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Original Research

Expression of Nav1.5 in the pathogenesis of temporal lobe epilepsy

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Abstract: To investigate the expressions of $Na_v1.5$ mRNA at different time points in a rat model of temporal lobe epilepsy (TLE), and to assess the potential contribution of $Na_v1.5$ to epileptogenesis. Male Sprague-Dawley rats (72) weighing 230 to 250 g were used for this study. They were randomly assigned to six groups (12 rats/group): control and five TLE groups. The TLE groups were day 1 (acute period), days 7 and 14 (latent period), and days 30 and 60 (chronic period). With the exception of control, epilepsy was induced in the rats with an intraperitoneal (*i.p.)* injection of aqueous solution of lithium chloride 18 h prior to pilocarpine injection (*i.p.*) at a dose of 125 mg/kg body weight (b.wt). Rats in the control group were injected *i.p.* with 0.9 % sodium chloride (125 mg/kg b.wt.) in place of pilocarpine. A total of 84 out of 112 rats developed status epilepticus (SE). The expression of $Na_v1.5$ in the brains of rats was assessed using quantitative real-time polymerase chain reaction (qRT-PCR), immunohistochemistry and Western blot analysis. The expressions of Scn5a mRNA in the hippocampus during the latent and chronic periods were significantly higher than in the control group ($p < 0.05$), but there were no significant differences in the corresponding expressions between the two different time points in the latent and chronic period groups ($p > 0.05$). The expression peaked 30 days post-SE, and was sustained for 60 days. There was no significant difference in the expression of Scn5a mRNA in the acute group, when compared to control. Immunohistochemical staining showed that expression levels of $Na_v1.5$ in the CA3 region during latent and chronic periods were significantly higher than those in control group ($p < 0.05$), and the expressions peaked at day 30. However, there was no significant difference in the expression of $Na_v1.5$ in the latent group, relative to the chronic period group. These results show that $Na_v1.5$ might be involved in the pathogenesis of TLE.

Key words: Temporal lobe epilepsy; Voltage-gated sodium channel; Hippocampus; Seizures; Expression.

Introduction

Epilepsy is a chronic neurological disorder characterized by recurrent, unprovoked seizures and it affects about 1 % of the world's population. Temporal lobe epilepsy (TLE), the most common and devastating type of epilepsy, is characterized by focal seizures of temporal structures (1). Studies have shown that neuronal death, aberrant synaptic plasticity and neuro-inflammation are three important mechanisms involved in epileptogenesis (2). Synaptic plasticity is crucial for the self-reinforcing cycle in epilepsy, but the mechanisms of enhanced excitability is still unknown (3). Sodium ion channels are transmembrane proteins which are widely distributed in skeletal muscle, myocardium and neurons, and voltage-gated sodium channel (VGSC) is essential for the initiation and propagation of transient and persistent sodium currents (INa_v) (4). An abnormality in the structure and function of Na_v plays a crucial role in epileptogenesis (5). Sodium ion channel contains a large α subunit and one or more smaller β subunits. The α subunit which is the functional subunit is highly conserved throughout evolution. The β subunit is a transmembrane protein which influences the voltage-dependency, gating, and cell surface distribution of the α subunit. Nine isoforms of mammalian α subunit of Na_v ($Na_v1.1$/Scn1a, $Na_v1.2$/Scn2a, $Na_v1.3$/Scn3a, $Na_v1.4$/Scn4a, $Na_v1.5$/Scn5a, $Na_v1.6$/Scn8a, $Na_v1.7$/Scn9a, $Na_v1.8$/Scn10a, and $Na_v1.9$/Scn11a) have been cloned so far (6). Changes in the biophysical properties of the Na_v current have been reported in different animal models of epilepsy (7, 8). In 2009, it was reported that $Na_v1.6^{+/-}$ mice caused a significant resistance to initiation and development of kindling when compared to the wild type mice (9). The $Na_v1.1$,1.2,1.3 and 1.6 are expressed mainly in the central nervous system (CNS) (10). Mutations in these VGSCs can lead to genetically acquired epilepsy. The $Na_v1.5$ was initially thought to be the principal cardiac subtype, but it has also been found to be expressed in limbic regions of the brain (11). In rat hippocampal progenitor stem cell line (HiB5), a novel splice variant of $Na_v1.5$ was found with a high sensitivity to lamotrigine (12). Missense Scn5a mutation may be expressed in the brain and heart, leading to a predisposition to seizures and cardiac arrhythmias (13). However, there is presently insufficient evidence to show the connection between missense Scn5a mutation and epilepsy. The aim of this study was to investigate the expressions of $Na_v1.5$ mRNA at different time points in a rat model of TLE, and to assess the potential contribution of $Na_v1.5$ to epileptogenesis.

Materials and Methods

Materials and Chemicals

Lithium chloride and pilocarpine were products of Sigma, St. Louis, USA; Trizol reagent was obtained from Invitrogen, California, USA, while gDNA Eraser

and SYBR® Premix Ex Taq™ were products of Takara, Dalian, China. The qRT-PCR Mastercycler was obtained from Eppendorf, Connecticut, USA; ABI StepOnePlusTM real-time PCR system was purchased from ABI, California, USA, while the primers were products of BGI Co., Shanghai, China. Freezing microtome, light microscope and image analysis system were products of Leica CM 1850, Wetzlar and Mannheim, Germany, while horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG and 3, 3'- diamininobenzidine (DAB) kit were purchased from Zhongshan Golden Bridge Biotechnology Co, Beijing, China. Radio-immunoprecipitation assay lysis buffer and BCA assay kit were obtained from Beyotime, Shanghai, China; Protease inhibitor PMSF was purchased from Solarbio, Beijing, China, while β-actin was a product of Santa Cruz, Texas, USA. The HRP-conjugated secondary antibody was a product of Proteintech, Chicago, USA. Enterochromaffin-like cells (ECLs) were obtained from Thermo pierce, California, USA, while Quantity one system was purchased from Bio-Rad, California, USA. This research was approved by the Animal Ethical Committee of Xiangya Hospital according to "Principles of Laboratory Animal Care" (NIH publication no. 85-23, revised 1985), the approval number is 229173 (14).

Sprague-Dawley rats

A total of 72 male Sprague-Dawley rats weighing 230 to 250 g were purchased from the Animal Experimental Centre of Central South University, China and randomly assigned to six groups of 12 rats each. The rats had free access to food and water and were maintained under controlled conditions (12 h light/dark cycle; 18 - 25 °C; and 50 - 60 % humidity). The experimental procedures were performed at the same time of the day (morning) in order to minimize the possible effects of circadian variation. The study was carried out in accordance with the Guide for Care and Use of Laboratory Animals and the guidelines of the Animal Care Committee of Central South University, China.

Induction of TLE

Rats in the epileptic groups were injected *i.p.* with an aqueous solution of lithium chloride 18 h prior to pilocarpine injection *i.p.* at a dose of 125 mg/kg b.wt. The initial dose of pilocarpine solution was 20 mg/kg b.wt., and it was gradually increased by 10 mg/kg b.wt. every 30 min until status epilepticus (SE) was established. Status epilepticus was defined as the onset of generalized convulsive seizures accompanied by rearing and falling (Racine's scale stages 4-5). An hour after the onset of SE, 10 % chloral hydrate (3 mg/kg b.wt.) was injected *i.p.* to terminate convulsions in the responsive rats. All the rats received injections of 1 ml of 0.9 % sodium chloride *i.p.* following the termination of SE in order to prevent dehydration. Rats in the control group were injected *i.p*. with 0.9 % sodium chloride (125 mg/kg b.wt.) in place of pilocarpine.

Rat behavior and grouping

Immediately after the injection of 125 mg/kg b.wt. of lithium chloride, the rats were carefully observed and monitored. After the rats were injected *i.p*. with pilocarpine (18 h later), 84 out of 112 rats developed SE. A total of 12 rats were randomly chosen as the acute period group (day 1),while 16 died a day after SE were excluded from the acute period group. We observed the animals from 8:00 am to 8:00 pm, if the animals did not show any seizures in this time, we considered the animals as being in the latent period, 24 seizure-free rats were randomly assigned to the latent period group (day 7 and day 14). We only chose the rats that develop epilepsy as the chronic period group, 24 rats were randomly chosen as the chronic period group (day 30 and day 60). Rats in the control and the five epileptic groups were sacrificed at the different time points (1, 7, 14, 30 and 60 days) post-seizure.

Quantitative real-time polymerase chain reaction (qRT-PCR)

In order to identify the function of Scn5a, the levels of expressions of Scn5a mRNA in the hippocampus at days 1, 7, 14, 30, and 60 post-SE were measured using qRT-PCR. Total hippocampal RNA was extracted using Trizol reagent and the RNA concentration was determined spectrophotometrically at 260 and 280 nm. A portion of the RNA (1 μg) was reverse-transcribed to cDNA using PrimeScriptTM RT reagent kit with gDNA Eraser using a Mastercycler. The qRT-PCR was performed with ABI StepOnePlusTM real-time PCR system using SYBR® Premix Ex Taq™. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the reference gene. The following primers were used: Scn5a-forward: 5'-GTCTTCAAGCTGGCCAAGTC-3';Scn5a-reverse: 5'-CCGAGTAGTTCTTGCCGAAG-3'; GAPDH-forward: 5'-CTCATGACCACAGTCCATGC-3', GAPDH-reverse: 5'-TTCAGCTCTGGGATGACCTT-3'.

The cycling conditions were: activation for 5 min at 95 °C, 40 cycles of amplification at 95 °C for 20 sec, and 60 °C for 30 sec. The levels of expressions of Scn5a at different time points were calculated as ratios against those of GAPDH. The threshold cycle (ct) was used to quantify the PCR product. The Δct was calculated from the difference of the ct of Scn5a and GAPDH. The mean Δct of the reference group was used to calculate ΔΔct. The data were calculated using the $2^{-\Delta\Delta Ct}$ method.

Immunohistochemistry

The rats were anesthetized with 10 % chloral hydrate (3 ml/kg b.wt., *i.p*.) and perfused transcardially with 400 ml of 0.9 % sodium chloride, followed by 400 ml of 4 % paraformaldehyde in 0.1 M phosphate-buffered saline (PBS) (pH 7.4). The brains were removed and post-fixed overnight. The brain tissues were coronally cut at 10-μm thickness using a freezing microtome after cryoprotection with 30 % sucrose in PBS. Several sections (1-in-5) were collected on polylysine-coated slides and preserved in the refrigerator at -80 °C for immunohistochemical staining. Before the staining procedure, the tissue sections were re-warmed at room temperature for 45 min and washed thrice with PBS for 5 min. The activity of endogenous peroxidase was inhibited with 0.3 % H_2O_2 at 37 °C for 15 min. The tissue sections were subsequently incubated overnight with primary antibodies (polyclonal antibody against Scn5a, 1:500 dilution) at 4 °C overnight. They were further incubated for 30 min with a mixture of HRP-conjugated goat anti-rabbit IgG at 37 °C. The reaction product was detected

using DAB kit. For each rat, five sections were observed under a light microscope, while ten visual fields were randomly chosen from the hyperfield (×400), and the images were processed using an image analysis system.

Western blotting

The total protein was extracted from the hippocampus using radio-immunoprecipitation assay lysis buffer containing protease inhibitor PMSF. The protein concentration was thereafter determined using a modified BCA assay kit. The proteins were separated with 10 % polyacrylamide gel electrophoresis and transferred onto PVDF membrane. The membrane was washed with a solution of Tris-buffered saline and tween 20 (TBST) after blocking with 5 % non-fat milk for 1 h at room temperature. The internal control was β-actin. Subsequently, the primary antibody (polyclonal antibody against Scn5a, 1: 200 dilution; and monoclonal antibody against β-actin, 1 : 4000 dilution, were applied at 4 °C overnight. After washing for 30 min (3 × 10 min), the membrane was subsequently incubated with HRP-conjugated secondary antibody at 1:3000 dilution at room temperature for 45 min. The membrane was finally incubated with ECLs. The immunoblotting analyses was performed using Quantity one system and the relative expression of Scn5a was calculated as the ratio of Scn5a to β-actin.

Statistical analysis

Data are expressed as means ± SEM. Statistical analysis was performed using SPSS (SPSS 13.0) and groups were compared using one-way ANOVA. Values of $p < 0.05$ were considered statistically significant.

Results

General observations

Rats in the control group did not show any signs of seizure or tremor, and they all survived. Following injection of 125 mg/kg b.wt. of lithium chloride, there were no differences in their behaviors, but after they were injected *i.p.* with pilocarpine (18 h later), some of them had seizures which consisted of facial convulsion, nodding of the head, clonic movements of forelimbs, rearing and falling. A total of 84 out of 112 rats developed SE, while 16 died a day after SE was established. Three of the remaining rats died during the procedure. Spontaneous seizure was observed during the chronic period and lasted 10 to 60 sec. The frequency of spontaneous seizure varied among the rats.

Outcomes of qRT-PCR

The expressions of Scn5a mRNA in the hippocampus during the latent and chronic periods were significantly higher than their corresponding expressions in the control group ($p < 0.05$), but there were no significant differences in their expressions between the two different time points in the latent and chronic period groups ($p > 0.05$). The expression peaked 30 days post-SE, and was sustained for 60 days. There was no significant difference in the expression of Scn5a mRNA between the acute group and the control group ($p > 0.05$; Figure 1).

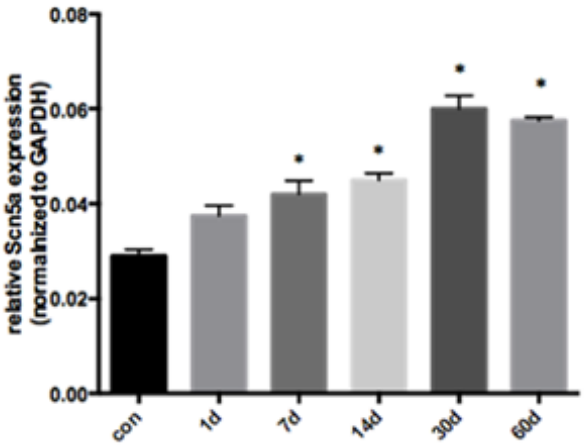

Figure 1. Levels of expression of Scn5a mRNA in the hippocampus of the control and TLE groups. *$p < 0.05$ when compared to control.

Expressions of Nav1.5 in the CA3 region of the hippocampi of control and TLE groups

The levels of expressions of $Na_v1.5$ in the CA3 region during latent and chronic periods were significantly higher than those in the control group ($p < 0.05$), and the expressions peaked at day 30. However, there was no significant difference in the expression of $Na_v1.5$ in the latent group, relative to the chronic period group ($p > 0.05$: Figure 2).

Expression of Nav1.5 protein (250kDa) in the hippocampi of the control and the different time point groups

From the results of semi-quantitative Western blotting $Na_v1.5$ presented a single band of approximately 250 kDa. The expression of $Na_v1.5$ was normalized by calculating the ratios of optical density (OD) of the bands for $Na_v1.5$ and β-actin. The OD ratios in the latent and chronic period TLE group were significant higher than that in the control ($p < 0.05$). The levels of expressions of $Na_v1.5$ in the latent and chronic period

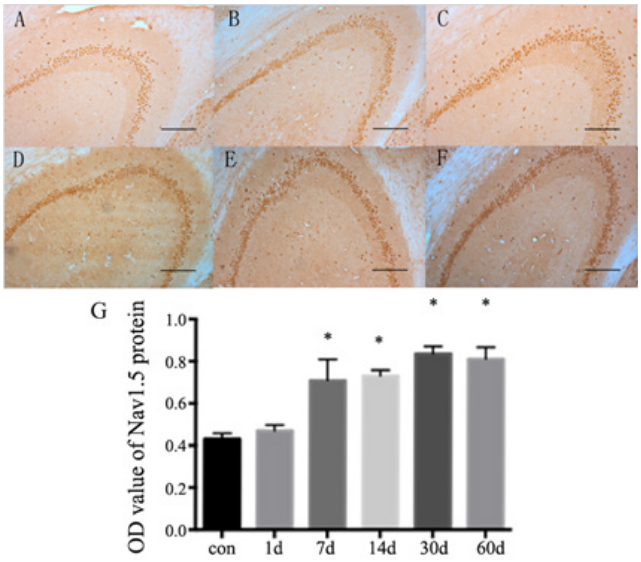

Figure 2. Expressions of Nav1.5 in the CA3 region of the hippocampi of control and TLE groups. (A: control; B: day 1; C: day 7; D: day 14; E: day 30; F: day 60; G: quantitative analysis *$p < 0.05$, compared to control.)

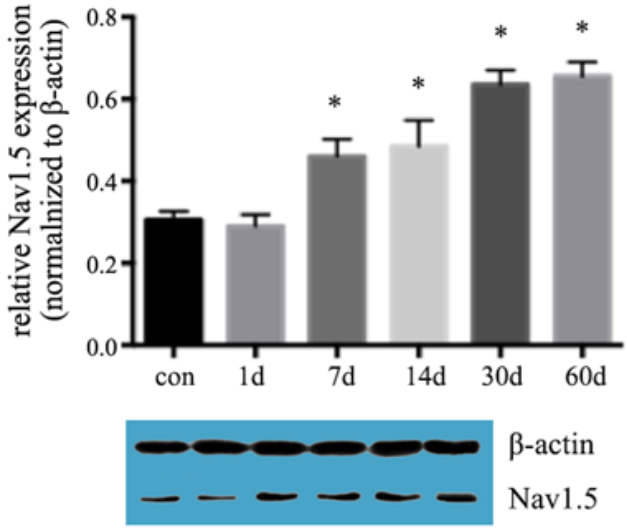

Figure 3. Expressions of Nav1.5 protein (250kDa) in the hippocampi of the control and the different time point groups. *$p < 0.05$ when compared to the control group.

were not significantly different ($p > 0.05$). There was also no significant difference in the levels of expressions of $Na_v1.5$ in acute period group relative to the control group ($p > 0.05$; Figure 3).

Discussion

Studies have shown that disordered membrane excitability which is a major factor in epilepsy is due to lack of homeostasis of VGSCs. The first connection between VGSCs and epilepsy was the discovery of mutation in the β1 subunit (15). This mutation produces a wave of membrane depolarization and hyper-excitability. So far, thousands of such mutations have been discovered in epileptic patients. Studies have shown that changes in VGSCs occur in different animal models of epilepsy. Scientific reports have suggested that mutations, changes in expressions, or inappropriate modulation of VGSCs lead to electrical instability in the cell membrane and inappropriate spontaneous activity during epilepsy (9, 16-19). The $Na_v1.5$ was thought to be the principal cardiac subtype, but it has been shown to be expressed in the limbic regions of the brain. In 2013, Parisi *et al.,* described a known mutation in the Scn5a gene in a family with Brugada syndrome and epilepsy. Their observation confirmed the possibility that mutation in Scn5a may confer susceptibility to recurrent seizure (20). It has been observed that $Na_v1.5$ plays a central role in the physiological processes of microglia (21). Microglia are considered as macrophages in the central nervous system (CNS) and they participate in the neuro-inflammatory reactions which contribute significantly to epileptogenesis (22).

In the present study, the expressions of Scn5a mRNA in the hippocampus during the latent and chronic periods were significantly higher those in the control group, but there were no significant differences in the corresponding expressions between the two different time points in the latent and chronic period groups. The expression peaked 30 days post-SE, and was sustained for 60 days. There was no significant difference in the expression of Scn5a mRNA in the acute group when compared to control. The levels of expressions of $Na_v1.5$ in the CA3 region during latent and chronic periods were significantly higher than those in the control group, and the expressions peaked at day 30. However, there was no significant difference in the expression of $Na_v1.5$ in the latent group, relative to the chronic period group. These results suggest the possibility of significant alteration in the transcription of $Na_v1.5$ gene (Scn5a) in pilocarpine-induced TLE rat model.

Presently, only little is known about the expression of $Na_v1.5$ in different animal models of epilepsy. However, several studies have reported the pathophysiological characteristic of $Na_v1.5$ in different cell lines (23, 24). In a previous study, it was demonstrated that βIV-spectrin (a key structural component required for VGSC clustering) creates a platform for regulation of neuronal excitability through direct phosphorylation of $Na_v1.5$ by Ca 2+ /calmodulin-dependent protein kinase II (CaMkII) (25). We found an upregulation of $Na_v1.5$ after SE, but whether $Na_v1.5$ participates in a self-reinforcing cycle of abnormally enhanced network activity needs further study. It is generally believed that VGSCs are therapeutic targets for most antiepileptic drugs (AEDs) such as carbamazepine and lamotrigine (26). In this study, there was an upregulation of $Na_v1.5$ after SE induction. Thus, our findings provide hypothesis that $Na_v1.5$ might be a novel therapeutic target in TLE.

The results of this study have demonstrated that $Na_v1.5$ is involved in the pathogenesis of TLE.

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None.

Conflict of interest
No conflict of interest associated with this work.

Contribution of authors
All work was done by the author named in this article and the authors accept all liability resulting from claims which relate to this article and its contents. The study was conceived and designed by Bo Xiao; Zhaohui Luo, Fangfang Bi, Jing Li collected and analysed the data; Yi Zhou wrote the text and all authors have read and approved the text prior to publication.

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