

Research Paper

Inhibitory effects of scorpion venom heat resistant protein on the excitability of acutely isolated rat hippocampal neurons

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Abstract: The effects of scorpion venom heat resistant protein (SVHRP) (National invention patent of China, 2004-10-20, No. ZL01 1 06166.92) on the excitability of acutely isolated rat hippocampal neurons were observed by whole-cell recording and the potential molecular mechanisms underlying its antiepileptic effect were investigated further. The results showed that SVHRP could decrease the excitability of hippocampal neurons. SVHRP (1×10^{-2} $\mu\text{g/mL}$) altered the action potential (AP) firing mode and decreased the AP firing frequency. Out of 52 neurons observed, 45 (86.54%) generated phasic firing, and 7 (13.46%) generated repetitive firing. Among the 45 neurons generating phasic firing, 8 (17.78%) neurons could still be induced phasic firing after treatment with 1×10^{-2} $\mu\text{g/mL}$ SVHRP and 37 (82.22%) neurons had no responses to the stimulation. The AP firing of neurons was dramatically different after treatment with SVHRP ($P < 0.01$, $n = 45$). Among the 7 repetitive firing neurons, all of them could only generate 1 or 0 AP instead of repetitive firing when SVHRP was applied. The number of APs was 14.57 ± 1.00 and 0.57 ± 0.20 before and after SVHRP treatment ($P < 0.01$, $n = 7$). The AP rheobase was (75.10 ± 8.99) pA and (119.85 ± 12.73) pA before and after 1×10^{-4} $\mu\text{g/mL}$ SVHRP application, respectively ($P < 0.01$, $n = 8$). The AP threshold was increased from (-41.17 ± 2.15) mV to (-32.40 ± 1.48) mV after 1×10^{-4} $\mu\text{g/mL}$ SVHRP treatment ($P < 0.01$, $n = 8$). The peak amplitude of AP was (68.49 ± 2.33) mV for the neurons before treatment with 1×10^{-4} $\mu\text{g/mL}$ SVHRP and (54.71 ± 0.81) mV after treatment ($P < 0.01$, $n = 8$). These results showed that SVHRP could decrease the AP firing frequency, increase the AP rheobase and threshold, but decrease the AP peak amplitude of hippocampal neurons. In other words, SVHRP can decrease the excitability of hippocampal neurons. SVHRP probably alters the excitability of hippocampal neurons by affecting sodium channels and this may be one of the underlying molecular mechanisms for its antiepileptic effect.

Key words: scorpion venom heat resistant protein; whole-cell patch clamp; excitability; hippocampal neuron

蝎毒耐热蛋白对大鼠急性分离海马神经元兴奋性的影响

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摘要: 应用全细胞膜片钳记录技术在电流钳模式下观察经持续高温等特殊处理后分离纯化的 30~50 kDa 蝎毒耐热蛋白 (scorpion venom heat resistant protein, SVHRP) (国家发明专利, 专利号 ZL01 1 06166.92) 对急性分离大鼠海马神经元兴奋性的影响。结果发现 SVHRP 可致海马神经元兴奋性降低。神经元经 1×10^{-2} $\mu\text{g/mL}$ SVHRP 处理后动作电位发放模式改变, 发放频率减少。在 52 个受检细胞中, 有 45 个细胞产生位相放电 (占 86.54%); 7 个细胞产生重复放电 (占 13.46%)。在产生位相放电的 45 个细胞中, 有 8 个细胞在 SVHRP 处理后仍可以诱发出位相放电 (占 17.78%); 37 个细胞在 SVHRP 处理后无法诱导出位相放电 (占 82.22%), SVHRP 处理后动作电位的产生与处理前相比, 有显著差异 ($P < 0.01$, $n = 45$); 在产生重复放电的 7 个细胞中, 在 1×10^{-2} $\mu\text{g/mL}$ SVHRP 作用后均不能再次诱发出重复放电, 而是产生一个动作电位或不再产生动作电位, 药物处理前产生的动作电位个数为 14.57 ± 1.00 , SVHRP 处理后产生动作电位的个数为 0.57 ± 0.20 , 二者之间有显著性差异 ($P < 0.01$, $n = 7$)。 1×10^{-4} $\mu\text{g/mL}$ SVHRP 处理后, 诱发动作电位产生的基强度由 (75.10 ± 8.99) pA 增加到 (119.85 ± 12.73) pA ($P < 0.01$, $n = 8$); 阈电位由 (-41.17 ± 2.15) mV 升至 (-32.40 ± 1.48) mV ($P < 0.01$, $n = 8$); 动作电位峰值由 (68.49 ± 2.33) mV 下降至 (54.71 ± 0.81) mV ($P < 0.01$, $n = 8$)。由于神经元超兴

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奋性被认为是癫痫发作的基本机制之一,因此上述结果表明SVHRP有可能通过降低海马神经元兴奋性发挥其抗癫痫作用,这为蝎毒药物的进一步开发提供理论依据。

关键词: 蝎毒耐热蛋白; 全细胞膜片钳技术; 兴奋性; 海马神经元

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Epilepsy is a chronic neurological disorder, which is characterized by the recurrent appearance of spontaneous seizures due to abnormal, paroxysmal electrical discharge of neurons in the cerebral cortex^[1-3]. Increasing evidence suggests that even though epilepsy is not a single syndrome, common pathogenetic processes either as basic mechanisms or as final common factors may be responsible as etiologic causes^[4]. Nonetheless, to date the hyperexcitability of neurons is considered as the principle mechanism underlying epilepsy.

Scorpion has been used to treat various neurological disorder symptoms for over two thousand years in China and it is the foremost choice for epileptic treatment as a traditional Chinese medicine^[5]. The therapeutically effective part of scorpion is scorpion venom (SV). SV has been proven to have antiepileptic effect, but its neurotoxicity greatly restricts its further clinical application^[6]. In the present study, we used a kind of SV obtained by means of special methods (persistent high temperature) named a 30-50 kDa scorpion venom heat resistant protein (SVHRP)(National Invention Patent of China. No. ZL01 1 06166.92). The results from animal experiments showed that SVHRP has obvious antiepileptic effect but its neurotoxic side effects diminish significantly^[7]. SVHRP may reduce the behavioral response of epileptic rats, inhibit proliferation and hypertrophy of astrocytes in the hippocampus, therefore reduce the degree of lesion in the neurons^[8]. SVHRP may selectively prevent loss of GABAergic interneurons in the ventral hippocampus of rats susceptible to epileptic seizures and increase the release of GABA^[9].

Because hippocampus is susceptible to epilepsy, we observed the effects of SVHRP on the excitability of acutely isolated rat hippocampal neurons and further investigated the potential molecular mechanisms underlying the antiepileptic effect of SVHRP.

1 MATERIALS AND METHODS

1.1 Cell preparation

The rats were obtained from the Animal Center of Dalian Medical University. All experiments were conducted in accordance with the National Institutes of Health Guide

for the Care and Use of Laboratory Animals. All efforts were made to minimize the number of animals used and their suffering. Neurons were acutely isolated from the hippocampal slices of rat brain according to the method of publications^[10,11] with some modifications. Briefly, Sprague-Dawley (SD) rats of post-neonatal days (PND) 7-10 were rapidly decapitated. The hippocampus was dissected and transverse slices (thickness 400-500 μm) were cut. The slices were incubated at 32 °C for 2 h in the bathing solution containing (in mmol/L): NaCl 150, KCl 5, KH_2PO_4 1.2, MgSO_4 1.3, CaCl_2 2.4, NaHCO_3 26, glucose 10, pH 7.4, osmotic pressure 320 mOsm/L.

The bathing solution was continuously bubbled with 95% O_2 + 5% CO_2 to ensure adequate oxygenation of slices and a pH of 7.4. After incubation, the tissue pieces were gently transferred to the bathing solution containing 1.5 mg/mL pronase E (Merk, Germany) at 32 °C for 20 min. The solution was continuously bubbled with 95% O_2 + 5% CO_2 . Thereafter, the slices were washed three times with the bathing solution without Ca^{2+} containing (in mmol/L): NaCl 124, KCl 5, EGTA 2, KH_2PO_4 1.2, MgSO_4 1.3, NaHCO_3 26, glucose 10, pH 7.4, osmotic pressure 320 mOsm/L, and then three times with the bathing solution. Slices were then transferred to the standard external solution containing (in mmol/L): NaCl 150, KCl 5, MgCl_2 1.1, CaCl_2 2.6, HEPES 10, glucose 10, pH 7.4, osmotic pressure 320 mOsm/L. Neurons were isolated by triturating the tissue pieces through a series of Pasteur pipettes with decreasing tip diameter. Hippocampal neurons were placed in six dishes (35 mm in diameter). Twenty minutes later, when the neurons were attached to the bottom, those neurons with bright and smooth appearance and no visible organelles were used for recording at room temperature (22-24 °C).

1.2 Electrophysiological recording

Isolated neurons that were bright with distinct outlines and dendrites without rounding up and clubbing were chosen for electrophysiological recording. Electrodes were pulled from borosilicate glass capillaries on a micropipette puller (Narishige, PP 830, Japan) with an open-tip resistance of 3-6 M Ω when filled with intracellular solution (in mmol/L): KCl 65, KOH 5.0, KF 80, HEPES 10, EGTA 10, $\text{Na}_2\text{-ATP}$

2.0, pH 7.2. We used an EPC-10 amplifier, Pulse and PulseFit 8.6 software (HEKA Elektronik, Germany) for data acquisition and analysis. Filter 1 was set to 10 kHz and Filter 2 to 2.9 kHz. After seal formation ($>5\text{ G}\Omega$) and membrane rupture, the cells were allowed to stabilize for 3-5 min. Under current clamp, action potentials (APs) were recorded. Neurons were considered for recording when satisfied the following criteria: (1) The seal resistance was larger than $5\text{ G}\Omega$; (2) The input resistance was less than $400\text{ M}\Omega$; (3) The resting potential (RP) was less than -40 mV ; (4) Overshoot must occur in AP. Neurons were given a long-term depolarizing current (500 ms, 100 pA) to induce AP firing and the effect of SVHRP on AP firing was recorded. In addition, a series of 10 ms depolarizing currents (from 0 pA to 200 pA, 40 pA increment each time) were given to neurons to induce AP firing. AP rheobase, AP threshold ($V_{\text{threshold}}$), AP peak amplitude (V_{peak}) and AP duration at half amplitude ($\text{APD}_{0.5}$) were recorded before and after the application of SVHRP. SVHRP dissolved in standard extracellular solution was locally applied to cells via BPS-8 superfusion system (ALA Scientific Instruments, USA)

1.3 Reagent

30-50 kDa SVHRP was supplied by Department of Physiology of Dalian Medical University. It was obtained by means of special methods (persistent high temperature) and showed single amplitude after filtration in chromatography column loaded with superdex 75 gel (48 cm \times 18 cm). It shows smear in SDS-PAGE electrophoresis (probably because SDS makes the protein denature), but in the range of 30-50 kDa there occur two bands. SVHRP was stored at $10\text{ }\mu\text{g/mL}$ and diluted to the concentrations needed before each experiment.

1.4 Data Analysis

All electrophysiological recording data were analyzed by PulseFit 8.6, Igor, SigmaPlot software. All data were represented as $\text{means}\pm\text{SEM}$ and statistically analyzed with t -test and χ^2 test. Statistical significance was set at $P<0.05$.

2 RESULTS

2.1 Morphological and electrophysiological properties of hippocampal neurons

Observed under the inverted microscope (Olympus, IX 70, Japan), the acutely isolated rat hippocampal neuron had a clean smooth surface with a well-kept long axon and some dendrites. The intact neurons were pyramidal or bipolar in shape. The axons of some neurons were longer

than $200\text{ }\mu\text{m}$. RP measured in current mode of EPC-10 amplifier was $(-55.36\pm 7.46)\text{ mV}$ ($n=40$). Membrane capacitance (C_m) was obtained from the value of C-Slow. Series resistance (R_s) is composed of the resistance of electrode and that of the ruptured membrane. In our study, R_s was $(12.65\pm 5.98)\text{ M}\Omega$ ($n=35$) and C_m was $(16.62\pm 3.7)\text{ pF}$ ($n=35$).

2.2 Effect of SVHRP on AP firing

Long-term depolarizing current can induce two modes of AP firing: phasic firing (number of AP=1, Fig.1A) and repetitive firing (number of AP >4 , Fig.2A). To observe the effect of SVHRP on AP firing, a long-term depolarizing current (500 ms, 100 pA) was given to the neurons in current clamp mode and the AP firing before and after SVHRP ($1\times 10^{-2}\text{ }\mu\text{g/mL}$) treatment was recorded. Out of 52 neurons observed, 45 (86.54%) of them generated phasic firing and the remaining 7 neurons generated repetitive firing. Among the 45 neurons generating phasic firing, 8 (17.78%) neurons could still be induced phasic firing after treatment with $1\times 10^{-2}\text{ }\mu\text{g/mL}$ SVHRP, and the other 37 (82.22%) neurons had no responses to the stimulation. The AP firing of neurons was dramatically different after treatment with SVHRP ($P<0.01$, $n=45$). Among the 7 repetitive firing neurons, all of them could only generate 1 or 0 AP instead of repetitive firing when SVHRP was applied. The number of APs was 14.57 ± 1.00 and 0.57 ± 0.20 before and after SVHRP treatment for the neurons. The difference was significant ($P<0.01$, $n=7$) (Fig.2, Table 1).

2.3 Effect of SVHRP on the excitability of rat hippocampal neurons

2.3.1 Effect of SVHRP on AP rheobase

A series of 10 ms depolarizing currents (from 0 pA to 200 pA, 40 pA increment each time) were given to a neuron to

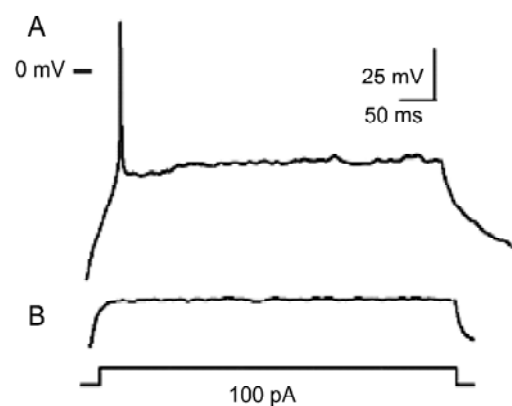


Fig.1. Phasic firing in hippocampal neuron before (A) and after (B) application of $1\times 10^{-2}\text{ }\mu\text{g/mL}$ SVHRP.

induce AP firing. Different concentrations of SVHRP were applied to the neurons for 10 s when they recovered to the resting state. We found that AP could not be evoked again after $1 \times 10^{-2} \mu\text{g/mL}$ and $1 \times 10^{-3} \mu\text{g/mL}$ SVHRP application, so we chose a lower concentration of $1 \times 10^{-4} \mu\text{g/mL}$ in the

following experiment. The rheobase was $(75.10 \pm 8.99) \text{ pA}$ and $(119.85 \pm 12.73) \text{ pA}$ before and after SVHRP application respectively. The rheobasic current required to generate one AP was significantly larger after the neurons were treated with SVHRP ($P < 0.01$, $n = 8$) (Fig.3, Table 1).

Table 1. Electrophysiological properties of the neurons before and after treatment of SVHRP

	Before treatment (n=8)	After treatment (n=8)
Rheobase (pA)	75.10 ± 8.99	$119.85 \pm 12.73^{**}$
V_{peak} (mV)	68.49 ± 2.33	$54.71 \pm 0.81^{**}$
$V_{\text{threshold}}$ (mV)	-41.17 ± 2.15	$-32.40 \pm 1.48^{**}$
APD _{0.5} (ms)	4.16 ± 0.18	3.96 ± 0.23
Number of APs in repetitive firing	14.57 ± 1.00	$0.57 \pm 0.20^{**}$

means \pm SEM. $^{**}P < 0.01$ vs before treatment of SVHRP.

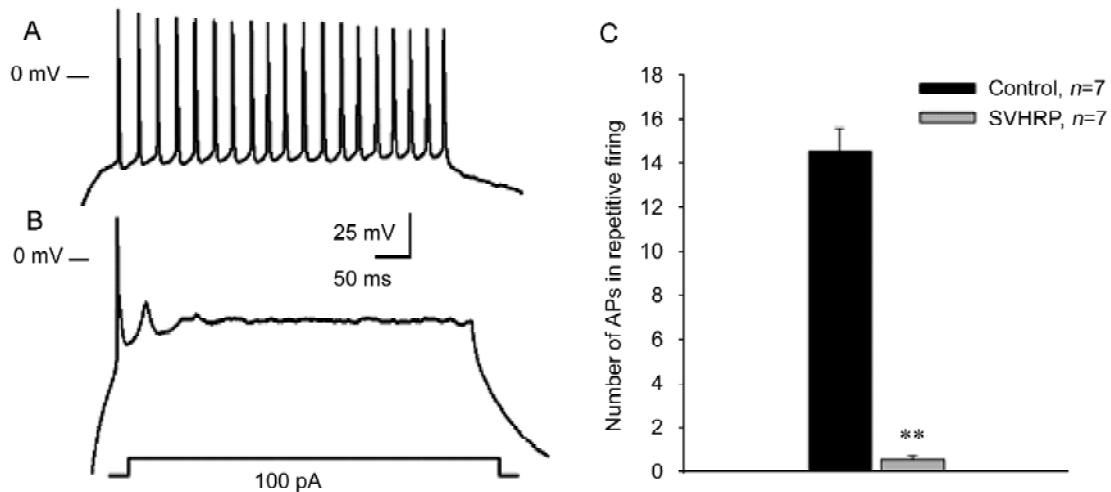


Fig.2. Repetitive firing in hippocampal neuron before (A) and after (B) treatment of $1 \times 10^{-2} \mu\text{g/mL}$ SVHRP. C: The number of APs in repetitive firing was compared before and after $1 \times 10^{-2} \mu\text{g/mL}$ SVHRP treatment. $^{**}P < 0.01$ vs control.

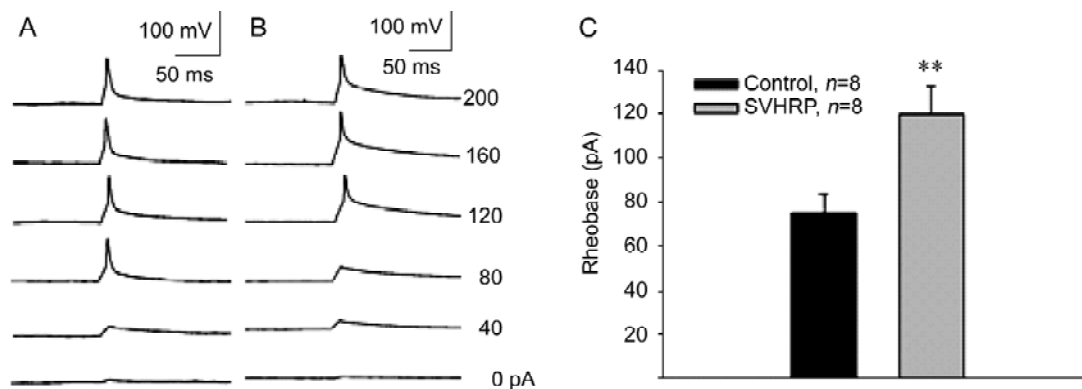


Fig.3. Voltage traces with evoked APs in the hippocampal neurons before (A) and after (B) application of $1 \times 10^{-4} \mu\text{g/mL}$ SVHRP. As shown in A and B, evoked APs were collected in the current clamp mode at -70 mV with 6 depolarizing currents (from 0 pA to 200 pA, 40 pA increment each time). Voltage traces in A and B started at the bottom and moved upward incrementally. C: Comparison of the mean rheobase of APs in the hippocampal neurons before and after treatment of $1 \times 10^{-4} \mu\text{g/mL}$ SVHRP. $^{**}P < 0.01$ vs control.

2.3.2 Effect of SVHRP on $V_{\text{threshold}}$

Any factor altering the difference between RP and threshold can affect the excitability of neurons. We observed the APs before and after SVHRP treatment in the same neuron, thus the RP was kept at the same level and the alteration of threshold would represent the change of excitability. In current clamp mode, a depolarizing current (160 pA, 10 ms) was exerted on a neuron to induce firing of AP. SVHRP ($1 \times 10^{-4} \mu\text{g/mL}$) was not applied until the neuron recovered to the resting state. $V_{\text{threshold}}$ was (-41.17 ± 2.15) mV before SVHRP treatment and (-32.40 ± 1.48) mV after that. $V_{\text{threshold}}$ was significantly increased when the neurons were treated with SVHRP ($P < 0.01$, $n=8$) (Fig.4, Table1).

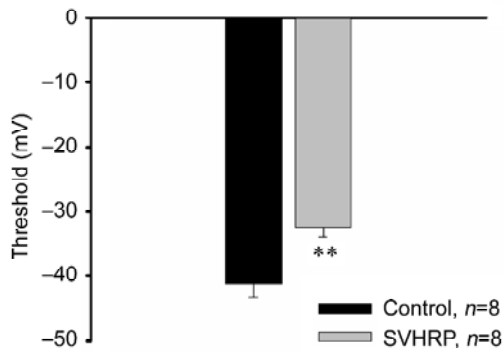


Fig.4. Comparison of threshold of APs in the hippocampal neurons before and after treatment of $1 \times 10^{-4} \mu\text{g/mL}$ SVHRP. ** $P < 0.01$ vs control.

2.3.3 Effect of SVHRP on V_{peak}

V_{peak} is near to the equilibrium potential of sodium ion and is the characteristic index of AP, so we compared the variation of V_{peak} . In current clamp mode, a neuron was given a depolarizing current (160 pA, 10 ms) to induce AP. V_{peak} before and after treatment with $1 \times 10^{-4} \mu\text{g/mL}$ SVHRP was recorded. V_{peak} was (68.49 ± 2.33) mV before SVHRP treatment and (54.71 ± 0.81) mV after that. V_{peak} decreased significantly ($P < 0.01$, $n=8$) (Fig.5, Table1).

2.3.4 Effect of SVHRP on $\text{APD}_{0.5}$

$\text{APD}_{0.5}$ is the duration of AP when it reaches half of the peak amplitude. It is an important index representing the duration of a whole AP. To determine whether SVHRP can affect the duration of AP, we applied stimulation (160 pA, 10 ms) to neurons and measured $\text{APD}_{0.5}$ before and after the neurons were treated with SVHRP ($1 \times 10^{-4} \mu\text{g/mL}$). $\text{APD}_{0.5}$ was (4.16 ± 0.18) ms before SHVPR treatment and (3.96 ± 0.20) ms after that. There was no significant difference (Table1).

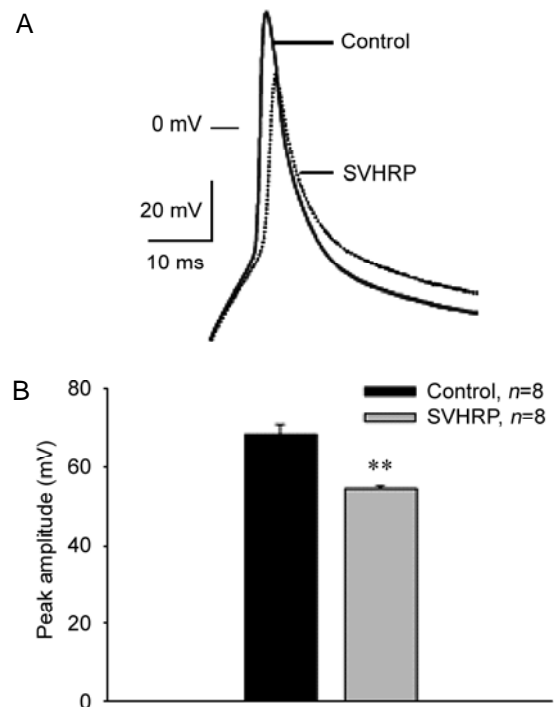


Fig.5. Comparison of the peak amplitude of APs in the hippocampal neuron before and after application of $1 \times 10^{-4} \mu\text{g/mL}$ SVHRP. A: APs were induced by a depolarizing current (160 pA, 10 ms) in the hippocampal neuron before and after application of $1 \times 10^{-4} \mu\text{g/mL}$ SVHRP. B: Comparison of mean peak amplitude of APs in the hippocampal neurons before and after treatment of $1 \times 10^{-4} \mu\text{g/mL}$ SVHRP. ** $P < 0.01$ vs control.

3 DISCUSSION

Epilepsy is the most common primary neurological disorder known^[12]. The pathogenesis of epilepsy is very complicated. Up to now, it is widely accepted that the hyperexcitability of neurons caused by the imbalance between excitatory and inhibitory modulators is one of the pivotal mechanisms.

Based on these mechanisms, there are kinds of antiepileptic drugs that can decrease the excitability of neurons such as SV. However, the neurotoxicity restricts the clinical application. Methods in obtaining antiepileptic drugs with high efficiency and fewer side effects are the key problem at present. The study of the utilization of SV for epilepsy treatment in our laboratory experienced several stages: BmK—SV—SVHRP. 30-50 kDa SVHRP shows single amplitude after filtration in chromatography column loaded with superdex 75 gel. Our previous work showed that SVHRP can clear free radicals and has neural protective and nutrition effect^[13]. Now it is proved that SVHRP is less neurotoxic and its antiepileptic effect is preserved. The

present study was aimed to explore whether 30-50 kDa SVHRP can affect the excitability of epilepsy susceptible hippocampal neurons after it was treated by persistent high temperature and further to explain the potential mechanism of its antiepileptic effect.

Our results showed that the AP firing frequency decreased, the rheobasic current required to generate one AP was significantly larger after neurons were treated with SVHRP and the threshold of APs increased. It is suggested that SVHRP can decrease the excitability of hippocampal neurons. Another significant change of APs is that the peak amplitude was decreased and adjacent to the equilibrium potential of sodium ions. The concentration of sodium ions was kept constant in our experiment, so we can imagine that SVHRP may exert effects on sodium channels.

Neurons exhibit a RP between discharges. Discharge—the generation of a more positive response (AP) involves a complex process in which ions (Na^+ , K^+ , Mg^{2+} , Ca^{2+} , Cl^-) flow across membranes under the influence of both inhibitory and excitatory modulators and the voltage over the neuron's membrane. Later the cell must regenerate its RP difference before it can discharge again. It is the disturbance of this fundamental physiological mechanism that actually leads to the generation of seizure activity^[2]. The movement of anions, cations and the other larger organic ions, in and out of the cells, is achieved by the presence of large, selective, transmembrane glycoproteins called “ion channels”.

In recent years, ion channels were widely investigated in epileptogenesis. The abnormality of ion channels in involved neurons was considered as one of the key mechanisms of epileptogenesis. The first connection between sodium channels and epilepsy was the discovery of a $\beta 1$ subunit gene mutation in sodium channel in a large Australian family with GEFS+^[14]. More sodium channels mutation were discovered to be associated with epilepsy^[15]. In fact, blockade of voltage-dependent sodium channels is a primary action of antiepileptic drugs phenytoin (PHT), carbamazepine (CBZ), oxcarbazepine (OXC), lamotrigine (LTG), topiramate (TPM), zonisamide (ZNS) and felbamate (FBM)^[16].

At the cellular level, three basic antiepileptic mechanisms are clearly understood: modulation of voltage-dependent ion channels (Na^+ , K^+ , Ca^{2+}); enhancement of γ -amino butyric acid (GABA)-mediated inhibitory neurotransmission; and alteration of excitatory (specially glutamate-mediated) transmission. Among the voltage-dependent sodium channel blockers in antiepileptic drugs, LTG can alter the excitability of neurons by increasing the level of GABA in brain or synapse, but CBZ, OXC and PHT do not have this ac-

tion^[17]. Our previous works showed that SVHRP also could increase the level of GABA in the hippocampus of epileptic rats^[9]. Neuropeptide Y (NPY) is important antiepileptic substance in brain^[18]. Our work also found that SVHRP could increase the expression of NPY-immunoreactivity in the cultured primitive hippocampal neurons in a dose- and time-dependent manner. The results of RT-PCR showed that SVHRP could promote the expression of NPY mRNA^[13]. Therefore SVHRP may affect the excitability of neurons through NPY. So we extrapolate that SVHRP can decrease the excitability of neurons in multiple mechanisms and exert its potent antiepileptic effect. Further study on brain slice should be done to confirm our hypothesis.

In summary, our data show that SVHRP can directly decrease the excitability of the hippocampal neurons, which may be one of the mechanisms underlying its antiepileptic effect. Meanwhile, SVHRP decreases the peak amplitude of APs, indicating that SVHRP probably alters the excitability by affecting sodium channels. The effects of SVHRP on sodium channels need to be further studied.

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