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Up-regulation of GABA transporters and $GABA_A$ receptor α1 subunit in tremor rat hippocampus

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ABSTRACT

The loss of GABAergic neurotransmission has been closely linked with epileptogenesis. The modulation of the synaptic activity occurs both via the removal of GABA from the synaptic cleft and by GABA transporters (GATs) and by modulation of GABA receptors. The tremor rat (TRM; tm/tm) is the parent strain of the spontaneously epileptic rat (SER; zi/zi, tm/tm), which exhibits absence-like seizure after 8 weeks of age. However, there are no reports that can elucidate the effects of GATs and $GABA_A$ receptors (GABARs) on TRMs. The present study was conducted to detect GATs and GABAR α1 subunit in TRMs hippocampus at mRNA and protein levels. In this study, total synaptosomal GABA content was significantly decreased in TRMs hippocampus compared with control Wistar rats by high performance liquid chromatography (HPLC); mRNA and protein expressions of GAT-1, GAT-3 and GABAR α1 subunit were all significantly increased in TRMs hippocampus by real time PCR and Western blot, respectively; GAT-1 and GABAR α1 subunit proteins were localized widely in TRMs and control rats hippocampus including CA1, CA3 and dentate gyrus (DG) regions whereas only a wide distribution of GAT-3 was observed in CA1 region by immunohistochemistry. These data demonstrate that excessive expressions of GAT-1 as well as GAT-3 and GABAR α1 subunit in TRMs hippocampus may provide the potential therapeutic targets for genetic epilepsy.

γ-Aminobutyric acid (GABA) is the predominant inhibitory neurotransmitter in the mammalian central nervous system (CNS). The modulation of the synaptic activity occurs both via the removal of GABA from the synaptic cleft and by GABA transporters (GATs) and by modulation of GABA receptors.

GATs located on the plasma membrane of neurons and astrocytes contribute to determining GABA level in the synaptic cleft [16,13]. Up to date, four subtypes of GATs, designated GAT-1, GAT-2, GAT-3 and betaine/GABA transporter (BGT)-1 have been cloned in rat brain [11]. GAT-1 and GAT-3 are the most copiously expressed GATs in the brain and the two types of membrane GATs are most likely to be responsible for regulating the ambient level of GABA [6], finally contributing to epileptogenesis. The fact that changes of GATs activity are closely related both to pathology and therapy of epileptic seizure has been approved in clinical practice [1].

$GABA_A$ receptors (GABARs) mediate the majority of fast inhibitory synaptic transmission in the CNS. Mammalian GABARs are composed of pentameric, ligand-gated ion channels assembled from various (α1–α6, β1–β3, γ1–γ3, δ, ε, π, θ) polypeptide subunits [12]. The α family is the largest with six different subtypes and contributes significantly to the functional characterization of the GABARs. GABAR α1 is the most widely expressed of all the α subtypes and shows a high level of expression in most regions of the brain [19]. The importance of the α1 subunit for inhibitory synaptic transmission is exemplified in α1 knock-in mice that are more susceptible to having anticonvulsant properties of benzodiazepines [23].

Synaptosome is an isolated nerve terminal of a neuron which is adequate for exploring neural function in vitro. Alterations of synaptosomal neurotransmitter levels have been implicated in the cause of neurological diseases, such as epilepsy.

The tremor rat (TRM) (tm/tm) is the parent strain of the spontaneously epileptic rat (SER: zi/zi, tm/tm), which exhibits both absence-like and convulsive seizures without any external stimuli [31]. Previous work has demonstrated that the electroencephalograms (EEGs) recording can show 5–7 Hz spike-wave-like complexes synchronously in hippocampus accompanied by absence-like seizures after 8 weeks (data not shown) [26]. So far, the effects of GATs and GABAR α1 in TRMs have not yet been well elucidated. We speculate that the etiopathogenesis and hypoinhi-

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bition of TRM in genetic epilepsy might be involved in changes of GATs and GABAR α1. Therefore, we measured the synaptosomal GABA concentration in TRM hippocampus by high performance liquid chromatography (HPLC). Furthermore, we investigated the expressions of GATs and GABAR α1 at the mRNA and protein levels of TRM through real time PCR, Western blot and immunohistochemical analysis.

Normal Wistar rats and TRMs at the age of 9–12 weeks were housed in individual cages under a controlled environment (12:12 h light/dark cycle, 50%–70% humidity, 24 °C). Food and water were available *ad libitum*.

The GAT-1, GABAR α1 and β-actin antibodies were purchased from Santa Cruz. The GAT-3 antibody was obtained from Abcam technology. Other reagents were from Sigma–Aldrich.

Purified synaptosomes were isolated according to Dunkley et al [7]. Briefly, the hippocampus was homogenized in 0.32 M sucrose buffer. After the two centrifugations at $1000 \times g$ for 2 min and $15{,}000 \times g$ for 12 min, resultant pellet was resuspended in 0.32 M sucrose and layered on top of Percoll discontinuous gradients (23%, 10% and 3%) in sucrose buffer. Synaptosomal fraction was obtained from the 23%/10% interface and synaptosomal protein was determined by Bradford method [3]. The Synaptosomal (1 mg/ml) sample was separated by HPLC method. The result was measured through the peak areas.

Total RNA was extracted from hippocampus using Trizol (Invitrogen Technology). PCR primers for GAT-1, GAT-3, GABAR α1 and β-actin were as follows: (GAT-1) forward: 5′-TGC AAA CAC GTA CGC ACA TAG AA-3′ and reverse: 5′-AGA TGC CTC AGC CAC ACG AC-3′; (GAT-3) forward: 5′-CGG TCA CTG GAA CAA CAA GGT G-3′ and reverse: 5′-AAC ACC ACG TAA GGA ATC AGG AAT G-3′; (GABAR α1) forward: 5′-CCT GGA CCC TCA TTC TGA GCA-3′ and reverse: 5′-ATC CTC GTG AAG ACA GTG GTG TTG-3′ and (β-actin) forward: 5′-AGG CCC CTC TGA ACC CTA AG-3′ and reverse: 5′-CCA GAG GCA TAC AGG GAC AAC-3′. They produced the PCR products of 144 bp, 135 bp, 94 bp and 118 bp, respectively. Total RNA was reverse-transcribed at 37 °C for 15 min and 85 °C for 5 s. SYBR Green I-based detection was carried out on a real-time PCR instrument with thermal cycler conditions of: 95 °C for 30 s, followed by 40 cycles (95 °C for 5 s and 62 °C for 34 s). Data were expressed as a ratio: relative quantity of GAT-1, GAT-3 and GABAR α1 mRNA/relative quantity of β-actin mRNA, respectively.

Samples were homogenized in RIPA lysis buffer. An equal amount of 60 μg proteins were loaded to each lane, separated electrophoretically by SDS-PAGE and electroblotted onto PVDF membranes. After blockage of 1 h in 5% nonfat dry milk, the membranes were probed respectively with the anti-GAT-1 (1:600 dilution), GAT-3 (1:600 dilution), GABAR α1 (1:500 dilution) or β-actin (1:1000 dilution) antisera conjugated goat anti-rabbit IgG and were detected with enhanced chemiluminesecence (ECL). β-actin was used as an internal reference for relative quantification.

Rats were anesthetized and perfused with 4% paraformaldehyde. The brains were further immersed into 30% sucrose. After blockage with 10% goat serum, free-floating sections were incubated respectively with rabbit anti-GAT-1 (1:150 dilution), anti-GABAR α1 (1:100 dilution), GAT-3 antisera (1:100 dilution) and goat anti-rabbit IgG (1:200 dilution). The reactions were visualized with 3,3-diaminobenzidine tetrahydrochloride (DAB) method.

Statistical analyses were performed using Student's *t*-test, values were expressed as mean ± SD, $p < 0.05$ was considered significant.

It has been recognized that the basal levels of neurotransmitters in the synaptosome were more closely linked with epileptogenesis than those in the whole brain tissue [5,25]. To explore the alteration of GABA in TRM, the GABA concentration was detected in TRMs hippocampal synaptosomes by HPLC. Obviously, the content of synaptosomal GABA in TRMs was definitely decreased compared with that in Wistar rats (59.55 ± 6.38 μg/mg protein in TRMs vs. 102.36 ± 21.90 μg/mg protein in Wistar rats, $p < 0.01$).

In order to evaluate the roles of GAT-1, GAT-3 and GABAR α1 in GABAergic inhibition of TRMs, we subsequently made an analysis of the mRNA expressions of GAT-1, GAT-3 and GABAR α1 in TRMs hippocampus using real time PCR. The data showed that the mRNA expression of GAT-1 in hippocampus was markedly increased than that of control groups (1.58 ± 0.42 in TRMs vs. 0.45 ± 0.33 in control rats; $p < 0.01$; Fig. 1a). In addition, GAT-3 mRNA in the hippocampus of TRMs was expressed much higher compared with that in control rats (1.40 ± 0.13 in TRMs vs. 0.77 ± 0.29 in control rats; $p < 0.05$; Fig. 1b). Moreover, the mRNA level of GABAR α1 in TRMs hippocampus was significantly higher than that in control groups (2.09 ± 0.57 in TRMs vs. 0.90 ± 0.41 in control rats, $p < 0.01$; Fig. 1c).

To investigate whether GAT-1, GAT-3 and GABAR α1 protein expressions in TRMs hippocampus were different from those in control rats, Western blot analysis was further performed. Fig. 1d showed the result with antibody specific to GAT-1 by Western blot. The protein expression of GAT-1 in TRMs hippocampus was significantly higher than that in control groups (0.64 ± 0.06 in TRMs vs. 0.32 ± 0.10 in Wistar rats, $p < 0.01$; Fig. 1g). Our finding also illustrated that GAT-3 protein was detectable as 74-kDa-immunostained polypeptide in experimental and control groups (Fig. 1e). In comparison with normal Wistar rats, increased expression of GAT-3 was apparently observed in TRMs hippocampus (0.94 ± 0.06 in TRMs vs. 0.51 ± 0.15 in Wistar rats, $p < 0.01$, Fig. 1h). It was noteworthy that the Western blot with GABAR α1 antibody exhibited an anticipated single band of 51 kDa (Fig. 1f) and the increased expression of GABAR α1 protein was obviously found in TRMs hippocampus compared with Wistar rats (0.75 ± 0.08 in TRMs vs. 0.44 ± 0.05 in control rats, $p < 0.01$, Fig. 1i). Thus, these results by Western blot analysis further confirmed the distinct expressions of GAT-1, GAT-3 and GABAR α1 at protein level in TRMs hippocampus compared with Wistar rats, which were in accordance with our real time PCR results.

Immunohistochemical analysis was employed in TRMs and Wistar rats hippocampus by using the anti-GAT-1, anti-GAT-3 and anti-GABAR α1. The positive reactions appeared brown on the membrane. It was obviously observed that GAT-1 and GABAR α1 proteins in TRMs and Wistar rats were both localized widely in hippocampus including CA1, CA3 and DG regions (Fig. 2(A) and (C) for GAT-1 and GABAR α1, respectively). However, in terms of GAT-3, a weak immunoreaction appeared in CA3 and DG regions except for the evident observation in CA1 region (Fig. 2(B)). With the help of the resultant analysis of immunohistochemistry, the integrated optical density values of GAT-1 in TRMs hippocampus were much higher than those in Wistar rats including CA1, CA3 and DG regions (Fig. 3(A): CA1 and DG regions, $p < 0.05$; CA3 region, $p < 0.01$). Meanwhile, it was quite evident for the integrated optical density of GAT-3 in the CA1 region of TRMs hippocampus compared with Wistar rats (Fig. 3(B): $p < 0.01$), however no significant changes were shown in CA3 and DG regions. Besides, the integrated optical density of GABAR α1 in the CA1 region of TRMs hippocampus was more pronounced than that in control groups (Fig. 3(C): $p < 0.01$).

Extracellular GABA was decreased in cerebrospinal fluid (CSF) obtained from patients during seizures [22]. Similarly, low levels of GABA were found in several animal models following induction of seizures [8,14]. In this study, we have employed the synaptosomal sample to measure the concentration of GABA in TRMs hippocampus. The synaptosome has the advantage of maintaining original synaptic function under proper conditions and thus it is a suitable subcellular fraction for investigating the mechanism of epileptogenesis. Several reports support the notion that alterations of synaptosomal neurotransmitter levels are more neuroactive than those of whole brain tissue in seizure-susceptibility or -severity