



Excitatory and Inhibitory Amino Acids in Cerebrospinal Fluid of Familial Spontaneous Epileptic Cats

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Abstract

Objective: To evaluate the excitatory and inhibitory amino acids in the cerebrospinal fluid (CSF) from cats with familial spontaneous epilepsy (FSEC).

Animals: Thirteen epileptic cats (suspected homozygotes: Affected) and nine non-epileptic cats (suspected heterozygotes: Unaffected) from FSEC line, and six clinical healthy cats which were unrelated FSEC line (Healthy).

Procedures: CSF samples were collected before and after vestibular stimulation, which induced generalized seizures only in the affected cats. Excitatory/inhibitory amino acids (aspartate, glutamate, serine, glycine taurine, and gamma-aminobutyric acid (GABA)) levels in the CSF were analyzed by high performance liquid chromatography. In addition, CSF samples from five Affected cats in the interictal phase, four Unaffected cats, and three Healthy cats were used for metabolomics.

Results: There was no difference in CSF glutamate concentrations between Affected and Healthy groups, while significantly lower glutamate concentrations were detected in the unaffected group. Following vestibular stimulation, glutamate concentrations were significantly elevated in the Unaffected and Healthy groups, but not in the Affected group. Moreover, the affected group exhibited the lower glutamine concentrations than the Healthy group. There was no difference in GABA concentrations between the groups, although some Affected cats showed elevated GABA.

Conclusions and clinical relevance: In FSEC, an imbalance between excitatory and inhibitory neurotransmitters may not be the direct cause of epileptic seizures, while alterations in the glutamate-glutamine cycle may be a causative mechanism. These findings provide new insight into the pathogenesis of FSEC and familial mesial temporal lobe epilepsy in human and animals.

Keywords

Cats; cerebrospinal fluid; Migration time; GABA

Introduction

Imbalance between excitatory and inhibitory neurotransmitters in the brain is considered a causative mechanism of epileptic seizures in humans and animal models [1–3]. In veterinary medicine, several reports have described the amino acid concentrations in the cerebrospinal fluid (CSF) from dogs with idiopathic epilepsy [4–8]. Glutamate is a major excitatory neurotransmitter in the mammalian central nervous system (CNS), and is key for higher brain functions such as learning and memory [9,10]. However, excess glutamate in the brain induces excitotoxicity, which causes numerous CNS pathogenesis including epilepsy [10,11]. For example, glutamate excitotoxicity plays an important role in the initiation, propagation, and continuance of epileptic activity [10–14]. By contrast, γ -aminobutyric acid (GABA) is a major inhibitory neurotransmitter in the CNS [15]. GABA is synthesized primarily from glutamate by glutamate decarboxylase in the soma and terminal of neurons [16]. In both in humans and animals models, GABA has also been shown to play a critical roles in the pathogenesis and treatment of epilepsy [5,17,18] and GABA concentrations in the CSF are reduced in human epileptic patients and animal seizure models [3,19]. In addition to glutamate and GABA, other amino acids such as aspartate, glycine, serine, and taurine also work as neurotransmitters and may modify the pathophysiology of epilepsy [20,21].

Cats have been widely utilized as experimental animals in the neurosciences, including epileptic seizure models. Neurotransmitters in the CSF and brain extracellular fluids were originally determined in feline seizure models [22,23]. However, the changes in neurotransmitters in cats with spontaneous (naturally occurring) epilepsy are poorly understood. We previously reported the model of FSECs, which show an autosomal recessive mode of inheritance [24]. FSEC represents a typical form of feline epilepsy [25] and is considered a natural model of familial mesial temporal lobe epilepsy [26,27].

Metabolomics is a tool that provides exhaustive analysis of metabolites in vivo. Metabolomic approaches have been widely used for identification of disease-related biomarkers and understanding the molecular mechanisms of disease [28–30]. Thus, we also employed a metabolomic approach to confirm our chromatographic analysis of CSF amino acids, and to assess changes in endogenous low molecular weight metabolites, in FSECs.

The purpose of this study reported here was to evaluate CSF levels of excitatory/inhibitory amino acids to further our understanding of the pathophysiology of FSEC. We hypothesized that the abnormal imbalance of excitatory and inhibitory amino acid concentrations in the CSF are the cause of epilepsy in FSEC. That is 1) higher excitatory amino acid and lower inhibitory amino acid during interictal phase, and then change into 2) lower excitatory amino acid and higher inhibitory amino acid during postictal phase.

Materials and Methods

Animals

This study was approved by the Animal Care and Use Committee of Nippon Veterinary and Life Science University (accession

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nos. 09-1, 10-3, 11-51, 12-42; representative researcher is D.H.). FSECs were maintained in the Nippon Veterinary and Life Science University and a commercial animal breeding facility (Chiba, Japan) [24]. These housings were conventional animal facilities. Cats were kept individually in the stainless clean cages (length × width × depth=around 1 × 1 × 1 m) with a water cup, bedding box and pet sheets. Light/dark cycle was loosely maintained 12L/12D, and room temperature was kept $23 \pm 3^\circ\text{C}$ by the air conditioner. Abundant tap water and necessary and sufficient amount of pouched cat food were served according to the manufacturer's recommendation (Royal Canin, Aimargues, France). Welfare-related assessments were carried out before, during and after the experiment. From the family, thirteen epileptic cats (19–84 months old, 2.5–4.2 kg), which showed recurrent seizures and were predicted homozygote (Affected group) by pedigree analysis, and nine non-epileptic cats (69–114 months old, 2.6–4.5 kg), which were parents of the Affected cats and were predicted heterozygote (Unaffected group), were used in this study. In addition, six clinically healthy cats (9–15 months old, 2.2–3.8 kg), which were maintained in the same conditions and were unrelated family line to FSECs, were used as the Healthy group. All experiments were open studies and experimenters were not blinded while analyzing the samples.

Collection of CSF samples

CSF samples were collected by cisternal puncture with 23 G needles under sedation by 0.07–0.08 mg/kg body weight intramuscular injection of medetomidine. In the Affected group, CSF samples were collected during the interictal phase, defined as a seizure-free time period of at least two weeks, and during the postictal phase, defined as the time period within 30 min after vestibular stimulation-induced generalized seizures. In all groups, vestibular stimulations were induced by manually swinging the carry cage up, down, right, and left for 2 min. In the Unaffected group and Healthy group, CSF samples were collected during the normal state and within 30 min after vestibular stimulation. CSF samples were stored -80°C until analysis.

High-performance liquid chromatography (HPLC) analysis of CSF amino acids

Excitatory/inhibitory amino acids (aspartate, glutamate, serine, glycine, taurine, and GABA) levels in the CSF were analyzed by HPLC with o-phthalaldehyde precolumn derivatization. The HPLC system consisted of two pumps, an autosampler, a column oven, a fluorescence detector and an integrator. Two Octa Decyl Silyl (ODS) columns (Pegasil ODS; 4.6 mm × 30 mm and 4.6 mm × 250 mm) were used in-line at 40°C . CSF samples were thawed immediately before use. Each of the CSF samples (40 μl) were deproteinized by mixing with 120 μl of 0.6 M HClO_4 and 40 μl of 2 M K_2CO_3 . The mixture was centrifuged at $10,000 \times g$ for 5 min. The supernatant was used for amino acids analysis. For pre-column derivatization, 60 μl of sample was mixed with 15 μl of OPA solution. The OPA solution is prepared by adding 1 ml of o-phthalaldehyde (27 mg/1 ml methanol) and 25 μl of 2-mercaptoethanol in 4 ml of 0.1 M borate buffer (7.63g $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}/200$ ml ultra pure water). After 30 s reaction, 10 μl of sample solution was injected into a HPLC column. The resolution of the amino acids was accomplished by use of gradient elution. The mobile phase consisted of solvent A (50 mM CH_3COONa , pH 5.80, 3.0% N,N-dimethylformamide) and solvent B (methanol), and was eluted at a flow rate of 1.0 ml/min. A gradient protocol was performed as follows: a linear gradient from 85% to 15% of solvent A for 55 min. Fluorescence of the eluent was monitored for 55 min with excitation wavelength set at 340 nm and emission wavelength set at 455 nm. To equilibrate the column between each run, the 85% solvent A was

isocratically eluted for 9 min. Values of amino acids were calculated by analyzing amino acid standards with known levels.

Metabolomics of CSF

The CSF samples from five Affected cats in the interictal phase, and CSF from four Unaffected cats and three Healthy cats, were used for metabolomics. Metabolome measurements were performed through a facility service at Human Metabolome Technologies Inc. (Yamagata, Japan). CSF 100 μl was mixed with 900 μl of methanol including 10 μM of internal standard. Next, 1,000 μl of chloroform and 400 μl of ultra-pure water were added to the mixture, and were mixed thoroughly and centrifuged (2,300 × g, 4°C , 5 min). The 400 μl of the supernatant was centrifugally filtered through a cutoff filter to remove proteins. The filtrate was lyophilized, resuspended in 50 μl ultra-pure water, and analyzed by capillary electrophoresis time-of-flight mass spectrometry (CE-TOFMS). Peaks were extracted using automatic integration software to obtain peak information including m/z, migration time for CE-TOFMS measurement (MT), and peak area. Signal peaks corresponding to isotopomers, adduct ions, and other product ions of known metabolites were excluded, and remaining peaks were annotated with putative metabolites from the Human Metabolome Technologies metabolite database based on their MTs and m/z values determined by TOFMS. The tolerance range for peak annotation was configured at ± 0.5 min for MT and ± 10 ppm for m/z. In addition, peak areas were normalized against those of the internal standards, and the resultant relative area values were then further normalized by sample amount.

Statistical analysis

Differences of amino acid levels in the CSF among groups were compared using Kruskal-Wallis test followed by post hoc test. Paired t-test was used to compare the CSF levels of amino acids between the interictal (normal) state and the postictal (after vestibular stimulation) in each group. Results of metabolomics were tested by Kruskal-Wallis test, followed by post hoc test. Statistical analysis was carried out using software program. $P < 0.05$ was considered statistically significant.

Results

HPLC analysis of CSF amino acids

Figure 1 shows CSF concentrations of aspartate, glutamate, serine, glycine, taurine, and GABA during the interictal (Affected) or normal (Unaffected and Healthy) state. Glutamate level in the Unaffected group was significantly lower than in the Healthy group, while there was no significant differences in the CSF levels of aspartate, serine, glycine, or taurine. Several cats in the affected group also showed trends for elevated GABA concentrations.

Figure 2 shows the changes in CSF amino acid levels following vestibular stimulation. Data are shown as absolute value difference between interictal (normal) and postictal (after vestibular stimulation). As vestibular stimulation induced epileptic seizures in five of 13 cats in the affected group, CSF samples of the five cats were collected immediately after the seizure, while the remaining eight cats were not included. CSF samples of eight of nine Unaffected cats and all of the six Healthy cats were collected after vestibular stimulation.

The CSF glutamate concentrations were significantly elevated after vestibular stimulation in the Unaffected and Healthy groups. By contrast, there was no significant change in glutamate concentrations in the Affected group. Further, there were no changes in other CSF amino acid following vestibular stimulation.

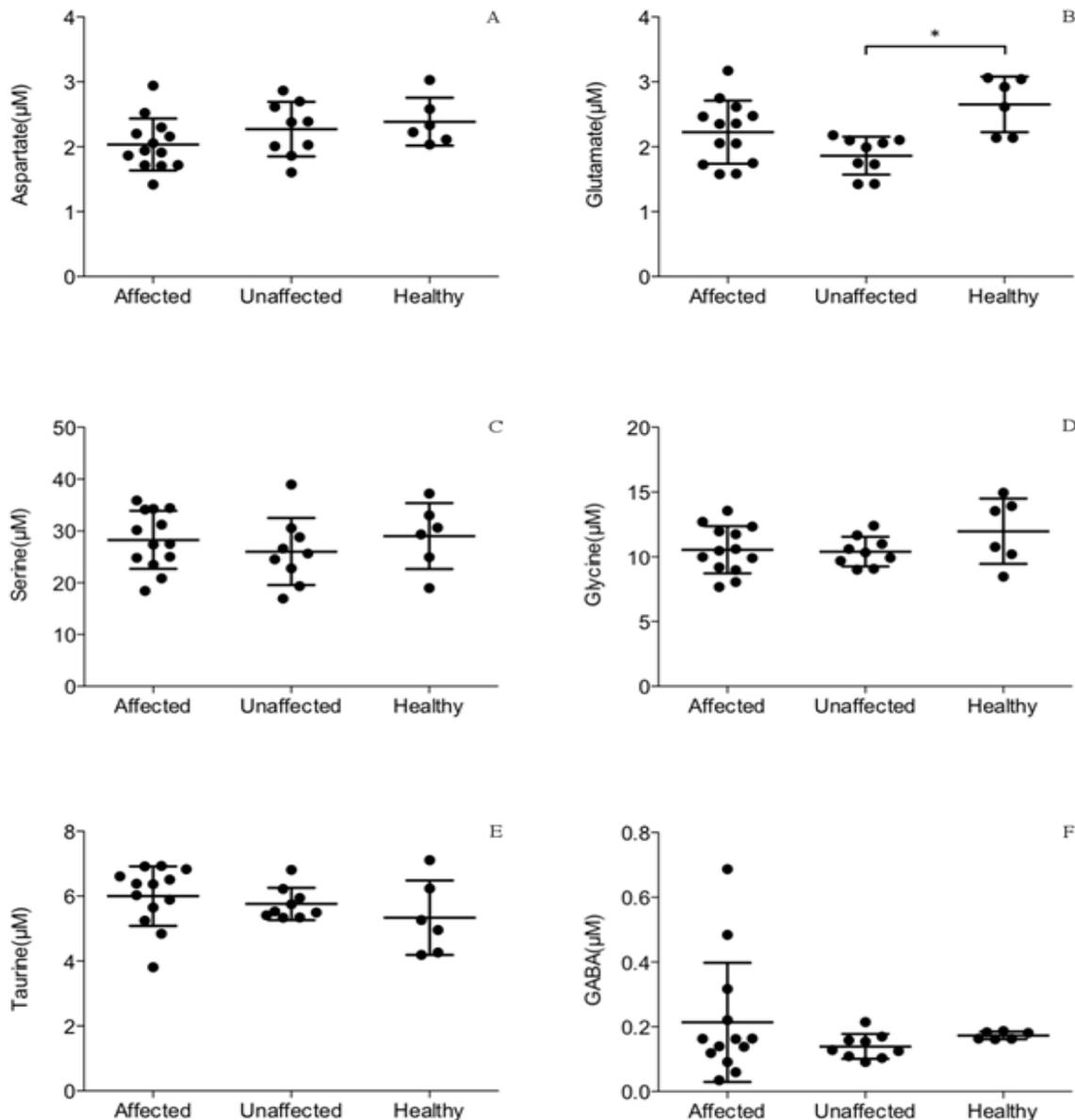


Figure 1: Levels of aspartate (A), glutamate (B), serine (C), glycine (D), taurine (E), and GABA (F) in the cerebrospinal fluid from Affected cats (n=13), Unaffected cats (n=9), and Healthy cats (n=6). Each point represents the mean of duplicate assays. Bars indicate the mean \pm SD * p<0.05.

Metabolomics of CSF

Metabolomic analysis resulted in detection of 95 metabolites, which consisted of 21 amino acids and 74 low molecular weight compounds. The relative amounts of the 21 amino acids (Affected/Healthy and Unaffected/Healthy) are shown in Table 1. CSF glutamine and 2-aminobutyric acid concentrations were significantly lower in the Affected group compared with the Healthy group. Among the 74 low molecular weight compounds, five compounds were significantly different between groups.

Discussion

In the present study, we evaluated levels of excitatory/inhibitory amino acid in the CSF of FSECs. During the interictal state, the Affected group showed similar levels of CSF glutamate to those in

the Healthy group. Moreover, during the normal (without vestibular stimulation) state, the Unaffected group showed significantly lower glutamate levels than the Healthy group. Several studies on spontaneous epilepsy in humans and animals reported increased levels of CSF glutamate during the interictal state and hypothesized that increased glutamate triggers epileptic seizures [6,18,19]. However, this hypothesis does not explain the CSF glutamate levels in our familial epileptic cats. In human patients with temporal lobe epilepsy, extracellular glutamate levels in the epileptogenic hippocampus are elevated before and during seizures, and then quickly return to basal levels after seizures resolution, Chapman et al.[13] suggesting that elevation of extracellular glutamate levels contributes to generation and evolution of seizures.

To evaluate the dynamic changes in CSF amino acids, we used vestibular stimulation to trigger epileptic seizures in affected cats.

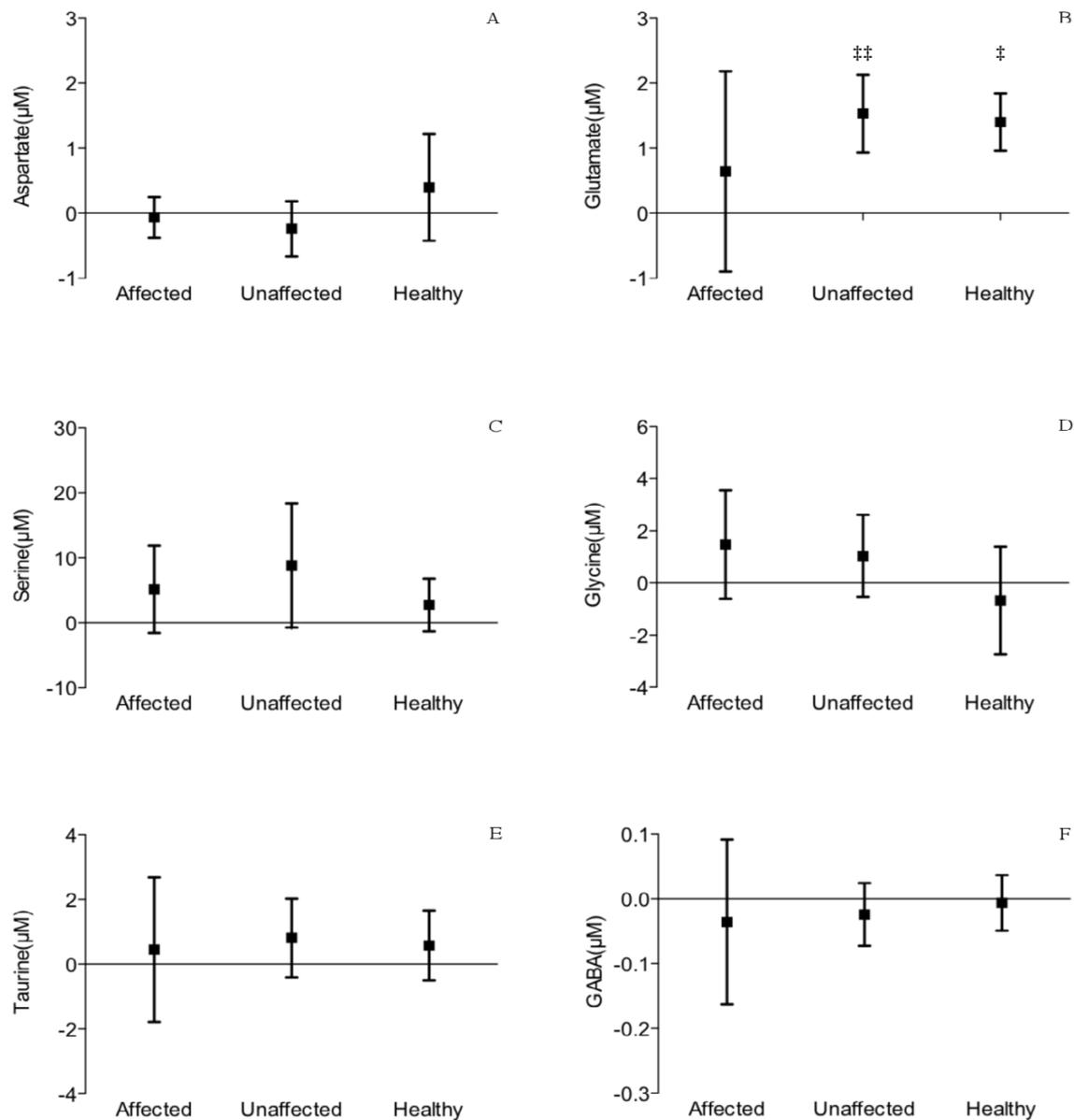


Figure 2: Absolute value difference of CSF aspartate (A), glutamate (B), serine (C), glycine (D), taurine (E), and GABA (F) levels in the interictal phase from the postictal phase in the Affected group. The absolute value differences of normal state compared with those after vestibular stimulation are shown for the Unaffected and Healthy group. ^{‡‡} $p < 0.01$, [‡] $p < 0.05$.

In Unaffected and Healthy cats, CSF glutamate concentrations were significantly elevated by vestibular stimulation, suggesting that increased glutamate release and/or decreased glutamate uptake in the brain occurred after vestibular stimulation. By contrast, CSF glutamate in Affected cats remained at basal levels after epileptic seizures induced by vestibular stimulation. It is possible that glutamate levels in the CSF of Affected cats were elevated, but then quickly returned to the basal levels before CSF sampling. In rat kindling models, expression of excitatory amino acid transporters on glial cells (EAAT1/GLAST and EAAT2/GLT-1) was decreased by seizures [31]. On the other hand, expression of the neuronal glutamate transporter EAAT3/EAAC1 was increased by seizures [31-33]. A similar mechanism may stabilize CSF glutamate levels after seizures in the Affected group in the present study.

We also found that CSF glutamine levels were significantly lower in the affected group than in the Healthy group. Further, in the unaffected group, glutamine levels tended to be lower than in the Healthy group. Glutamine is a major precursor of glutamate [34]. Glutamate released from presynaptic terminals is taken up by astrocytes and converted to glutamine by glutamine synthetase. The newly synthesized glutamine is transferred to neurons and hydrolyzed into glutamate by mitochondrial glutaminase. This cycle is called the 'glutamate-glutamine cycle' [35]. The low CSF glutamine levels in the Affected and Unaffected groups suggests that decreased substrates of the glutamate-glutamine cycle, which may result in the low CSF glutamate levels observed in the Unaffected group. In addition, increased glutamate uptake into neurons may lead to reduced glutamate uptake and glutamine synthesis by glial cells.

Table 1: Levels of metabolites and amino acids in the various groups.

| | Ratio (Mean ± SD) | |
|--------------------------|-------------------|--------------------|
| | Affected/Healthy | Unaffected/Healthy |
| <i>Amino acids</i> | | |
| Aspartate | 0.82 ± 0.11 | 0.95 ± 0.25 |
| Glutamate | 0.80 ± 0.12 | 0.85 ± 0.17 |
| Serine | 0.74 ± 0.13 | 0.90 ± 0.14 |
| Glycine | 0.90 ± 0.06 | 1.03 ± 0.13 |
| Methionine | 0.79 ± 0.14 | 1.12 ± 0.88 |
| Glutamine | 0.80 ± 0.05* | 0.86 ± 0.07 |
| Lysine | 2.10 ± 0.44 | 1.89 ± 0.67 |
| Arginine | 1.60 ± 0.40 | 1.68 ± 0.32 |
| Tryptophan | 1.17 ± 0.09 | 1.18 ± 0.19 |
| Phenylalanine | 1.02 ± 0.08 | 1.00 ± 0.16 |
| Leucine | 0.94 ± 0.10 | 1.15 ± 0.20 |
| Isoleucine | 0.91 ± 0.11 | 1.15 ± 0.18 |
| Histidine | 0.90 ± 0.14 | 1.00 ± 0.38 |
| Valine | 0.82 ± 0.10 | 0.83 ± 0.11 |
| Threonine | 0.74 ± 0.18 | 0.74 ± 0.12 |
| Asparagine | 0.88 ± 0.10 | 0.86 ± 0.14 |
| Tyrosine | 0.83 ± 0.12 | 0.78 ± 0.15 |
| Proline | 0.77 ± 0.11 | 0.77 ± 0.15 |
| Alanine | 0.70 ± 0.07 | 0.69 ± 0.19 |
| Ornithine | 1.36 ± 0.26 | 1.54 ± 0.27 |
| 2-Aminobutyric acid | 0.59 ± 0.11* | 0.93 ± 0.31 |
| <i>Other metabolites</i> | | |
| Pyridoxal | 1.35 ± 0.18† | 0.86 ± 0.12 |
| 2-Hydroxybutyric acid | 0.71 ± 0.11†† | 0.94 ± 0.12 |
| 3-Hydroxybutyric acid | 0.64 ± 0.12* | 0.76 ± 0.14 |
| Cyridine | 0.76 ± 0.08 | 0.66 ± 0.10* |
| Saccharopine | 0.61 ± 0.07* | 0.68 ± 0.06 |

* p<0.05, ** p<0.01, *** p<0.001, significantly different from Healthy group cats. † p<0.05, †† p<0.01, significantly different from Unaffected group cats.

In the present study, elevated GABA levels were observed in several cats in the affected group. In the CNS, GABA is mainly synthesized from glutamate by glutamate decarboxylase in GABAergic neurons. GABA is an inhibitory neurotransmitter that reduces neural hyperexcitability and seizures [36–38]. In the Affected group, the elevated GABA suggests a compensatory reaction to epileptic activity in individual cats.

Our metabolomic analysis detected 21 CSF amino acids, with similar changes to that in the HPLC analysis. Additionally, there were significant differences in five low molecular weight compounds (pyridoxal, 2-hydroxybutyric acid, 3-hydroxybutyric acid, cyridine, and saccharopine) between the groups. However, our metabolic pathway analysis could not identify the definite relationship to CNS metabolism. Further metabolomics studies are required to examine these compounds in more detail.

The results obtained in this study were contrary to our initial hypothesis. Interestingly, the levels of excitatory amino acids in the affected group did not change during interictal or postictal phase, and the level of GABA, the main inhibitory amino acid, tended to be higher during interictal phase. In addition, in the unaffected group, the level of glutamate was lower during the interictal phase. These results may lead us to elucidation seizure mechanisms in FSECs.

Conclusion

In conclusion, we found characteristic changes in CSF glutamate, glutamine, and GABA levels in FSECs. These findings further our

understanding of the pathogenesis of FSEC, as well as familial mesial temporal lobe epilepsy.

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