Changes in Extracellular Neurotransmitters in the Cerebrum of Familial Idiopathic Epileptic Shetland Sheepdogs Using an Intracerebral Microdialysis Technique and Immunohistochemical Study for Glutamate Metabolism

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ABSTRACT. Intracerebral microdialysis combined with electroencephalographic recordings was performed on 4 dogs of a familial idiopathic epileptic Shetland sheepdog colony to identify the kinds of neurotransmitters responsible for seizure activity. Immunohistochemistry using glutamate (Glu), glutamate transporter (GLT-1 and GLAST), and glutamine synthetase (GS) antibodies was also carried out on the cerebrum of four familial dogs that died of status epilepticus (SE). High values for extracellular levels of Glu and aspartate (ASP) were detected in association with an increased number of spikes and sharp waves during hyperventilation in 3 of 4 the familial epileptic dogs. The values of other amino acids analyzed were not altered in any of the familial epileptic dogs. Immunohistochemically, Glu-positive granules were occasionally found in the perineuronal spaces of the cerebral cortex in 3 of the familial epileptic dogs that died of SE. Immunostains for GLT-1 antibody predominantly decreased in the cerebral cortex and lateral nucleus of the thalamus in all the dogs that died of SE, whereas there were no differences detected in immunolabellings for GLAST and GS antibodies between familial epileptic dogs and controls. These results suggest that an extracellular release of both Glu and Asp may play an important role in the occurrence of seizure activity in this epileptic colony, and that a decreased expression of astrocytic GLT-1 may be related to development of SE.
KEY WORDS: canine, epilepsy, GLT-1, glutamate, neurotransmitter.

Idiopathic epilepsy is one of the most common causes of seizure in dogs and humans. Although inheritance has been suggested as a possible etiology of idiopathic epilepsy in dogs [6, 9, 16] and humans [1, 17], the pathophysiology of this syndrome is not fully understood. Electroencephalographic (EEG) epileptic discharges may result from a mismatch between excitatory (e.g., aspartate and glutamate) and inhibitory (e.g., γ-aminobutyrate) amino acid systems [18]. Excitatory amino acids could also act as factors causing the seizure-related brain damage according to the concept of excitotoxicity [12–14, 24]. Recently, we reported a familial idiopathic epileptic dog that is considered to belong to the frontal lobe epilepsy category [15]. The neurochemical events in the canine epileptic focus have not previously been monitored *in vivo* since no technique has been available for collecting neurotransmitters directly from the canine brain. However, this can be achieved with intracerebral microdialysis, which has recently been applied to human patients [4, 18, 31] and experimental animals [3, 10, 19, 31]. We used this sampling method together with recording of the electrical cortical activity using EEG.

In the central nervous system (CNS), glutamate (Glu) is a major neurotransmitter involved in fast excitatory synaptic transmission, plasticity and development, and pathophysiologies of neurodegenerative diseases [5, 11]. The Glu transporter has been considered to play an important role in

inactivation of synaptically released Glu in the CNS [21, 26]. To date, four isoforms of Glu transporter have been identified, including the GLAST (EAAT1) and GLT-1 (EAAT2) astroglial transporters and the EAAC1 (EAAT3) and EAAT4 neuronal transporters. GLT-1 is mainly present in the cerebral cortex and hippocampus, whereas GLAST, which is a Glu and Asp transporter, is exclusively located in the cerebellum. The general concept is that astrocytes have a more efficient uptake system for Glu than neurons [21, 26]. On the other hand, glutamine synthetase (GS) is a ubiquitous enzyme that catalyzes the ATP-dependent conversion of Glu to glutamine (Gln) using ammonia as the nitrogen source [30]. After release from neurons into the synaptic cleft, Glu is taken up by GLT-1 and catabolized by astrocytes. Glu in astroglial cytoplasm can be transformed by GS to Gln, which is available for neurons as a precursor amino acid of Glu [26]. There have been, to our knowledge, few reports on immunohistochemical study of Glu, GLT-1, GLAST, and GS in spontaneously familial epileptic dogs.

The purpose of the present study is to identify neurotransmitters responsible for the occurrence of abnormal EEG waves, such as spikes and sharp waves, stimulated by hyperventilation (HV), and to investigate whether the metabolism of Glu, which is a main excitatory neurotransmitter in the CNS, would be affected in a familial epileptic Shetland sheepdog colony.

MATERIALS AND METHODS

Electroencephalography and hyperventilation: Four familial epileptic Shetland sheepdogs (Nos. 4 to 7) that had clinically shown no episode of seizures, ranging in age from 1 year and 1 month to 3 years and 8 months, and 3 age-matched control Shetland sheepdogs (Nos. 1 to 3), ranging from 2 years and 11 months to 4 years, were used. All dogs were sedated with a 1.0 mg/kg of body weight intramuscular injection of xylazine (Celactal, Bayer AG, Germany). Under anesthesia with 1–2% halothane (Fluothane, Takeda Pharmaceutical Company Limited, Osaka, Japan), dogs were submitted to a single recording using standard 10–20 surface electrodes [15]. A 2-channel electroencephalograph in the internal-dorsal areas of the frontal and parietal lobes was used for EEG recording and a polygraph (No. 1A52, NEC San-ei, Tokyo, Japan) was used with a time constant of 0.3 seconds, an amplification sensitivity of 50 μV/5 mm, and a high-frequency filter of 60 Hz. The paper speed was 3.0 cm/second as the voltage calibration was maintained constantly at a deflection of 5 mm/50 μV. The electrodes were 27-gauge tungsten-platinium alloy subdermal needle electrodes. Hyperventilation (HV) to define sharp waves and spikes was carried out with a ventilator (Igarashi Ika Kogyo, Japan) using a standardized procedure while monitoring arterial pH and carbon dioxide pressure ($PaCO_2$). The HV condition was attained when the $PaCO_2$ values ranged from 10 to 20. The HV condition lasted at least 30 min.

Microdyalysis and analysis of extracellular amino acids: Microdialysis probes were inserted into the internal-dorsal areas of the frontal and parietal lobes. Steril microdialysis probes with membrane lengths of 5 mm (EICOM Corporation, Japan) were inserted into the internal-dorsal area near the EEG electrode positions in the frontal and parietal lobes, and were perfused with sterile Ringer's solution (140 mM Na^+, 2.7 mM K^+, 1.2 mM Ca^{2+}, and 147 mM Cl^- per 1,000 ml) at a flow rate of 2 μl/min by a microinjection pump. To avoid the damaging effect of probe insertion, sample collection was started at 2 hr after insertion of the probe. Samples were collected every 10 min and frozen at –80°C. They were then analyzed for amino acids by high-performance liquid chromatography using an electrochemical detection (HPLC-ED) instrument (Microdialysis detection system, DTA-300, EICOM Corp., Japan). The HPLC-ED system was optimized for analysis of aspartate (Asp), glutamate (Glu), γ-aminobutyrate (GABA), arginine, glycine, and taurine at the expense of glutamine (Gln) and asparagine (Asn). The values of all the amino acids were changed into ratios for the value of amino acids during normal ventilation in each case. This study was carried out under the control of the Animal Research Committee in accordance with the Guidelines for Animal Experimentation of the Faculty of Agriculture, Tottori University [15].

Immunohistochemistry: Four familial epileptic dogs that died of SE, ranging in age from 1 year and 8 months to 4 years and 8 months, were examined. Three clinically healthy Shetland sheepdogs, ranging in age from 2 years and 4 months to 6 years old were used as controls. Complete postmortem examination was performed, and systemic organs were immediately fixed in 10% phosphate-buffered formalin, embedded in paraffin, and cut at 5 μm. Sections were stained with the hematoxylin and eosin (HE) and Klüver-Barrera (KB) stains. Three polyclonal antibodies, one for GLT-1 (Wako, Japan diluted 1:100), one for GLAST [23], and one for Glu (Chemicon, U.S.A., diluted 1:5), and one monoclonal antibody for GS (Chemicon, U.S.A., diluted 1: 300) were used for immunohistochemistry. Sections were employed for immunohistochemical analysis using the labeled streptavidin procedure (DAKO, Glostrup, Denmark). Antigen recovery was accomplished for the used antibodies by exposure to microwaves. After blocking endogenous peroxidase activity with 3% hydrogen peroxide (H_2O_2), sections were transferred into 10% normal goat serum, used as a blocking reagent and blocking was performed for 7 min in the micro oven. This was followed by incubation with the respective primary antibodies for 20 min in the microwave oven. Sections were sequentially incubated with biotinylated anti-mouse or anti-rabbit IgG antiserum (diluted 1:200) for 7 min in a microwave oven, and then incubated with streptavidin complex. A peroxidase reaction product was developed by incubating sections for approximately 5 min in a solution of 3,3-diaminobenzidine tetrahydrochloride. After rinsing in distilled water, sections were weakly counterstained with Mayer's hematoxylin. During staining for the negative control, sections were incubated with non-immune serum instead of each primary antibody.

RESULTS

Electroencephalographic findings: A high frequency of sharp waves was observed in the frontal lobe of the familial epileptic dogs, whereas a small number of sharp waves was detected in the parietal lobe. No abnormal EEG waves were deteceted in the 3 control dogs. The three control cases (Nos. 1 to 3) showed no abnormal waves on the EEG under normal ventilation and HV (Fig. 1a). A mild increase in the number of sharp waves was infrequently detected during HV. The frequency of sharp waves varied between 1 and 2.6 events/min in the frontal lobe and 0 and 1.8 in the parietal lobe.

Three (Nos. 4 to 6) of the four familial epileptic dogs showed abnormal waves, such as sharp waves and spikes, in the frontal and parietal lobes under normal ventilation and developed seizure patterns, including spikes and sharp waves on the EEG under HV (Fig. 1b). While in the HV state, the maximum frequency of sharp waves for case 4 was 20 (events/min) in the frontal lobe and 107 (events/min) in the parietal lobe (Tables 1 and 2). The maximum frequency of spikes was 0.6 (events/min) in the frontal lobe and 13 (events/min) in the parietal lobe during HV. The maximum frequency of spikes and sharp waves in the epileptic familial dogs (Nos. 4 to 6) are shown in Table 2. One familial epi-

Normal ventilation

P

F

Hyperventilation

P

F

a

$\underline{\qquad}$| 100 μ V
$\overline{1 \text{ sec}}$

Normal ventilation

P

F

Hyperventilation

P

F

b

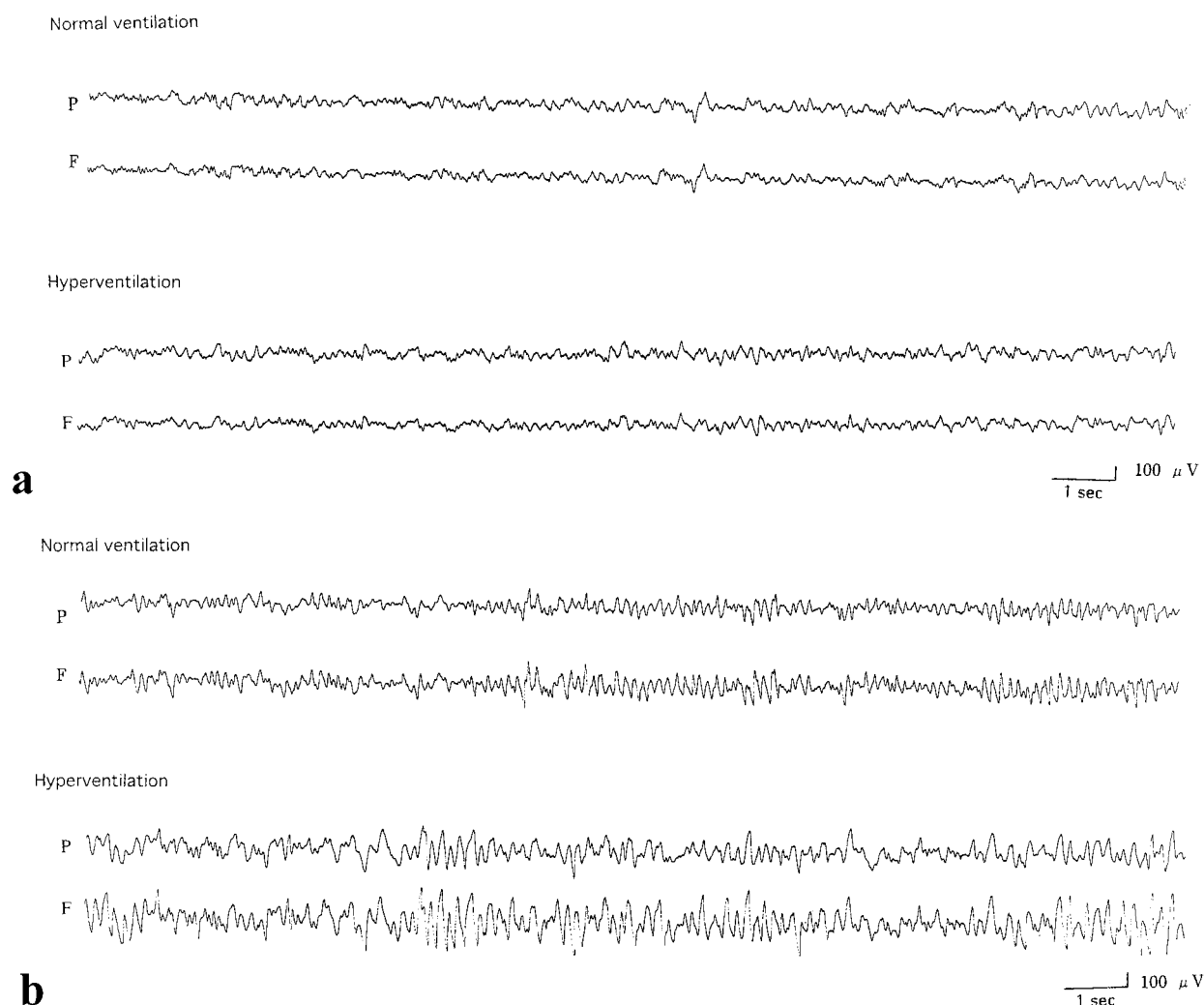$\underline{\qquad}$| 100 μ V
$\overline{1 \text{ sec}}$

Fig. 1.   (a): Control case (No. 1). Typical EEG findings under normal ventilation and hyperventilation. No abnormal waves were detected in the hyperventilation state. (b): Familial case (No. 4). Typical EEG findings under normal ventilation and hyperventilation. Small numbers of sharp waves were detected in the stage of normal ventilation, and large numbers of sharp waves and some spikes were observed during the hyperventilation state. P: parietal lobe, F: frontal lobe.

Table 1.   Frequency of spikes and sharp waves in the parietal lobe of a familial case (No. 4)

	Normal ①		Normal ②		Transitional		Hyper ①		Hyper ②		Hyper ③		Transitional		Normal	
Spike	0	0.8	0	0	0.2	0	0	5.2	13	0.8	1.4	0	0	0	0.2	0.2
Sharp wave	28.4	13.6	12	16.4	23.4	28.8	0.6	67	107.2	95.2	63.2	50.2	26.4	14.2	19.6	19

Normal: normal ventilation, Hyper: hyperventilation.
The numbers of spikes and sharp waves were calculated as the average per minute (event/minute).

leptic dog (No. 7) also showed abnormal waves, such as sharp waves and spikes, in the frontal and parietal lobes under normal ventilation, but no alteration in the frequency of abnormal waves was detected during HV.

Levels of extracellular amino acids: During normal ventilation after insertion of the dialysis probe, extracellular levels of amino acids became stable in both the frontal and parietal lobes of the familial and control cases.

During HV, few prominent changes in amino acid values were observed in the 3 control cases (Nos. 1 to 3; Fig. 2 a). In familial case 4, the Glu concentration increased by up to 3 folds compared to those during normal ventilation, and Asp was up to twice as high in the frontal lobe, corresponding to an increased number of spikes and sharp waves during

Table 2. The maximum frequency of spikes and sharp waves in the familial epileptic dogs

	Location	Spikes	Sharp waves
Case 4	Frontal	0.6	20
	Parietal	13	107
Case 5	Frontal	1.6	5.6
	Parietal	0.6	3.8
Case 6	Frontal	2.1	4.5
	Parietal	1.2	4.2

Frontal: frontal lobe, Parietal: parietal lobe
The numbers of spikes and sharp waves were calculated as the average per minute (event/minute).

HV. In the parietal lobe, the Glu and Asp concentrations increased by up to 6 folds compared to those during normal ventilation, corresponding to an increased number of spikes and sharp waves (Fig. 2b). The concentrations of other amino acids were not altered during HV. In familial case 5, the Glu concentration increased by up to 1.8 folds and Asp was up to twice as high in the frontal lobe, corresponding to an increased number of spikes and sharp waves during HV. In the parietal lobe, the Glu concentration increased by up to 1.7 folds and Asp was up to 9 folds higher during the transitional stage to HV. The values of other amino acids were not altered during HV. In Familial case 6, the Glu concentration increased by up to 1.5 folds and Asp was up to twice as high in the frontal lobe, corresponding to increased number of spikes and sharp waves during HV. In the parietal lobe, the Glu concentration increased by up to 1.5 folds and Asp was up to 5 folds higher during the transitional stage to HV. The values of other amino acids were not altered during HV. In familial case 7, the concentrations of all amino acids, including Glu and Asp, did not change during HV.

Immunohistochemistry: In the control cases, GLT-1 imminoreactivity was diffusely demonstrated in the cyto-
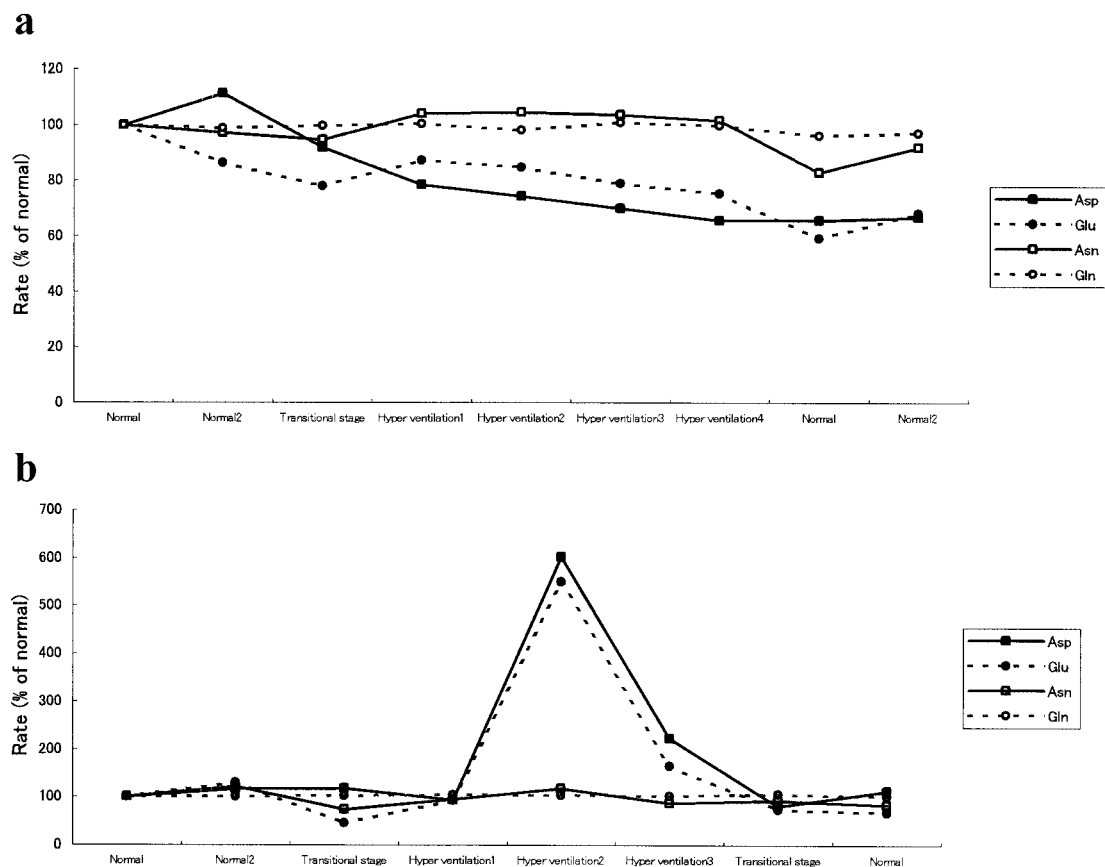

Fig. 2. (a): Control case (No. 1). Typical findings of extracellular amino acid (glutamate, aspartate, glutamine, and asparagine) values in the frontal lobe. Few changes in amino acid values were detected for both normal ventilation and hyperventilation. (b): Familial case (No. 4). Typical findings of extracellular amino acid (glutamate, aspartate, glutamine, and asparagine) values in the parietal lobe. During the hyperventilation state, values of glutamate and aspartate increased by up to 6 folds compared to those during normal ventilation. The alteration of glutamate and aspartate values corresponded to increased number of spikes and sharp waves (Table 1). Samples were collected every 10 min; time of normal ventilation ① and ②, the transitional stage, and hyperventilation ①, ② and ③ are 10 min, respectively. Glu: glutamate, Asp: aspartate, Gln: glutamine, Asn: asparagine.
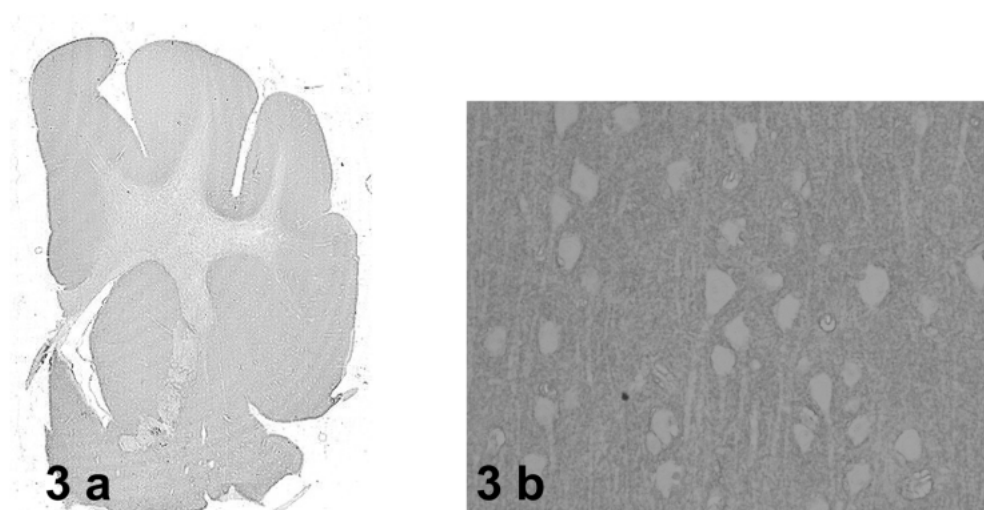
Fig. 3. Cerebral cortex of a control case. GLT-1 immunohistochemistry. (a): GLT-1 immunoreactivity was diffusely observed in cytoplasm and the processes of astrocytes. × 3.5 (b): Fine granular GLT-1 immunopositive stains were noted in the neuropil. × 460.
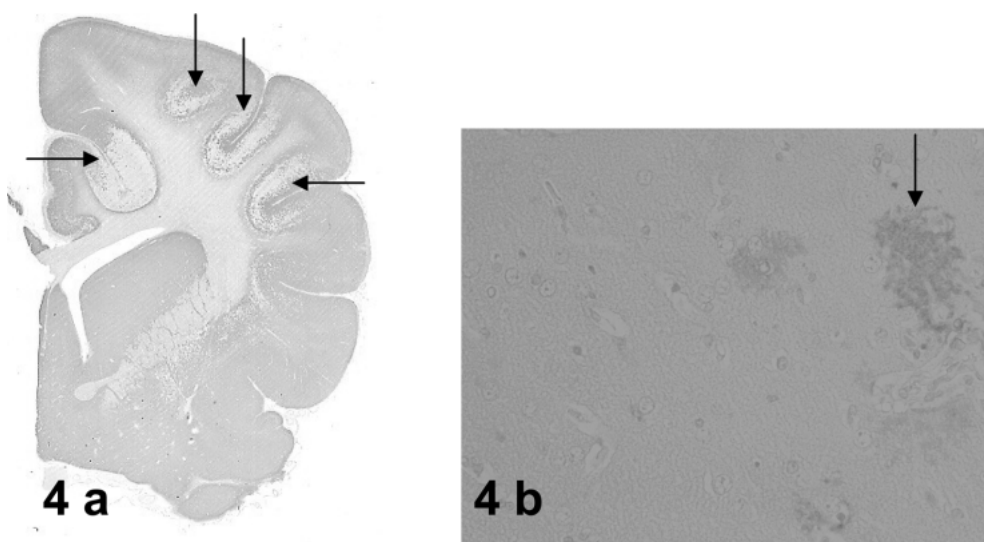

Fig. 4. Cerebral cortex of a familial case. GLT-1 immunohistochemistry. (a): A decrease in GLT-1 immunostaining was apparent in the cortex, which was prominent in the area surrounding the sulcus (arrows). × 3.5. (b): Loss of fine granular immunopositive stains was found. Some astrocytic processes were intensely stained with GLT-1 antibody (arrow). × 460.

plasm and processes of astrocytes in the cerebral cortex and central grey matter (Fig. 3). In the familial epileptic dogs affected by SE, immunostaining for GLT-1 antibody predominantly decreased in the cerebral cortex, which was exclusively identified in the area surrounding the sulcus (Fig. 4a) and lateral nucleus of the thalamus. Some astrocytic processes were intensely stained with GLT-1 antibody (Fig. 4b). In control cases, GLAST immunoreactivity was diffusely demonstrated in the cytoplasm and processes of astrocytes in the cerebral cortex and central grey matter.

Also, in the familial epileptic dogs affected by SE, astrocytic cytoplasms and processes were diffusely stained. In control cases, Glu immunoreactivity was weakly demonstrated in neuronal cell bodies, dendrites, and axons. In two of the familial epileptic dogs, Glu-positive granules were occasionally found in the perineuronal spaces of the cerebral cortex (Fig. 5). In the control cases, GS immunoreactivity was diffusely demonstrated in the cytoplasm and processes of astrocytes in the cerebral cortex and central grey matter. In the familial epileptic dogs with SE and the control cases,
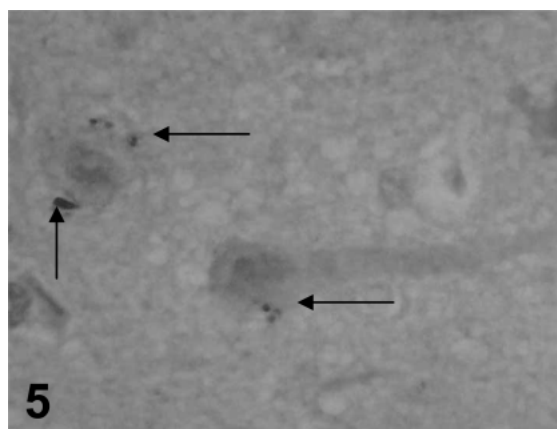
Fig. 5.   Cerebral cortex of a familial case. Glu immunohistochemistry. Glu immunopositive granules were found in the perineuronal spaces (arrows). Neurons were atrophic and had ischemic changes × 660.

astroglial processes were stained with GS antibody.

DISCUSSION

Many different methods have been presented to induce epileptiform activity in the EEGs of epileptic patients. Only a few of them are easy and safe enough to be used in the daily clinical routine. One of the methods most often used is HV [25, 32]. Although the detailed mechanisms for provoking epileptiform activity by HV have not been clearly documented, it has been considered that abnormal EEG discharges induced by HV occur due to elevated pH, rather than ischemia with vascular contraction [2, 7]. In 3 of the familial epileptic dogs, a significant elevation in Glu and Asp concentrations was observed during HV. This data suggests that hyperventilation environment may provoke an increased concentration of these excitatory neurotransmitters in the extracellular spaces of the cerebral cortex. During HV, the familial epileptic dogs showed an increased number of sharp waves and spikes, and the mean number of events was much greater than observed during normal ventilation. Concurrently, elevated levels of Glu and Asp were detected. The numbers of sharp waves and spikes increased predominantly in one of the familial epileptic dog (case 4). These findings suggest that elevated levels of these excitatory neurotransmitters in the extracellular spaces may cause abnormal EEG activities. Although the mechanism behind the elevation of these amino acids remains to be elucidated, epileptogenesis in these animals may be associated with a preparatory state for the release of these amino acids or with a disturbance in the uptake of these amino acids from extracellular spaces. In one of the familial epileptic dogs (case 7), neither an alteration of amino acids levels nor an abnormal EEG were induced following hyperventilation. This may be related to the differences in the brain condition of each familial case. Examination of the extracellular values of amino acids using intracerebral microdialysis has been

used to study possible neurotransmitter changes in human patients with epilepsy [4, 18, 31] and experimental animal models of epilepsy [3, 10, 19]. The results for extracellular levels of amino acids in human epileptic patients and various epilepsy models have not been consistent, although these studies have demonstrated altered levels of some kinds of amino acids, including Glu and Asp. These heterogeneous results can probably be ascribed to different epileptogenic mechanisms in each epilepsy type. A significant increase in the extracellular levels of Glu and Asp, however, has been reported in the kindling rat, post-traumatic epilepsy models and the epileptic human brain. In kindled rats induced by pentylenetetrazole, a significant and sustained increase in the Glu value was observed [10]. Epileptic brain tissue displayed significantly higher extracellular levels of Glu and Asp in the models of posttraumatic epilepsy induced by intracortical iron injection [19]. On the other hand, in epileptic human patients with focal seizures, a dramatic increase in the extracellular Asp, Glu, glycine, and serine concentrations was observed [18]. Marked elevations of Asp (79-fold), glycine (21-fold), Glu (16-fold), and serine (8-fold) concentrations occurred in association with the onset of seizures in human focal epilepsy [4]. In the human hippocampus with epilepsy, an increased value of Glu, Asp, and GABA during seizures was detected [31]. Our data suggest that the high values of Glu and Asp seem to be factors related to the development of seizures, although these findings apparently do not distinguish whether they are causative factors for seizure episodes or the results of abnormal EEG waves occurrence. This is the first report describing changes in the extracellular values of Glu and Asp associated with the detection of spikes and sharp waves in a canine epileptic model.

In this study, we examined immunoreactivity for GLT-1, GLAST, Glu, and GS in the cerebrum of dogs affected by SE. GLT-1 immunostains were markedly decreased in the cerebral cortex, which was exclusively found in the area surrounding the sulcus and lateral nucleus of the thalamus. These findings suggest that decreased levels of astrocytic GLT-1 occur in spite of astrogliosis in familial epileptic dogs with SE. Also, Glu immunopositive granules were demonstrated in some perineuronal spaces. It was considered possible that a decrease of GLT-1 levels may induce the elevation in extracellular Glu levels, which would evoke neuronal hyperexcitability. The levels of Glu uptake proteins and their corresponding mRNA have been studied in several animal models of epilepsy with varying results, and a decreased GLT-1, the same as our data has been reported. Decreased cortical protein levels for GLT-1 have been reported in a rat model of post-traumatic epilepsy [22]. Further, it has been found that expression of the mRNA and protein of GLT-1 and GLAST were down-regulated in chronic seizures induced by kainic acid [29]. Our results suggest that epileptogenesis in this familial epileptic dog colony with SE may be associated with the collapse of extracellular Glu regulation caused by a functional failure of Glu transport. We think it is a critical finding relating to the

development of recurrent seizures and neurotoxicity relating to acute neuronal necrosis, which has been previously described [15]. It has been reported that under certain pathophysiological circumstances, such as acute cerebral ischemia and epilepsy, Glu neurotoxicity is propagated due to failure or reversal of glial glutamate transport; genetically GLT-1 deficient mice die from SE [20, 27].

In this study, we examined GS immunoreactivity in the cerebrum of dogs with SE. In epileptic canine specimens, mild increase of GS immunolabelings, which was visible to sharp astroglial dendritic ramifications were found compared to control. Some significant findings for GS activity have been described in different experimental models of epilepsy; in the PTZ-stimulated cortical focal seizure model induced by $FeCl_2$, an increase in GS activity has been observed, whereas a decrease in GS activity has been found in the cortex in kindled amygdala seizures [28]. Alternatively, GS protein expression was slightly decreased in only the thalamus of young genetic absence epilepsy rat (GAERS), but GS mRNA showed no differences [8]. Our data provides evidence of seizure-induced alterations in the Glu-Gln cycle of the astrocytes and neurons, and indicates that the sequence of alterations may be correlated to the progressive development of SE. Furthermore, these findings indicate that an elevation of extracellular Glu levels in extracellular spaces may occur, and we surmise that a functional failure of GS is not related to epileptogenesis in these familial epileptic dogs.

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