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Research report

Altered expression of $GABA_A$ receptors (α4, γ2 subunit), potassium chloride cotransporter 2 and astrogliosis in tremor rat hippocampus

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ABSTRACT

Impaired GABAergic inhibitory neurotransmission plays an essential role in the pathogenesis of epilepsy. $GABA_A$ receptor ($GABA_AR$), potassium chloride cotransporter 2 (KCC2) and astrocytes are of particular importance to GABAergic transmission and thus involved in the development of increased seizure susceptibility.

The tremor rat (TRM: *tm*/*tm*), a genetic mutant discovered in a Kyoto–Wistar colony, can manifest both absence-like seizures and tonic convulsions without any external stimuli. So far, there are no reports that can elucidate the effects of $GABA_AR$ (α4, γ2 subunit), KCC2 and astrocytes on TRMs. The present study was undertaken to detect the expressions of $GABA_AR$ α4, $GABA_AR$ γ2 and KCC2 in TRMs hippocampus at mRNA and protein levels. In this work, mRNA and protein expressions of $GABA_AR$ α4 were significantly elevated while $GABA_AR$ γ2 and KCC2 were both evidently decreased in TRMs hippocampus by real-time RT-PCR and western blot, respectively. Furthermore, a dramatic elevation of KCC2 protein level was found after cerebroventricular injection with K252a to TRMs than that in the DMSO-treated TRMs. Besides, our present study also demonstrated that GFAP (a major component of astrocyte) immunoreactivity was much more intense in TRMs hippocampal CA1, CA3 and DG regions than that in control group with immnohistochemistry and confocal microscopic analyses. The protein expression of GFAP was also markedly elevated in TRMs hippocampus, suggesting that astrogliosis appeared in the TRM model. These data demonstrate that altered expressions of $GABA_AR$ (α4, γ2) and KCC2 and astrogliosis observed in TRMs hippocampus may provide us good therapeutic targets for the treatment of genetic epilepsy.

1. Introduction

Epilepsy, which is characterized by the recurrent seizures, is among the most prevalent neurological disorders, affecting over 50 million people worldwide [25]. Nearly 30% of them are inadequately controlled or controlled with severe side effects. It is well established that epileptic seizures are associated with excessive excitation or insufficient inhibitory neurotransmission [15,24]. γ-Aminobutyric acid (GABA) is a central inhibitory neurotransmitter in the adult brain [8,29]. A disruption of the inhibition mediated by GABA is a principal reason for the occurrence of neuronal hyperexcitability during epileptic seizures [29].

$GABA_A$ receptors ($GABA_ARs$) mediate most fast inhibitory synaptic transmission in the central nervous system (CNS). The evidence that altered function of $GABA_AR$ subtypes is closely related to epileptogenesis has been elucidated in animal models and human epilepsy [5,27]. Pentameric $GABA_ARs$ are ligand-gated ion channels assembled from a diversity of polypeptide subtypes (α1–α6, β1–β3, γ1–γ3, δ, ε, π, θ and ρ1–ρ3) [20]. They maintain two forms of inhibition, namely, phasic inhibitory synaptic transmission and tonic perisynaptic inhibition. Both forms of GABA signaling are modulated by distinct $GABA_AR$ subunits that typically comprise two α and two β subunits together with the γ2 subunit [9]. Receptors containing α1 and γ2 subunits are preferentially targeted

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to the synapse and involved in phasic inhibition [38], whereas α4 isoform is predominantly located at extrasynaptic site and is critical for tonic inhibition in hippocampus [32].

In addition, $GABA_AR$-mediated fast-hyperpolarizing inhibition is dependent upon the low intracellular concentration of chloride. The neuron-specific potassium chloride cotransporter 2 (KCC2) is the major neuronal chloride extruder in the adult CNS [18]. Cumulative evidence has elucidated that KCC2 is responsible for an inwardly directed electrochemical gradient of chloride and hence for the generation of hyperpolarizing $GABA_A$ receptor-mediated postsynaptic currents in the adult brain [10,34]. The expressional change of KCC2 was described previously in the pilocarpine-induced epilepsy as well as in cortical epilepsy-associated malformations [1,23]. However, the underlying mechanism that regulates this change in KCC2 expression is not clear.

Astrocytes, one class of glial cells, also play a critical role in the regulation of synaptic transmission throughout the brain [30]. Previous report has demonstrated that hippocampal astrocytes also respond to GABA, finally regulating GABAergic inhibitory synaptic transmission [42]. Reactive astrocytosis is pathologically characterized by gross hyperplasia and hypertrophy of astrocytes in injured or diseased areas of the brain, accompanied by the up-regulation of glial fibrillary acidic protein (GFAP), a predominant component of neurofilaments [31]. The enhanced expression of GFAP has been observed in temporal lobe epilepsy [43], however, the potential mechanism involved in the reactive gliosis linked with epilepsy remains to be elucidated.

The tremor rat (TRM: *tm*/*tm*), a genetic mutant discovered in a Kyoto–Wistar colony, can manifest both absence-like seizures and tonic convulsions without any external stimuli [26,36]. Previous study has illustrated that the absence-like seizures in the TRM are characterized by paroxysmal occurrence of 5–7 Hz spike-wave complexes in hippocampal electroencephalograms (EEGs) after 8 weeks (data not shown) [37]. Furthermore, it was reported that the spike and wave complex appeared 0.8–1.9 times per minute and lasted for 1–17 s. However, in the intervening periods, normal EEG recording free of absence-like seizure was noted in the TRM examined [37]. Thus, this TRM is regarded as a very useful model for the research of human absence seizures. So far, the generating effects of $GABA_AR$ α4 and $GABA_AR$ γ2 subunits as well as KCC2 in TRMs have not yet been well elucidated. Besides, although recent studies have demonstrated that there are evident alterations in the expression of KCC2 in the hippocampus of pilocarpine-induced epilepsy and temporal lobe epilepsy patients [23,28], the underlying mechanism that regulates KCC2 expression in epileptogenesis remains unclear. Furthermore, as to the changes of astrocytes in TRMs, there are no relevant publications. Analysis of $GABA_AR$ α4 and $GABA_AR$ γ2 isoforms and KCC2 expression patterns together with the alterations of astrocytes will help to explore the mechanisms of GABAergic inhibitory neurotransmission in the epileptogenesis. In the current study, we studied the expression of $GABA_AR$ α4, $GABA_AR$ γ2 and KCC2 at transcript and protein levels in TRM hippocampus by real-time quantitative RT-PCR and Western blot, respectively. In addition, the role of BDNF/TrkB signaling pathway in regulating KCC2 expression in TRM hippocampus was also investigated. Meanwhile, the protein distribution and expression of GFAP were evaluated using this TRM model.

2. Materials and methods

2.1. Experimental animals

Wild control rats and TRMs at the age of 9–12 weeks were used in this study. The rats were housed under a controlled environment (12:12 h light/dark cycle, 50–70% humidity, 24 °C), with free access to food and water. All procedures involving animals were in strict accordance with the guidelines established by the NIH in the USA and approved by Animal Care Committee of China Medical University. Efforts were made to minimize suffering and reduce the number of animals used.

2.2. Real-time quantitative RT-PCR

Total RNA was extracted from the whole hippocampus tissues of TRMs ($n = 6$) and control rats ($n = 6$) using Trizol reagent (Invitrogen, CA) according to manufacture's instructions. The concentration of RNA was measured by spectroscopy, with an expected A_{260}/A_{280} ratio close to 1.8–2.0, denoting an acceptably pure nucleic acid sample. 500 ng of RNA from each sample was reverse-transcribed in a volume of 10 μl to produce cDNA using Takara RNA PCR Kit (AMV) Ver.3.0 (TaKaRa Bio Inc). Reverse transcription (RT) reactions were carried out at 37 °C for 15 min and 85 °C for 5 s. SYBR Green I-based detection was conducted using ABI PRISM 7300 instrument with thermal cycler conditions of: 95 °C for 30 s, followed by 45 cycles (95 °C for 10 s and 62 °C for 31 s). Standard curves plotting the threshold amplification cycle number (*Ct*) values against input quantity for each gene were constructed using five-fold serial dilutions of RT product. GAPDH was served as an endogenous internal standard control. All experiments were repeated twice and, in each experiment, samples were assayed in duplicate. Data were expressed as a ratio: relative quantity of $GABA_AR$ α4, $GABA_AR$ γ2 and KCC2 mRNA/relative quantity of GAPDH mRNA, respectively. Primer sequences for $GABA_AR$ α4, $GABA_AR$ γ2, KCC2 and GAPDH were as follows: ($GABA_AR$ α4) forward: 5′-GCC CGG AAA ATT TTA CCC GTA TC-3′ and reverse: 5′-GAG CTG TCA TGT TAT GGG AGA C-3′; ($GABA_AR$ γ2) forward: 5′-CTT CTG GAA GGG TAC GAC AAC-3′ and reverse: 5′-AGC ATC CTG TTG GGA GTC G-3′; (KCC2) forward: 5′-AGG TGG AAG TCG TGG AGA TG-3′ and reverse: 5′-CGA GTG TTG GCT GGA TTC TT-3′; (GAPDH) forward: 5′-GCA TTG CTC TCA ATG ACA ACT T-3′ and reverse: 5′-GGC CTC TCT CTT GCT CTC AGT-3′.

2.3. Western blot analysis

Western blot analysis was performed on samples of the whole hippocampus of TRMs ($n = 6$) and control rats ($n = 6$). In brief, samples were washed twice with PBS buffer and homogenized in cold lysis buffer (50 mM Tris–HCl, 150 mM NaCl, 10% glycerol, 1% Nonidet P-40, 5 mM EDTA and 1 mM phenylmethylsulfonyl fluoride). The lysates were spun at 13,200 × *g* for 20 min at 4 °C. The supernatant was collected and quantified for total protein levels using a BCA protein assay kit with bovine serum albumin as the standard (Beyotime Institute of Biotechnology, China).

Equal amounts of protein (60 μg) were subjected to SDS-PAGE using 8% or 10% acrylamide gels and electroblotted onto nitrocellulose membranes (Millipore, MA). The membranes were blocked with 5% fat-free milk in TBS containing 0.1% Tween-20 for 1 h at room temperature. Blots were incubated with the following primary antibodies: rabbit anti-$GABA_AR$ α4 (1:300, Santa Cruz, USA), goat anti-$GABA_AR$ γ2 (1:200, Santa Cruz, USA), rabbit anti-KCC2 (1:1500, Upstate Biotechnology, NY), rabbit anti-GFAP (1:200, Santa Cruz, USA), mouse anti-β-actin (1:2000, Santa Cruz, USA) and mouse anti-GAPDH (1:2000, Kang Chen, China), respectively, overnight at 4 °C. After the membranes were washed, they were incubated with horseradish peroxidase-conjugated second antibody (1:5000, Santa Cruz, USA) for 2 h at room temperature. Immunolabeled protein bands were detected using an enhanced chemiluminescence kit (Pierce, CA) and exposed on an X-ray film. β-Actin or GAPDH was used as an internal reference for relative quantification. Immunoblots scanned by the densitometer were subjected to the grey value analysis using Quantity One software (BioRad, USA).

2.4. Intracerebroventricular drug application

Rats were anesthetized by intraperitoneal injection of sodium pentobarbital (50 mg/kg) and placed in a stereotaxic device. Each rat was prepared with a 0.4 mm external diameter hypodermic needle extending into the left lateral cerebral ventricle (coordinated from bregma, AP: 0.8 mm, ML: 1.5 mm, DV: 4.2 mm) [7]. The guide cannula was fixed well with dental cement through the surface of the skull. Animals were cerebroventricular injected with K252a (2 μg dissolved in 10 μl PBS with 0.01%DMSO; Sigma, USA) or 10 μl phosphate-buffered saline (PBS) with 0.01%DMSO as a vehicle control once a day for two days. The position of injection cannula tip in the ventricle was verified after experiment by injecting blue ink and checking for distribution.

2.5. Tissue preparation for immunohistochemical and confocal laser scanning microscopic analysis

TRMs ($n = 6$) and control rats ($n = 6$) were deeply anesthetized with an intraperitoneal injection of chloral hydrate (300 mg/kg) and were transcardially perfused 0.1 M PBS (pH 7.4), followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). After perfusion, the brains were dissected, immersed in the same fixative for 12 h and cryoprotected in 30% sucrose solution for 3 days at 4 °C. 8 μm frozen sections containing the hippocampus were cut coronally using Cryostat (CM1900 UV, Leica, Germany).