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ENHANCED EXPRESSION OF GLUTAMATE-ASPARTATE TRANSPORTER (GLAST) mRNA IN THE HIPPOCAMPUS AND FRONTAL CORTEX OF RATS DURING KAINIC ACID-INDUCED SEIZURE ACTIVITY

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Abstract

In vivo microdialysis measurement of extracellular amino acids during kainate (KA)-induced seizure shows an increase of glutamate. Glutamate transporters may regulate the extracellular glutamate concentration during epileptogenesis. We used Northern blotting to measure sequential changes of the glutamate-aspartate transporter (GLAST) mRNA expression in rat hippocampus and frontal cortex during KA-induced epileptogenesis. Systemic administration of KA to induce acute seizures increased GLAST mRNA expression in both the hippocampus and cortex. Enhancement of GLAST mRNA expression was also observed in a chronic seizure model induced by unilateral microinjection of kainic acid into the amygdaloid body. The level of expression increased just after injection of KA and continued to increase in rats sacrificed 1 day, 7 days and 30 days after injection. Thus we found that GLAST mRNA expression is induced by either a single seizure or by repetitive seizure activity. These results suggest that the failure to maintain glutamate and/or the GLAST protein at a normal level could be one of the causes of the molecular and cellular events responsible for the selective vulnerability of neurons during the process of KA-induced epileptogenesis.

Introduction

Kainic acid (KA)-induced seizures result in the selective degeneration of vulnerable neuronal populations in limbic structures including the hippocampus and piriform cortex (Bruce and Baudry, 1995). Although an excessive increase of the extracellular glutamate concentration has been suggested to be important, the molecular and cellular events responsible for this selective vulnerability remain unknown. Extracellular glutamate levels increase rapidly in the KA-induced epileptic model (Takazawa, Murashima *et al.*, 1995; Wade, Samson *et al.*, 1987). It is thought that the glutamate increase in the extracellular hippocampal space activates *N*-methyl-D-aspartate (NMDA) receptors with a subsequent Ca^{++} influx into neurons (Siesjö, 1993; Van Reempts, Haseldonckx *et al.*, 1986). Following release of glutamate, its removal from the synaptic cleft is an essential component of the transmission processes in glutamatergic synapses. To date, three types

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of cDNAs, encoding high-affinity sodium-, potassium-dependent glutamate transporters, have been cloned with their distribution estimated and their roles proposed (Kanai, Smith *et al.*, 1993). These glutamate transporters are: excitatory amino-acid carrier (EAAC1) (Kanai and Hediger, 1992), glutamate-aspartate transporter (GLAST) (Tanaka, 1993) and glutamate transporter-1 (GLT-1) (Pines, Danbolt *et al.*, 1992). GLAST, based on its high Km value compared to EAAC1 and GLT-1, appears to function as a reserve transporter, protecting neurons by preventing glutamate from accumulating to toxic levels (Kanai, Smith *et al.*, 1993).

Both experimental ischemia and epilepsy cause a remarkable increase of glutamate (Takazawa, Murashima *et al.*, 1995; Wade, Samson *et al.*, 1987). Glutamate transporters, especially GLAST, may have an important role in regulating the extracellular glutamate concentration in the process of epileptogenesis. In this work, we studied the sequential changes of GLAST mRNA expression during the process of KA-induced epileptogenesis by Northern blotting. The aim of the study was to clarify the role of GLAST expressed in the hippocampus and frontal cortex in this model of epilepsy.

Materials and Methods

Materials

[^{32}P]dCTP (111 TBq/mmol, 370 MBq/ml) was purchased from Dupont New England Nuclear. Nitrocellulose membranes were obtained from Micron Separations (Westboro, MA). Cesium chloride and guanidine thiocyanate were from Nacalai Tesque. Deionized formamide and reverse transcriptase from Gibco BRL. The random primers DNA labeling system was from TaKaRa, Co. Ltd., Japan. Agarose (electrophoresis grade) was purchased from Funakoshi, Co. Ltd., Japan, and Kainic acid obtained from Sigma Chemical Co., (St. Louis, MO).

Thirty-six male Wistar rats (200-230 g) were used for this study. The rats were allowed free access to food and water and were housed under a 12 hr light/dark cycle.

Experiment I: Systemic injection of KA

To induce acute seizures in rats, we injected KA (1% solution in 0.9% saline, 15 mg/kg) intraperitoneally (I-Sz, n = 6). The animals were killed 3 hr after injection and the bilateral hippocampi and frontal cortexes were removed from the brain on ice. Tissues were stored at -80°C until mRNA isolation. Control rats received an equivalent volume of 0.9% saline injected intraperitoneally (I-C, n = 6).

Experiment II: Focal microinjection of KA into the amygdaloid body

To make a chronic epileptic model of experimental complex-partial seizure, we used a method reported elsewhere (Tanaka, Tanaka *et al*, 1992). Twenty-four male Wistar rats were anesthetized with sodium pentobarbital (37.5 mg/kg i.p.). Kainic acid (0.5 μg) was dissolved in 0.5 μl phosphate buffered saline (0.2 M PBS at pH 7.4), and sterilized with Millex-HA microfilters (0.45 μm filter unit). Stereotaxic coordinates were determined with the rat brain atlas (Paxinos and Watson, 1986). The incisor bar was set on the intraaural line. While under anesthesia, a polyethylene tube that served as an external guide cannula (1.09 mm o.d., 0.55 mm i.d., 2.5 cm in length) was stereotaxically implanted with a stylet, and anchored to the skull with miniature screws and dental cement. This external cannula was fixed at 6.0 mm anterior and 5.0 mm to the right of the lambda, and 8.5 mm below the surface of the skull. Seven days after the operative procedure, the stylet was replaced with an internal delivery cannula (0.5 mm o.d., 0.25 mm i.d.). The KA solution (0.5 μl) was microinjected through the inner cannula connected with a microinfusion pump at a rate of 1 μl/min (BRC, Japan), and then EEG changes and behavior were observed for at least 6 hr. The external guide cannula was also used for electroencephalographic recording.

Rats used for chronic seizures were divided into four groups: II-S rats (n = 6) were injected with 0.5 μl of 0.2 M PBS without KA and killed 30 days later. II-D (n = 6), II-W (n = 6) and II-M (n = 6) rats were injected with KA and sacrificed at the following times: II-D, one day after injection, II-W, 7 days after injection, II-M, 30 days after injection. Bilateral hippocampi and frontal cortexes were removed from each rat and stored at -80°C until RNA isolation.

RNA isolation and Northern hybridization

Total RNA was isolated from the bilateral hippocampi and frontal cortexes by the guanidinium thiocyanate-cesium chloride method (GIT-CsCl method) (Chirgwin, Przbyla *et al.*, 1979). Twenty μg of RNA, as determined by absorbance at 260 nm, was electrophoresed on a 1.0% agarose gel containing 2.2 M formaldehyde, and then blotted onto a nitrocellulose membrane by capillary transfer in 10 x SSC (NaCl 87.5 g/l, sodium citrate 44 g/l, pH 7.4). Hybridization probes were labeled according to the random priming method described previously (Feinberg and Vogelstein, 1983) using the random primers labeling system. [^{32}P]-labeling of the GLAST cDNA probe was carried out under highly stringent conditions. The filters were hybridized at 42°C overnight, washed and autoradiographed as previously described (Cleveland, Lopata *et al.*, 1980). To facilitate rehybridization with additional probes, hybridization probes were removed by washing in

0.1% SDS for 10 min at 65°C. To detect problems with RNA transfer or unequal loading of RNA in different lanes of the gel, filters were rehybridized with ^{32}P-labeled β-actin cDNA. Autoradiograms of Northern blots were analyzed using a Bio-imaging analyzer (Fujix Bas 2000, Japan).

Preparation of cDNA probes

A cDNA probe (631 bp) specific to GLAST mRNA complimentary to bases 689-1299 (Tanaka, 1993) was synthesized using RT-PCR. Total RNA was extracted from rat brain by the GIT-CsCl method. First strand cDNA was synthesized using 20 μg RNA, 800 ng of random hexamer primers, 8 μl of each dNTP (2.5 mM), and 800 units of reverse transcriptase. The first strand cDNA was then amplified by PCR using the following primers: sense, 5'-AGATGCCTTCCTGGATTTAAT-3'; antisense, 3'-TAGTGAAAGTT-CACGGACCTT-5'. The PCR conditions were as follows: denaturation at 94°C for 1 min, annealing at 55°C for 2 min, and extension at 72°C for 3 min. The number of cycles used for amplification was 40. The PCR product was sequenced to confirm its identity after subcloning into INVaF'. DNA sequence of the probe was confirmed by the DNA sequence technique.

Statistical analysis

Statistical analysis was carried out using one-way (Group effect) ANOVA with post hoc Tukey's test for multiple comparisons. $P < 0.05$ was regarded as a significant difference.

Results

EEG recording and behavior of KA-induced seizures

In accordance with a previous report (Evans, Griffiths *et al.*, 1984), all rats developed partial seizures about 20-40 min after administration of KA. Secondary generalized seizures occurred 60-80 min after KA injection. All of the rats had epileptiform discharges from 60-120 min on EEG. About 3 hr after KA injection these animals had limbic seizures followed by secondary generalized seizures.

Expression of GLAST mRNA in the hippocampus and frontal cortex

The % ratio of GLAST/β-actin in each lane was calculated. GLAST mRNA expression in the frontal cortex and hippocampus of I-Sz rats induced by systemic administration of KA was increased (about a 180% increase in each region) compared with that of I-C rats (see Figure 1, lane A1-2, and lane C1-2, Figure 2).

GLAST mRNA expression in the frontal cortex of the II-D (about a 150% increase), II-W (about a 356% increase) and II-M (about a 163% increase) rats was mark-

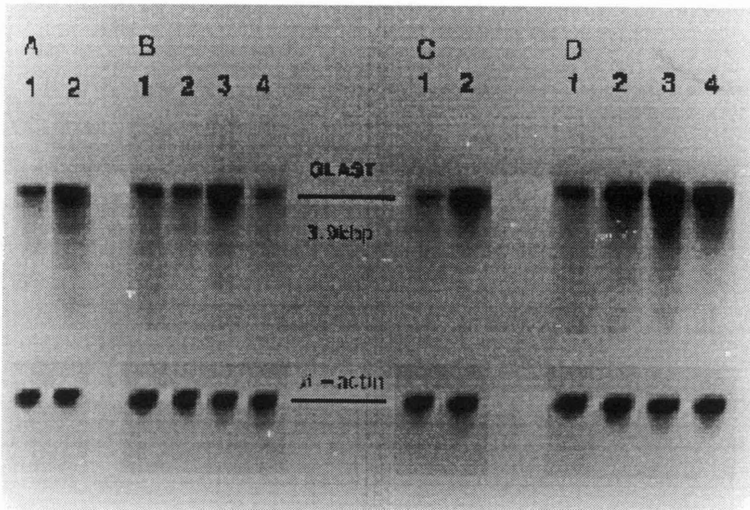

Figure 1. Northern blot analysis of GLAST mRNA. Lane A1-2 results of Exp. I in the frontal cortex; lane C1-2 results of Exp. I in the hippocampus; lane B1-4 results of Exp. II in the frontal cortex; lane D1-4 results of Exp. II in the hippocampus. Total RNA was isolated from the brain of rats sacrificed during seizure (lane A2; I-Sz in the frontal cortex; lane C2; I -Sz in the hippocampus) and at 1 day (lane B2; II-D in the frontal cortex, lane D2; II-D in the hippocampus), 7 days (lane B3; II-W in the frontal cortex, lane D3; II-W in the hippocampus), and 30 days (lane B4; II-M in the frontal cortex; lane D4; II-M in the hippocampus). Lane A1, B1, C1 and D1 are the control lanes for each experiment. Total RNA was separated on a 1.0% agarose gel, transferred to a nitrocellulose membrane, and hybridized to ^{32}P-labeled cDNA probe as described under Materials and Methods. These blots were analyzed using a Bio-imaging analyzer for numeric estimates of the GLA ST mRNA content after normalizing to the β-actin signal.

edly increased (see Figure 1, lane B1-4 and Figure 2) compared with II-S. In the hippocampus, GLAST mRNA expression in the II-D (about a 190% increase), II-W (about a 325% increase) and II-M (about a 256% increase) rats also was marked increased (see Figure 1, lane D1-4 and Figure 2) compared with II-S. Most increases occurred 7 days after unilateral injection into the amygdaloid body in both regions.

Discussion

In vivo microdialysis shows that the extracellular glutamate concentration is increased in the hippocampus during seizures (During and Spencer, 1993; Lallement, Car-

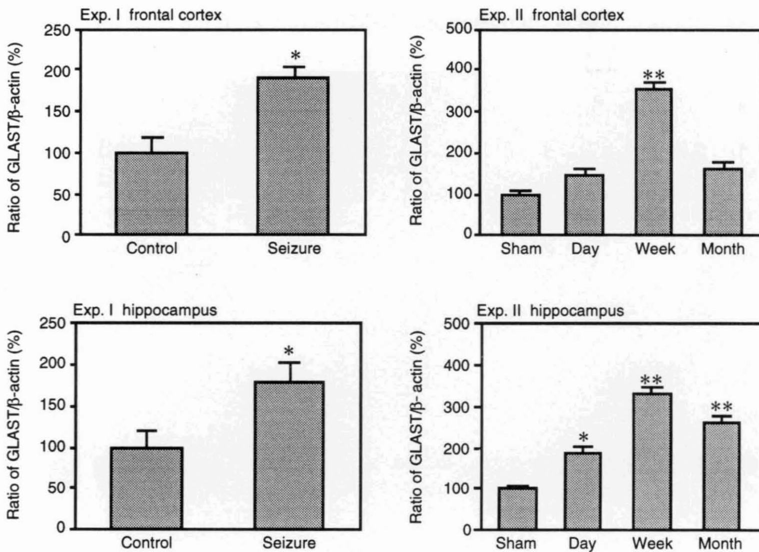

Figure 2. The expression of GLAST in the hippocampus and the frontal cortex increased following seizure, and also 1 day, 1 week and 1 month after unilateral injection of KA into the amygdaloid body. The ratio between GLAST and β-actin has been calculated to correct for differences in the total amount of mRNA loaded onto each lane. The ratio between GLAST and β-actin in each control animal is set to 100%. Each value represents the average of 6 animals and the standard error of the mean is indicated (*$P < 0.05$ **$P < 0.01$ difference statistically significant: one-way ANOVA with post hoc Tukey's test for multiple comparisons).

pentier *et al.*, 1991; Rowley, Martin *et al.*, 1995; Takazawa, Murashima *et al.*, 1995; Wade, Samson *et al.*, 1987). In a kindled model, the repetitive transient stimulus-induced enhancement of glutamate in the extracellular space causes excessive propagation of seizure activity (Ueda and Tsuru, 1995). Increases in the extracellular glutamate level is linked to reverse transport of glutamate, which occurs by disintegration of ion gradients. Thus, glutamate transporters are thought to be linked with the seizure-related elevation in the extracellular glutamate concentration. Among the three known glutamate transporters, GLAST has a Km value that is higher than that of EAAC1 and GLT-1. GLAST is therefore, proposed to function as a reserve transporter, protecting neurons by preventing glutamate from accumulating to toxic levels. Under pathologic conditions, such as ischemia, GLAST is expected to be effective in limiting the detrimental rise in the glutamate concentration and lesion expansion (Kanai, Smith *et al*, 1993).

The results of out first experiment support the conclusion that GLAST mRNA was induced by acute seizure activity. GLAST mRNA expression was increased in II-D rats that were sacrificed at a relatively acute phase of the experiment. The increasing ratio of GLAST mRNA in rats at the chronic phase, Such as II-W and II-M, was greater than that of II-S and II-D rats. Unilateral microinjection of KA into the amygdaloid body induced transient limbic seizures for 24-48 hr, and then, 3-5 weeks later, spontaneous secondary generalized convulsions (Tanaka, Tanaka *et al.*, 1992). The Sequential changes in the levels of GLAST mRNA expression observed in Exp. II, are probably related to changes in the convulsive pattern.

These molecular biological results are thought to correlate with seizure-induced elevation of extracellular glutamate levels in the hippocampus, as determined by *in vivo* microdialysis. Induction of epileptogenic changes in the hippocampus induced by microinjection of KA into the amygdaloid body, is thought to model human complex partial seizures. Spontaneous seizures with transient glutamate release in the hippocampus occurs in humans with the epileptogenic focus (During and Spencer, 1993). This increase of glutamate induced by seizures is transient. Therefore, GLAST could operate in reverse mode during a seizure, and then, during the interictal phase of the seizure operate normally by taking up extracellular glutamate. The increased expression of GLAST mRNA we found in both acute and chronic seizures suggests dual roles for GLAST, not only of neuro-protection, but also the potential to contribute to the neurotoxicity of glutamate as well.

The increase of GLAST mRNA expression might compensate for the increase in the extracellular glutamate level in the hippocampus and act to reduce excess extracellular glutamate. This explanation is appropriate to the result of Exp. I, which indicates that elevation of GLAST mRNA expression could be induced simply by acute seizure activity.

An anatomical connection exists between the frontal lobe and the limbic portion of the temporal lobe. Seizures originating in the limbic temporal regions spread to the frontal lobe in humans. We found that GLAST mRNA expression in the frontal cortex and in the hippocampus generally resembled each other in the duration we observed. However, the elevation of GLAST mRNA expression of II-M rats in the hippocampus tended to be greater than that in frontal cortex. Thus, it is possible that there is a different mechanism for the expression of GLAST mRNA in the frontal cortex and hippocampus following KA-induced epileptogenicity.

Alternatively, some other mechanism may be involved in the chronic phase, such as constructive changes in the hippocampus. Proliferation of glial cells in response to

repetitive seizures could be related to the elevated GLAST mRNA expression as shown in Exp. II. Indeed, it has been reported that KA microinjection into the brain causes mitosis of both glial cells and neurons (Tanaka, Kondo *et al.*, 1988).

In conclusion, GLAST mRNA expression which regulates the normal level of extracellular glutamate in the hippocampus is increased by simple and/or repetitive seizure. Altered GLAST expression may be the molecular and cellular mechanism responsible for the selective vulnerability of neurons during the process of KA-induced seizure.

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