Overexpression of Synaptic Vesicle Protein 2A Inhibits Seizures and Amygdaloid Electroencephalogram Activity in Pilocarpine-induced Pharmacoresistant Epileptic Rats

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Abstract

Purpose: The present study investigated the role of upregulation of synaptic vesicle protein 2A (SV2A) in seizure control and electroencephalogram (EEG) activity in pilocarpine-induced pharmacoresistant epileptic rats.

Methods: A total of 100 healthy adult male Sprague-Dawley rats were used to establish the pilocarpine-induced model of epilepsy. The successful epilepsy model was then used to select for pharmacoresistance by testing seizure responses to phenobarbital and carbamazepine. The selected pharmacoresistant rats were assigned to a pharmacoresistant epileptic group (PRE group, 10 rats) or a SV2A upregulation group (PRU group, 8 rats). Ten pharmacosensitive epileptic rats (PSE group, 10 rats) selected randomly and 10 normal rats (NCR group, 10 rats) served as controls. Immunohistochemistry and western blots were performed to assess SV2A expression in hippocampal tissue samples from all 4 groups. EEG changes and epileptic seizures were recorded by video-EEG and compared among the groups.

Results: Immunohistochemical staining showed that SV2A levels increased slightly in the PSE group (0.26 ± 0.018) compared with the NCR group (0.24 ± 0.031). However, SV2A decreased remarkably in the PRE group (0.11 ± 0.121). Western blot analysis yielded similar findings. SV2A increased in the PSE group (4.10 ± 1.127) compared with the NCR group (3.26 ± 0.699) and decreased in the PRE group (1.86 ± 0.421). Frequency and duration of seizures increased in the PRE group as compared with the PSE group. After overexpression of SV2A, levels of SV2A protein increased in the PRU group. In addition, seizure severity, frequency, and duration were decreased compared with the PRE group.

Conclusion: SV2A might be associated with epileptogenesis of pharmacoresistance in pilocarpine-induced epileptic rats.

Keywords

Synaptic vesicle protein 2A(SV2A), Overexpression Pharmacoresistant epilepsy, Pilocarpine
Introduction

Epilepsy has a prevalence of 5 to 7 per 1000 persons in the United States and affects about 1% of the population worldwide [1]. It is also associated with diminished health-related quality of life and is thus considered a major public health problem [2]. Despite considerable efforts towards pharmacological control of seizures, approximately 30% of epileptic patients do not achieve complete seizure control [3]. In human epilepsy, pharmacoresistance to antiepileptic drug therapy is a major problem affecting a substantial fraction of patients [4]. Although new antiepileptic drugs are being introduced into clinical practice, the percentage of drug-resistant epilepsy cases remains stable [5]. The mechanism underlying pathogenesis of pharmacoresistant epilepsy remains unclear.

Synaptic vesicle protein 2 (SV2) is a neuronal vesicle membrane glycoprotein that appears to be an important target in treatment of partial and generalized epilepsies [6]. It is thought to decrease neuronal excitability [7] since knockout of synaptic vesicle protein 2A (SV2A) in mice leads to seizures [7]. In addition, SV2A-deficient mice show rapid development of kindling, which suggests accelerated epileptogenesis. Gene expression of SV2A is reduced during epileptogenesis in the rat, and changes in expression of SV2A may have consequences for progression of epilepsy [8]. Recent studies have found downregulation of SV2A expression in brain tissue obtained from patients with intractable temporal lobe epilepsy [6,9]. Several lines of evidence suggest that SV2A is the binding site for the antiepileptic drug levetiracetam (LEV) [10,11]. Clinically, there is a group of intractable patients who show an impressive response to LEV after failing many antiepileptic drugs [12,13]. Multiple retrospective studies have reported good efficacy and tolerability of LEV in human patients with epilepsy [14]. LEV was developed for use as an add-on therapy in patients with intractable epilepsy [12]. Expression of SV2A is correlated with the clinical response to LEV and predicts LEV efficacy in patients with epilepsy secondary to tumor [15]. The novel LEV analogs, brivaracetam (BRV) and seletracetam, which are effective as an adjunctive treatment in epilepsy, also bind to SV2A [16,17].

These findings suggest that decreased SV2A may play a role in epileptogenesis and pharmacoresistance of epilepsy. Our previous studies have demonstrated that SV2A expression is significantly decreased in pharmacoresistant epileptic rats when compared with pharmaco-sensitive epileptic rats [18]. Moreover, reduced expression of SV2A can contribute to increased epileptogenicity in animal models of experimental epilepsy [8]. Therefore, increasing SV2A levels might be effective in controlling epilepsy. However, there are no data on seizure counts and time course of epileptic discharge after upregulating SV2A. In this study, we analyzed changes in SV2A expression in the pilocarpine model of epilepsy and effects of upregulating SV2A on epileptic seizures.

Experimental Procedures

Materials

Experimental subjects

All procedures were performed with protocols approved by the Animal Care and Use Committee of Guizhou Medical University, Guizhou China. A total of 100 healthy adult male Sprague-Dawley rats (200–250 g, grade II, certificate no. SCXK2007-0005) were maintained under controlled standard conditions (12 h/12 h light/dark cycle, lights on 7:00 a.m.). The rats were moved to the laboratory 1 week before the experiment to allow adaptation to the environment. They were individually housed in galvanized wire mesh cages with free access to food and water. Laboratory room temperature ranged from 21 to 25°C, and there was natural lighting. The number of animals was kept as small as possible, and the ARRIVE (Animal Research: Reporting In Vivo Experiments [19]) guidelines were followed.

Main equipment

For the study, the main apparatus and reagents were as follows: Stimulating electrodes(Made of Nichrome enameled wire with diameter of 0.1mm and Teflon-coated except for 0.03mm at the tip, made by the authors), BL-420 biological functional system (Chengdu Thai League Science and Technology Co., Ltd, Chengdu, China), DY-1 stereotaxic apparatus(Shanghai Chuansha Huamu Agricultural Machinery Factory, Shanghai, China), Real time PCR apparatus(Applied Biosystems, California, USA), DYY-6C electrophoresis apparatus(Beijing Liuyi Instrument Factory, Beijing, USA), EQU307 bio-rad trans(Bio Basic Inc, Canada). Barnstead/
Research

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pure water filter (Hangzhou Ferrotec Electronics Co., Ltd., Hangzhou, China), electronic balance (Göttingen, Sartorius, Germany), Beckman Coulter (Göttingen, Sartorius, Germany), Constant temperature circulating water tank (Hangzhou Ferrotec Electronics Co., Ltd., Hangzhou, China), Mini double-sided vertical cell (The Bio-Rad Laboratories, Inc., California, USA), All/full wet transfer device (The Bio-Rad Laboratories, Inc., California, USA), Gel imaging analysis system/alpha innotech (The Bio-Rad Laboratories, Inc., California, USA).

Main reagents

The main reagents consisted of the following: Carbamazepine (CBZ, Shanghai New Asiatic Pharmaceuticals Co., Ltd., Shanghai, China) and Phenobarbital sodium injection (PB, Shanghai New Asiatic Pharmaceuticals Co., Ltd., Shanghai, China), Absolute ethyl alcohol (Bio Basic Inc., Canada), TRIZol (Invitrogen, California, USA), Chloroform (Bio Basic Inc., Toronto, Canada), Isopropanol (Bio Basic Inc., Canada), Green-2-Go qPCR Mastermix-ROX (2x) (Bio Basic Inc., Toronto, Canada), BCA protein assay kit (Shanghai Sangon Biotech Co., Ltd., Shanghai, China), PVDF membrane (Bio Basic Inc., Toronto, Canada), Anti-SV2A antibody (Abcam company, Massachusetts, USA), Anti-rabbit secondary antibody (Beijing Zhongshan Goldenbridge Bio Co., Ltd., Beijing, China), Bovine serum albumin (Shanghai Sangon Biotech Co., Ltd., Shanghai, China), Tween-20 (Shanghai Sangon Biotech Co., Ltd., Shanghai, China), Glycine (Shanghai Sangon Biotech Co., Ltd., Shanghai, China), RIPA Lysis Buffer (Beijing Dingguo Changsheng Biotechnology Co. Ltd., Beijing, China), SDS-PAGE gel preparation kit (Beijing Solarbio life sciences, Beijing, China), SDS-PAGE loading buffer (Beijing Dingguo Changsheng Biotechnology Co. Ltd., Beijing, China), Western antibody dilution (Beijing Dingguo Changsheng Biotechnology Co. Ltd., Beijing, China).

Spontaneous chronic epileptic model induction

Spontaneous chronic epileptic model induction

Induction of status epilepticus (SE)

The pilocarpine model of temporal lobe epilepsy was used as previously described [20]. Animals were given LiCl (127 mg/kg) intraperitoneally (i.p.) 24 h before the pilocarpine treatment. Animals were then intraperitoneally treated with pilocarpine (30 mg/kg, i.p.) 30 min after atropine methyl bromide (5 mg/kg, i.p.). Within 30 min after the pilocarpine injection, most animals developed SE, which was featured by continuous tonic-clonic seizures lasting for several hours. Diazepam (20 mg/kg, i.p.) was administered 90 min after onset of SE and repeated as needed.

For 2 weeks after SE, animals were video monitored 24 h per day for seizure severity and occurrence of spontaneous seizures. Rats showing spontaneous recurrent seizures (SRS) were used as chronic epileptic animals.

Seizure severity was assessed using the Racine scale: 0, no response; stage 1, grooming, hyperactivity; stage 2, head nodding, tremor; stage 3, unilateral forelimb clonus; stage 4, clonus with rearing; stage 5, generalized tonic-clonic seizure with loss of righting reflex [21].

After chronic epileptic model induction, 57 out of 100 rats were chronic epileptic animals showing SRS, 9 rats died from SE, and 34 rats showed no epileptic seizures.

Electrode implantation

Electrodes were implanted into the right amygdala, and electroencephalography was performed to acquire seizure counts and time course of epileptic discharge over 24 h in chronic epileptic rats.

The methods used were the same as our previously published studies [22,23]. Briefly, chronic epileptic animals were anesthetized and placed in a stereotaxic frame. Next, animals were implanted with depth electrodes in the right amygdala (stereotaxic coordinates were obtained from the rat brain atlas of Paxinos and Watson [24]) as follows: 2.8 mm posterior to the bregmatic fontanel, 4.9 mm lateral to midline on the right, and 8.6 mm ventral to the skull surface. The incisor bars were placed 3.3 mm under the plane of the ear. A screw was fixed in the skull over the left frontal cortex as a reference electrode. The electrodes were connected to 3-hole wire connectors and fixed to the surface of the skull using 502 superglue blended with denture acrylic. Penicillin (40,000 U/kg, i.p.) was injected for 3 consecutive days to prevent infection, and the rats were allowed to recover from surgery for at least 7 d before being subjected to the experiment.
Pharmacoresistance selection

For selection of pharmacoresistance, rats were subjected to prolonged treatment with PB and CBZ [25].

Selection of PB responders and nonresponders

Based on these preliminary experiments and a previous PB selection trial in another group of epileptic rats [26,27], the following PB dosing protocol was used in the 57 rats with SRS: a bolus dose of 25 mg/kg (i.p.) in the morning of the first treatment day, followed 10 h later by 15 mg/kg (i.p.), and then 15 mg/kg (i.p.) twice daily for 13 consecutive days. Before onset of drug treatment, baseline seizure frequency was determined over 2 weeks (pre-drug control period). In all rats, seizures were continuously monitored (24 h/d, 7 d/week) by video/electroencephalogram (EEG) recording over the 4 weeks of the experiment as described below.

Responders were defined by complete seizure suppression during treatment or seizure suppression of at least 50% compared with seizure frequency in the pre-drug and/or post-drug control periods [27]. In all, 29 responders were considered pharmacosensitive, and 28 nonresponders were considered pharmacoresistant. When the epileptic rats were found pharmacoresistant to PB, the process of selection was continued using CBZ.

2.3.2 Selection of CBZ responders and nonresponders

Following the 2-week post-drug period after PB, a second drug trial was performed with daily administration of CBZ. CBZ was dissolved in Tween-80 saline at a dose of 40 mg/kg and injected i.p. 3 times per day for 2 weeks. Dose and posology of CBZ were the same as previously described [28-30]. Seizures were continuously monitored (24 h/d, 7 d/week) by video/EEG recording over the 2 weeks of the experiment as described below.

Finally, 9 responders served as pharmacosensitive epileptic rats, and 19 nonresponders served as pharmacoresistant epileptic rats.

Construction of recombinant SV2A lentivirus

For SV2A experiments, the sequences of rat SV2A (NM-057210) lentivirus were designed and synthesized as follows: SV2A (8304-11)-P1 sense oligonucleotide, 5'-GAGGATCCCCGAGACCACCATGGAAGAAGGCTTTCGAGACC-3'; antisense oligonucleotide SV2A (8304-11)-P2, 5' - TCCTTGTAGTCCATACCTGCACACCTGTCCCCGGGTCT -3'. The lentivirus vector was GV287. The same vector backbone but carrying GFP protein was used as a negative control lentivirus (NC-LV). Lentivirus vector construction and SV2A lentivirus were completed by Shanghai Jikai Gene Chem Co. Ltd. (Shanghai, China). The titer of the viral stocks was 1.0 × 10⁹TU/mL.

Microinjection of SV2A lentivirus into the hippocampal CA3 region

Animals were anesthetized with 10% chloral hydrate (300–400 mg/kg, i.p.) and received bilateral injections (5 μL lentivirus solution per side) into the CA3 region with the following stereotaxic coordinates: 3.6 mm posterior to the bregmatic fontanelle, 2.4 mm lateral to midline, and 3.0 mm ventral to the skull surface. After surgery, the rats were returned to the Mesh cages. Penicillin (40,000 U/kg, i.p.) was injected for 3 consecutive days to prevent infection.

Experiment grouping

Six weeks after i.p. injection of PB, of the 57 induced epileptic rats, 29 rats were pharmacosensitive to PB, and 28 rats were pharmacoresistant to PB, but 8 out of the 28 rats were sensitive to CBZ. Finally, 18 rats were pharmacoresistant to both PB and CBZ, which yielded a pharmacoresistance rate of 31.58%.

Ten pharmacoresistant epileptic rats served as the pharmacoresistant control group (PRE group, 10 rats) to assess SV2A expression compared with pharmacosensitive epileptic rats (PSE group, 10 rats). The other 8 pharmacoresistant epileptic rats were assigned to the upregulated SV2A group, (PRU group, 8 rats), and they were subjected to upregulation of hippocampal SV2A experiments. Ten rats were selected randomly from the normal rats and were used as controls (NCR group, 10 rats).

Hippocampal tissue immunohistochemistry

Tissue collection

The methods used were the same as in our previously published studies [23]. Briefly, the heart was exposed and perfused with 100 mL cold normal saline before perfusing with 500 mL...
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4% paraformaldehyde in 0.1 mol/L phosphate buffer solution. The hippocampal brain tissues were removed from the skull and placed into the perfusate solution overnight at 4°C. The tissues were then placed into a 20% sucrose solution for 12 h. Standard tissue dehydration, clearing, infiltration, and embedding were performed on the isolated tissues. Finally, paraffin blocks were prepared, sections of hippocampus (4 mm thickness) were cut and mounted on slides.

Immunohistochemical staining

For staining with hematoxylin-eosin, slides were placed into hematoxylin for 6 min, followed by 1% HCl alcohol for 10 s and eosin for 1 min. Slides were then placed in 70% alcohol for 1.5 min, 80% alcohol for 2 min, 90% alcohol for 2 min, 95% alcohol for 2 min, 100% alcohol for 4 min, and 100% alcohol for 5 min. Finally, slides were cleared with 2 exposures (5 min per exposure) to dimethylbenzene, and the coverslip was sealed with Permount.

For each rat, 2 brain tissue slices were used for SV2A expression analysis. Paraffin sections were routinely deparaffinized in water, then placed into 0.01 M citrate buffer and heated in a microwave for 10 min. To each section, a 50 mL drop of 0.1% Triton-X was applied followed by incubation at 37°C for 10 min. The sections were then flushed with phosphate-buffered saline (PBS) 3 times for 3 minutes each. Then, a drop of rats serum was applied for blocking, and sections were incubated at 37°C for 15 min. A drop of rabbit anti-SV2A (1:100 dilution) was applied, and sections were incubated overnight at 4°C. The sections were then flushed with PBS 3 times for 5 min each. Then, a drop of biotin was applied to mark or rabbit-IgG, and sections were incubated overnight at 37°C for 20 min. Sections were again flushed with PBS for 3 minutes each, incubated at 37°C for 20 min in streptavidin peroxidase, and flushed with 3 rinses of PBS (3 min per rinse). Finally, the 3,3′-diaminobenzidine color reaction, alcohol dehydration series, and dimethylbenzene clearing were performed, and slides were sealed with Permount.

Determination of SV2A-positive neurons

Neurons were considered SV2A-positive when brown-yellow granules were observed in the cytomembrane. After the hippocampal tissue sections were stained, images were collected using a SONY camera (Sony Corporation, Tokyo, Japan) and entered into the Biomias2001 image analysis system (Sichuan University, Chengdu, China) to count SV2A-positive neurons per unit area. Five areas (size of the visual field = 0.49296 mm²) in each section were randomly selected to determine the number of SV2A-positive neurons per unit area using the mouse click method. The average gray value of the SV2A-positive neurons was also determined in 5 visual fields selected randomly using the mouse segmentation method.

Western blot analysis of SV2A protein expression

The hippocampal samples were homogenized in a solution of Tris-HCl, NaCl, Triton X-100, and double-distilled water using a homogenizer. The mixture was centrifuged at 12000 rpm for 15 min at 4°C to isolate the supernatant. After protein quantification, the samples were separated by SDS-PAGE and transferred onto a PVDF membrane. The membrane was blocked with blocking buffer overnight at 4°C and then incubated with anti-SV2A antibody (1:1000) 1 h at room temperature, followed by incubation in secondary antibody (1:5000). Finally, bands were visualized using chemiluminescence and exposure to film. β-actin was probed using the same procedures as SV2A and used as an internal control to normalize results. Densitometric analysis of bands was conducted with an image analysis system for Windows (National Institutes of Health, USA).

Statistical analysis

The experimental data are expressed as mean ± standard deviation (X ± S). All data were analyzed using SPSS 16 (SPSS Inc., Chicago, IL, USA). Comparisons between 2 sample means were made using a t-test, and p < 0.05 was taken as the criterion for statistical significance. A two-way analysis of variance was used to make comparisons among multiple groups. Statistical analysis was performed in consultation with the Department of Biostatistics of Guizhou Medical University.

Results

Effect of pharmacoresistance on seizure severity and EEG parameters

Spontaneous seizures were frequently observed after the pilocarpine model of epilepsy was established successfully. Seizure severity, frequency, and duration were compared between the PSE and PRE groups. Significant increases in these parameters were noted in the PRE group.
compared with the PSE group (Table 1). Ictal EEGs displayed transient spike waves with frequencies of 10–30 Hz and amplitudes of 100–1000 μV. In the PRE group, the EEGs showed higher frequency and amplitude epileptiform discharges compared with the PSE group (Figure 1).

- **Hippocampal SV2A expression**

Immunohistochemical staining showed SV2A immunoreactivity (IR) in the neuropil throughout all hippocampal subfields. The somata of the granule and pyramidal cells were devoid of staining. The strongest IR was observed in the dentate gyrus and the stratum lucidum of the CA3 region (mossy fiber terminals).

A significant difference in SV2A expression was observed among the NCR, PSE, and PRE groups (F = 7.83, P = 0.003). Further analysis indicated that the relative quantity of SV2A increased slightly in the PSE group compared with the NCR group. In contrast, SV2A levels decreased significantly in the PRE group compared with the PSE and NCR groups. These findings suggest that PRE rats have decreased SV2A expression (Figure 2).

Western blot analysis was used to quantify expression of SV2A in the hippocampus. A significant difference in relative optical density (OD) was observed among the groups (F = 29.84, P = 0.009). The OD of SV2A/β-actin was significantly increased in the PSE group compared with that from the NCR group. In contrast, it decreased remarkably in the PRE group compared with the PSE group (Figure 3).

- **Changes in seizure severity and EEG parameters after upregulating SV2A**

To observe the effects of SV2A on seizure activity and EEGs, we increased the quantity of SV2A by lentivirus-mediated overexpression. Both immunohistochemical (Figure 4) and Western blot (Figure 5) analysis demonstrated that SV2A levels increased dramatically in the PRU group compared with the PRE group; the differences were significant.

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**Table 1: Comparison of seizure severity between the PRE and PSE groups (x ± s).**

<table>
<thead>
<tr>
<th>Group</th>
<th>(n) Seizure duration (s)</th>
<th>Seizure frequency (times/day)</th>
<th>Racine degree</th>
</tr>
</thead>
<tbody>
<tr>
<td>PSE group</td>
<td>21.28 ± 3.39</td>
<td>0.35 ± 0.29</td>
<td>6.70</td>
</tr>
<tr>
<td>PRE group</td>
<td>71.25 ± 3.39*</td>
<td>3.34 ± 0.57*</td>
<td>14.30*</td>
</tr>
<tr>
<td>t (p)</td>
<td>11.326 (0.000)</td>
<td>6.72 (0.000)</td>
<td>0.003</td>
</tr>
</tbody>
</table>

*Compared with the PSE group, the difference was significant (p < 0.050)
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**Figure 2:** SV2A immunohistochemistry in the rat hippocampus of the NCR group (A), PSE group (B), and PRE group (C).

A: Histologically normal hippocampal CA3 region showing diffuse neuropil synaptic vesicle protein 2A (SV2A) immunoreactivity (IR) with punctuate labeling throughout all hippocampal layers.

B: Strong SV2A IR was observed along the cell borders and processes (arrows in B) of neurons in the PSE group.

C: SV2A IR in the hippocampus CA3 region from the PRE group showing reduced neuropil staining and disruption of the regular punctate pattern of SV2A IR.

D: Bar graph showing quantitation of immunohistochemical SV2A expression (OD value). Compared with the NCR group, SV2A expression slightly increased in the PSE group. In contrast, SV2A expression significantly decreased in the PRE group.

**Figure 2:** SV2A immunoreactivity (IR) in the rat hippocampus from the NCR, PSE, and PRE groups. (OD values represent the mean OD ratio of SV2A/β-actin).

A: Representative immunoblot of SV2A (93 kDa) in total hippocampal homogenates from the NCR, PSE, and PRE groups.

B: Densitometric analysis. Values represent OD units relative to OD of the reference protein β-actin. #significant difference compared with the NCR group (one-way ANOVA, p < 0.05), *significant difference compared with the PSE group (one-way ANOVA, p < 0.05).
Figure 4: SV2A immunohistochemistry in the hippocampus from the PRE and PRU groups.
A: Representative image showing reduced SV2A immunoreactivity (IR) in the neuropil throughout the hippocampus in the PRE group.
B: Strong SV2A expression was observed in residual neurons with IR around the somata and dendrites (arrows) in the PRU group.
C: Bar graph showing quantitation of SV2A expression (OD value). Compared with the PRE group, SV2A levels increased significantly ($p < 0.05$) in the PRU group.

Figure 5: Western blot analysis of the hippocampus using the monoclonal SV2A antibody identified the 93 kDa protein in the PRE and PRU groups.
A: Representative immunoblot of SV2A (93 kDa) in total hippocampal homogenates from the PRE and PRU groups.
B: Densitometric analysis. The values represent OD units relative to OD of the reference protein β-actin. There is the dramatic increase in SV2A level in the PRU group.
*significant difference compared with the PRE group values (Student’s $t$ test, $p < 0.05$).

After upregulation of SV2A, the Racine stages decreased, and the frequency and duration of seizures were reduced. A significant difference was observed compared with the PRE group, which suggests that upregulation of SV2A has an inhibitory effect on seizure activity in pharmacoresistant epileptic rats (Table 2). In the PRU group, EEGs displayed transient spike waves and sharp waves with frequencies of 10–20 Hz and amplitudes of 100–200 μV. However, the EEGs showed lower frequency and amplitude epileptiform discharges compared with the PRE group (Figure 6).

**Table 2: Comparison of seizure severity between the PRE and PRU groups ($\bar{x} \pm s$).**

<table>
<thead>
<tr>
<th>Group</th>
<th>Seizure duration (s)</th>
<th>Seizure frequency (times/day)</th>
<th>Racine degree</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRE group 8</td>
<td>70.89 ± 2.44</td>
<td>3.21 ± 0.33</td>
<td>11.00</td>
</tr>
<tr>
<td>PRU group 8</td>
<td>34.57 ± 2.51*</td>
<td>0.98 ± 0.47 *</td>
<td>6.00*</td>
</tr>
<tr>
<td>t (p)</td>
<td>-1.392 (0.011)</td>
<td>5.977 (0.000)</td>
<td>0.038 (Wilcoxon W 48.00)</td>
</tr>
</tbody>
</table>

*Compared with the PRE group, $P < 0.05$
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Discussion

In the present study, SV2A expression increased slightly in the PSE group compared with the NCR group. However, expression decreased remarkably in the PRE group compared with the PSE and NCR groups. In the PRE group, increases in both seizure severity and frequency were observed. After increasing levels of SV2A in the hippocampus, the seizures were inhibited as evidenced by decreased seizure severity and frequency as well as shortened duration of seizures. These results suggest that SV2A plays an important role in development of pharmacoresistance. Thus, increasing the quantity or function of SV2A might be effective in reducing epileptic pharmacoresistance.

SV2A is an integral membrane protein necessary for proper function of the central nervous system and is associated with the physiopathology of epilepsy. SV2A is the molecular target of the anti-epileptic drug LEV and its racetam analogs, BRV and seletracetam [31]. LEV has only modest affinity for SV2A, but BRV is a selective, high-affinity SV2A ligand with promising antiepileptic properties and fast onset of action. BRV, which has rapid brain entry and fast brain SV2A occupancy [17], provides potent and complete seizure suppression in animal models of partial, generalized, and drug-resistant seizures [32].

LEV is a widely prescribed antiepileptic drug with demonstrated efficacy against focal and generalized seizures [33]. Several studies have reported good efficacy and tolerability of LEV in human patients with epilepsy [14]. Recent experimental data demonstrated that LEV accesses SV2A through vesicular endocytosis [10,34]. Taken together, these results suggest that SV2A is an important and novel target for intractable antiepileptic drug discovery.

Decreased SV2A was observed in brain tissues of patients with intractable epilepsy and may also contribute to the progression of epilepsy [8,9,35]. Similarly, SV2A was decreased throughout the hippocampus of epileptic rats, particularly in the chronic epileptic phase. In contrast, in control tissue, SV2A was robustly expressed in presynaptic terminals throughout the hippocampus [8,36]. These results are consistent with our present study, in which a significant decrease in SV2A expression was observed in the hippocampus of the pharmacoresistant epileptic rats. Mice lacking SV2A are characterized by a decrease in the calcium-dependent exocytotic burst, which is a measure of availability of neurotransmitter vesicles ready to release their content [37]. Furthermore, a homozygous mutation in the SV2A gene results in intractable epilepsy, involuntary movements, microcephaly, and developmental and growth retardation [38].

It is reasonable to hypothesize that upregulation of SV2A expression may have consequences for control of intractable epilepsy. This idea is supported by results of our present study. Compared with previously published studies, the current study focused on dynamic changes of...
SV2A in normal and pharmacoresistant epileptic rats as well effects of upregulation of SV2A on seizure activity and frequency.

Based on the present study, we hypothesize that increased SV2A expression in pharmacosensitive epileptic rats is a compensatory mechanism after seizures are induced by pilocarpine. Once SV2A is depleted, the seizures then become pharmacoresistant. Thus, the pharmacoresistant epileptic rats displayed significant decreases in levels of SV2A. These findings are consistent with those obtained from brain tissues of patients with pharmacoresistant epilepsy [8,9]. Given that the absence of SV2A in knockout mice is associated with development of seizures, we speculate that the observed decrease of SV2A expression might be the mechanism underlying progression of refractory epilepsy. In addition, because SV2A is the binding site for Antiepileptic drugs, promoting function of SV2A might be effective in treating pharmacoresistant epilepsy, and decreased SV2A expression may potentially interfere with efficacy of drugs targeting this protein.

The mechanism through which SV2A underlies pharmacoresistance was not investigated, which might be a limitation of this study. Experimental and clinical studies have found that SV2A levels are decreased in pharmacoresistant subjects. Increasing levels of SV2A inhibited seizure severity, frequency, and duration in our study. Our results, taken together with the known physiological roles of SV2A, suggest that downregulation of SV2A may be associated with progression of pharmacoresistant epilepsy. The present study provides evidence indicating that upregulation of SV2A might be a strategy for treatment of pharmacoresistant epilepsy.

There is an interaction between the SV2A and the soluble N-ethylmaleimide-sensitive factor attachment protein receptor proteins (SNARE) . SNARE was associated with the release of neurotransmitter GABA and development of experimental epilepsy [39-41]. The SNARE would be decreased in animal model of epilepsy and its decrease might be associated the development of pharmacoresistance. Increasing its amount should be also effective in the treatment of intractable epilepsy.However, we were unable to observe the changes of the SNARE. This might be a great limitation of the present study. Future more detailed researches are required to address such problems.

**Conflict of Interest**

The authors declare that they have no any actual or potential conflict of interest including any financial, personal or other relationships with other people or organizations within three years of beginning the submitted work.

**Source of Funding**

This research was supported by the Natural Science Fund of China (81560222/H0913, 81760245/H0913), Program for Changjiang Scholars and Innovative Research Team in University (IRT10358) and Guizhou Province Governor Special Funds (1065-09) as well as Guizhou High-level Personnel Scientific Funds (TZJF-2010-054)

**Acknowledgements**

We are indebted to the Guizhou Centre of Disease Control and Prevention for providing us with the laboratory platform. We also wish to thank all the postgraduates involved for their hard work during the study.

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**References**

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25. Loscher WC, Brandt, High seizure frequency prior to antiepileptic treatment is a predictor of pharmacoresistant epilepsy in a rat model of temporal lobe epilepsy. *Epilepsia* 51(1), 89-97 (2010).


