Epilepsy & Behavior 69 (2017) 18–23

Contents lists available at ScienceDirect

Epilepsy & Behavior

journal homepage: www.elsevier.com/locate/yebeh

# Effect of synaptic adhesion-like molecule 3 on epileptic seizures: Evidence from animal models

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ARTICLE INFO

*Article history:*
Received 12 September 2016
Revised 10 November 2016
Accepted 11 November 2016
Available online 14 March 2017

*Keywords:*
Intractable epilepsy
Synaptic adhesion-like molecule 3
Rat model

ABSTRACT

Axonal sprouting and synaptic reorganization are the primary pathophysiological characteristics of epilepsy. Recent studies demonstrated that synaptic adhesion-like molecule 3 (SALM3) is highly expressed in the central nervous system and plays important roles in neurite outgrowth, branching, and axon guidance, mechanisms that are also observed in epilepsy. However, the expression of SALM3 in the epileptic brain and the effect of SALM3 in the pathogenesis of epilepsy remain unclear. The aims of this study were to investigate SALM3 expression in rat models of epilepsy and to explore the functional significance of SALM3 in epilepsy. We demonstrated that SALM3 was expressed at significantly higher levels in epileptic rats compared with controls. Inhibition of SALM3 by SALM3 shRNA inhibited status epilepticus in the acute stage of disease and decreased spontaneous recurrent seizures in the Lithium-pilocarpine model of chronic stages of epilepsy. Consistent with these findings, SALM3 shRNA significantly prolonged the latent period in the PTZ kindling model. Our study suggests that the overexpression of SALM3 might be associated with epileptogenesis and that selectively inhibiting SALM3 may have therapeutic potential in treating epilepsy.



## 1. Introduction

Epilepsy is characterized by recurrent abnormal neuronal discharges and is the most prevalent chronic neurological disorder, affecting approximately 50 million people worldwide [1]. Despite the approval and availability of numerous effective antiepileptic drugs, >30% of newly diagnosed patients will develop medically intractable or drug refractory epilepsy [2,3]. Temporal lobe epilepsy (TLE) is the most common type of medically intractable epilepsy; however, the precise mechanisms underlying the pathogenesis of TLE remain unclear. Alterations in brain plasticity, including axonal sprouting, neurogenesis, and synaptic reorganization, were recently implicated in the development of medically intractable epilepsy [4].

Synaptic adhesion-like molecules (SALMs), also referred to as Lrfns (leucine rich repeat and fibronectin type III domain-containing), are primarily expressed in the brain. Synaptic adhesion-like molecules function as synaptic adhesion molecules and regulate neurite outgrowth and branching, and synapse formation, maturation, and plasticity [5–7]. The reported functions of SALMs are similar to the mechanisms observed in patients with epilepsy and in animal models of epilepsy. The SALM family of proteins consists of five members (SALM1–5) [8,9]. Synaptic adhesion-like molecule 3 induces both excitatory and inhibitory presynaptic induction in coculture assays [10]. Similarly, overexpression of SALM3 in cultured neurons induces increases in the number of excitatory and inhibitory presynaptic contacts. Wang et al. reported that all SALMs (SALMs 1–5) possess the ability to promote neurite outgrowth and branching, as demonstrated by overexpression and knockdown experiments. The enhanced neurite outgrowth was observed both in dendrites and axons [11]. Synaptic adhesion-like molecule 3, which plays important roles in the formation and maturation of dendrites and axons, contains six leucine-rich repeats (LRRs) followed by a single transmembrane domain and an intracellular region that terminates in a C-terminal PDZ-binding motif [9,12]. A recent study that tested all of the known members of the SALM family found that SALM3 and SALM5 were the only SALM proteins capable of inducing presynaptic differentiation in axons [10]. Synaptic adhesion-like molecule 3 overexpression in cultured neurons increases the number of excitatory and inhibitory presynaptic contacts. Synaptic adhesion-like molecule 3 recruits PSD-95 or SAP102 to the sites of early synaptic adhesion [10,13]. In contrast, SALM1 and SALM2, which lack the ability to interact with presynaptic ligands, cannot be directly recruited to sites of early synaptic adhesion. Synaptic adhesion-like molecule 3

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http://dx.doi.org/10.1016/j.yebeh.2016.11.023
1525-5050/

signaling also induces the elongation of monocytic cells by modulating actin cytoskeletal rearrangements [14]. Interestingly, synaptic reorganization and mossy fiber sprouting as pathologic mechanisms of chronic seizures in epilepsy models and proteins associated with these pathologic changes are also associated with the pathogenesis of epilepsy.

An intriguing possibility would be that the axon sprouting in epilepsy is caused by SALM3 overexpression, but nothing is known about the relation of SALM3 and epilepsy. We hypothesized that SALM3 plays a role in the pathophysiology of epilepsy. To address this possibility, we evaluated the impact of the specific inhibition of endogenous SALM3 in vivo in an animal model of epilepsy using a lentiviral vector expressing a short hairpin RNA (LV-shRNA) targeting SALM3. We explored the expression of SALM3 in the lithium-pilocarpine (Li-Pilo) model of epilepsy and evaluated the impact of SALM3 shRNA on behavioral changes in two distinct models of epilepsy.

## 2. Materials and methods

### 2.1. Lithium-pilocarpine model

Adult male Sprague-Dawley rats weighing 190–230 g (6–8 weeks) were used as subjects. All animals were obtained from the Experimental Animal Center of Xinxiang Medical University, China. Rats were housed and maintained in a temperature-controlled room with a 12-h light–dark cycle and were given free access to standard food and water. All experimental procedures were conducted in accordance with international standards [15]. The rats were randomly divided into the experimental group ($n = 15$) or the normal control group ($n = 5$). The experimental group was randomly divided into 3 sub-groups according to the time after intraperitoneal (i.p.) injection: 1 day, 10 days or 30 days. These stages were selected to represent the acute stage of epilepsy and the chronic stage with spontaneous recurrent seizures. Pilocarpine-induced seizures were classified according to Racine's standard criteria [16]. Rats that experienced convulsive seizures (stages 4 or 5) according to Racine's scale were used for further analysis. Rats in the epilepsy group were injected intraperitoneally (i.p.) with lithium chloride (127 mg/kg; Sigma, USA) followed by pilocarpine administration (50 mg/kg; i.p.; Sigma) 24 h later. Atropine sulfate (1 mg/kg) was given 30 min prior to the administration of pilocarpine to reduce peripheral cholinergic side effects. Pilocarpine (10 mg/kg; i.p.) was injected every 30 min. These animals were injected with diazepam (10 mg/kg, i.p.) 1 h after the onset of status epilepticus (SE). Animals in the control group were intraperitoneally injected with the same volume of normal saline.

### 2.2. Pentylenetetrazole (PTZ) chronic kindling model

Sprague-Dawley rats were intraperitoneally injected with subconvulsive doses of PTZ (35 mg/kg) at 10:00 A.M. every day as previously described [17]. The Racine score was recorded and convulsive behaviors were observed for at least 20 min after each PTZ injection. The animals were considered to be kindled after experiencing four consecutive class 4 or 5 seizures.

### 2.3. Surgical procedures and intrahippocampal injection of lentivirus

All animals were anesthetized by intraperitoneal injection of chloral hydrate (0.35 g/kg) (Sangon biotech, Shanghai, China) and placed in a stereotaxic apparatus (Stoelting, Wood Dale, IL, USA) for the operation. A midline skin incision was made to expose the dorsal surface of the skull. To inject the lentivirus, a 0.5 mm hole was drilled into the skull at the following stereotaxic coordinates according to the brain atlas of Paxinos and Watson: 2.0 mm posterior to the bregma and 2.0 mm lateral to the midline [18]. All animals were maintained at a warm temperature until fully awake and were allowed to recover from surgery for 7 days before further experimental manipulations, as previously described [19].

The SALM3-targeting oligonucleotide sequence was designed as described by Morimura [8]. The lentiviral vectors expressing GFP alone (LVGFP) were used as controls. The lentivirions were generated by Genechem Corporation (GeneChem, Shanghai, China). The viruses were prepared as previously described [20]. The lentivirus vectors were used at a titer of $2 \times 10^9$ Tu/mL. On the day of the experiment, SALM3 shRNA was injected into the hippocampus. A 5 μL Hamilton syringe (Hamilton, Bonaduz, Switzerland) was slowly inserted according to the stereotaxic coordinates, and then SALM3 shRNA or vehicle shRNA (2 μL) was slowly injected (0.5 μL/min) into the hippocampus (2.5 mm below the skull surface), and the needle remained in place for 1 min to allow the drug to diffuse from the needle. Synaptic adhesion-like molecule 3 shRNA or vehicle shRNA was injected into the hippocampus 3 days before the administration of pilocarpine or PTZ.

### 2.4. Animal behavior investigations

After the administration of the drugs, the rats were maintained in a standard 12 h light-dark cycle and allowed free access to food and water. In the acute stage of the Li-Pilo epilepsy model, the latent period was calculated as the time from the first pilocarpine injection to the time when a class 4 seizure occurred. In the chronic stage of disease with spontaneous recurrent seizures (SRS) in the pilocarpine epilepsy model, animal behavior was recorded using a closed-circuit video system 24 h a day to detect class 4 and 5 seizures during week 6. Spontaneous recurrent seizures resembled class 4 or 5 limbic motor seizures induced by PTZ kindling [16]. We recorded SRS times in each animal for 1 week. For the chronic PTZ kindling model, the latency period was defined as the time from the first PTZ injection to the day when four consecutive class 4 or 5 seizures occurred.

### 2.5. Tissue preparation

The rats were sacrificed 1, 10 or 30 days after the onset of status epilepticus. The hippocampus and cortex were quickly resected using RNase-free instruments. One portion of the brain tissue was immediately placed in liquid nitrogen and later used for protein extraction. Another portion was fixed with 4% formalin for 1 day and embedded in paraffin. Five-μm thick sections were prepared for immunohistochemistry assays, and 10-μm thick sections were used for double immunofluorescence staining as previously described [19].

### 2.6. Western blot

SALM3 protein expression was examined using western blot analysis as previously described [21]. Total protein was extracted according to the manufacturer's instructions (Keygen Biotech, Nanjing, China). Then, 50 μg of total protein was loaded in each lane and separated using SDS-PAGE (5% spacer gel; 10% separating gel) for 50 min at 80 V. The protein was then electrotransferred to a polyvinylidene fluoride membrane (PVDF, Millipore, Darmstadt, Germany) at 250 mA for 2 h. The PVDF membrane was then blocked at 37 °C for 60 min in 5% skim milk. The membranes were incubated with the goat anti-SALM3 (1:200) (Santa Cruz Biotechnology, Inc., CA, USA) and rabbit anti-GAPDH (1:4000) (Beijing 4A Biotech Co., Ltd., Beijing, China) primary antibodies overnight at 4 °C. After washing with Tween-20 Tris-buffered saline (TTBS) three times for 15 min each, the membranes were incubated with a horseradish peroxidase-conjugated secondary antibody (1:4000) (Zhongshan, Beijing, China) for 60 min at 37 °C and washed with TTBS three times for 15 min each. The resultant pixel density of the images was quantified using the Quantity One software (Bio-Rad Laboratories) [22].