Aberrant glutamate receptor 5 expression in temporal lobe epilepsy lesions

Jin-Mei Li, Yi-Jun Zeng, Fang Peng, Li Li, Tian-Hua Yang, Zhen Hong, Ding Lei, Zhong Chen, Dong Zhou

1. Introduction

Temporal lobe epilepsy (TLE) is the most common form of epilepsy in humans. Patients present with recurrent complex partial seizures and/or secondary generalized seizures originating from mesial structures of the temporal lobe. Kainic acid evokes hippocampal epileptiform activity and has been used extensively to induce animal models of TLE (Ben-Ari, 1985; Ben-Ari and Cossart, 2000; Westbrook and Lothman, 1983). Notably, studies examining the toxicity of kainic acid identify kainate receptors as its main target in animal models of TLE (Pinheiro and Mulle, 2006).

The kainate receptor subunit, GluR5, binds kainic acid with high affinity. It was cloned in 1994 along with other ionotropic glutamate receptors (Hollmann and Heinemann, 1994). As an important member of this receptor family, GluR5 has been reported to regulate the release of excitatory neurotransmitters in mesial structures, such as the hippocampus and amygdala. Kainate receptors, including GluR5, are distributed in temporal lobe structures and play important roles in physiological processes underlying synaptic transmission, integration and plasticity (Bettler and Mulle, 1995; Nakanishi, 1992; Pinheiro and Mulle, 2006). Recent experimental evidence has implicated GluR5 in triggering epileptiform activity and as potential targets for antiepileptic drugs such as Topiramate (Gryder and Rogawski, 2003; Kaminski et al., 2004; Rogawski et al., 2003; Smolders et al., 2002); however, the specific role of GluR5 in TLE remains obscure and controversial.
Despite numerous studies investigating the role of GluR5 in epilepsy in vitro and in vivo, it is unknown whether this receptor becomes activated in the brain of TLE patients. Therefore, we investigated the GluR5 expression in resected brain tissue from patients with pharmacoresistant TLE using real-time PCR and Western blotting. The level of GluR5 protein was also investigated in hippocampal lesions from a macaque model of TLE induced by Coriaria lactone.

2. Results

2.1. Demographic and clinical features of the patients

The mean age of 32 patients with TLE was 23.88±6.23 years (range from 15 to 45 years), with 20 male and 12 female. The mean duration of epilepsy was 9.94±6.96 years (range from 2 to 24 years). All patients presented with complex partial seizure and/or secondary generalized seizure. Table 1 shows the clinical features of the TLE patients. The biopsy samples as the control group were obtained for brain trauma, brain hernia due to cerebral hemorrhage, or neurospongioma (adjacent to the hippocampus). The control group consisted of 12 patients, with 9 male and 3 female. The mean age of 12 patients was 37.75±12.56 (19–57). Statistical analysis showed there was no significant difference in age/gender between the epileptic group and the control group (P>0.05). Table 2 shows the clinical features and regions of resected tissues of the controls.

2.2. Experimental data of macaque

Six male macaques were included in this study. The mean age of macaque with TLE model was 16.7 months. The mean age of control macaque was 15.7 months. Table 3 summarizes the detailed data for the macaques.

In three macaques used in establishment of the TLE model, seizures were induced by Coriaria lactone. Different dosages of Coriaria lactone and the same volume of normal saline were administered in each group until stage 5 or more consecutive seizures were elicited according to Racine seizure classification scale (Racine,1972). The Macaques in experimental group presented with blinking or facial movements followed generalized tonic–clonic seizures. Deep electrodes implanted in hippocampus and temporal neocortex exhibited spikes from

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F, female; M, male; CPS, complex partial seizure; SGS, secondarily generalized seizure; 1, phenytoin; 2, valproate; 3, carbamazepine; 4, clonazepam; 5, lamotrigine; 6, levetiracetam; 7, topiramate; 8, phenobarbital; 9, oxcarbazepine; 10, left; 11, right; SP, spikes; Bi, bilateral; TN, temporal neocortex; H, hippocampus; a, trophy; ac, astrocytosis; g, gliosis; gc, gangliocytoma; gg, ganglioglioma; la, lateral atrophy; aa, architectural abnormalities; nl, neuron loss; nd, neuron degeneration; s, satellitosis.
the hippocampus (data not shown) at the end of procedure before sacrificing the macaque.

2.3. Electron microscopic analysis

Electron microscopic analysis of hippocampal tissues from epilepsy patients revealed decreased number of rough endoplasmic reticulum, swollen mitochondria and disarrayed myelin sheaths compared to controls. The control hippocampal tissues exhibited an almost normal structure. In the macaque model of TLE, swollen mitochondria were also seen (Fig. 1).

2.4. H&E and Timm’s staining

H&E staining of samples from epilepsy patients and the macaque model of TLE revealed neuron loss, balloon degeneration, and dispersion of granule cells in the dentate gyrus. However, normal structures were found in the control samples (Fig. 2). In Timm’s staining, mossy fibers mainly in the supragranular layer in the hilar region of the dentate gyrus in tissue from TLE patients. Mossy fiber sprouting was not observed in the hippocampus of human controls, the macaque model of TLE, and its controls (Fig. 3).

2.5. Real-time PCR for GluR5

The mean 260/280 OD of all samples was 1.827 (range: 1.783–1.905). REST analysis gave an R value of 4.896 for GluR5 amplification products from human epileptic hippocampal samples compared to control (P=0.038). The R value was 0.262 for GluR5 amplification products from human epileptic temporal neocortex samples compared to control (P=0.408). Agarose gel electrophoresis showed that GluR5 amplification product was 161 bp.

2.6. Western blot analysis for GluR5

In Western blot, relatively strong GluR5 expression was seen in human hippocampal samples from TLE patients versus controls, whereas no significant difference was found between temporal neocortex samples from TLE patients and controls. The relative ratio of GluR5/β-actin protein density is shown as mean±SEM. GluR5 protein level was upregulated in hippocampal tissues from TLE patients (2.611±0.382) versus controls. There was no significant difference in temporal neocortex between TLE patients (1.506±0.633) and controls (1.556±0.594) (P>0.05). There was no significant difference in hippocampus between the macaque TLE (1.007±0.034) and control (1.001±0.032) (P>0.05). GluR5 was detected as a band at 105-kDa protein (Fig. 4).

3. Discussion

The involvement of GluR5 in the brains of patients with TLE is poorly understood. In a previous study (Mathern et al., 1998), GluR5-7 mRNA levels in hippocampal tissue from patients with TLE were examined using in situ hybridization; autopsy samples were used as controls for comparison. However, total

<table>
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<th>CL dosage of subthreshold (mg/kg)</th>
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M, male; nl, neuron loss; nd, neuron degeneration; N, normal.
mRNA levels in the hippocampus likely decline immediately after death due to hypoxia. In the present study, the control hippocampus samples were collected from biopsies to minimize hypoxic effects. We demonstrated that GluR5 mRNA and protein levels were upregulated in the hippocampus of patients with TLE compared to controls, whereas GluR5 expression in the neocortex of TLE patients did not differ from that of controls. The latter result is similar to the findings of DeFelipe (DeFelipe et al., 1994). Taken together, these data suggest that GluR5 upregulation in the hippocampus, but not in the neocortex, is involved in the pathological mechanism of pharmacoresistant TLE in humans.

It is known that Coriaria lactone extracted from the Chinese herb Coriaria Sinica Maxim is a mixture of neurotoxin. An animal model with partial seizure type kindled by peripheral injection or cortical administration of Coriaria lactone has been widely used to study epileptogenic mechanisms in China (Zhou et al., 2006; Liao et al., 1987). In previous studies, a rat model of TLE was established by intramuscular injection of Coriaria lactone. In this model, seizures induced by Coriaria lactone originate from the hippocampus, spread to other limbic structures, and can be followed by neuronal loss in selected regions of the brain reminiscent of the brain damage seen in patients with TLE (Wang et al., 2003; Wang-Tilz et al., 2006). In the present study, the macaque model of TLE was also established by intramuscular injection of Coriaria lactone. Seizures episodes, scalp EEG finding, electrocorticogram (ECoG) including hippocampal (EHG) recording and hippocampus pathological examination were observed: blinking or facial movements followed generalized tonic-clonic seizures (stage 5 seizure or consecutive fully kindled seizures according to Racine seizure classification scale), spikes from both temporal region in scalp EEG and from deep electrodes implanted in hippocampus, and brain damage in the hippocampus are similar to that in human TLE. The mechanism by which Coriaria lactone induces seizures is not fully known. It may increase the release of glutamate, activate the N-methyl-D-aspartate (NMDA) receptor on the postsynaptic membrane, and block the synthesis of γ-aminobutyric acid (GABA) in hippocampal neurons (Yu et al., 1996; Wu et al., 1996; Kudo et al., 1984). In vitro studies have revealed that Coriaria lactone increases intracellular calcium in rat hippocampal neurons (Zhang et al., 2009).

Fig. 1 - Electron microphs showing similar profiles in human and macaque TLE samples (arrows) and in controls (arrowheads). (A) Decreased number of rough endoplasmic reticulum, swollen mitochondria cristae in neuron from hippocampus of human TLE (magnification 12,000×). (B) Normal ultrastructure of neuron from hippocampus of human control (magnification 16,000×). (C) Swollen mitochondria in neuron from hippocampus of macaque TLE (magnification 8000×). (D) Normal structure of neuron from hippocampus of macaque control (magnification 20,000×).
In this model of TLE, GluR5 expression in hippocampal tissue was not upregulated compared to controls. The duration of seizures in macaque model lasted for 3 months with spontaneous spikes from hippocampus; however, neither spontaneous seizure nor mossy fiber sprouting was observed. This indicates that GluR5 upregulation is not

Fig. 2 – H&E staining of dentate gyrus shows similar pathological findings in human and macaque TLE samples. (A) Neurons loss, degenerating cells in sample from TLE patient. (B) Normal structure from human control. (C) Marked neuron loss, balloon degeneration, and dispersion of granular cells in sample from macaque TLE model. (D) Normal structure from control macaque. Scale bar in all figures is 100 μm.

Fig. 3 – Timm’s staining of dentate gyrus from TLE patients and macaque TLE models. (A) Mossy fiber sprouting in the supragranular layer in human TLE sample. (B–D) No mossy fibers in samples from human control (B), macaque TLE model and macaque control (D). SGL, supragranular layer; GCL, granule cell layer; PML, polymorphic layer. Scale bar in all figures is 100 μm.
involved in all epileptic activity generated from the hippocampus. In contrast, the duration of seizures in the TLE patient ranged from 2 to 24 years. Neuron loss and mossy fiber sprouting in the hippocampus of TLE patients, and the lack of mossy fiber sprouting in the hippocampus of the short-term macaque model suggests that upregulation of GluR5 is involved in mossy fiber sprouting and may be specifically associated with human chronic epilepsy of long duration.

GluR5 reportedly enhances induction of long-term potentiation at mossy fiber synapses in the CA3 region of the hippocampus by facilitating synaptic release of L-glutamate (Bortolotto et al., 1999; Contractor et al., 2001; Lauri et al., 2001). LY382844, a selective antagonist of GluR5, reportedly has no effect on NMDA receptor-dependent long-term potentiation but prevents the induction of mossy fiber sprouting (Bortolotto et al., 2003). Thus, GluR5 may be involved in the mechanism underlying chronic TLE through activating mossy fiber sprouting that leads to the formation of a recurrent excitatory circuit, which then cause repeated seizures.

Kainate receptors including GluR5 are widely distributed in presynaptic boutons and accelerate glutamate activation. However, the role of GluR5 in GABAergic inhibition of neurotransmission is somewhat controversial in epilepsy studies. Some experimental evidence has implicated GluR5 receptors in triggering epileptiform activity by downregulating GABAergic synaptic inhibition (Clarke et al., 1997; Rodriguez-Moreno et al., 1997). However, two studies revealed that kainate receptors enhance the release of GABA in the interneurons of CA1 (Cossart et al., 2001; Khalilov et al., 2002). These studies suggest that the function of GluR5 in GABAergic synapses is bidirectional and that distinct GluR5 functions may be associated with specific cells of the hippocampus. The limitation of this study is that we could not investigate the expression of GluR5 in different cells in subregions of the hippocampus due to difficulties of distinguishing them in the hippocampus. The role of GluR5 in TLE is complex and further investigation is required to determine the precise mechanism.

Fig. 4 - Western blotting shows GluR5 expression in human with TLE and macaque of TLE. GluR5 expression in the hippocampal tissue (A) and temporal neocortex tissue (B) from TLE patients and controls. In (A) and (B), lanes 1–4 are epileptic samples, and lanes 5–7 are control samples. (C) GluR5 expression in hippocampal tissue of macaque, lanes 1–3 are epileptic samples, and lanes 4–6 are control samples. (D) Histogram of the normalized GluR5 band intensities in three groups.
Conclusion: In summary, upregulation of GluR5 expression was found in the hippocampus of pharmacoresistant TLE patients but not in the neocortex of these patients or in the hippocampus of a macaque TLE model. These results indicate that GluR5 function in the affected region may be associated with the degree of neuronal damage and mossy fiber sprouting.

4. Experimental procedures

4.1. TLE patients and controls

Archived tissue samples from 32 patients with pharmacoresistant TLE who underwent resection of the temporal neocortex and/or hippocampus were obtained from the Department of Neurosurgery of the West China Hospital of Sichuan University. Informed consent was obtained from patients and their direct relatives for the use of brain tissue in research, and the study was approved by the Ethics Committee of the West China Hospital. The diagnosis of seizure type was confirmed according to the 1981 International Classification of Epileptic Seizures of the International League Against Epilepsy (1981). All TLE diagnoses and data were submitted to the Epilepsy Research Center of the West China Hospital. The diagnoses were independently confirmed by at least two neurologists.

All 32 TLE patients exhibited pharmaco-resistance to at least two antiepileptic drugs, including phenobarbital, phenytoin, valproate, carbamazepine, clonazepam, lamotrigine, levetiracetam, topiramate, and oxcarbazepine. Before surgery, the epileptic lesion was localized by magnetic resonance imaging, video electroencephalogram (video-EEG), and/or positron emission tomography. Depth electrodes were embedded in patients whose lesions could not be localized by the above techniques. Intraoperative electrocorticography was performed on all patients before resection. Two neuropathologists independently reviewed all samples.

The control samples were obtained from those patients who accept neurosurgery and abided by ethic requirement. Informed consents also were obtained from all control patients and/or their direct relatives.

4.2. Macaque model of TLE and the control

Six male macaques (5.1–6.6 kg, Certificate No. SYXX 2003-030, NCCSED/West China-Frontier PharmaTech) were maintained in individual cages in a room at 24 °C and 50–60% humidity. The research was carried out in accordance with the ethical guidelines and the requirements for use of laboratory animals of Sichuan University Animal Experimentation Committee.

Standard laboratory food and water were given to the macaques. Three macaques were treated by intramuscular injection with Coriaria lactone every 3 days for 3 months (3 mg/ml, mixture extracted by the Pharmacology Department of Chengdu University of Chinese Medicine). The behavioral seizures were classified according to the Racine scale (Racine, 1972) as: Class I, facial automatisms; II, head nodding; III, unilateral upper limb clonus; IV, rearing and bilateral upper limb clonus; and V, falling over with convulsive seizures. The seizure behaviors, the dose of CL of each injection, the latency from injection to seizure and the seizure duration were recorded after every injection.

After 3 months the animals were sacrificed. Under sodium pentobarbital anesthesia (3%, 1 ml/kg, i.v.), macaques were fixed on an operating table, and electrodes were placed on the scalp and implanted into the temporal lobe cortex and hippocampus (montage reference, EB/Mizar, EBBNeuro S.p.A., Florence, Italy). Then, bilateral hippocampus resection was performed when the macaques were under deep anesthesia. For the three healthy macaques, the animals were sacrificed by anesthetic overdose at the end of the procedures.

4.3. Tissue preparation

A portion of each resected sample was immediately placed in liquid nitrogen for subsequent rt-PCR and Western blot analyses, and another portion was fixed in 10% buffered formalin and 2.5% glutaraldehyde for subsequent hematoxylin and eosin (H&E) staining and electron microscopy.

4.4. Electron microscopy

Samples were fixed in 2.5% glutaraldehyde for 3 h and then in a solution containing 1% osmium tetroxide, pH 7.3, at room temperature for 1 h. The samples were dehydrated in a graded alcohol series and embedded in epoxy resin. Thin sections (70 nm) were cut and viewed under an electron microscope (Hitachi H-7650, Japan).

4.5. H&E and Timm’s staining

The tissue samples were embedded in paraffin after fixation in 10% buffered formalin. For H&E staining, 4-μm sections were prepared from paraffin-embedded samples. For Timm’s staining, 20-μm sections were prepared, and staining was performed as described by Danscher (1983). For Timm’s staining, tissue sections were immersed in a 0.1 M sodium phosphate buffer containing 0.4% sodium sulfide for 30 min and then fixed overnight in 0.1 M phosphate buffer containing 1% paraformaldehyde, 1.25% glutaraldehyde and 20% sucrose. The sections were then immersed in a 12:6:2 mixture of gum arabic/hydroxyquinone/citric acid–sodium citrate buffer, pH 4, containing 34% silver nitrate.

4.6. Real-time PCR for GluR5

Total RNA was extracted from each sample and purified by RNA Catcher (Invitrogen, USA) according to the manufacturer’s instructions. The concentrations of RNA were estimated spectrophotometrically at 260 and 280 nm. The integrity of the RNA was checked by electrophoresis on 1% agarose gels. Primers for PCR amplification were designed as: forward: 5′-GAAGACAGCACAGGTCTAAT-3′; reverse, 5′-CACTCAAAATATCACA-TAGAATCT-3′; TaqMan probe: 5′-CTCATAAAGCCTCCCT-CAGA3′ (GluR5); 5′-GCCAACACAGGTCTGCT-3′; reverse, 5′-AGAGCAATTGTCTTATCTT-3′. TaqMan probe: 5′-CTGCAC-CACCAACTGTCTAGG-3′ (β-actin) (Shanghai Sango). After reverse transcription to cDNA, PCR amplification was performed (FTC2000 PCR system, Funglyn, Canada) as follows: 94 °C for 2 min; 45 cycles at 94 °C for 20 s, 55 °C for 30 s, and 60 °C for 40 s.
PCR amplification products were examined at random by 1.5% agarose gel electrophoresis. REST software (Pfaffl et al., 2002) was used to analyze the fluorescence data using the following equation:

\[
R = \left( \frac{E_{\text{target}}}{E_{\text{ref}}} \right)^{\Delta C_{\text{ref}}-\Delta C_{\text{target}}} \text{Mean control–Mean sample}
\]

\[
/ \left( \frac{E_{\text{ref}}}{E_{\text{ref}}} \right)^{\Delta C_{\text{ref}}-\Delta C_{\text{target}}} \text{Mean control–Mean sample}
\]

(R, ratio; E, efficiency; Cp, crossing point).

4.7. Western blot analysis for GluR5

Tissue samples (100 mg) were homogenized in 10 mM Tris–HCl buffer (pH 7.5) containing 1% Triton X-100, 0.5 mM EDTA, 0.5% NP-40, 4 mM NaF, and 1 mM phenylmethanesulfonfyl fluoride. The lysates were centrifuged at 20,000×g at 4 °C for 15 min. The protein concentration of lysates was determined by BCA and adjusted to 2 μg/μl. Lysates (60 μg) were subjected to 8% SDS-PAGE analysis and electrotransferred to a PVDF membrane (DuPont, USA). The membrane was blocked with PBS, pH 7.2, containing 3% bovine serum albumin, washed with PBS, and then incubated with rabbit anti-human GluR5 (1:1000, Millipore, USA) at 4 °C overnight. After washing with PBS, membranes were incubated with horseradish peroxidase-conjugated secondary antibody (1:1000; Pierce, USA), and bound antibody was detected using a chemiluminescent reagent (Pierce) followed by exposure to X-ray film. Western blot data were quantified using Labworks Analysis Software (USA). Values are presented as mean ± SEM. Statistical analyses were performed using SPSS 13.0 software for Windows (Chicago, USA). One-way ANOVA followed by Games–Howell test was performed. Tests were two-tailed and P<0.05 was considered statistically significant.

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Appendix A. Supplementary data


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