 days. Lymphoblastic cell lines were established by infecting peripheral blood lymphocytes with the Epstein Barr virus. Cells were grown in suspension in an SC flask (Greiner 658190) in an upright position, in 10 ml of PRMI-1640 medium that contained 12.5% FCS, maintained at 37 °C. Fluid was routinely changed every 2 days by removing the medium above the settled cells and replacing it with an equal volume of fresh medium.

4.3. Analysis of CPT II activity

CPT II activities of patients' lymphoblasts were analyzed as previously described [14]. To prepare whole cell extracts, cells were harvested and washed twice with PBS (−) at $250 \times g$ for 10 min and then lysed with 0.5 mL of ice-cold lysis buffer (5 mM Tris–HCl buffer, pH 7.4, containing 1% Tween-20 and 0.5 M KCl), then centrifuged at $147,600 \times g$ for 1 h at 4 °C. To analyze the heat stability of CPT II, cell lysates were pre-incubated at 30, 37 and 41 °C for 0–120 min. Protein concentrations in the cell lysates were measured using the BCATM Protein Assay Kit (Thermo SCIENTIFIC).

4.4. Measurement of blood ATP levels

ATP concentrations in whole blood lysate were measured by an ENLITEN® ATP assay system bioluminescence detection kit (Promega) according to the instructions provided by the manufacturer and the values were expressed as ATP levels in whole blood.

5. Results

5.1. CPT II polymorphism in the patients

As shown in Table 1, among the 15 patients studied, seven had a thermolabile F352C CPT II variant (1 F352C only and six [F352C + V368I]), four V368I only,

two [V368I + M647 V], and two no polymorphisms. In 12 patients with acute encephalopathy (Cases 1–12), six (Cases 1–3 and 5–7) had a thermolabile F352C CPT II variant (1 F352C only and five [F352C + V368I]), and five (Cases 8–12) had the V368I CPT II variant (4 V368I only and one [V368I + M647 V]) and one (Case 4) showed no CPT II variant. Two patients with acute encephalopathy who died (Cases 1 and 2) had a thermolabile F352C CPT II variant (1 F352C only and the other [F352C + V368I]). In three patients with febrile delirium associated with influenza infection (cases 13–15), only case 13 (brief febrile seizure and unusually long febrile delirium) had the [F352C + V368I] CPT II variant. No other reported CPT II mutations or polymorphisms were detected.

There was no significant difference in the age at onset (41.0 ± 23.3 vs. 24.3 ± 12.7 months of age, $p = 0.18$), duration of high fever (52.0 ± 35.3 vs. 63.0 ± 44.9 h, $p = 0.28$), and duration of seizures (40.5 ± 40.1 vs. 56.7 ± 23.4 h, $p = 0.12$) between the six patients with acute encephalopathy with a thermolabile F352C CPT II variant (Cases 1–3,5–7) and six patients with acute encephalopathy without this thermolabile variant (Cases 4,8–12) (Mann–Whitney U-test).

5.2. Lymphocyte CPT II activity in the patients

As shown in Fig. 2(b), CPT II activity using peripheral lymphocytes of a patient with a thermolabile F352C CPT II variant was significantly reduced to about 50% during incubation for 120 min at 41 °C as compared to those at 30 and 37 °C. All patients with a thermolabile F352C CPT II variant showed a significant reduction of CPT II activity at 41 °C.

Fig. 2(a) shows CPT II activity in a patient with the V368I CPT II variant without reduction even at 41 °C.

5.3. Blood ATP levels in patients with acute encephalopathy

As shown in Fig. 3, ATP levels in the extracts of whole blood in the acute phase of encephalopathy during high fever were significantly low (0.58 ± 0.16 mM, $n = 10$) compared with those in the convalescent phase (1.08 ± 0.27 mM, $n = 5$) and with those of patients with febrile seizure status (1.01 ± 0.36 mM, $n = 9$). The blood ATP levels in the acute phase of encephalopathy revealed no significant difference when compared to those of patients with mitochondrial disease exhibiting several symptoms (0.79 ± 0.39 mM, $n = 6$).

6. Discussion

Although the precise pathomechanisms of acute encephalopathy have yet to be clarified, it is postulated that some genetically-determined factors might be involved, because some types of acute encephalopathy are more frequent in Japanese than in Caucasians. Chen et al. [12] demonstrated that the thermolabile phenotype of CPT II variations such as the F352C CPT II variant or complex [F352C + V368I] CPT II variant might be a principal genetic background of IAE in Japanese. On the basis of the analysis of fatty acid oxidation and cellular ATP production in COS-7 cells transfected with wild-type and variant *CPT2* cDNAs at 37 and 41 °C, Yao et al. [14] suggested that the compound *CPT2* variants with thermolabile phenotypes are the main cause of multiple-organ failure, particularly in high ATP-consuming organs as well as endothelial cells and play a major role in the etiology of IAE.

In the 12 patients with acute encephalopathy studied, six patients (Cases 1–3 and 5–7) had thermolabile F352C CPT II variants (F352C CPT II variant alone in one case and complex [F352C + V368I] CPT II variants in five cases), which were reported to be frequently noted in severe IAE patients [12,14]. Of the six patients, two patients (Case 1, IAE and Case 2, *Hemophilus influenzae*-associated septic encephalopathy) died despite intensive care. Case 2, who died of fatal septic encephalopathy [16], showed a high acylcarnitine ratio ((C16 + C18:1)/ C2:0.203) on admission. This value corresponded to the ratio (>0.09) of the high-risk group of patients with IAE showing a fatal outcome, thus reflecting the disorder of mitochondrial ●-oxidation. [12]. The remaining six patients (Cases 4 and 8–12) with acute encephalopathy without a thermolabile F352C CPT II variant followed a relatively mild clinical course (Table 1). Out of the six patients, five had a V368I CPT II variant.

As shown in Fig. 2, the CPT II activities of lymphocyte in patients with the F352C CPT II variant showed thermal instability, that is, a marked activity reduction at 41 °C, while those in patients with the V368I CPT II variant did not. There was no significant difference in the age at onset, duration of high fever, and duration of seizures between the six patients with the F352C CPT II variant (Cases 1–3 and 5–7) and six patients without this variant (Cases 4 and 8–12). Therefore, taken together, it seems likely that a thermolabile F352C CPT II variant might be related to the severity of disease, that is, the rapidity of progression of brain edema. In Caucasians, two polymorphisms of CPT II, p.V368I and p.M647 V, occur with a frequency of 0.5 and 0.25, respectively, exhibiting a Hardy–Weinberg equilibrium. A third polymorphism, p.F352C, occurs with a frequency of 0.21 exclusively in the Japanese population [17]. Therefore, this thermolabile F352C CPT II variant might be one of the predisposing factors to trigger the pathomechanism of acute encephalopathy in Japanese.

The CPT system regulates the entry of long-chain fatty acids into the mitochondrial matrix for ●-oxidation. Fatty acid oxidation is an important source of acetyl-CoA for maintaining the tricarboxylic acid cycle.
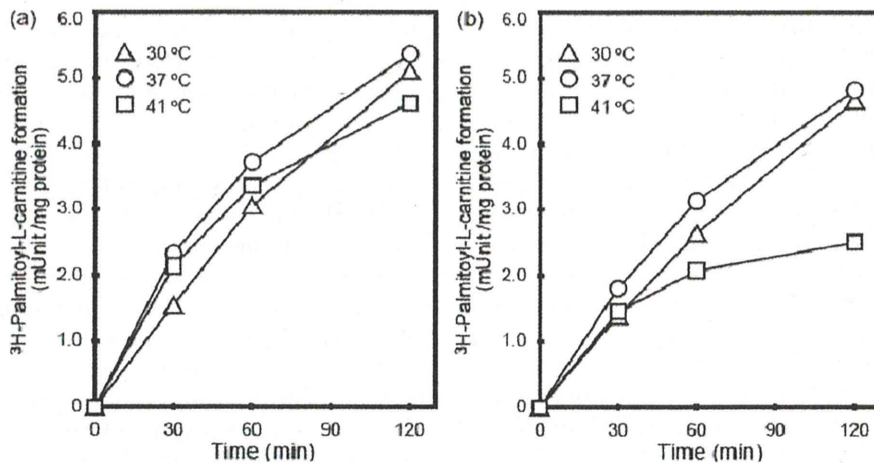
Fig. 2. (a) Lymphocyte CPT II activity in case 12 (influenza-associated encephalopathy) with V368I CPT II variant at 30, 37 and 41 °C. No definite reduction of CPT II activity was observed at 41 °C. (b) Lymphocyte CPT II activity in Case 1 (influenza-associated encephalopathy) with a thermolabile F352C CPT II variant at 30, 37 and 41 °C. At 41 °C, the CPT II activity decreased to about 50% of that at 37 °C after 2-h-incubation.
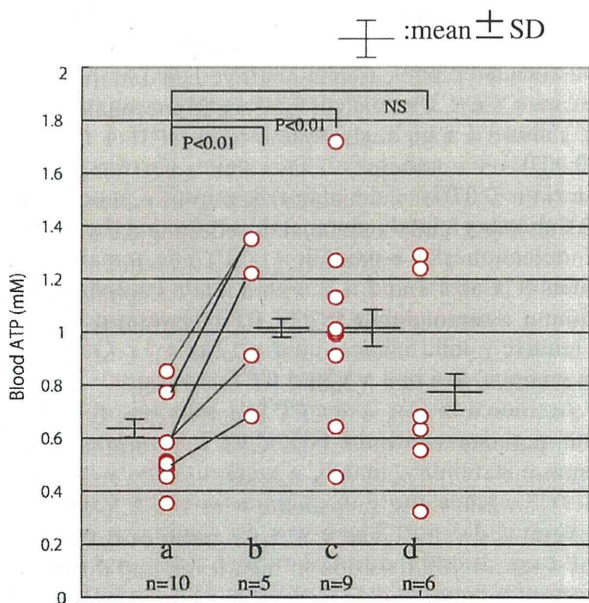

Fig. 3. ATP levels in whole blood in patients with acute encephalopathy (acute (a) and convalescent phase (b)), febrile seizure status (c) and mitochondrial disease (d). In five patients with acute encephalopathy, blood ATP level recovered at convalescent phase.

The CPT II is ubiquitously expressed in all tissues that require fatty acid oxidation as an energy-producing pathway [18]. CPT II deficiency is a disorder of long-chain fatty acid oxidation. It is classified into three clinical types based on the age at onset and disease severity: lethal neonatal form, severe infantile hepatocardiomuscular form, and myopathic form. It is clear that our patients' clinical manifestations did not correspond to any of these three types. The thermolabile instability of the F352C CPT II variant in our cases explains the situation whereby impaired energy metabolism could

occur during high fever due to a secondary CPT II deficiency in spite of the absence of symptomatic manifestations of CPT II disorder in daily life at a normal temperature [12,14].

Olpin et al. [19] reported based on mutation analysis that when CPT II activities are above 20% of controls, fatty acid oxidation in fibroblasts is usually within the normal range (>70% of controls). However, under heat stress, fasting, acidosis, and seizures, moderately lowered CPT II activity due to the thermolabile F352C CPT II variant may accelerate the disease process of acute encephalopathy.

Blood ATP levels in the acute phase of encephalopathy during high fever were significantly lower than those in the convalescent phase and also with those of patients with febrile seizure status. This suggests that mitochondrial energetic failure may be more severe in patients with acute encephalopathy, and the pathological process of acute encephalopathy should differ from the febrile seizure status. The low levels of ATP in the acute phase of encephalopathy were normalized in the convalescent phase in line with clinical recovery. Interestingly, blood ATP levels in the acute phase of encephalopathy corresponded to those of mitochondrial disease with several symptoms. Yao et al. [14] showed that COS-7 cells transfected with thermolabile [F352C + V368I] CPT II variants exhibited significantly decreased fatty acid oxidation and subsequent intracellular ATP reduction at 41 °C. The decreased ATP levels seemed to reflect systemic mitochondrial dysfunction including the blood brain barrier (BBB) at the acute phase of encephalopathy in our cases. The ATP demand per body weight is so high in infants that a thermolabile CPT II variant induced-ATP reduction might lead to a greater susceptibility to the pathophysiology of encephalopathy in children than in adults.

The brain capillary endothelium is characterized by a greater density of mitochondria than that of peripheral capillaries [20]. This greater mitochondrial density is required to maintain the significant active transport mechanisms, electrochemical gradients, autoregulatory adjustments, and regulation of tight junctional complexes. As such, the requirement of a constant ATP supply may make the BBB particularly susceptible to acute hypoxic insult [21]. From a similar perspective, BBB breakdown may occur at an initial stage of encephalopathy under the condition of ATP reduction, thus leading to subsequent brain edema due to complex cascade of hypercytokinemia, excitotoxicity, and oxidative stress. Although there is one hypothesis that cytokine storm due to virus–glial cell interaction might cause endothelial cell damage (BBB breakdown) leading to brain edema and neuronal injury [11], we consider that endothelial cell damage might induce in turn cytokine production resulting in neuronal damage in patients with thermolabile F352C CPT II variant irrespective of encephalopathy type .

In three patients with febrile delirium associated with influenza virus infection (Cases 13–15), Case 13 with a thermolabile F352C CPT II variant developed a short seizure and an intermittent confused state with visual hallucinations and agitation lasting 6 h. Cases 14 and 15 without F352C CPT II variant showed short-term consciousness alteration and abnormal behavior without seizures. All patients' brain MRIs were normal, and they fully recovered. Although more extensive study is needed, the grade of febrile delirium associated with influenza virus was more severe in a case with a thermolabile F352C CPT II variant when compared with that in cases without F352C CPT II variant.

Given that a thermolabile CPT II variant might be one of the predisposing factors for acute encephalopathy, we should revise the therapeutic strategy from the acute phase. Considering the rapid progression of encephalopathy and associated low CPT II activity during high fever, immediate hypothermia, sufficient glucose infusion, and L-carnitine supplementation should be adopted as treatment options. We speculate that the immediate hypothermia led to the recovery of the lowered CPT II activity and, thus, mitochondrial energy failure became minimal in many tissues including the brain capillary endothelium, leading to less severe damage to the central nervous system.

Acknowledgment

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ORIGINAL RESEARCH

Oseltamivir reduces hippocampal abnormal EEG activities after a virus infection (influenza) in isoflurane-anesthetized rats

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Background: Oseltamivir phosphate (OP, Tamiflu®) is a widely used drug in the treatment of influenza with fever. However, case reports have associated OP intake with sudden abnormal behaviors. In rats infected by the influenza A virus (IAV), the electroencephalogram (EEG) displayed abnormal high-voltage amplitudes with spikes and theta oscillations at a core temperature of 39.9°C to 41°C. Until now, there has been no information describing the effect of OP on intact brain hippocampal activity of IAV-infected animals during hyperthermia.

Objective: The aim of the present study was to examine the effect of OP on abnormal EEG activities in the hippocampus using the rat model of influenza-associated encephalopathy.

Methods: Male Wistar rats aged 3 to 4 weeks were used for the study. Influenza A/WSN/33 strain (1×10^5 plaque forming unit in PBS, 60 µL) was applied intranasally to the rats. To characterize OP effects on the IAV-infected rats, EEG activity was studied more particularly in isoflurane-anesthetized IAV-infected rats during hyperthermia.

Results: We found that the hippocampal EEG of the OP-administered (10 mg/kg) IAV-infected rats showed significant reduction of the high-voltage amplitudes and spikes, but the theta oscillations, which had been observed only at >40°C in OP non-administered rats, appeared at 38°C core temperature. Atropine (30 mg/kg) blocked the theta oscillations.

Conclusion: Our data suggest that OP efficiently reduces the abnormal EEG activities after IAV infection during hyperthermia. However, OP administration may stimulate ACh release in rats at normal core temperature.

Keywords: influenza A virus, oseltamivir, electroencephalogram, slow oscillations, theta oscillations, hippocampus

Introduction

Influenza A virus (IAV) is a common infectious pathogen in humans, which occasionally causes influenza-associated encephalopathy (IAE). IAE is characterized by severe neurological complications, such as convulsive seizures, loss of consciousness, and abnormal behaviors.[1,2]

Oseltamivir phosphate (OP) is a selective neuraminidase inhibitor that prevents influenza virus replication. It is prescribed for seasonal influenza and was the recommended drug for treating the anticipated pandemic of swine influenza (H1N1) in 2009.[3–5] OP works effectively in humans when used within 48 hours following the first appearance of symptoms (fever).[6] However, case reports have associated OP intake with sudden abnormal behaviors.[7–9]

Recent studies have shown that OP and its metabolite, OP carboxylate (OC), cross the blood-brain barrier.[10,11] They have been shown to induce neuronal excitability

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and synchrony in hippocampal slices and slice cultures.[7,12] Moreover, OP increases dopamine levels in the medial prefrontal cortex of rats.[13] Most animal model studies of the action of OP on the brain have been conducted in normal rather than IAV-infected rats or mice, and the animals functioned at the normal core temperature. Until now, there has been no information describing the effect of OP on intact brain hippocampal activity of IAV-infected animals during hyperthermia.

Despite the efficacy of OP in clinics, the putative side effects associated with OP have made the use of this drug controversial. In clinics, IAE patients' electroencephalograms (EEG) are characterized by abnormal high-voltage EEG activity with spikes and theta oscillations during high fever.[14–16] These clinical abnormal EEG activities (high-voltage amplitudes, increased EEG spikes, and theta oscillations) were reproduced in the rat model of IAE under hyperthermia (39.9°C–41°C).[17]

Rats and mice do not normally develop fever after IAV infection.[17,18] In a previous study,[17] abnormal EEG activities were not observed in non-anesthetized IAV-infected rats at the normal core temperature (37°C–38°C). Thus, hyperthermia was a precondition to observe abnormal EEG activities and was induced using a heating system, as previously reported.[17] In the present study, we examined the effect of OP on abnormal EEG activities in the hippocampus of isoflurane-anesthetized rats during hyperthermia, using the rat model of IAE.[17]

Methods
Animals, virus infection and electrophysiology

This study was performed in accordance with the guidelines for animal care and use approved by the animal care committee of The University of Tokushima. Since our goal was to model IAE in children, male Wistar rats aged 3 to 4 weeks were used because rats in this range correspond roughly to the human age range of 2.5 to 3 years.[19]

For viral infection, the rats were anesthetized with ketamine-xylazine (62.6 mg/kg–12.4 mg/kg). Influenza A/WSN/33 strain was stocked (frozen) at −82°C and diluted to obtain the dose (1 × 10^5 plaque forming unit in PBS, 60 μL), which was then applied intranasally to the rats, as previously reported.[17] The rooms for virus stockage, viral infection, and handling of the infected animals were designed in accordance with the guidelines for animal care and use of The University of Tokushima.

Anesthesia and hyperthermia

The in vivo electrophysiology experiments were carried out under isoflurane anesthesia. EEG activity in isoflurane anesthetized animals is characterized by slow bursting activity followed by a burst suppression period.[17,20]

The rats were first anesthetized by low-dose of ether, then fitted to a stereotaxic frame (model SN-6N, Narishige, Tokyo). The animals were gas-anaesthetized with 1.5–1.7 minimum alveolar concentration (MAC) of isoflurane mixed with 30% O$_2$ and 70% N$_2$, which allowed spontaneous respiration. During the EEG recordings, a heating pad system (model 21051-00; Fine Science Tools Inc, Foster City, CA) with a feedback control probe was inserted rectally. The body temperature of rats placed on the heating pad gradually increased from 37°C to 41°C in a period of 20 minutes, as previously reported.[17]

EEG recordings

EEG activity was recorded using enamel-coated tungsten wire electrodes with an uncoated diameter of 120 μm (MT Giken Co, Tokyo, Japan). Craniotomy was performed without damaging the underlying dura using a standard miniature drill equipped with 0.5 mm diameter drill bit. The electrodes were inserted based on the rat brain in stereotaxic coordinates of Paxinos and Watson.[21] To record the hippocampal activity, an electrode was lowered into the CA1-CA3 area (left hemisphere) at 2.8 to 3.0 mm posterior to the bregma, 2.7 to 2.9 mm lateral from midline, and 2.6 to 3.0 mm below the dura. Signals were recorded using a dual microprobe system (WP Manufacturing, Inc, Longmont, CO), and a homemade amplifier (×1000). The baseline was adjusted to zero-level with a slow voltage clamp system with a time constant of 2.2 seconds. The signal was low-pass filtered at 0.5 to 3 kHz, sampled at 1 kHz, and recorded using Axopatch software (Axon Instruments, Palo Alto, CA). To verify the electrode position, the electrode tip was coated with a lipophilic tracer dissolved in dimethylsulfoxide at a concentration of 1 mg/mL to 2.5 mg/mL before insertion into the brain. After removal of the electrodes, the rats were anesthetized with ketamine-xylazine (62.6 mg/kg–12.4 mg/kg) and were transcardially perfused with saline, followed by fixation (4% paraformaldehyde). The brain was removed and immediately put in sucrose and kept in a 4°C room. Sections 800 μm thick were then prepared. Red traces of the dye left by the electrodes were observed under the microscope and photographed.

The electrophysiology EEG data analysis was performed using IGOR Pro 4 (WaveMetrics, Inc, Lake Oswego, OR)

and the fast Fourier transformation (FFT) of EEG activity was computed for 30-second periods.

Slow and distinctive EEG waves were monitored continuously during the experiments to ensure that the rat was well anesthetized and without pain. At the end of experiments, the rats were given a lethal dose of pentobarbital (50 mg/kg).

Characterization of abnormal EEG activities

In order to examine the efficacy of OP on abnormal EEG activities during hyperthermia, we focused particularly on the following observed after IAV infection in the rats during hyperthermia:[17] abnormal high-voltage EEG activity, enhanced low-amplitude fluctuation (LAF) during burst suppressed periods, increased EEG spikes, and theta oscillations. The EEG activity was recorded in the hippocampus of the IAV-infected rats at 8 to 12 hours, 26 to 30 hours, and 50 to 56 hours, corresponding to the periods during which the peak abnormal EEG activities are observed in the rat model of IAE.[17]

The EEG amplitude was measured by quantifying the number of bursts (n = 15 bursts average/point) and the EEG spike was identified as a sharp wave that usually sprouts randomly within the burst and during the burst suppressed periods, as previously described.[17] Theta oscillations amplitude was determined by measuring theta wave amplitude (from the positive to the peak, n = 15 waves average/points). The details of the EEG components and parameters are shown in Figure 1A.

Drug and antagonist treatment

All the rats were infected with IAV. OP was not administered (control) to some of the rats, but was administered to the remaining rats. Tamiflu® capsules (75 mg) were purchased commercially from Chugai Pharmaceutical Co. (Tokyo, Japan), and the OP in the contents of the capsules was dissolved in water. OP mixture with stabilizing additives from the capsules or recrystallized OP was orally administered to the rats and they were monitored (for 1 hour postadministration) in a cage prior to the EEG recording. OP was administered to the rats in a single dose or in two doses per day (Figure 2A).

Atropine (30 mg/kg; Nacarai, Kyoto, Japan), an mAChR agonist, was dissolved in saline (0.9% NaCl) and was given via intraperitoneal (ip) injection.

Statistical analyses

Numerical values were expressed as mean ± SD. P value was obtained by Student's paired t-test (using SigmaPlot 10; Systat Software Inc, San Jose, CA), and $P < 0.05$ was considered statistically significant.

Results

In single dose cases, OP was administered 3 hours after infection (n = 5) and EEG was recorded at 8 hours to 12 hours after infection. In the control rats (IAV-infected rats not given OP), the EEG showed slow oscillations at 38°C core temperature; the corresponding slow component (<1 Hz) is depicted in the FFT plot (Figure 1B, top left). At 40°C core temperature, the EEG showed high-voltage slow EEG activity (or theta-like oscillations in two of the five rats) with increased spikes (arrowheads in the EEG traces) and enhanced amplitudes of the LAF, as shown in the expanded trace (segment 1) (Figure 1B, left bottom). In this study and a previous study,[17] theta-like oscillations in the control rats were observed only during hyperthermia (39.9°C–41°C). In contrast, in the OP-administered rats, the EEG displayed theta-like oscillations at the normal core temperature (38°C) in two out of the five rats (Figure 1B, top right). The FFT plot shows the corresponding theta frequency distributions (Figure 1B, bottom right). At 40°C, the EEG amplitude was reduced and the rhythmic activity of the LAF was disrupted while the amplitude of the LAF was reduced, as shown in the expanded trace (segment 2) shown in Figure 1B. There was a significant difference (n = 3, $P < 0.001$) between the amplitude of LAF values of the OP-administered rats (0.14 ± 0.05 mV) and that of the control rats (0.39 ± 0.06 mV). The quantified abnormal EEG amplitudes (left) and EEG spikes (right) were reduced in the OP-administered rats compared with the control rats (Figure 1B, bottom).

The EEG was then recorded at 26 to 30 hours after infection. Abnormal EEG amplitudes in OP-administered rats were reduced (n = 5, supplementary Figures 1), but the reduction was not significant. However, the amplitude of LAF values (0.26 ± 0.04) was significantly (n = 3, $P < 0.05$) reduced in the OP-administered rats compared to the control rats (0.14 ± 0.04), and the EEG spikes were significantly ($P < 0.001$) reduced in OP-administered rats. This suggests that the efficiency of the single-dose OP administration may be weakened after 26 hours of infection.

When two doses of OP were administered per day, abnormal EEG amplitudes ($P < 0.05$) and EEG spikes ($P < 0.001$) recorded at 26 to 30 hours after infection were more significantly reduced compared with the control rats (n = 3; Figure 2B, top). Those at 50 to 60 hours after infection were also reduced in comparison with the control rats (n = 3, Figure 2B, bottom). This finding suggests that two doses of OP administered per day were more efficient than a single dose in reducing abnormal EEG activities in IAV-infected rats.
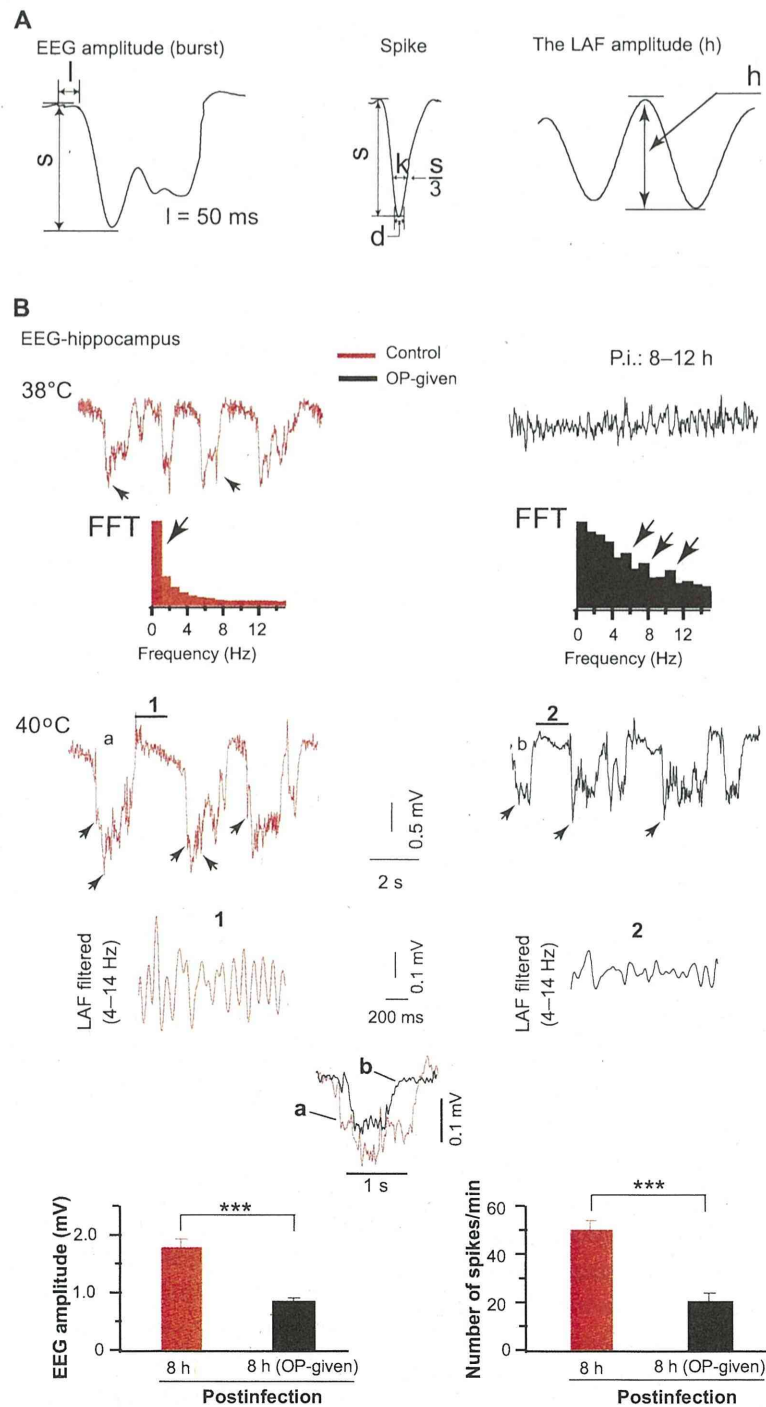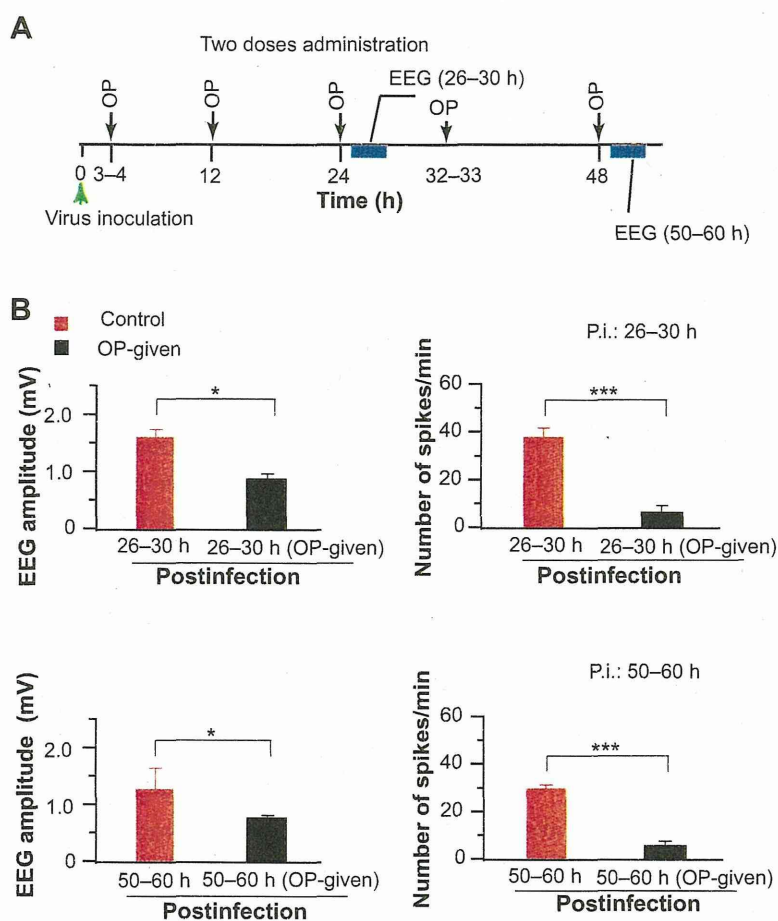
Figure 1 Abnormal EEG activities were reduced in infected rats treated with OP soon after infection. (**A**) The characterization of abnormal EEG components (S, amplitude in mV, from the base line to depth negative, I = 50 ms); spike (1 ms < d ≤ 15 ms, 1 < k ≤ 35 ms); LAF amplitude (h, amplitude in mV). (**B**) The recorded abnormal EEG activities at 8 to 12 hours after infection are displayed at 38°C and 40°C body temperatures. Left: At 38°C in the control rats, EEG displayed slow activity and its corresponding dominant slow component (arrow) is depicted in the FFT plot. At 40°C, EEG displayed high-voltage slow EEG activity with enhance LAF amplitude as depicted in the filtered (4–14 Hz) and expanded segment (1, right). In the OP-administered rats, EEG shows theta oscillations (top) at 38°C and the EEG corresponding FFT plot peaked at various theta band frequencies indicated by the arrows (bottom). At 40°C, EEG displayed a low-voltage slow EEG activity. The LAF is shown in the expanded segments (2) filtered at 4–14 Hz. Note a disrupted rhythmic activity and reduce amplitude in the trace (2). Superimposed traces of bursts of the control EEG (**a**) and of OP-administered EEG (**b**) displaying a clear reduced amplitude of OP-administered trace. Bottom: Quantification of EEG amplitude (left) and EEG spikes (right) are plotted.
Notes: A significantly decreased EEG amplitudes and number of spikes. Arrowhead in the EEG traces indicates the spike. OP-administered is replaced by OP-given. FFT of EEG activity was computed for periods of 30 seconds. ***$P < 0.001$, ns; n = 3.
Abbreviations: EEG, electroencephalogram; LAF, low-amplitude fluctuation; OP, oseltamivir phosphate; FFT, fast Fourier transformation; ns, nonsignificant.

Figure 2 OP significantly reduced abnormal EEG activities. OP applied two doses per day. (**A**) OP oral administration and EEG recording time course is schematized. The green arrowhead indicates the virus inoculation time, dark arrows show the OP administration time, and the blue rectangle indicates the EEG recording time. (**B**) Top: EEG activity recorded at 26 to 30 hours after infection.
Notes: The quantification plot shows reduced EEG amplitudes (left) and EEG spikes (right) in the OP-given rats. Bottom: Similar findings and arranged as in top for EEG activity recorded at 50 to 60 hours after infection. OP-given means OP-administered. Data are mean value ± standard deviation of the mean. *$P < 0.05$; ***$P < 0.001$; n = 3.
Abbreviations: EEG, electroencephalogram; OP, oseltamivir phosphate.

Two types of theta are known: atropine sensitive (a muscarinic receptor blocker) and atropine resistant.[22,23] We studied the effect of atropine on the OP-induced theta oscillations in IAV-infected rats. We first confirmed that EEG displayed theta oscillations at 38°C. Then, maintaining this temperature, a saline ip solution was injected, and 30 minutes after the saline injection, atropine (30 mg/kg, ip) was administered. As can be seen in Figure 3, atropine blocked the theta oscillation in all the rats examined (n = 3), revealing that the OP-induced theta oscillation is atropine sensitive.

Discussion

The present work is the first study to report the effect of OP on rats infected with IAV under hyperthermia in vivo.

We found that one or two orally administered doses of OP were efficient when given soon (~3 hours) after IAV infection. OP efficiently reduced the abnormal EEG activities (ie, enhanced amplitude of the LAF, high-voltage EEG amplitudes, increased EEG spike) in the IAV-infected rats. However, we also observed theta oscillations at 38°C core temperature in the OP-administered rats.

Although the mechanisms by which OP reduces abnormal EEG activity are not clear, we presume at least that the N-methyl-D-aspartate (NMDA) receptor and the blockage of the virus spread from the host cells could play important roles in reducing the high-voltage amplitudes and EEG spikes, as explained below.

Izumi[7] reported that OP induces neuronal excitability via NMDA receptor activation. Our recent study also found that in uninfected rats the activation of NMDA
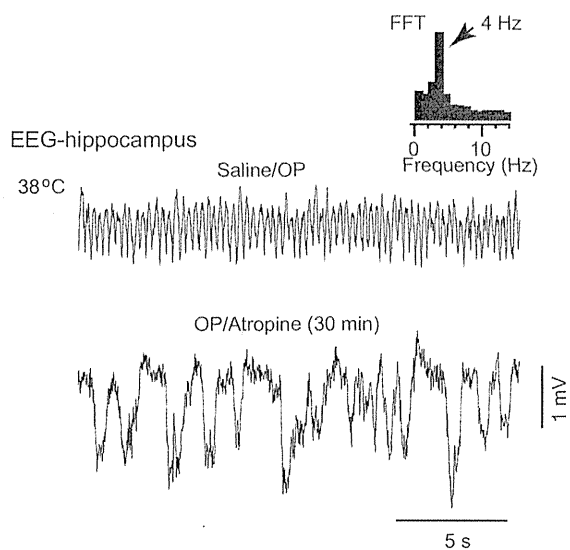
Figure 3 Atropine blocked theta oscillations. The EEG activity was recorded in the hippocampus at 38°C rat body temperature. OP-induced theta oscillations were suppressed by intraperitoneal injection of atropine.
Notes: The FFT plot indicating the theta frequency peak at 4 Hz (top). Atropine blocked theta oscillation in all administered (n = 3) rats. FFT of EEG activity was computed for periods of 30 seconds.
Abbreviations: EEG, electroencephalogram; OP, oseltamivir phosphate; FFT, fast Fourier transformation.

receptors peaked at 2 hours after OP administration and declined at 4 hours after OP administration (data not shown). This suggests the inactivation of the NMDA receptors at 4 hours after OP administration, and this inactivation may prevent calcium influx into the neurons. It is also known that OP is a sialic acid analogue, which inhibits the influenza neuraminidase enzyme and prevents the release and spread of the virus from infected host cells during budding.[4,6] OP treatment (1 hour after infection at daily base) significantly reduces the infection rate in multiple cell types and reduces the progression of the virus in mice.[24] These previous findings suggest that the early administration of OP after IAV infection within a period before the virus proliferates, in addition to the inactivation of the NMDA receptors at a later time (>4 hours) after OP administration, could be possible factors in explaining how OP works to reduce the abnormal EEG activities.

The observation of theta oscillations at 38°C in OP-administered rats is an important finding for confirming the possible action of OP on muscarinic acetylcholine (mACh) receptors. We have observed a similar finding in normal uninfected rats; the OP administered to these rats induced theta oscillations (2 Hz to 6 Hz) at 38°C core temperature (data not shown). Theta oscillations were atropine sensitive

and prevented fast hippocampal activity, which resulted in a slow EEG activity (Figure 3). This result is in full agreement with a previously reported in vitro study in which OP was found to activate mACh receptors.[12] The activation of mACh receptors may suggest the stimulation of acetylcholine containing cholinergic neurons, which have been known to play important roles in cortical activation and in regulating states of consciousness.[25,26] ACh is a major excitatory neurotransmitter in the central nervous system. It plays key roles during synaptic transmission, and constitutes a system with other neurotransmitter/modulators to regulate brain states of vigilance.[25]

Because the EEG recording from the hippocampus in nonanesthetized rats was not possible, the effect of anesthesia could be an issue to consider during the interpretation of the data. Isoflurane has a neuroprotective effect on the brain. It reduces excitatory synaptic transmission in the hippocampus,[27–29] and it enhances inhibitory synaptic potentials at concentrations above 0.5 MAC (1%).[29] Isoflurane alters the ACh release in a dose-related manner, and that at 1.5 MAC, it significantly decreases ACh release in the cortex and striatum of rats.[30,31] With such concentrations, EEG activity is generally characterized by slow (bursting) activity followed by burst-suppressed periods.[17,20] In the present and previous studies,[17] 1.5–1.7 MAC isoflurane was used. Thus, it is unlikely that the theta oscillations were isoflurane dependent.

The physiological significance of high-voltage slow and theta oscillations in influenza patients in the clinic is not well documented. However, in the rat model of IAE, we speculated that the alternation between these two oscillations may lead to brain instability, and that this may explain the abnormal behaviors observed in some patients.[17] In the present study, the OP-induced theta oscillations were similar to those observed during hyperthermia in the IAV-infected rats not given OP. Thus, both theta oscillations suggest the stimulation of ACh release. Excessive release of ACh may affect synaptic transmission and oscillation patterns in the brain, which may lead to abnormal behavior in influenza patients. Under such conditions, atropine may play a therapeutic role in stabilizing the brain states from fast (2–6 Hz) to slow (<1 Hz) oscillations.

In the present study, because hyperthermia was a precondition for observing EEG abnormalities in the control rats, the effect of OP on core temperature before and during EEG recording was not investigated. However, recent studies showed that OP induces hypothermia in mice[32] and OP administered in ethanol-injected rats significantly augmented