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Electrographic seizures are significantly reduced by in vivo inhibition of neuronal uptake of extracellular glutamine in rat hippocampus

Keiko Kanamori* and **Brian D. Ross**

Huntington Medical Research Institutes, 660 South Fair Oaks Avenue, Pasadena, CA 91105, USA

Summary

Rats were given unilateral kainate injection into hippocampal CA3 region, and the effect of chronic electrographic seizures on extracellular glutamine (GLN_{ECF}) was examined in those with low and steady levels of extracellular glutamate (GLU_{ECF}). GLN_{ECF} , collected by microdialysis in awake rats for 5 h, decreased to $62 \pm 4.4\%$ of the initial concentration ($n = 6$). This change correlated with the frequency and magnitude of seizure activity, and occurred in the ipsilateral but not in contralateral hippocampus, nor in kainate-injected rats that did not undergo seizure ($n = 6$). Hippocampal intracellular GLN did not differ between the Seizure and No-Seizure Groups. These results suggested an intriguing possibility that seizure-induced decrease of GLN_{ECF} reflects not decreased GLN efflux into the extracellular fluid, but increased uptake into neurons. To examine this possibility, neuronal uptake of GLN_{ECF} was inhibited in vivo by intrahippocampal perfusion of 2-(methylamino)isobutyrate, a competitive and reversible inhibitor of the sodium-coupled neutral amino acid transporter (SNAT) subtypes 1 and 2, as demonstrated by 1.8 ± 0.17 fold elevation of GLN_{ECF} ($n = 7$). The frequency of electrographic seizures during uptake inhibition was reduced to $35 \pm 7\%$ ($n = 7$) of the frequency in pre-perfusion period, and returned to $88 \pm 9\%$ in the post-perfusion period. These novel in vivo results strongly suggest that, in this well-established animal model of temporal-lobe epilepsy, the observed seizure-induced decrease of GLN_{ECF} reflects its increased uptake into neurons to sustain enhanced glutamatergic epileptiform activity, thereby demonstrating a possible new target for anti-seizure therapies.

Keywords

Epileptic seizure; Extracellular glutamine; Neuronal uptake; 2-(Methylamino) isobutyrate; Rat hippocampus; Kainate

Introduction

Temporal-lobe epilepsy, which accounts for 40% of epilepsy, is associated with an epileptic focus in the hippocampus resulting from local brain injury. The mechanism of the onset of chronic recurrent seizures, following a latent period, is a subject of intense investigation (reviewed by (Bradford, 1995; Jefferys, 2010; Morimoto et al., 2004). Among numerous animal models, the chronic kainate-induced rodent model resembles most closely the EEG and biochemical abnormalities of temporal-lobe epilepsy (Mathern et al., 1993; Riban et al., 2002; Tanaka et al., 1992). The rat develops chronic recurrent seizures between 3 and 90 days after unilateral intrahippocampal injection of kainate (KA) (Bragin et al., 1999, 2005), which is an agonist of the KA receptor of the ionotropic glutamate receptor family (Vincent and Mulle, 2009).

Our recent study using this model showed that chronic recurrent seizures with no or mild behavioral components (electrographic seizures) caused not only elevation of the excitatory neurotransmitter glutamate in the extracellular fluid (GLU_{ECF}), but also a significant decrease in the concentration of its precursor glutamine in the extracellular fluid (GLN_{ECF}) (Kanamori and Ross, 2011). Decrease of GLN_{ECF} associated with chronic epileptiform activity was a novel finding that had not been reported previously.

The physiological basis of our study is illustrated in Fig. 1 which shows schematically the major metabolic pathways of GLU, GLN and γ -aminobutyric acid (GABA) and their transport pathways between synaptic vesicles, extracellular fluid (ECF), glia and the neuron, according to a suggested model of GLN/GLU/GABA cycle (Erecinska and Silver, 1990; Hertz, 1979; Shank and Aprison, 1981) (see section “Role of GLN_{ECF} in sustaining epileptiform activity in vivo” for discussion of an alternative model).

The questions that we address in the present paper are the following:

1. Does the decrease in GLN_{ECF} , associated with seizure, also occur in those KA rats that exhibit no detectable change in GLU_{ECF} ?
2. If so, does the change in GLN_{ECF} correlate with the frequency and magnitude of chronic electrographic seizures?
3. If a correlation is found, does the decrease in GLN_{ECF} reflect decreased efflux of glial GLN to ECF or its increased uptake into neurons (Fig. 1)?
4. If the latter is likely, how does inhibition of neuronal uptake of GLN_{ECF} in vivo affect the frequency of electrographic seizures?

To address questions #1 and #2, we examined GLN_{ECF} in those KA rats that exhibit no significant change in GLU_{ECF} . Specifically, we examined the frequency and magnitude of EEG seizures and the time-course of GLN_{ECF} in (a) the ipsilateral (KA-injected) hippocampus of seizure-exhibiting vs seizure-free rats, and also (b) in the seizure-prone ipsilateral vs seizure-free contralateral hippocampus of the same rat. To address question #3, we measured the concentrations of hippocampal intracellular GLN (as well as of GLU and GABA) in seizure-exhibiting vs seizure-free KA rats. To address question #4, we took a novel approach of inhibiting in vivo the SNAT1/SNAT2 mediated neuronal uptake of

GLN_{ECF} (Fig. 1) by intrahippocampal perfusion of 2-(methylamino)isobutyrate (MeAIB), a competitive, non-metabolizable and reversible inhibitor of System A transporter (Christensen, 1990; Christensen et al., 1965), and examined the effect of this inhibition on the frequency of electrographic seizures.

We report here the results of these studies which strongly suggest that (a) the observed GLN_{ECF} decrease in response to epileptiform activity reflects increased neuronal uptake, and (b) uptake inhibition significantly reduces the frequency of electrographic seizures in vivo. Implications of the results for a major role of GLN in sustaining excitatory neurotransmission during enhanced seizure activity are discussed.

Material and methods

Kainic acid injection

Adult male Wistar rats (240–340 g) were anesthetized with pentobarbital (40 mg/kg wt) and placed on a stereotaxic instrument. Kainic acid (KA) was injected unilaterally into CA3 region of the right hippocampus at AP = −5.6 mm, L = +4.5 mm and V = 5.5 mm (Paxinos and Watson, 1997). Sodium kainate (Sigma–Aldrich, St. Louis, MO, USA), dissolved in 0.1 M phosphate buffer, was injected with a 0.5 µL syringe at a dose of 0.4 µg/0.2 µL for a 325 g rat and adjusted according to body weight, as described previously (Kanamori and Ross, 2011). The rat, which awoke from anesthesia within 1 h, was continuously monitored for behavioral seizures for 6 h after injection (acute phase).

Chronic-phase procedures

During the chronic phase at 37–46 days after KA injection, we implanted EEG recording and grounding electrodes (Plastics One, Roanoke, VA, USA) and microdialysis guide cannula with a stylet (BioAnalytical systems, West Lafayette, IN, USA), as described previously (Kanamori and Ross, 2011). Three experimental protocols, with objectives described in section “Overview of experimental design”, were used in the present study. The surgical and experimental procedures for each protocol are described below.

EEG/microdialysis (Experiment I)

In Experiment I, EEG recordings were taken bilaterally with microdialysis only in the ipsilateral hippocampus, in overnight-fasted rats. The EEG electrode was implanted at AP = −5.6 mm, L = −4.5 mm and V = 5.5 mm in the contralateral hippocampus. In the ipsilateral hippocampus, the coordinates were AP = −5.6 mm, L = 4.5 mm and V = 5.5 mm for the electrode, and for the guide cannula attached to the electrode, V = 3.5 mm. This places the electrode tip in CA3 region and the tip of the microdialysis guide cannula, just above the CA1 region (Paxinos and Watson, 1997). For preliminary bilateral EEG recording from an awake rat one week after the surgery, the electrode contacts on the skull were connected through a commutator to an amplifier (A-M systems, Carlsborg, WA, USA). Recordings were taken wide-band 0.1 Hz to 1 KHz, sampled at 10 KHz/channel and with a gain of 10 K, and processed with DATAPAC 2K2 software (Run Technology, Mission Viejo, CA, USA). When EEG recordings taken for several hours showed chronic-phase recurrent seizures

(Bragin et al., 1999, 2005), the rat was considered ready for the microdialysis/EEG experiment.

On the day of microdialysis, the rat was lightly anesthetized with pentobarbital and the guide stylet was replaced with the microdialysis probe 320 μm in OD and 2 mm in length (BioAnalytical systems, West Lafayette, IN, USA). The probe spans CA1–CA3 region, with the bottom of the probe in the CA3 region and the top of the probe in the CA1 region within 0.5 mm of the dentate gyrus. The probe collects extracellular fluid from hippocampal tissue $\sim 700 \mu\text{m}$ in diameter and 2 mm in length at the site of KA injection. The probe was perfused at a rate of 2 $\mu\text{L}/\text{min}$ with artificial cerebrospinal fluid (aCSF) containing the following equivalents of electrolytes (mM): 150 Na^+ , 3.0 K^+ , 1.4 Ca^{2+} , 0.8 Mg^{2+} , 1 PO_4^{3-} and 155 Cl^- at pH 7.4. The dialysis tubings were connected to a liquid swivel for collection from an awake animal. A 3-h stabilization period was allowed before the collection of dialysates for analysis. The rat usually woke within 1 h after probe insertion and hence 2 h before the start of dialysate collection and simultaneous EEG recording. The dialysates were collected every 2 min for 5 h, and stored at -20°C till analysis. In correlating EEG activity with changes in GLU_{ECF} and GLN_{ECF} , we took into account the fact that it takes 180 s for dialysate to flow from rat brain to the collection vial under our experimental condition. The microdialysis time is reported at the center of each 2-min collection time.

EEG/microdialysis/i.v. infusion (Experiment II)

In Experiment II, dialysates were collected from both hippocampi with concomitant EEG recording and i.v. infusion of glucose. Microdialysis guide cannula fixed to the recording electrode was implanted bilaterally at the coordinates used in Experiment I. Two days before the EEG/microdialysis experiment, an indwelling silastic catheter was placed in the right external jugular vein for i.v. infusion of glucose (Kanamori et al., 1998). The distal end of the catheter (90 cm long), exiting at the nape, was placed in a backpack worn by the rat (Kondrat et al., 2002).

On the day of the experiment, the EEG electrode contacts on the skull of the lightly anaesthetized rat were connected to a 90-cm cable which is mesh-covered on proximal end and equipped with solder lugs on the distal end for connection to the amplifier. The rat was placed in RATURN (BioAnalytical Systems) with its collar attached to a balance arm. The EEG cable, the inlet and outlet dialysis tubings and the i.v. infusion catheter were passed through an opening in the sensor of the RATURN. This arrangement permits free movement of the awake rat without twisting the EEG cable, dialysis tubings or the infusion catheter which was connected to a peristaltic infusion pump. Dialysates were collected bilaterally in 15-min fractions for 2 h without i.v. infusion, then every 5 min with concomitant EEG recording and i.v. infusion of glucose for 3 h. D-Glucose was given according to the protocol developed by (Fitzpatrick et al., 1990) and shown to achieve steady-state blood glucose concentration in 9 min and brain glucose concentration in 30 min (Kanamori and Ross, 2001). Thus, the *total microdialysis time* in Experiment II is 5 h as in Experiment I.

MeAIB perfusion (Experiment III)

In Experiment III, dialysates were collected from, and MeAIB perfused into, only the ipsilateral hippocampus with bilateral EEG recording in glucose-infused or fed rats, which were prepared as in Experiment II. 2-(Methylamino)isobutyric acid (MeAIB) (Sigma–Aldrich, St. Louis, MO, USA) was dissolved in aCSF at the concentration indicated in the “Result” section. At 175 mM (the optimized dose), the pH of this solution was 7.1, and the pH was adjusted to 7.4 (the same pH as that of aCSF) with a few drops of 25 mM NaOH before perfusion. After 88 min of microdialysis combined with i.v. infusion of glucose, MeAIB was perfused into ipsilateral hippocampus at a flow rate of 2 μ L/min for 30 min.

After the microdialysis experiment, the locations of the microdialysis probe and the electrodes were confirmed in each rat as described previously (Kanamori and Ross, 2011). The confirmed coordinates (mean $\pm$ SE) are shown in Table 1. The brain was then removed from the anesthetized rat and frozen in liquid nitrogen.

The protocol was approved by the Institute Animal Care and Use Committee and is in conformance with the US Public Health Service's Guide for the Care and Use of Laboratory Animals.

Identification of seizure and quantification of EEG activity

EEG seizure was identified according to the definition of (Bragin et al., 2005), as a period of consistent and repetitive changes in amplitude and frequency of electrical activity that was clearly different from interictal activity and which persisted for >10 s. Representative EEG recordings of ictal events in our KA rats were shown in Fig. 2 of (Kanamori and Ross, 2011). To examine possible correlation between EEG activity and changes in GLN_{ECF} , we quantified EEG activity in Experiment I by measuring (a) peak areas of EEG waves and (b) peak amplitudes of EEG spikes as described previously (Kanamori and Ross, 2011). Then peak areas in dialysate fractions (1–150) were consecutively added to determine cumulative peak areas for the 300-min observation period. The same method was used to obtain cumulative peak amplitudes of spikes. The height of a population spike, over a wide range, can be used as a measure of the number of discharging neurons (Andersen et al., 1971), and hence as an approximate measure of the quantity of neurotransmitter GLU that is released into ECF as hypersynchronized population bursts in electrographic seizures.

HPLC assay of extracellular GLU, GLN and TAU

Amino acids in brain dialysate were assayed, after pre-column derivatization with ortho-phthalaldehyde (OPA) and 2-mercaptoethanol and separation on a reverse-phase column, by fluorometric detection as described previously (Kanamori and Ross, 2011), with the following minor modification. To achieve baseline separation of GLU, GLN and TAU from adjacent peaks, the following chromatographic program was used; elution with 25% methanol and 75% aqueous sodium phosphate buffer (50 mM, pH 5.38) for 10 min followed by increase in the percentage of methanol to 49.6% in 15 min and to 100% in 8 min.

Tissue concentrations of GLU, GLN and GABA in the hippocampal region sampled by microdialysis

Hippocampal tissue was sectioned at -10°C from the brain that had been snap frozen at the end of the microdialysis experiment and stored in liquid nitrogen. The tissue was homogenized and intracellular metabolites were extracted as described previously (Kanamori and Ross, 2011). Tissue concentrations of GLU and GLN were measured by HPLC as described above. For measurement of GABA, the chromatographic program was modified as follows for baseline separation of GABA from the adjacent peaks; elution with 25% methanol and 75% aqueous sodium phosphate buffer (50 mM, pH 5.38) for 10 min, followed by increase in the percentage of methanol to 40% in 29.5 min, to 84% in 22.3 min and to 100% in 3 min.

K⁺ assay

K⁺ concentration in the dialysate was measured with an ion-selective electrode in the Clinical Laboratory at Huntington Hospital, Pasadena, California.

Statistical analysis

ANOVA was used to determine whether the mean value of GLN_{ECF} and GLU_{ECF} at the indicated time t during microdialysis differed significantly from the initial value, and whether percentage decrease in GLN_{ECF} differed between ipsilateral and contralateral hippocampi. It was also used to determine whether the mean values of hippocampal tissue concentrations of GLU, GLN and GABA in KA rats differed between the Seizure and No-Seizure groups, and whether the mean value of the frequency of seizures during MeAIB perfusion and post-perfusion periods differed from that in the preperfusion period.

Results

EEG characteristics of KA rats

Upon unilateral KA injection, all rats developed acute-phase behavioral seizures (Löscher et al., 1989) lasting up to 6 h. At the low dose of kainate used (section “Kainic acid injection”), there was no mortality during the acute or the subsequent latent and chronic phases. Preliminary EEG recordings in the chronic phase (section “EEG/microdialysis (Experiment I)”) showed that 68% of the KA rats, out of a total of 44, exhibited chronic-phase recurrent electrographic seizures during several hours of recording. Those 30 rats were used for EEG/microdialysis experiments.

Overview of experimental design

Table 1 shows EEG characteristics of KA rats in three Experimental Groups used in the present study. Experiments I and II address the question of possible correlation between seizure activity and changes in GLN_{ECF} as outlined in the Introduction. In Experiment I, GLU_{ECF} and GLN_{ECF} were collected only from the ipsilateral hippocampus (site of KA injection) for 5 h. In Experiment II, after 2 h of microdialysis, intravenous infusion of glucose was started and dialysates were collected from both the ipsilateral and contralateral hippocampi for additional 3 h. Experiment II was designed (1) to examine whether the

seizure-induced decrease of GLN_{ECF} observed in Experiment I was dependent on energy status and (2) to compare their occurrence in the seizure-prone ipsilateral vs seizure-free contralateral hippocampus. Experiment III was designed to address the question of whether the observed decrease of GLN_{ECF} reflects increased neuronal uptake of GLN_{ECF} , as outlined in the Introduction. Dialysates were collected from, and uptake inhibitor 2-(methylamino)isobutyrate (MeAIB) perfused into, the ipsilateral hippocampus of glucose-infused or fed KA rats and the effect of inhibition on seizure frequency was examined.

As shown in Table 1, all KA rats underwent microdialysis/EEG experiments during the chronic phase between 44 and 58 days post KA. Out of the 30 KA rats that showed recurrent seizures during preliminary EEG recording (section “EEG characteristics of KA rats”), those that showed frequent spontaneous seizures during the 5 h microdialysis experiments are called Seizure Group and those that did not are called No-Seizure Group. In the Seizure Group of Experiment I ($n = 6$), the number of seizures ranged from 5 to 15, with mean $\pm$ SE of 9 ± 1.9 . The No-Seizure Group ($n = 6$) had no seizure during the microdialysis experiment. In Seizure Group of Experiment II ($n = 5$), the number of seizures (mean $\pm$ SE) observed during 3 h of EEG/microdialysis/glucose infusion was 5 ± 1 , while the No-Seizure Group ($n = 4$) showed no seizure. For Experiment III ($n = 9$), the result is shown later in Table 3. As shown in Table I, the coordinates of the EEG electrodes and the microdialysis probes, confirmed after the experiment in every rat, as described previously (Kanamori and Ross, 2011), were virtually the same in all groups.

GLU_{ECF} during resting and seizure periods

Fig. 2 shows the time-course of GLU_{ECF} in KA rats of Experiment I. The concentration of dialysate GLU (in μM) at each time point was measured and expressed as percentage of the basal concentration measured at the start of microdialysis in each rat. Then the mean $\pm$ SE of the percentage change at each time point was calculated for $n = 6$. With significance defined as $p < 0.05$, these mean $\pm$ SE values did not differ significantly from the initial value, indicating that GLU_{ECF} in Seizure Group of Experiment I underwent no significant change during the 5-h microdialysis experiment. The figure also shows the time-course of GLU_{ECF} for glucose-infused KA rats of Experiment II ($n = 5$) during the last 3 h of microdialysis. In these rats too, the mean $\pm$ SE values did not differ significantly from the initial value, indicating that there was no significant change in GLU_{ECF} .

Effect of EEG seizure on GLN_{ECF} in the ipsilateral hippocampus

As shown in Table 1, the basal concentration of dialysate GLN in KA rats of Seizure Group of Experiment I, $31.5 \pm 3.8 \mu M$, was not significantly different from that, $30.3 \pm 5.0 \mu M$ in the No-Seizure Group.

Fig. 3A (top) shows, for one rat (R1051) of Seizure Group, the time of occurrence of EEG seizures (arrows). The seizures consisted of low-voltage fast (LVF) onset and hypersynchronous (HYP) onset seizures that are characteristic of chronic-phase seizures in this model (Bragin et al., 1999, 2005). Representative EEG recordings are shown later (Figs. 5, 6 and 10). The top graph shows cumulative peak areas of EEG waves (left coordinate) and cumulative peak amplitudes of EEG spikes (right coordinate), determined as described

in section “Identification of seizure and quantification of EEG activity”. Six seizures were detected during the 300 min observation period, with frequent seizures occurring between 180 and 253 min. During this period, the cumulative spike amplitude increased sharply. Fig. 3A (bottom) shows, for the same rat, the time course of GLN_{ECF} expressed as dialysate GLN concentration. Comparison of the two time-courses shows that GLN_{ECF} decreases significantly, when seizures occur frequently and cumulative spike amplitude increases sharply. When seizures ceased ($t > 253$ min), GLN_{ECF} increased slightly. During $t = 231$ – 295 min, GLN_{ECF} was $23.8 \pm 1.4 \mu\text{M}$ which is significantly different from the initial concentration of $37.5 \mu\text{M}$ with $p < 0.0001$. Fig. 3B shows the result for another rat (R1052) of Seizure Group, which underwent 7 seizures (LVF and HYP). Seizures between 85 and 125 min were accompanied by gradual increase in cumulative peak areas and spike amplitudes (top). During this period, GLN_{ECF} decreased slightly (bottom). More dramatic is the effect of frequent seizures between 237 and 275 min which caused sharp increase in cumulative peak areas and spike amplitudes. During this period, GLN_{ECF} decreased significantly (bottom figure); GLN_{ECF} was $14.7 \pm 0.5 \mu\text{M}$ during $t = 237$ – 295 min, which is significantly different from the initial concentration of $26.4 \mu\text{M}$ with $p < 0.0001$. Taken together, these results show that (a) the timing of GLN_{ECF} decrease correlates with the frequency and magnitude of seizure activity, and (b) when seizures occur frequently, persistent reduction in GLN_{ECF} is observed. Similar results were observed in other KA rats of the Seizure Group.

Fig. 4A compares the time-course of GLN_{ECF} between the Seizure Group ($n = 6$) and the No-Seizure Group ($n = 6$) of Experiment I, in which the rats were fasted overnight. Dialysate concentration at each time point during the microdialysis experiment was first expressed as the percentage of initial concentration in each rat, then the mean $\pm$ SE ($n = 6$) was calculated. The result clearly shows that, in rats of Seizure Group, GLN_{ECF} decreased progressively and at $t = 295$ min, was $62 \pm 4.4\%$ of the initial value. This is significantly different from the initial value with $p < 0.01$. By contrast, in rats that underwent no seizure during microdialysis, the mean $\pm$ SE values were not significantly different from the initial value, indicating that there was no change in GLN_{ECF} . Fig. 4B shows changes in GLN_{ECF} in the ipsilateral hippocampus of KA rats of Experiment II which underwent i.v. glucose infusion for the last 3 h. As shown in Fig. 4B, GLN_{ECF} in the Seizure Group ($n = 5$) decreased progressively and at $t = 295$ min, was $75 \pm 2.6\%$ (significantly different from the initial value with $p < 0.01$). By contrast, in the No-Seizure group ($n = 4$), the mean $\pm$ SE values were not significantly different from the initial value, indicating that there was no change in GLN_{ECF} . These results clearly show that the decrease of GLN_{ECF} in response to seizure activity occurs in glucose-infused as well as in overnight-fasted rats. The difference in the mean percentage decrease of $62 \pm 4.4\%$ in Experiment I and $75 \pm 2.6\%$ in Experiment II was not statistically significant.

Effect of seizure on GLN_{ECF} in ipsilateral vs contralateral hippocampus

For KA rats ($n = 5$) of Experiment II, basal dialysate GLN concentration was $32.2 \pm 4.4 \mu\text{M}$ in the ipsilateral hippocampus and $25.3 \pm 4.0 \mu\text{M}$ in the contralateral hippocampus in the Seizure Group, while in the ipsilateral hippocampus of the No-Seizure Group, it was $30.3 \pm$

8%. Dialysate GLN in the normal rat was $28.4 \pm 1.5 \mu\text{M}$ ($n = 6$ for two hippocampi from 3 rats). There was no statistically significant difference among those mean values.

Fig. 5 shows a representative result for changes in GLN_{ECF} in the ipsilateral vs contralateral hippocampus. Fig. 5A shows EEG recordings from the ipsilateral (top trace) and the contralateral hippocampus (bottom trace) in a KA rat of this group (R1119). The ipsilateral hippocampus showed two seizure episodes with high-amplitude ictal spikes; each episode lasting about 20 min. These were electrographic seizures detected by EEG but not accompanied by behavioral or motor seizures. By contrast, the contralateral hippocampus showed little EEG activity. As shown at the top of Fig. 5B, these seizures in the ipsilateral hippocampus occurred during 220–295 min of microdialysis. Fig. 5B shows changes in GLN_{ECF} collected by microdialysis from the ipsilateral and contralateral hippocampi before and during the seizure, and expressed as percentage of the initial concentration. In the ipsilateral hippocampus, GLN_{ECF} decreased to ~70% of the initial concentration during seizure, while in the seizure-free contralateral hippocampus, GLN_{ECF} remained at 88–100%. The mean GLN_{ECF} level in percentage in the ipsilateral hippocampus during $t = 257\text{--}287$ min when the level has stabilized, $70.4 \pm 0.8\%$, is significantly different from the level, $87.2 \pm 0.9\%$, in the contralateral hippocampus during the same period with $p < 0.01$.

Fig. 6 compares the effect of hypersynchronous seizures on GLN_{ECF} in the two hippocampi in another KA rat (R1111) of Experiment II. In the ipsilateral hippocampus, the seizures caused decrease of GLN_{ECF} to 70–80% of the initial value while GLN_{ECF} was unchanged in the seizure-free contralateral hippocampus. The mean level of GLN_{ECF} in percentage in the ipsilateral hippocampus, $78.7 \pm 1.8\%$, is significantly different from that in the contralateral hippocampus, $99.8 \pm 0.9\%$, with $p < 0.01$. A similar result was observed in another KA rat (result not shown). Table 2 summarizes changes in GLN_{ECF} in response to seizure activity in KA rats of Experiments I and II. Taken together, the results strongly suggest that significant decrease in hippocampal GLN_{ECF} is associated with electrographic epileptiform activity in those KA rats that show no detectable change in GLU_{ECF} .

Tissue concentrations of GLU, GLN and GABA in the ipsilateral hippocampal region sampled by microdialysis

To examine whether the observed decrease of GLN_{ECF} is due to lower concentration of glial GLN which can decrease the rate of SNAT3-mediated efflux of GLN to ECF (Fig. 1), we examined the tissue concentration of GLN, as well as those of GLU and GABA, in the ipsilateral hippocampal tissue that contained the CA1 and CA3 subfields sampled by microdialysis and the KA injection site. The weight of tissue (mean $\pm$ SE) was 10.7 ± 0.99 mg and 9.5 ± 1.5 mg for the Seizure and No-Seizure Groups of Experiment I respectively. The tissue concentrations are shown in Fig. 7. In KA rats of Seizure Group, GLN concentration was 4.3 ± 0.27 mM which was not significantly different from that in No-Seizure Group, 4.4 ± 0.47 mM with significance defined as $p < 0.05$. GLU concentration in this region was 7.4 ± 0.55 mM in Seizure Group and 7.3 ± 0.55 mM in No-Seizure Group, while GABA concentration was 1.77 ± 0.43 mM in the Seizure Group and 1.38 ± 0.39 mM in the No-Seizure Group. The difference in the mean values between Seizure and No-

Seizure Groups was not statistically significant for either metabolite, with significance defined as $p < 0.05$.

The results strongly suggest that the observed decrease of GLN_{ECF} in the Seizure Group is unlikely to be due to reduced concentration of glial GLN which lowers the rate of GLN efflux to ECF. (On the basis of immunocytochemical studies, at least 80% of brain tissue GLN is estimated to be in glia (Ottersen et al., 1992; Nagelhus et al., 1996; Kanamori and Ross, 1997)). Because the result raised an intriguing possibility that the observed decrease of GLN_{ECF} reflects its increased uptake into neurons, we inhibited neuronal uptake of GLN_{ECF} by perfusion of the competitive, non-metabolizable and reversible inhibitor MeAIB *in vivo*, and examined the effect on frequency of electrographic seizures.

Inhibition of neuronal uptake of GLN_{ECF} by MeAIB perfusion: Optimization of dose and duration

Our previous study in normal rat hippocampus showed that perfusion of 50 mM MeAIB elevates GLN_{ECF} 1.6 fold *in vivo* in 2 h (Kanamori and Ross, 2004); this concentration is close to that of 12.5 and 50 mM used respectively in hippocampal slices and neuronal cultures (Bacci et al., 2002). For the present study, a more rapid elevation by this nonmetabolizable and non-toxic inhibitor (section “Inhibition of neuronal uptake of GLN_{ECF} *in vivo*”) was required to study its effect on seizure frequency. The minimum concentration of MeAIB that achieves *in vivo* inhibition of neuronal uptake of GLN_{ECF} within 10 min, as indicated by elevation of GLN_{ECF} , was examined by intrahippocampal perfusion at MeAIB concentrations of 125, 175 and 250 mM. At 125 mM, no elevation was observed within this period. At 175 and 250 mM, GLN_{ECF} was elevated by 1.7–2 fold. Accordingly, a concentration of 175 mM was used in subsequent experiments. The duration of MeAIB perfusion that elevates GLN_{ECF} without change in extracellular taurine (TAU_{ECF}), an indicator of osmotic stress, was examined by perfusion for 30 or 55 min. Perfusion of 175 mM MeAIB for 30 min achieved GLN_{ECF} elevation without elevation of TAU_{ECF} during that period. Accordingly, this experimental condition was used in all subsequent experiments.

Elevation of GLN_{ECF} by MeAIB perfusion

Fig. 8 shows a representative time course of GLN_{ECF} during *in vivo* inhibition of its neuronal uptake by MeAIB perfusion. In this rat (R1156), GLN_{ECF} was elevated 1.8 fold when the perfusate was changed from aCSF to 175 mM MeAIB for 30 min. When the perfusate was changed back to aCSF, GLN_{ECF} returned to the initial concentration. GLU_{ECF} remained at low concentrations throughout the experiment. TAU_{ECF} did not change significantly during the 30-min MeAIB perfusion, showed a slight elevation after the perfusate was changed back to aCSF but eventually returned to the basal concentration.

To examine the time-courses of these extracellular metabolites in all KA rats of Experiment III, the following analysis was performed. As shown in Fig. 9, the start of MeAIB perfusion was set at perfusion time $T = 0$ (top abscissa) in all rats. The basal concentration of metabolites was measured at 40 ± 3 min (mean $\pm$ SE for $n = 7$) before the start of MeAIB perfusion, which corresponds to 48 ± 3 min of microdialysis time t (bottom abscissa). For

GLN_{ECF} and TAU_{ECF}, the concentration at each subsequent time point was expressed as percentage of the basal concentration in each rat, then the mean $\pm$ SE at each time-point was calculated for $n = 7$ (the left coordinate). For GLU_{ECF}, the mean $\pm$ SE of the dialysate GLU concentration (as shown on the right coordinate) was calculated at each time point for n ranging from 4 to 7 (n was less than 7 at some time points because a 2- μ L dialysate aliquot, necessary for accurate measurement of the low concentration GLU_{ECF}, was not available from all rats of this group). The basal concentrations (mean $\pm$ SE) were $32.5 \pm 3.0 \mu\text{M}$ for GLN_{ECF} and $0.64 \pm 0.24 \mu\text{M}$ for GLU_{ECF} (Table 1), and $5.9 \pm 0.33 \mu\text{M}$ for TAU_{ECF}.

As shown in Fig. 9, upon MeAIB perfusion, GLN_{ECF} was rapidly elevated 1.6 fold in 10 min and achieved a maximum elevation of 1.8 ± 0.17 fold (significantly different from the basal value with $p < 0.01$). Upon changing the perfusate back to aCSF, GLN_{ECF} returned to the basal value. TAU_{ECF} was unchanged during the 30-min MeAIB perfusion, but upon changing the perfusate to aCSF, was transiently elevated 1.6 fold (significantly different from the basal value with $p < 0.05$). By $T = 73$ min, TAU_{ECF} returned to the basal concentration. This interval of 43 ($=73 - 30$) min corresponds to the time required for the non-metabolizable inhibitor, MeAIB, to be cleared from ECF, as shown by our previous results and described in section “Inhibition of neuronal uptake of GLN_{ECF} in vivo”. For dialysate GLU, the mean concentration remained low without significant change ($p > 0.05$) throughout the experiment.

There was no difference in the time-courses of these extracellular metabolites between KA rats that were overnight-fasted then given i.v. infusion of glucose during microdialysis ($n = 6$) and those KA rats that were fed ad libitum ($n = 3$). Blood glucose concentrations at end point were 152 ± 9.6 mg/dL for the former and 137 mg/dL for the latter. K⁺ concentration in the effluxing dialysate during MeAIB perfusion was 2.7 mM compared to 2.9 mM in pre-perfusion period; both are close to the normal concentration of 3 mM in CSF.

The effect of inhibition of neuronal uptake of GLN_{ECF} on seizure frequency

This was examined by comparing the frequency of electrographic seizures observed (a) during the 30-min preperfusion period, (b) during the 30-min MeAIB perfusion and (c) in the 30-min post-perfusion period ($T = 78$ – 108 min) after restoration of GLN_{ECF} and TAU_{ECF} to pre-perfusion levels (as shown at the top of Fig. 9). Fig. 10A shows EEG recordings from the ipsilateral hippocampus of a representative KA rat (R1156) of Experiment III during those three periods. Seizure activity was significantly reduced during inhibition of neuronal uptake of GLN_{ECF} by MeAIB perfusion. The number of hypersynchronous seizures, identified by the criteria described in section “Identification of seizure and quantification of EEG activity”, was 6 in the pre-perfusion period, decreased to 2 during the inhibition of neuronal uptake of GLN_{ECF} by MeAIB perfusion and returned to 6 in the post-perfusion period. During seizure, the amplitude of ictal spikes often exceeded 1.6 mV, but the wave pattern, shown in the inset, has characteristics of hypersynchronous population bursts from pyramidal glutamatergic neurons. The time of occurrence of these seizures in the respective period is shown by arrows in Fig. 8. Similar analyses were performed for EEG recordings from the other rats. An EEG recording, processed with a faster sweep time, of a hypersynchronous seizure from the ipsilateral hippocampus of

another rat in Experiment III is shown in Fig. 10B. The observed electrographic seizures in the majority of KA rats (71%) in this group showed hypersynchronous (HYP) ictal onset pattern that is observed in the majority (70%) of spontaneous seizures and start in the ipsilateral hippocampus adjacent to the lesion, as described by (Bragin et al., 2005). The HYP seizure pattern consisted of high-amplitude ictal spikes, often accompanied by fast-ripples (high-frequency oscillations in the range of 250–500 Hz (reviewed by (Bragin et al., 2010)) and fast-ripple tail-gammas. Seizures in the remainder of rats (29%) showed low voltage fast (LVF) onset pattern which is observed in 26% of recurrent seizures (Bragin et al., 2005). The LVF ictal activity began with low-voltage sinusoidal 5–30 Hz pattern followed by 30-Hz rhythmic waves. In each rat, the same ictal onset pattern and waveforms were observed during the microdialysis experiment. The average duration of ictal seizures was 85 ± 12 s for the rats listed in Table 1

The number of seizures in the pre-perfusion period ranged from 3 to 6 in seven KA rats of Experiment III. In each rat, the number of seizures in the pre-perfusion period was set to 100%, and the number of seizures recorded (a) during MeAIB perfusion and (b) in the post-perfusion period was expressed as the percentage of the number of seizures in the pre-perfusion period. Then the mean $\pm$ SE percentage in each period was calculated for $n = 7$. The result is summarized in Table 3. The frequency of electrographic seizures during in vivo inhibition of neuronal uptake of GLN_{ECF} by MeAIB perfusion is reduced to $35 \pm 7\%$ of the frequency observed in the 30 min pre-perfusion period. These frequencies are significantly different with $p < 0.01$. In the post-perfusion period, when the relevant metabolite concentrations returned to the basal values, the frequency of seizures returned to $88 \pm 9\%$ of the initial frequency. This is significantly different from the frequency during MeAIB perfusion with $p < 0.01$. There was no statistically significant difference in seizure frequency between the pre- and post-perfusion periods. In the two remaining KA rats in Experiment III, the number of seizures was 1 in the pre-perfusion period, 0 during MeAIB perfusion and 2 or 3 in the post-perfusion period. These were not included in the statistical analyses in Table 3 (because inclusion of a zero % frequency can lower the mean artificially), but when included, the frequency during MeAIB is $27 \pm 7\%$ of that during the pre-perfusion period; these are significantly different with $p < 0.01$.

Discussion

GLU_{ECF}

Electrographic seizures detected in KA rats in this study had characteristics of hypersynchronous discharge from glutamatergic neurons (Fig. 10A inset) which are abundant in hippocampal CA3 region. Concomitant microdialysis from CA1/CA3 regions in 2-min (Experiment I) or 5-min fractions (Experiments II and III) showed that the concentration of GLU_{ECF} remained low with no significant change (Figs. 2 and 9). The absence of seizure-induced change in GLU_{ECF} has been reported in animal models of epilepsy (Obrenovitch et al., 1996) including the KA model (Bruhn et al., 1992; Lehmann et al., 1985; Takazawa et al., 1995). Our result strongly suggests that GLU released into ECF was efficiently taken up by energy-dependent EAAT2-mediated transport into glia (Danbolt, 2001; Rothstein et al., 1996), and possibly also by EAAT3-mediated uptake into

GABAergic neuron (Stafford et al., 2010) (Fig. 1). Factors that influence the rate of uptake into glia include: (1) GLU_{ECF} concentration at the site of EAAT2 relative to its K_m value for GLU (1–100 μM (Danbolt, 2001)) and (2) the concentration of EAAT2 transporter protein which can be regulated in response to KA treatment (Ueda et al., 2001). With respect to the first factor, it is interesting to note that the basal dialysate GLU concentration was $0.64 \pm 0.06 \mu\text{M}$ ($n = 5$) in seizure-exhibiting KA rats of Experiment II and $0.64 \pm 0.24 \mu\text{M}$ in those of Experiment III. Both concentrations are close to those in the normal rat, $0.74 \pm 0.16 \mu\text{M}$ (Table 1). Hence, one reasonable explanation for the steady GLU_{ECF} concentration in KA rats of Experiments II and III in the present study is that GLU_{ECF} was taken up into glia at a rate comparable to that of release, because GLU_{ECF} concentration was low relative to the K_m value of EAAT2. Another potentially important factor is the concentrations of EAAT2 transporter (and of EAAT3 transporter) in KA rats of this study compared to those in KA rats that showed seizure-induced GLU_{ECF} elevation (Kanamori and Ross, 2011; Ueda et al., 2001). This question is beyond the scope of our present study and must await future investigation (see section “Effect of inhibition on seizure frequency”).

It is noteworthy that while our experimental method can detect significant (e.g. larger than 2 fold) increase in GLU_{ECF} , smaller changes (e.g. 30% decrease in GLU_{ECF}) is difficult to quantify reliably because the experimental method is designed to quantify accurately changes in GLN_{ECF} whose basal concentration is ~23–50 fold higher (Table 1), and the elevated concentration 37–80 fold higher, than that of basal GLU_{ECF} . This required the use of a 2 μL aliquot of each 5-min dialysate fraction for the HPLC assay, which also quantified TAU_{ECF} (~9 fold higher than GLU_{ECF}) and GLU_{ECF} itself. Because of the large dynamic range, small changes in GLU_{ECF} during MeAIB perfusion in Figs. 8 and 9 may have been difficult to detect even with the relatively sensitive fluorometric detector. Reliable examination of small (e.g. 30%) changes in GLU_{ECF} as well as in GABA_{ECF} (basal concentration 1/10th of GLU_{ECF}) must await future study, either by use of larger quantities of dialysate in separate HPLC assays, or by installation of the more sensitive electrochemical detector that can quantify ~10 femtomol of extracellular amino acids.

Seizure-induced decrease of GLN_{ECF}

In KA rats of Seizure Group of Experiment I, GLN_{ECF} decreased progressively during the 5-h microdialysis experiment to $62 \pm 4.4\%$ of the initial value (Fig. 4A; $n = 6$), while in KA rats of Experiment II which were given i.v. infusion of glucose, the final GLN_{ECF} concentration was $75 \pm 2.6\%$ (Fig. 4B and Table 2). Hence, seizure-induced decrease of GLN_{ECF} occurs in both glucose-infused and overnight-fasted rats. The timing of GLN_{ECF} decrease correlated with the frequency and magnitude of seizure activity (Fig. 3A and B). Further evidence that the decrease in GLN_{ECF} is associated with epileptic activity is provided by the observation (Fig. 5) that after intense electrographic seizures in the ipsilateral hippocampus, GLN_{ECF} decreased to 70%, while it showed little change in the seizure-free contralateral hippocampus. Whether a single seizure associated with release of modest quantities of glutamate into ECF causes detectable change in GLN_{ECF} under our experimental condition is a question that must await future investigation.

It is informative to consider whether the observed seizure-induced GLN_{ECF} decrease during frequent epileptiform activity is reversible. SNAT3 mediates the co-transport of GLN with Na^+ in exchange for H^+ (Chaudhry et al., 1999). In the intact brain, the trans-membrane GLN gradient, $\text{GLN}_{\text{glia}}/\text{GLN}_{\text{ECF}}$, is estimated to be 17–20 (Bröer and Brookes, 2001; Kanamori and Ross, 2005), and an increase in the ratio caused by GLN_{ECF} decrease is expected to induce efflux. Hence, during long seizure-free period when the rate of neuronal uptake of GLN_{ECF} is normal, increased release of glial GLN into ECF may restore GLN_{ECF} to equilibrium concentration. In our study, KA rats which showed recurrent seizures in preliminary EEG recording (section “EEG/microdialysis (Experiment I)”) were subsequently used for microdialysis (section “EEG characteristics of KA rats”). These rats were lightly anesthetized on the day of microdialysis for probe insertion, woke in about 1 h and were allowed additional 2 h for stabilization of extracellular metabolites before the start of simultaneous EEG recording and the collection of dialysates for analysis (section “EEG/microdialysis (Experiment I)”). Restoration to equilibrium concentration during this quiescent period may explain the finding that GLN_{ECF} concentrations measured at the start of microdialysis experiment (after the stabilization period) do not differ significantly between those that subsequently exhibited frequent seizures and those that did not (Table 1).

Inhibition of neuronal uptake of GLN_{ECF} in vivo

Neuronal uptake of GLN_{ECF} was inhibited in vivo by perfusion of MeAIB, as demonstrated by 1.8 fold elevation of GLN_{ECF} (Figs. 8 and 9). This maximal elevation is in good accord with our earlier result that, in the cortex of normal rat, maximum elevation was 1.8 fold regardless of the concentration of perfused MeAIB over 50–250 mM range (Kanamori and Ross, 2004). A possible cause for this upper limit is that elevation of GLN_{ECF} reduces or suppresses SNAT3-mediated GLN efflux from glia, because the rate of efflux depends on $[\text{GLN}]_{\text{glia}}/[\text{GLN}]_{\text{ECF}}$ ratio (Chaudhry et al., 1999; Kanamori and Ross, 2005).

The concentration of 175 mM used for in vivo inhibition appears to be high but was necessary to achieve inhibition within 10 min, as explained in section “Inhibition of neuronal uptake of GLN_{ECF} by MeAIB perfusion: Optimization of dose and duration”. Upon perfusion through the dialysis membrane, MeAIB diffuses through the hippocampal interstitial space, and is transported by SNAT1/SNAT2 into glutamatergic and GABAergic neurons, resulting in inhibition of GLN_{ECF} uptake. Because MeAIB has a lower affinity for these transporters (Albers et al., 2001; Su et al., 1997) than GLN (Mackenzie et al., 2003; Varoqui et al., 2000; Yao et al., 2000), a high concentration is required for effective inhibition. However, MeAIB is non-metabolizable and was non-toxic when administered intraperitoneally to rats at a dose of 10 mmol/kg (Christensen and Cullen, 1968) for study of its effect on hepatic retention of amino acids. Furthermore, in vivo inhibition occurs in a very small volume of the hippocampus, estimated to be 700 μm in diameter and 2 mm in length (Kanamori and Ross, 2004). The rat in the present study, observed during microdialysis, appeared to be in good physiological condition and showed no abnormal behavior. This was also true in our previous study (Kanamori and Ross, 2005) when 500 mM MeAIB was perfused through a 4 mm probe for 2 h. Taken together, these results strongly suggest that local perfusion of MeAIB in a small volume of the hippocampus achieves rapid inhibition without toxic or adverse physiological effect. It is worth noting

that, in hippocampal slices, the frequency of spontaneous epileptiform discharges was decreased by MeAIB-induced inhibition of GLN uptake, but the frequency was restored after MeAIB washout (Bacci et al., 2002). The same work also shows that MeAIB-induced reduction of the amplitude of population excitatory post-synaptic potential was reversible. Taken together, these results strongly suggest that the effect of this non-metabolizable inhibitor on neuronal activity is reversible.

During MeAIB perfusion for 30 min, TAU_{ECF} , a major osmoregulatory metabolite in the brain (Estevez et al., 1999; Gullans and Verbalis, 1993) remained at the basal level (Figs. 8 and 9). The result is in agreement with our previous report that, upon perfusion of 250 mM MeAIB in the cerebral cortex of normal rats which caused 1.8 fold elevation of GLN_{ECF} , TAU_{ECF} , measured 30 min after the start of perfusion, remained at the basal value (Kanamori and Ross, 2004). These results show that MeAIB-induced elevation of GLN_{ECF} in vivo is due to inhibition of neuronal uptake of GLN_{ECF} , and not due to osmoregulatory efflux of organic osmolytes. After MeAIB saturates SNAT1/SNAT2, residual MeAIB is in ECF. Upon change of perfusate to aCSF, MeAIB in ECF diffuses back into dialysate. In a previous study, we measured the concentration of MeAIB diffusing back into dialysate as function of CSF perfusion time; at 6 min after switch from 500 mM MeAIB to aCSF, MeAIB concentration in the dialysate was 6 mM and MeAIB was cleared from ECF in 30 min (Kanamori and Ross, 2006; Fig. 4). In the present study, during this clearance of MeAIB, TAU_{ECF} was transiently elevated, but returned to basal value in 30–40 min (Fig. 9). TAU efflux probably occurs mainly from glia (Junyent et al., 2011). However, the mechanism of the observed transient TAU_{ECF} elevation during MeAIB clearance is beyond the scope of the present paper, because the nature of the volume-activated taurine efflux pathway is still controversial (Shennan, 2008). The important points for the present study are (1) elevation of GLN_{ECF} resulting from MeAIB perfusion is due to inhibition of neuronal uptake of GLN_{ECF} and (2) the frequency of EEG seizure in the post-perfusion period is analyzed after MeAIB is cleared from ECF and TAU_{ECF} , as well as GLN_{ECF} , has returned to the basal value.

The concentrations of electrolytes Na^+ and K^+ in ECF are unlikely to be perturbed by MeAIB perfusion for the following reasons. K^+ concentration in the effluxing dialysate during MeAIB perfusion was 2.7 mM compared to 2.9 mM in the basal pre-perfusion dialysate (Unpublished result in this Laboratory), and is quite close to the normal K^+ concentration of 3 mM in CSF. Na^+ concentration in MeAIB perfusate, prepared as described in section “MeAIB perfusion (Experiment III)”, is at most 0.5% higher than the normal Na^+ concentration of 150 mM in ECF (Kanamori and Ross, 2004).

Effect of inhibition on seizure frequency

Inhibition of neuronal uptake of GLN_{ECF} in the ipsilateral hippocampus by MeAIB perfusion decreased the frequency of electrographic seizures to $35 \pm 7\%$ of the frequency observed in the pre-perfusion period (Fig. 10 and Table 3). After clearance of this non-metabolizable and reversible inhibitor from ECF, the frequency of seizures returned to $88 \pm 9\%$ of the initial frequency. These results strongly suggest that electrographic seizures are significantly reduced when pyramidal neurons in the CA3/CA1 region are deprived of

GLN_{ECF} as potential provider of neurotransmitter GLU. To the best of our knowledge, this is the first study that reports the effect of inhibition of neuronal uptake of GLN_{ECF} on seizure frequency in vivo.

It is informative to briefly summarize (a) novel aspects of this study in relation to our previous study, (b) suggested biochemical bases for our findings, and (c) remaining issues for future investigation. In our previous study (Kanamori and Ross, 2011), we focused on those KA rats which showed significant elevation of GLU_{ECF}, possibly due to impairment or down-regulation of glial glutamate transporter EAAT2. Such impairment of GLU uptake into glia reduced the availability of substrate GLU for GLN synthesis (Fig. 1), as suggested by the observed reduction of intracellular GLN in the relevant hippocampal region in the Seizure Group compared to the No-Seizure Group (Table 2, Kanamori and Ross, 2011). Hence, the observed seizure-induced decrease in GLN_{ECF} could reasonably be attributed to reduced GLN efflux from glia to ECF, although there remained an intriguing possibility that seizure-induced decrease of GLN_{ECF} also reflects its increased uptake into neurons to replenish neurotransmitter glutamate during enhanced epileptiform activity. Subsequently, we found that seizure-induced decrease in GLN_{ECF} also occurs in those KA rats that exhibit no significant elevation of GLU_{ECF}, which is the subject of the present study. A probable cause for the low and steady level of GLU_{ECF} is that glial glutamate transporter EAAT2 was not significantly impaired by KA injection (section “GLU_{ECF}”). We hope to examine the cause of the difference in these two groups of KA rats by measuring the levels of EAAT2 proteins by Western blotting. GLU_{ECF}, taken up into glia serves as an important substrate for glutamine synthesis. In support of this concept, the intracellular hippocampal GLN in the Seizure Group was virtually the same as that in the No Seizure group (Fig. 7). Accordingly, it is reasonable to assume that the rates of GLN efflux into ECF were similar in the two groups and unlikely to account for the observed seizure-induced decrease in GLN_{ECF}. Hence, in the present study, we examined more closely the possibility that the observed GLN_{ECF} decrease reflects increased uptake into neurons. We inhibited neuronal uptake of GLN_{ECF} in vivo and found that this causes significant reduction of seizure frequency. The novel findings in this study are (a) seizure-induced decrease in GLN_{ECF} occurs in those KA rats that exhibit no significant elevation of GLU_{ECF}, (as well as in those that do), and (b) neuronal uptake of GLN_{ECF} plays an important role in sustaining recurrent epileptiform activity.

Another factor that can influence the concentrations of GLU_{ECF} and GLN_{ECF} and of hippocampal intracellular GLU and GLN is the activity and concentration of glutamine synthetase (GS) (Fig. 1). In the hippocampal formation of patients with mesial temporal lobe epilepsy (MTLE), GS protein and activity are severely reduced (Eid et al., 2004), and this has been proposed as a possible mechanism for the elevated GLU_{ECF} reported in these patients (section “Role of GLN_{ECF} in sustaining epileptiform activity in vivo”). Eid et al. also report that in a rat model of MTLE, inhibition of GS activity by chronic perfusion of methioninesulfoxime into the hippocampal formation induces recurrent seizures (Eid et al., 2008); among proposed mechanisms (Eid et al., 2012) is elevation of astrocyte GLU due to GS inhibition which impairs glial uptake of GLU_{ECF} resulting in its elevation. These findings can have important implications for epileptogenesis in MTLE patients and provide a reasonable explanation for the occurrence of seizures in their animal model. However, in

chronic KA rats, it remains to be investigated whether changes in GS activity contribute to the seizure-induced GLN_{ECF} decrease reported in our study. In KA rats, proliferation of astroglia (gliosis) in the chronic phase can complicate interpretation of GS activity measurements (Hammer et al., 2008).

Role of GLN_{ECF} in sustaining epileptiform activity in vivo

In the suggested model of GLN/GLU/GABA cycle (Fig. 1), a controversial issue that is under intense investigation is whether GLN taken up from ECF by SNAT1/SNAT2 is important (a) for neurotransmitter generation (Chaudhry et al., 2002; Jenstad et al., 2009), or (b) is the precursor of the metabolic, but not the neurotransmitter pool, of GLU (Grewal et al., 2009; Melone et al., 2006). Evidence in support of the first concept includes the recent immunocytochemical study (Jenstad et al., 2009) which showed that production of GLU from GLN in hippocampal glutamatergic neurons expressing SNAT2 (González-González et al., 2005; Jenstad et al., 2009) can be accelerated in response to enhanced neuronal activity. A study of epileptiform discharges in hippocampal slices also suggests an important role for GLN as provider of GLU during stimulated neuronal activity (Bacci et al., 2002). Tani et al. (2007) report that, in slices from injured cortex as an in vitro model of epileptogenesis, addition of glutamine to the incubation medium increased abnormal spontaneous activity while MeAIB attenuated the effects of added glutamine; this work also suggests an important role of glutamine in sustaining seizure activity. Our present finding – the first one from an in vivo brain – that (a) GLN_{ECF} decreases in response to epileptiform activity and (b) upon inhibition of its uptake, the frequency of seizure is significantly reduced, also suggests that GLN_{ECF} , taken up into neurons, is important in sustaining enhanced excitatory neurotransmission in vivo. GLN_{ECF} is also a provider of the inhibitory neurotransmitter GABA (Brown and Mathews, 2010; Solbu et al., 2010) (Fig. 1). Results in the present study (Fig. 7) show that GABA concentrations in the ipsilateral hippocampus did not differ significantly between the Seizure and No-Seizure Groups of Experiment I, when they were measured in the brains that were snap-frozen at the end of microdialysis experiment. This shows that the seizure-induced decrease in GLN_{ECF} observed in the Seizure Group (Fig. 4A) did not cause significant reduction of GABA in the relevant hippocampal region, and suggests that the reduction of seizure upon inhibition of GLN_{ECF} uptake observed in the present study is more likely to occur through decreased glutamatergic neurotransmission, than through disinhibition due to reduction in GABA. However, further studies, including measurement of extracellular GABA, are needed to address this issue. Because basal GABA_{ECF} concentration is 1/10th of that of GLU_{ECF} , a sensitive detector such as the electrochemical detector is needed for such study (section “ GLU_{ECF} ”).

Microdialysis studies in temporal-lobe epilepsy patients have provided valuable insight into correlation of ictal and interictal GLU_{ECF} levels with (a) severity of spontaneous seizures and (b) hippocampal sclerosis, in conscious (Cavus et al., 2005; Cavus et al., 2008; During and Spencer, 1993; Wilson et al., 1996) or anesthetized (Ronne-Engström et al., 1992; Thomas et al., 2004) patients. However, many questions remain, including the cause of seizures in patients that exhibit no change in GLU_{ECF} (Wilson et al., 1996). To the best of our knowledge, GLN_{ECF} concentrations in temporal-lobe epilepsy patients have been measured only in the interictal periods when the patients are resting quietly at least 6 h away

from any seizure activity (Cavus et al., 2005, 2008). It is interesting to note that the mean GLN_{ECF} concentration was lower in the epileptogenic compared to the non-epileptogenic hippocampus (Cavus et al., 2005) and in atrophic compared to the non-atrophic hippocampus (Cavus et al., 2008), although the differences were not statistically significant. It is possible that progressive decrease in GLN_{ECF} in response to electrographic seizures, such as that reported in this study, is undetectable during interictal resting periods in epileptic patients. Interpretation of clinical data also requires caution because patients use anti-epileptic drugs with diverse effects on epileptogenesis. Animal models of temporal-lobe epilepsy permit examination of correlation of epileptiform activity with extracellular neurochemicals during awake ictal periods in the absence of anti-seizure treatment. It is hoped that our novel finding will contribute to a clearer understanding of the role of GLN in sustaining epileptiform activity in vivo, and possibly open the way to therapeutic intervention to suppress chronic recurrent seizures.

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Abbreviations

EAAT	excitatory amino acid transporter
ECF	extracellular fluid
GABA	γ -aminobutyric acid
GLN	glutamine
GLU	glutamate
KA	kainic acid
MeAIB	2-(methylamino)isobutyrate
SNAT	sodium-coupled neutral amino acid transporter
TAU	taurine

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Figure 1.

A schematic diagram showing major metabolic pathways of glutamate (GLU), glutamine (GLN) and gamma-aminobutyric acid (GABA) and their transport pathways between synaptic vesicles, extracellular fluid (ECF), glia and the neuron, according to a suggested model of GLN/GLU/GABA cycle (see “Discussion”). EAAT2, excitatory amino acid transporter subtype 2; EAAT3, subtype 3; GABA_{NT}, inhibitory neurotransmitter GABA; GLC, glucose; GLNase, glutaminase; GLU_{gl}, glial GLU derived from glucose by the tricarboxylic acid cycle; GLU_{NT}, excitatory neurotransmitter GLU; GS, glutamine synthetase; SNAT, sodium-coupled neutral amino acid transporter (SNAT1, SNAT2 and SNAT3 are also called SAT1, SAT2 and SN1 respectively, but the new nomenclature is used in the text for consistency). MeAIB: 2-(methylamino)isobutyrate, an inhibitor of the System A transporters SNAT1 and SNAT2.

Figure 2.

The time-course of GLU_{ECF} in the ipsilateral hippocampus of the Seizure Groups of KA rats in Experiments I and II. GLU_{ECF} is expressed as percentage of the initial concentration and shown as the mean $\pm$ SE for $n = 6$ (Experiment I) and $n = 5$ (Experiment II). The initial concentration was measured at $t = 6$ min for Exp. I, and for Exp. II (microdialysis concomitant with glucose infusion) at $t = 112$ min (section “EEG/microdialysis/i.v. infusion (Experiment II)”). As the initial concentrations were set at 100%, there is no error bar at these data points.

Figure 3.

The top figures show the time of occurrence of EEG seizures (arrows) and the cumulative peak areas and spike amplitudes (section “Identification of seizure and quantification of EEG activity”) for comparison with the time-course of GLN_{ECF} change, shown as dialysate GLN concentration (μ M) in the bottom figures, for KA rats of Seizure Group of Experiment I; (A) R1051 and (B) R1052.

Figure 4.

(A) The time-course of change in GLN_{ECF} , expressed as percentage of the initial concentration for KA rats of Seizure Group of Experiment I (filled square; mean $\pm$ SE for $n = 6$). The initial concentration refers to that measured in the first fraction after the start of microdialysis. The value at $t = 275$ min (shown by *) is significantly different from the initial value with $p < 0.01$. The time-course for KA rats of No-Seizure Group (mean $\pm$ SE for $n = 6$) is shown by filled triangle. (B) The time-course for Seizure Group of KA rats of Experiment II (mean $\pm$ SE for $n = 5$). The value at $t = 295$ min (shown by *) is significantly different from the initial value with $p < 0.01$. The No-Seizure Group ($n = 4$) showed no change. The initial value for the No-Seizure Group was measured at the same time point as that for the Seizure Group, but the filled triangle symbol overlaps with, and is hidden under,

the filled square symbol for the Seizure Group. In both A and B, the error bar is smaller than the symbol ($<2.5\%$) where not shown.

Figure 5.

Effect of EEG seizure on GLN_{ECF} in the seizure-prone ipsilateral vs seizure-free contralateral hippocampus. (A) EEG recording of electrographic seizure in the ipsilateral (KA injected) hippocampus, compared to the contralateral hippocampus, in a KA rat (R1119) of Experiment II. (B) The time-course of GLN_{ECF} from the ipsi- and contra-lateral hippocampus, expressed as percentage of the initial concentration measured at $t = 7.5$ min. The EEG seizures shown in (A) occurred during $t = 220$ – 295 min as shown at the top of (B).

Figure 6.

High-amplitude ictal discharges in the ipsilateral hippocampus compared to the contralateral in a KA rat (R1111) of Experiment II. The time-course of GLN_{ECF} shows significant decrease in the ipsilateral hippocampus and no change in the contralateral hippocampus during 3 h of dialysate collection concomitant with i.v. glucose infusion. The initial concentration refers to that collected 7.5 min before this 3 h period.

Figure 7.

Tissue concentrations of GLN, GLU and GABA in the ipsilateral hippocampus (in the region sampled by microdialysis and the site of KA injection) of Seizure (S) and No-Seizure (N-S) Groups of KA rats of Experiment I. The data are shown as the mean $\pm$ SE for $n = 6$. Tissue conc. in mM was calculated from the measured weight of hippocampal tissue and metabolite concentration in μmol , based on the usual assumption that 80% of tissue weight is water.

Figure 8.

The time-course of GLU_{ECF} , GLN_{ECF} and TAU_{ECF} in the ipsilateral hippocampus of a representative KA rat (R1156) of Experiment III. The bottom abscissa shows microdialysis time t . The top abscissa shows perfusion time T . At $T = 0$, the perfusate was changed from aCSF to MeAIB (an inhibitor of neuronal uptake of GLN_{ECF}) for 30 min, resulting in 1.8 fold elevation of GLN_{ECF} . Upon changing the perfusate to aCSF, GLN_{ECF} returned to basal value. GLU_{ECF} did not change and TAU_{ECF} showed only transient and modest change. The arrows show the time of occurrence of EEG seizures (see section “Elevation of GLN_{ECF} by MeAIB perfusion” for details).

Figure 9.

The time-courses of GLN_{ECF} and TAU_{ECF} , each expressed as percentage of pre-perfusion concentration (mean $\pm$ SE for $n = 7$) for KA rats of Experiment III (left coordinate). The time-course of GLU_{ECF} is expressed as dialysate concentration (mean $\pm$ SE for $n = 7$), as shown on the right coordinate, on a scale of 0–5 so that the symbols do not overlap with those of GLN_{ECF} and TAU_{ECF} . During MeAIB perfusion, GLN_{ECF} was elevated while GLU_{ECF} and TAU_{ECF} remained stable. TAU_{ECF} was transiently elevated after the perfusate was changed back to aCSF, but returned to basal value by $T = 73$ min. At the top, time periods shown as pre-, MeAIB, and post- (30-min each) refer to those used for comparison of the frequency of EEG seizures (see section “Elevation of GLN_{ECF} by MeAIB perfusion”).

Figure 10.

(A) EEG recordings from the ipsilateral hippocampus of a representative KA rat (R1156) of Experiment III during (a) pre-perfusion period (b) MeAIB perfusion and (c) post-perfusion period. The frequency of seizure is significantly reduced during MeAIB perfusion. The amplitude of ictal spikes often exceeded 1.6 mV, but the expanded plot (inset) shows a wave pattern characteristic of hypersynchronous population bursts from glutamatergic neuron. (B) An EEG recording of a hypersynchronous seizure from the ipsilateral hippocampus of another KA rat (R1155) of Experiment III during post-perfusion period, processed with a faster sweep time.

Table 1

Experimental design, EEG characteristics and basal dialysate GLU and GLN concentrations in KA rats.

Exp	RAT	Group	Days post KA	Seizure	Coordinates			Microdialysis in HC		Glc inf.	Basal dialysate GLU conc. (μM) ^a		Basal dialysate GLN conc. (μM) ^a	
					EEG electrode (mm)			MD probe top (mm)	Ipsi.		Contra.	Ipsi.		Contra.
					AP	L	V							
I	KA	Seizure (n = 6)	46 ± 2	9 ± 1.9	-5.6	4.5 ± 0.07	5.3 ± 0.16	3.56 ± 0.16	+	-	1.11 ± 0.17 (6)	31.5 ± 3.8 (6)		
I	KA	No-seizure (n = 6)	50 ± 4.2	None	-5.6	4.5 ± 0.08	5.6 ± 0.08	3.6 ± 0.1	+	-	1.29 ± 0.23 (6)	30.3 ± 5.0 (6)		
II	KA	Seizure (n = 5)	48 ± 0.3	5 ± 1	-5.6	4.46 ± 0.04	5.4 ± 0.19	3.7 ± 0.25	+	+	0.64 ± 0.06 (5)	32.2 ± 4.4 (5)	25.3 ± 4.0 (5)	
II	KA	No-Seizure (n = 4)	52 ± 6	None	-5.6	4.33 ± 0.05	5.4 ± 0.12	3.6 ± 0.23	+	+	0.85 ± 0.24 (4)	30.3 ± 8 (4)	Not measured	
III	KA	Seizure (n = 9)	53 ± 1.3	See Table 3	-5.6	4.37 ± 0.08	5.4 ± 0.08	3.67 ± 0.096	+	+	0.64 ± 0.24 (7)	32.5 ± 3.0 (7)		
	Normal	n = 6		None					+	+	0.74 ± 0.16 (6)	28.4 ± 1.5 (6)		

Table 2

Seizure-induced decrease in extracellular GLN in KA rats.

Exp.	RAT	Group	<u>Microdialysis in HC</u>		Glc inf.	<u>Extracellular GLN (% of initial conc.)^a</u>	
			Ipsi.	Contra.		Ipsi.	Contra.
I	KA	Seizure (<i>n</i> = 6)	+	–	–	62 ± 4.4	–
I	KA	No-Seizure (<i>n</i> = 6)	+	–	–	None	–
II	KA	Seizure (<i>n</i> = 5)	+	+	+	75 ± 2.6	
	R1119		+	+	+	70	88–100
	R1111		+	+	+	70–80	98–103
II	KA	No-Seizure (<i>n</i> = 4)	+	+	+	None	– ^b

Data are mean ± SE.

^aInitial concentration refers to that measured in the first dialysate fraction, as explained in the legends of Fig. 4 (Exp. I and II), Fig. 5 (R1119) and Fig. 6 (R1111).

^bNot measured because adequate control measurements were performed in the provided data.

Table 3

The frequency of seizures during MeAlB perfusion and the post-perfusion period, expressed as the percentage of the frequency in the pre-perfusion period.

Observation period (30 min each)	Seizure frequency
I. Pre-perfusion	100%
II. MeAlB perfusion	$35 \pm 7\%$ ^a
III. Post-perfusion	$88 \pm 9\%$ ^b

Data are mean $\pm$ SE (n = 7).

^aSignificantly different from I with $p < 0.01$.

^bSignificantly different from II with $p < 0.01$.