ARTICLE

Antiepileptic geissoschizine methyl ether is an inhibitor of multiple neuronal channels

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Geissoschizine methyl ether (GM) is an indole alkaloid isolated from *Uncaria rhynchophyll* (UR) that has been used for the treatment of epilepsy in traditional Chinese medicine. An early study in a glutamate-induced mouse seizure model demonstrated that GM was one of the active ingredients of UR. In this study, electrophysiological technique was used to explore the mechanism underlying the antiepileptic activity of GM. We first showed that GM ($1-30\,\mu mol/L$) dose-dependently suppressed the spontaneous firing and prolonged the action potential duration in cultured mouse and rat hippocampal neurons. Given the pivotal roles of ion channels in regulating neuronal excitability, we then examined the effects of GM on both voltage-gated and ligand-gated channels in rat hippocampal neurons. We found that GM is an inhibitor of multiple neuronal channels: GM potently inhibited the voltage-gated sodium (Na_V), calcium (Ca_V), and delayed rectifier potassium (I_K) currents, and the ligand-gated nicotinic acetylcholine (nACh) currents with IC_{50} values in the range of $1.3-13.3\,\mu mol/L$. In contrast, GM had little effect on the voltage-gated transient outward potassium currents (I_A) and four types of ligand-gated channels (γ-amino butyric acid (GABA), *N*-methyl-*D*-aspartate (NMDA), α-amino-3-hydroxy-5-methylisoxazole-4-propionate/kainite (AMPA/KA receptors)). The in vivo antiepileptic activity of GM was validated in two electricity-induced seizure models. In the maximal electroshock (MES)-induced mouse seizure model, oral administration of GM ($50-100\,mg/kg$) dose-dependently suppressed generalized tonic-clonic seizures. In 6-Hz-induced mouse seizure model, oral administration of GM ($100\,mg/kg$) reduced treatment-resistant seizures. Thus, we conclude that GM is a promising antiepileptic candidate that inhibits multiple neuronal channels.

Keywords: geissoschizine methyl ether; *Uncaria rhynchophyll*; antiepileptic drug; hippocampal neurons; action potential; nicotinic acetylcholine receptors; voltage-gated ion channels; mouse seizure model

INTRODUCTION

Uncaria rhynchophylla (UR) is a commonly used traditional Chinese medicine that has been used to treat epileptic seizures for centuries [1, 2]. It is also one of the dried medicinal herbs in yi-gan san (yokukansan), a traditional herbal preparation widely used in China and Japan [3, 4]. Yokukansan has been approved by the Japanese Ministry of Health, Labor, and Welfare as a prescription drug for neurosis, insomnia, and irritability in children [4]. Various chemical components, such as rhynchophylline, isorhynchophylline, and geissoschizine methyl ether (GM), have been isolated from UR [2, 5]. Among these components, GM is known to be an active component of UR and a major contributor to the psychotropic nature of yokukansan [2, 4].

In a preliminary study, GM was found to exhibit moderate antiepileptic effects in a glutamate-induced seizure mouse model [6]. However, the mechanism underlying its antiepileptic activity remains unclear. The neuroprotective effects of GM have been observed in several glutamate-induced neuronal death experiments [7–10], and these effects are probably due to the inhibition of Ca^{2+} influx, the reduction in mitochondrial respiration and the

suppression of the generation of reactive oxygen species (ROS) [11, 12]. Consistent with its neuroprotective effects, GM has also been found to ameliorate myelin deficiency in mature oligodendrocyte formation and to be involved in remyelination in the medial prefrontal cortex [13]. Moreover, GM may also behave as a partial agonist of the $5-HT_{1A}$ receptor and an antagonist of the $5-HT_{2A}$, $5-HT_{2C}$ and $5-HT_7$ receptors, which was thought to be the mechanism underlying the effects of yokukansan in the treatment of aggression and reduced sociality in mice [14–17]. The inhibitory activity on acetylcholinesterase could also contribute to the neuroprotective effects of GM against damage in Alzheimer's disease models [2, 18]. However, neither of these proposed activities of GM provides an appropriate explanation for its antiepileptic effects.

Epilepsy is commonly characterized by abnormal spontaneous recurrent electrical discharge in the central nervous system [19]. Damage to the hippocampus was found in more than 50% of patients with temporal lobe epilepsy; thus, hippocampal neurons have been widely used for mechanistic studies of antiepileptic drugs (AEDs) [19, 20]. Voltage-gated sodium (Na_V), potassium (K_V)

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630

and calcium (Ca$_V$) channels play pivotal roles in neuron firing, and mutations in these channels may cause genetic epilepsy [21]. Impaired GABAergic inhibition and increased glutamatergic excitation are correlated with the generation of seizures [22, 23]. In addition, abnormalities in neuronal nicotinic acetylcholine (nACh) receptors and cholinergic neurotransmission are also involved in the pathophysiology of epilepsy, such as autosomal dominant nocturnal frontal lobe epilepsy (ADNFLE) and juvenile myoclonic epilepsy (JME) [24–26]. All of these voltage-gated and ligand-gated channels have been demonstrated to be relevant in the therapeutic activities of many AEDs, such as topiramate, phenytoin, and carbamazepine [27, 28]. However, AEDs may encompass different antiepileptic treatment spectrums in animal models of epilepsy [19]. In the present study, we explored the molecular mechanism underlying the antiepileptic activity of GM using a custom electrophysiology setup in hippocampal neurons and evaluated its antiepileptic treatment spectrum in the maximal electroshock (MES) and 6-Hz seizure models.

MATERIALS AND METHODS

Materials
GM was purchased from Standard Technology Co., Ltd (Shanghai, China). The purity of the synthetic GM was ≥98%, as assessed by HPLC. The compound was dissolved and stored in dimethyl sulfoxide (DMSO) to produce 20 mmol/L stock solutions that were then diluted in bath solution to obtain the final concentrations. DMSO at the final concentrations (≤0.5%) was well tolerated with no observable toxic effects to the neurons. To conduct research on animals, GM was dissolved in a mixture of 5% DMSO and 95% Tween-80. TTX was purchased from Aquaculture Technical Developing Company, Qinhuangdao, China. Topiramate (TPM), levetiracetam (LEV), γ-amino butyric acid (GABA), N-methyl-D-aspartic acid (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA), kainic acid (KA) and acetylcholine chloride (ACh-Cl) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Animals
Sprague-Dawley rats at postnatal days 1–7, 18-day-old C57BL/6 mouse embryos and male KM mice weighing 20 ± 2 g were purchased from the Shanghai SLAC Laboratory Animal Co., Ltd (Shanghai, China). All animal procedures were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals under protocols strictly followed and approved by the guidelines of the IACUC (Institutional Animal Care and Use Committees). The IACUC checked all protocols and approved this study.

Primary cultured hippocampal neuron preparations
Hippocampal neurons were isolated from 18-day-old C57BL/6 mouse embryos. Briefly, the dissected hippocampal tissues were enzymatically dissociated with 3 mg/mL proteinase (Sigma-Aldrich) and incubated at 32 °C for 8 min. One mg/mL of bovine serum albumin (Sangon Biotech, Shanghai, China) and trypsin inhibitor (Sigma-Aldrich) was then added to the solution to stop digestion. The tissues were then triturated into single cells using a fire-polished glass Pasteur pipette. The neurons were collected by centrifugation, resuspended in DMEM/F12 (Gibco, Grand Island, USA) with 10% FBS (Gibco), and, finally, plated at a density of 150 000 cells/mL on poly D-lysine-coated glass coverslips in 24-well plates and cultured at 37 °C in 5% CO$_2$. The medium was replaced after 6 h with the same volume of serum-free Neurobasal-A (Gibco) containing 2% B-27 (Gibco), 0.5 mmol/L GlutaMAX (Gibco), and 1% penicillin and streptomycin (Gibco). Thereafter, half of the old medium was removed and replaced with the same volume of fresh medium every 3 days. Neurons were used in electrophysiological experiments after 12 days of culture.

Acutely isolated hippocampal neuron preparations
Hippocampal neurons were obtained from Sprague-Dawley rats at postnatal days 1–7. Similar to the mouse neurons, the hippocampal tissues were triturated into single cells with a dissociation solution after digestion with proteinase. The acutely isolated hippocampal neurons were used in the following 6 h. As calcium-free dissecting solution has been reported to be beneficial for dissociating the hippocampal tissues and reducing the activity of intrinsic proteases, calcium was omitted in the solution [29]. The dissecting solution contained (in mmol/L): 82 Na$_2$SO$_4$, 30 K$_2$SO$_4$, 5 MgCl$_2$, 1 NaPy, 10 HEPES, and 20 glucose at pH 7.4, adjusted with NaOH.

Electrophysiological recordings
Pipettes were pulled from borosilicate glass capillaries, and the resistances were 3–5 MΩ when filled with the intracellular solution. Current-clamp mode was used to record mouse and rat neuronal firing properties. The intracellular solution contained (in mmol/L) 140 KCl, 1 MgCl$_2$, 10 EGTA, and 10 HEPES, with the pH adjusted to 7.2 using KOH; the bath or extracellular solution contained (in mmol/L) 140 NaCl, 5 KCl, 1 CaCl$_2$, 1.25 MgCl$_2$, 10 glucose, and 10 HEPES (pH 7.4 adjusted with NaOH). During recording, the cells were held at a potential of −60 mV, and the bath solution was continuously perfused using a bath perfusion system. Ligand-gated ion channel currents (GABA, AMPA, NMDA, KA, and nACh) were activated by the corresponding ligands in rat hippocampal neurons kept at the holding potential of −60 mV. The preapplication of GM for 60 s followed by the coapplication of GM and each ligand for 2 s (GABA, AMPA, NMDA, and KA) or 5 s (ACh-Cl) resulted in a change in the amplitude of the peak. To elicit sodium, calcium, potassium currents in rat hippocampal neurons, the standard whole-cell voltage-clamp technique was used. Sodium currents were recorded in the intracellular solution that contained (in mmol/L) 120 CsCl, 10 NaCl, 10 TEA-Cl, 1 CaCl$_2$, 1 MgCl$_2$, 10 EGTA and 10 HEPES (pH 7.2 adjusted with CsOH); the extracellular solution contained (in mmol/L) 120 NaCl, 5 KCl, 1 CaCl$_2$, 1.25 MgCl$_2$, 20 TEA-Cl, 10 glucose, and 10 HEPES (pH 7.4 adjusted with NaOH). The holding potential was −90 mV and was depolarized to −20 mV for a duration of 50 ms. Calcium currents were recorded in an intracellular solution containing (in mmol/L) 130 CsCl, 10 TEA-Cl, 5 MgATP, 10 EGTA, and 10 HEPES, pH 7.2 adjusted with CsOH; the extracellular solution contained (in mmol/L) 130 NaCl, 5 CaCl$_2$, 20 TEA-Cl, 10 glucose, and 10 HEPES, with pH adjusted to 7.4 using NaOH. The calcium currents were evoked by steps from the holding potential of −90 to 0 mV. Potassium currents and Kir currents were recorded in an intracellular solution containing (in mmol/L) 140 KCl, 1 CaCl$_2$, 1 MgCl$_2$, 10 EGTA, and 10 HEPES (pH 7.2 adjusted with KOH); the extracellular solution contained (in mmol/L) 140 NaCl, 5 KCl, 1 CaCl$_2$, 1.25 MgCl$_2$, 10 glucose, and 10 HEPES, with pH adjusted to 7.4 using NaOH. The holding potential was −50 mV and was hyperpolarized to −110 mV for a duration of 500 ms. Then, whole-cell potassium currents were evoked by steps from −110 to 40 mV. To elicit delayed rectifier potassium currents (I_K), the potential was depolarized to −50 mV for a duration of 50 ms to deactivate the fast transient K$^+$ currents (I_A). The peak amplitude of I_A was measured. The amplitude of the I_K was measured with 300 ms latency. To record Kir2.1 currents, human embryonic kidney 293 (HEK-293) cells stably expressing hKir2.1 channels were clamped at a holding potential of −70 mV and a subsequent 500 ms test pulse to −140 mV. Data acquisition was achieved using a Axopatch 200B amplifier (Axon Instruments, Burlingame, CA, USA), and the signals were filtered at 2 kHz and digitized at 10 kHz with a Digidata 1440 A interface (Axon Instruments, Burlingame, CA, USA). Current and voltage recordings were made at room temperature.

MES-induced seizure assays
Electroconvulsions were generated by an alternating current (5.4 s stimulus duration, fixed current intensity of 4 mA, maximum stimulation voltage of 160 V) delivered via ear-clip electrodes through a physiological and pharmacological electronic stimulator

Geissoschizine methyl ether is a channel inhibitor
ZQ Xie et al.

631

(Ji-nan, China). The criterion for the onset of seizures was the presence of tonic-clonic generalized seizures that result in tonic hind-limb extension. The day before the experiment, mice who had experienced generalized tonic-clonic seizures induced by MES were selected to test the anticonvulsant effects of the compounds. Mice were separately administered GM (50, 80, or 100 mg/kg) or vehicle orally 1 h before electrical stimulation. Animals were thought to be protected from MES-induced seizures in the absence of the hind-limb tonic extension component of the seizure.

6-Hz corneal kindling epilepsy test

The acute 6-Hz seizure model was conducted as explained previously [30–32]. Corneal stimulation (0.2 ms rectangular pulses at 6 Hz for 3 s) was delivered by a constant-current device (CCU1) connected to an S48 Square Pulse Stimulator (Grass Technologies, West Warwick, RI, USA). A fixed current intensity of 32 mA was used to allow this data to be directly compared with data obtained in the aforementioned studies. Animals were randomly divided into three groups with ten animals in each group. Before stimulation, a drop of saline was applied to each eye to ensure optimal current conductivity. Seizures were characterized by a stunned or fixed posture often accompanied by rearing, forelimb clonus, and twitching. The duration of seizure activity was measured as the evaluation criterion for the protective effect of the compound. The animal was considered to be protected if it resumed its normal exploratory behavior within 7 s of the stimulation. In contrast, the compound had no protective effect if the above typical characteristic behaviors occurred in the animal after drug administration or if the duration of the seizure was longer than 7 s.

Data analysis

All patch clamp data were processed using Clampfit 10.4 (Molecular Device, Sunnyvale, CA, USA) and then analyzed using GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA). Voltage-dependent activation curves were fitted to the Boltzmann equation: $G = G_{min} + (G_{max}-G_{min})/(1 + \exp(V - V_{1/2})/S)$, where G_{max} is the maximum conductance, G_{min} is the minimum conductance, $V_{1/2}$ is the voltage to reach 50% of the maximum conductance, and S is the slope factor. Steady-state inactivation curves were constructed by plotting the normalized peak currents during the test pulses as a function of the prepulse potential. The data were fitted to the Boltzmann equation: $I/I_{max} = 1/\{1 + \exp[(V - V_{mid})/K_i]\}$, where I is the amplitude of peak current at each voltage, I_{max} is the maximal peak current value, V and V_{mid} are the prepulse potential and the half-maximal potential for inactivation, respectively, and K_i is the inactivation slope factor. Dose-response curves were fitted to a 3-parameter Hill equation: $Y = \text{Bottom} + (\text{Top-Bottom})/\left[1 + 10^{(X-\text{LogIC}_{50})}\right]$, where Bottom and Top are the minimum and maximum inhibition, respectively; X is the log of the concentration; Y is the value of $I_{Drug}/I_{Control}$; and IC_{50} is the drug concentration producing a half-maximum response. The data are presented as the means ± SEM, and the significance was estimated using paired two-tailed Student's t tests unless otherwise stated.

RESULTS

Inhibitory effects of GM on action potential firing in cultured hippocampal neurons

The effects of GM on action potential firing were individually examined in cultured mouse and rat hippocampal neurons using whole-cell current-clamp technology. For mouse neurons, under increasing doses of GM (1, 3, 10 and 30 μmol/L), the firing frequency decreased, with inhibition ratios of 45.3% ± 3.7%, 49.6% ± 5.1%, 82.3% ± 1.1% and 97.8% ± 0.5% (Fig. 1a, e, $n = 6$), respectively. GM also decreased the amplitude of single action
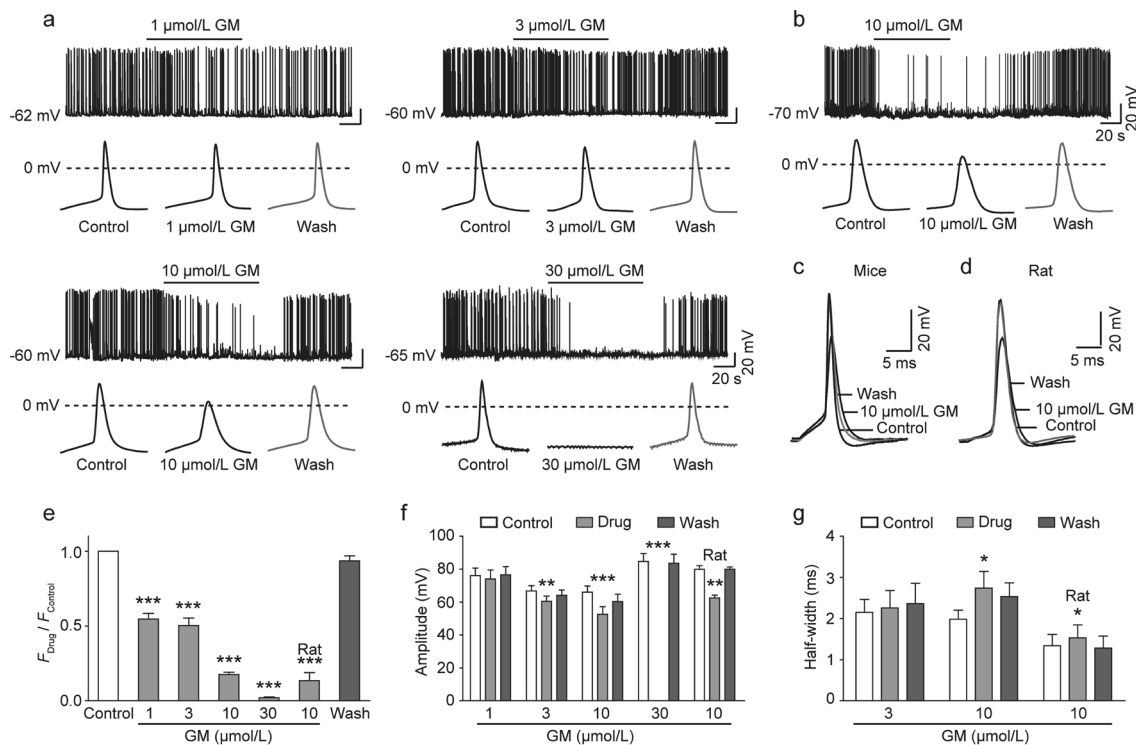

Fig. 1 GM inhibited the neuronal excitability of rat and mouse hippocampal neurons. Spontaneous firing and representative traces of action potentials recorded from cultured mouse **a** and rat **b** hippocampal neurons without or with GM at indicated concentrations. Representative traces showing the effects of 10 μmol/L GM on the half-width of action potential from cultured mouse **c** and rat **d** hippocampal neurons ($n = 6$). **e** Summarized data showing the inhibitory effects of GM on the firing frequency ($n = 6$). **f** Amplitudes of action potentials before and after the application of GM ($n = 6$). **g** Summarized data showing the effects of GM on the half-width of action potential ($n = 6$). Paired Student's t test, $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. control.

potentials in a dose-dependent manner. The amplitude was reduced by 9.5% ± 1.8%, 20.9% ± 3.2% and 100.0% in the presence of 3, 10 and 30 μmol/L of GM, respectively (Fig. 1a, f, $n = 6$). The inhibitory effects of GM on the firing frequency and the amplitude were reversible after washout (Fig. 1a, e, f, $n = 6$). Notably, in addition to a decrease in the amplitude, the duration of the action potential was also affected. In the presence of 10 μmol/L GM, the half-width of the action potential was prolonged to 2.7 ± 0.4 ms from 2.0 ± 0.2 ms in the controls (Fig. 1g, $n = 6$). Intriguingly, the resting membrane potential of the action potential did not change after GM perfusion (Fig. 1a). GM caused effects on action potential firing in rat neurons similar to those caused in mouse neurons, including a reduction in the firing frequency and the inhibition of the amplitude and prolongation of the duration, which supports the idea that there are no species differences in the activity of GM in mouse and rat neurons (Fig. 1d–g). Rat neurons were used in the following electrophysiological experiments unless otherwise stated.

Effects of GM on epilepsy-causing voltage-gated ion channels in hippocampal neurons

Na_V, Ca_V, and K_V channels form a superfamily of ion channels that allow hippocampal neurons to generate and propagate action potentials [33]. Mutations in the genes encoding these channels have been linked to various genetic epilepsy disorders, such as Dravet syndrome, benign familial neonatal-infantile epilepsy and episodic ataxia [34]. These channels have been proposed to be the targets of some AEDs, such as phenytoin, carbamazepine and LEV

[19]. To reveal the potential mechanisms underlying the antiepileptic activity of GM, the effects of GM on Na_V, Ca_V, and K_V channels were individually examined in acutely isolated rat hippocampal neurons.

GM inhibits Na_V currents. To elicit sodium currents, a 50 ms test pulse was applied to a holding potential of −90 mV, depolarizing it to −20 mV. The peak currents decreased as the concentration of GM increased in the perfusion solution (Fig. 2a). As shown in Fig. 2c, GM inhibited Na_V currents in a dose-dependent manner with an IC_{50} of 10.2 (9.3–11.2) μmol/L ($n = 7$). Thus, a concentration of 10 μmol/L was used in the following experiments. To understand how GM inhibits Na_V channels, the effects of GM on Na_V channel kinetics were further characterized. To plot the activation curves, Na_V currents were elicited by applying step pulses to a holding potential of −90 mV in 10 mV increments, ranging from −80 to +30 mV, for 50 ms. Representative current traces before and after the bath perfusion of 10 μmol/L GM are shown in Fig. 2d. Activation curves showed that no significant changes occurred in the half-activation voltage after the perfusion of GM (Fig. 2e). The values in the absence and presence of 10 μmol/L GM were −45.1 ± 1.0 mV and −42.7 ± 0.8 mV, respectively. Next, the influence of GM on steady-state inactivation was evaluated using a 500 ms conditioning pulse ramping from −120 to −20 mV in 10 mV increments, followed by a 20 ms test pulse at −20 mV (Fig. 2f). In contrast to its lack of effect on the activation curves, GM caused a hyperpolarizing shift in steady-state inactivation. The value of $V_{1/2}$ was shifted from −54.4 ± 0.5 mV
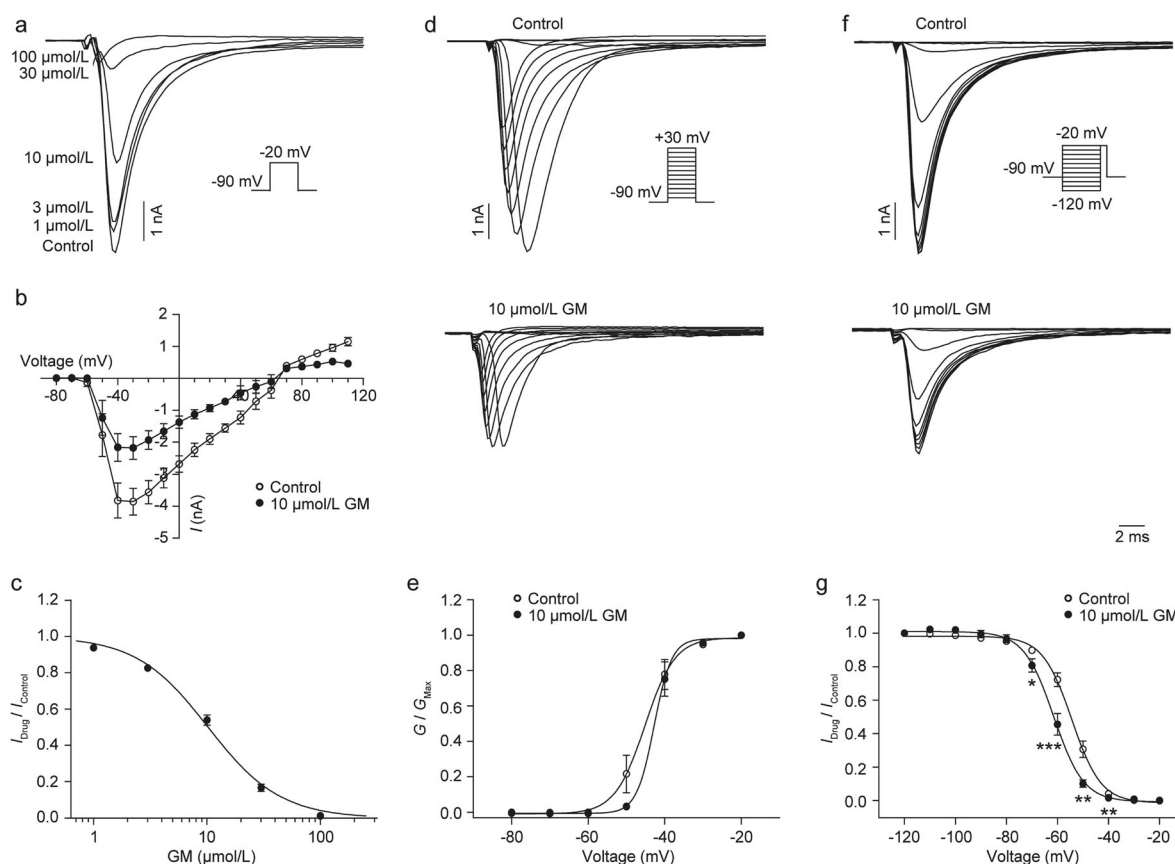

Fig. 2 Characterization of GM inhibition on Na_V currents in rat hippocampal neurons. a Representative Na_V current traces in the presence of GM at indicated concentrations. **b** Current–voltage relationship of Na_V currents with or without 10 μmol/L GM. **c** The dose-response curve of GM on Na_V currents. The IC_{50} value was 10.2 (9.3–11.2) μmol/L ($n = 7$). **d** Representative activation current traces of Na_V channels before and after 10 μmol/L GM. **e** Activation curves of Na_V currents before and after 10 μmol/L GM ($n = 6$). **f** Representative steady-state inactivation traces of Na_V currents before and after 10 μmol/L GM. **g** Steady-state inactivation curves of Na_V currents with or without 10 μmol/L GM ($n = 6$). Paired Student's t test, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. control.

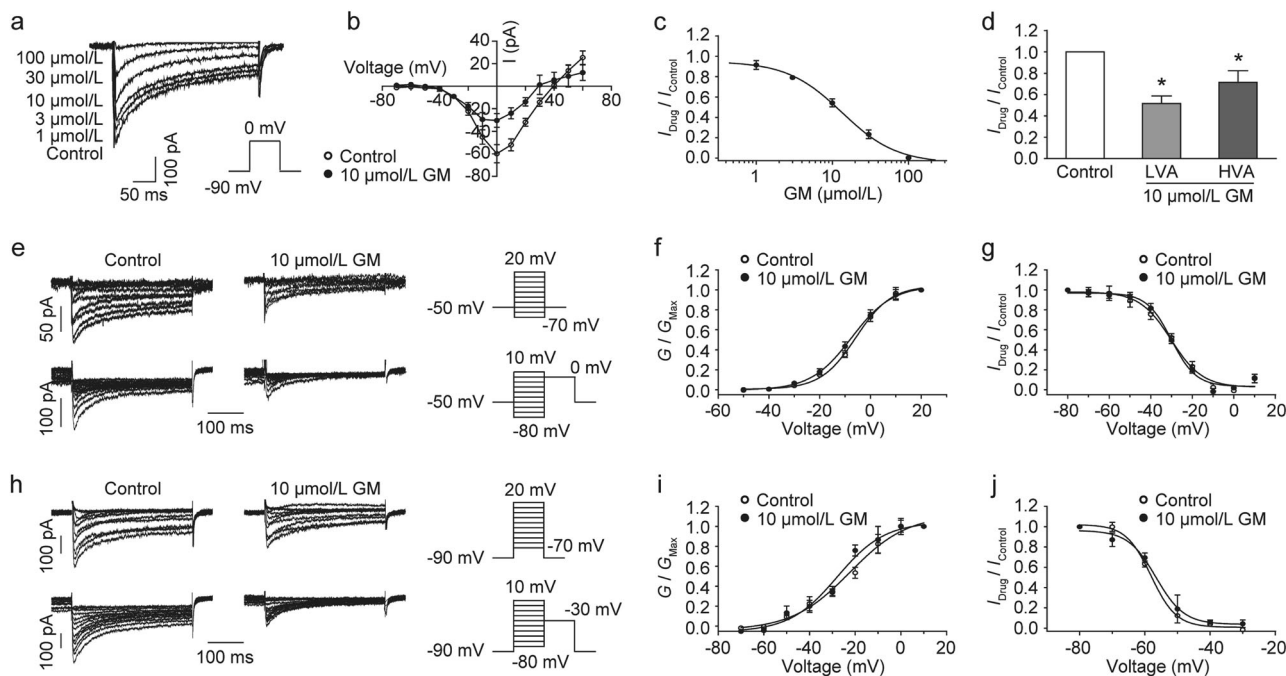Geissoschizine methyl ether is a channel inhibitor
ZQ Xie et al.

633

Fig. 3 **Inhibitory effects of GM on Ca_V currents in rat hippocampal neurons. a** Representative traces of total Ca_V currents measured under the increasing concentrations of GM. **b** Current-voltage relationship of HVA calcium currents in the absence and presence of 10 μmol/L GM. **c** The dose-response curve of GM for total Ca_V currents summarized from **a**. The fitted IC_{50} value was 13.3 (11.4–15.5) μmol/L ($n = 6$). **d** The inhibitory ratios of 10 μmol/L GM on HVA and LVA currents elicited at 0 mV ($n = 7$). **e** Original activation (top) and inactivation currents (bottom) of HVA channels. **f** Activation curves of HVA currents with or without 10 μmol/L GM ($n = 8$). **g** Inactivation curves of HVA currents with or without 10 μmol/L GM ($n = 9$). **h** Original activation (top) of total Ca_V currents and inactivation (bottom) of LVA currents. **i** Activation curves of LVA currents before and after the application of 10 μmol/L GM. The LVA currents were isolated by subtracting the current values of HVA from the values of total currents ($n = 5$). **j** Inactivation curves of LVA currents in the absence and presence of 10 μmol/L GM ($n = 5$). Paired Student's t test, *$P < 0.05$ vs. control.

in the control condition to -61.4 ± 0.6 mV in the presence of 10 μmol/L GM (Fig. 2g). These data showed that GM is an inhibitor of Na_V channels distributed in hippocampal neurons and preferentially affects channel inactivation.

GM inhibits Ca_V currents. Ca_V currents were elicited by a 300 ms test pulse from a holding potential of -90 mV to 0 mV. The peak currents decreased in a dose-dependent manner in the presence of GM, with an IC_{50} of 13.3 (11.4–15.5) μmol/L (Fig. 3a, c, $n = 6$). The Ca_V currents in the isolated rat hippocampal neurons could be separated into two categories: high-voltage-activated (HVA) and low-voltage-activated (LVA) calcium currents [35]. With two protocols, HVA and LVA calcium currents could be evoked in different proportions, allowing a separation of currents based on their biophysical characteristics [35]. From a holding potential of -90 mV, both LVA and HVA components were evoked by depolarizing stimulations, whereas the majority of HVA currents were evoked when using a holding potential of -50 mV. Following the subtraction of the HVA currents, LVA currents were isolated. After perfusion with 10 μmol/L GM, the evoked HVA and LVA currents were dramatically reduced by $28.5\% \pm 10.9\%$ and $48.4\% \pm 6.9\%$, respectively (Fig. 3d, $n = 7$). These data demonstrated that GM preferentially inhibits LVA currents. Kinetic analysis showed that the activation curves of both HVA and LVA currents did not exhibit a remarkable change after 10 μmol/L GM treatment (Fig. 3f, i). The effects of GM on the inactivation kinetics of HVA and LVA currents were then examined individually. To induce the inactivation of HVA currents, neurons were clamped at -50 mV, and then, a 300 ms prepulse ramping from -80 to $+10$ mV in 10 mV increments was applied, followed by a 300 ms test pulse to 0 mV. No significant shift in the inactivation curves of HVA currents was observed, despite the peak currents being significantly smaller after the

application of 10 μmol/L GM (Fig. 3e, g, $n = 9$). The inactivation of LVA currents was examined using a range of depolarization prepulses (from -80 to 10 mV in 10 mV increments) from a holding potential of -90 mV, followed by a 300 ms test pulse to -30 mV. The lack of an effect on LVA current inactivation was similar to that seen for HVA inactivation (Fig. 3h, j, $n = 5$). Taken together, GM dose-dependently inhibits Ca_V currents in hippocampal neurons without observable effects on channel kinetics.

GM inhibits K_V currents. Potassium currents in hippocampal neurons are composed of a rapidly inactivating component and a noninactivating, or only slowly inactivating, component, which are named the transient outward potassium current (I_A) and delayed rectifier potassium current (I_K), respectively. These two distinct components could be distinguished by applying voltage steps from a holding potential of -50 mV, at which I_A was almost completely inactivated and I_K remained. I_A could be isolated by subtracting I_K from the total outward current. According to these electrophysiological properties, the effects of GM on K_V currents were examined (Fig. 4a), revealing IC_{50} values of 1.6 (1.3–2.0) μmol/L and 50.0 (30.3–82.3) μmol/L for I_K and I_A, respectively (Fig. 4b, $n = 5$). The effects of GM on inwardly rectifying potassium (Kir) channels that lack voltage-sensing domains (VSD) were then investigated. In contrast to the potent inhibition of K_V currents, we found that 10 μmol/L GM did not inhibit human Kir2.1 channels heterologously expressed in HEK-293 cells (Fig. 4c, d). These data showed that GM is an inhibitor of K_V channels.

Effects of GM on ligand-gated ion channels associated with epilepsy in hippocampal neurons
In addition to voltage-gated ion channels, some ligand-gated ion channels, such as ionotropic nACh, GABAergic, and glutamatergic
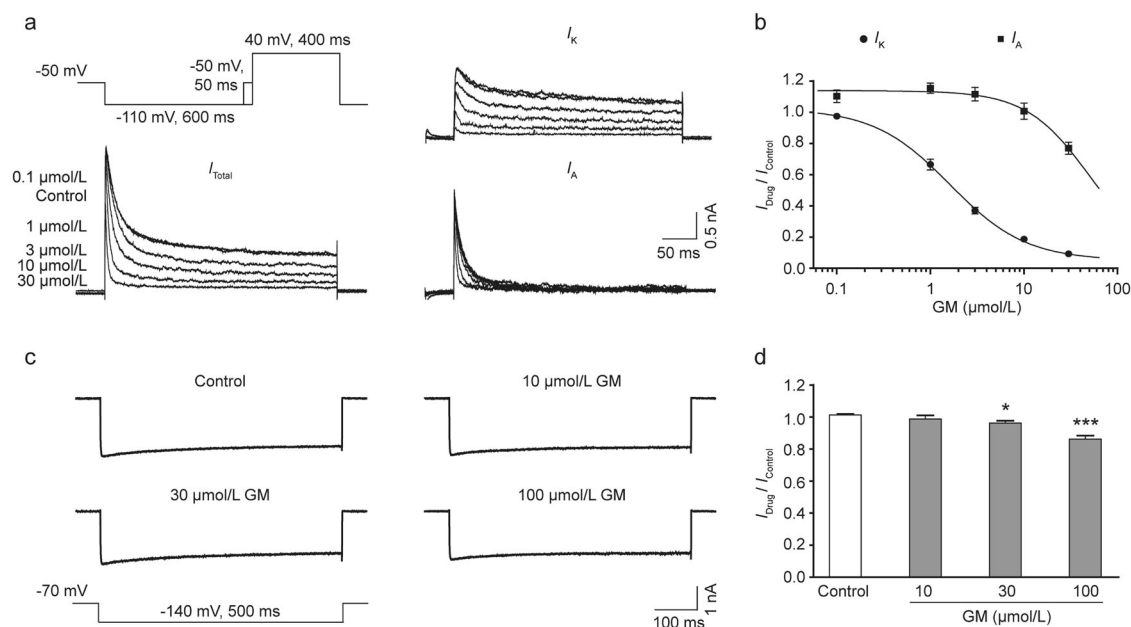
Geissoschizine methyl ether is a channel inhibitor
ZQ Xie et al.

634

Fig. 4 **Inhibitory effects of GM on the potassium currents in rat hippocampal neurons. a** Typical traces of the transient outward potassium currents I_A and the delayed rectifier potassium currents I_K in the presence of GM at indicated concentrations. **b** The dose-response curve of GM on I_K and I_A currents. The IC_{50} values were 1.6 (1.3–2.0) μmol/L and 50.0 (30.3–82.3) μmol/L, respectively ($n = 5$). **c** Inhibition of hKir2.1 channels heterologously expressed in HEK-293 cells by 10, 30, and 100 μmol/L GM. **d** Summarized data showing the inhibitory effects of GM (10, 30, and 100 μmol/L) on the Kir2.1 currents, the inhibition ratios were 1.3% ± 2.2% ($n = 5$), 3.8% ± 1.5% ($n = 7$) and 13.9% ± 2.2% ($n = 10$), respectively. Paired Student's t test, *$P < 0.05$, ***$P < 0.001$ vs. control.

systems, have also been associated with genetic epilepsy disorders [34]. The potential influences of GM on these three types of epilepsy-associated ligand-gated ion channels were individually examined in acutely isolated or cultured rat hippocampal neurons.

GM inhibits nACh receptors. To investigate the influence of GM on nACh receptors, cultured rat hippocampal neurons were used. As shown in Fig. 5a, a typical inward current was evoked by a rapid pulse of acetylcholine (1 mmol/L for 5 s) and then rapidly returned to baseline after the end of acetylcholine application. We found that acetylcholine-evoked currents could be completely inhibited by 30 μmol/L GM (Fig. 5a). A dose-response study further revealed an IC_{50} of 1.3 (1.1–1.6) μmol/L (Fig. 5b, $n = 4$).

Influences of GM on ionotropic GABA and glutamate receptors. Ionotropic glutamate receptors expressed in rat hippocampal neurons can be separated into three categories: NMDA, AMPA, and KA receptors. To activate each of these three types of glutamate receptors or to activate GABA receptors, acutely isolated rat hippocampal neurons were clamped at −60 mV and then exposed to their ligands individually at the indicated concentrations. No remarkable changes were observed for currents mediated by glutamate or GABA receptors in the presence of 30 μmol/L GM, and the modulation was very weak even after exposure to concentrations as high as 100 μmol/L GM (Fig. 5c–f, $n = 4$). These data argued against these ligand-gated channels contributing to the antiepileptic activity of GM. Taken together, GM potently inhibited nACh receptors but not ionotropic glutamate and GABA receptors.

Antiepileptic activity of GM in MES and 6-Hz seizure models
The potent inhibitory effects of GM on channels involved in epilepsy and neuronal firing in hippocampal neurons propelled us to evaluate its antiepileptic activities in other epilepsy models. First, a primary antiepileptic assay was performed in MES-induced seizure model mice. The incidence of hind-limb extension was

recorded. If extension did not occur during the assay, the animal was thought to be protected from MES-induced seizure [36]. In contrast to that in the vehicle group, tonic hind-limb extension decreased in mice orally administered GM. Protection against MES in mice was achieved at a rate of 0%, 10%, 50% and 80% when vehicle or 50, 80, and 100 mg/kg of GM was administered, respectively (Table 1, $n = 10$). Dose-dependent relationship analysis revealed that the ED_{50} of GM was 78.9 (60.3–103.3) mg/kg.

Different from the MES model, which represents generalized tonic-clonic seizures, the 6-Hz test is commonly regarded as a model for treatment-resistant seizures. To explore whether GM has broad-spectrum antiepileptic activity, the antiepileptic activity of GM was then evaluated in the 6-Hz psychomotor seizure model. LEV, an inhibitor of SV2A and Ca_V channels, was used as a positive control in the experiment [37]. After the oral administration of 100 mg/kg GM 60 min prior to stimulation, 70% of the tested mice were protected from 6-Hz-induced seizures. The vehicle group exhibited seizures, whereas no seizures were observed in the mice administered 100 mg/kg LEV (Table 2). The protective effects of GM in the 6-Hz model were comparable to those seen in the MES model. GM not only dose-dependently relieves generalized tonic-clonic seizures in the MES model but also reduces treatment-resistant seizures in the 6-Hz model, suggesting that GM is a promising antiepileptic drug candidate.

DISCUSSION
The antiepileptic activity of GM has been previously observed in a glutamate-induced seizure mouse model [6]. Consistent with its antiepileptic activity, GM also exhibited neuroprotective effects against glutamate-induced cell death in multiple types of neurons [6–10]. Most recently, GM was found to reduce ROS generation in mitochondria and enhance neuron glycolysis in a glutamate-induced neuron toxicity model, suggesting an indirect neuroprotective mechanism [11]. In the present study, we found that GM inhibited voltage-gated channels (Na_V, Ca_V, and K_V) and a ligand-gated channel (nACh) expressed in neurons, suggesting that the

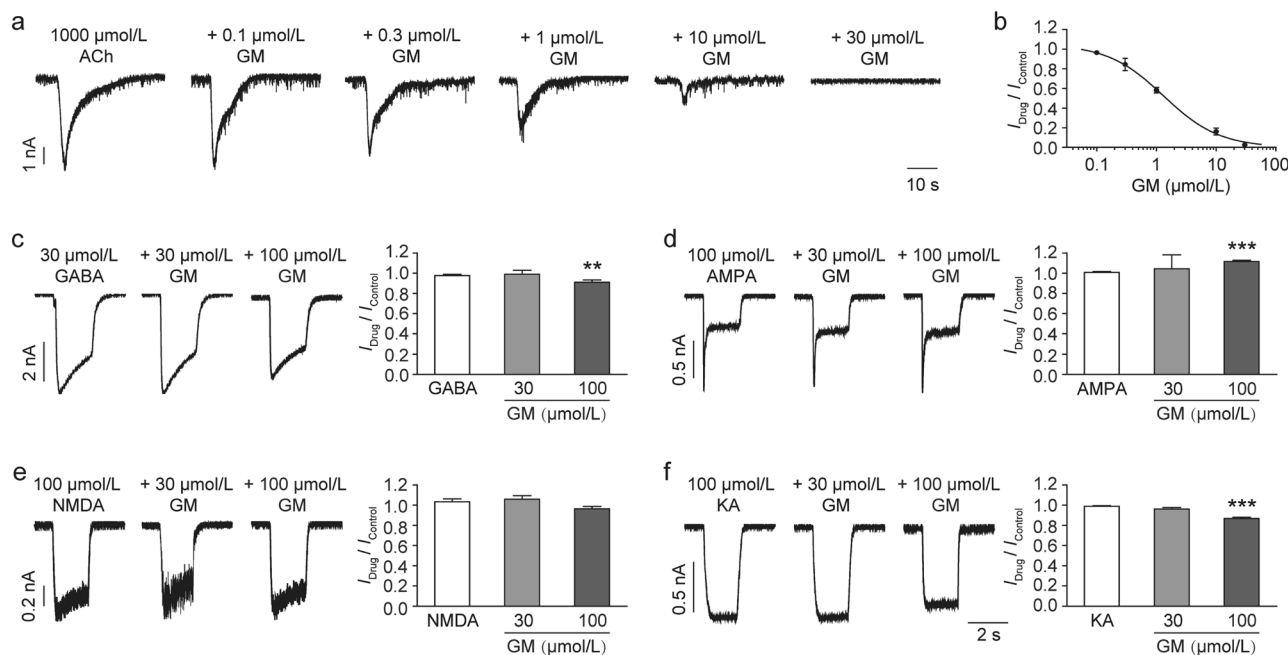Geissoschizine methyl ether is a channel inhibitor
ZQ Xie et al.

635

Fig. 5 Effects of GM on ligand-gated receptors associated with epilepsy in rat hippocampal neurons. a Representative acetylcholine-evoked current traces recorded from cultured rat hippocampal neurons without or with GM at indicated concentrations. **b** Dose-response curve of GM on nACh receptors. The IC$_{50}$ value was 1.3 (1.1–1.6) µmol/L ($n = 4$). **c–f** Representative traces of GABA (**c**), AMPA (**d**), NMDA (**e**) and KA (**f**) were shown. GM did not affect glutamate and GABA receptors in hippocampal neurons. The rat hippocampal neurons were clamped at −60 mV and the currents were induced by 2 s exposure to specific ligands at the indicated concentrations ($n = 4$). Paired Student's t test, **$P < 0.01$, ***$P < 0.001$ vs. control.

Table 1. Antiepileptic activities of GM in the MES-induced epilepsy mouse model ($n = 10$).

	Dosage (mg/kg)	Mortality n_{Dead}/n_{Test}	Tonic hind-limb extension $n_{Tonic-clonus}/n_{Test}$	Protection rate (%)
Vehicle	–	0/10	10/10	0
GM	50	0/10	9/10	10
	80	0/10	5/10	50
	100	0/10	2/10	80
TPM	40	0/10	0/10	100

Vehicle was 5% DMSO + 95% Tween-80; TPM (Topiramate) was used as a positive control

Table 2. Antiepileptic activity of GM in the 6-Hz corneal kindling epilepsy mouse model ($n = 10$).

	Dosage (mg/kg)	Mortality n_{Dead}/n_{Test}	Tonic hind-limb extension $n_{Tonic-clonus}/n_{Test}$	Protection rate (%)
Vehicle	–	0/10	10/10	0
GM	100	0/10	3/10	70
LEV	100	0/10	0/10	100

Vehicle were 5% DMSO + 95% Tween-80; LEV (Levetiracetam) was used as a positive control

antiepileptic activity of GM may arise through its modulation of multiple neuronal channels.

GM dose-dependently inhibited Na$_V$ currents with an IC$_{50}$ of 10.2 (9.3–11.2) µmol/L (Fig. 2c). During action potential firing, Na$_V$ channels are thought to mediate the explosive and regenerative inward current during the rising phase [33]. Consistent with its inhibition of Na$_V$ channels, the amplitudes of action potentials were indeed reduced after the administration of 10 µmol/L GM (Fig. 1f). A kinetics study showed that 10 µmol/L GM did not affect the voltage-dependent activation curve. In contrast, under identical experimental conditions, GM shifted the inactivation curve in the negative direction (the half-maximal inactivation changed from −54.4 mV in the control group to −61.4 mV after 10 µmol/L GM administration) (Fig. 2g), which is in line with the notion that increasing the proportion of inactivated Na$_V$ channels would reduce neuronal excitability [38]. Indeed, action potential firing in hippocampal neurons decreased in a dose-dependent manner after the administration of GM (Fig. 1a, b, g). Our study supported the idea that the inhibition of Na$_V$ channels in hippocampal neurons promotes GM-produced antiepileptic activity.

GM dose-dependently inhibited calcium currents with an IC$_{50}$ of 13.3 (11.4–15.5) µmol/L (Fig. 3c). Consistent with our finding in neurons, 10 µmol/L GM has been shown to be able to relax the calcium channel agonist Bay K8644- or Ca^{2+}-induced contractions in isolated rat aorta strips [39], suggesting an inhibitory effect of GM on Ca$_V$ channels expressed in vascular tissues. The inhibitory effect of GM on the LVA calcium current was stronger than that on the HVA calcium current. After the administration of 10 µmol/L GM, the reduction in LVA currents was 48.4% ± 6.9%, whereas the reduction in HVA was only 28.5% ± 10.9% (Fig. 3d). Considering the key roles of LVA during the generation of neuronal burst firing, a much more potent inhibition of LVA currents might be beneficial to ameliorate hyperexcitability disorders, including epilepsy [40]. Therefore, it is reasonable to speculate that the inhibitory effects of GM on Ca$_V$ currents, particularly on LVA currents, contribute to the inhibition of firing activity in hippocampal neurons and the antiepileptic activity of GM in vivo.

In addition to inhibiting Na$_V$ and Ca$_V$ currents, GM was found to inhibit neuronal K$_V$ currents (I_K and I_A). The IC$_{50}$ values for I_K and I_A

Geissoschizine methyl ether is a channel inhibitor
ZQ Xie et al.

636

were 1.6 (1.3–2.0) μmol/L and 50.0 (30.3–82.3) μmol/L, respectively (Fig. 4b). During action potential firing, the outward potassium currents mediated by K_V channels terminate the action potential by repolarizing the membrane potential [33]. Therefore, the inhibition of K_V channels may prolong the duration of an action potential. Consistently, the duration of action potentials recorded in hippocampal neurons was indeed significantly longer after perfusion with 10 μmol/L GM (Fig. 1c, d, g). In addition, the inhibition of K_V initially increases neuron firing frequency [41, 42]. However, we did not observe an increase in the firing frequency after GM administration. We already know that in addition to inhibiting K_V, GM shows strong inhibitory activity on Na_V and Ca_V channels (Figs. 2 and 3), resulting in the suppression of action potential firing. Mutations in human K_V genes, such as $K_V7.2$ and $K_V2.1$ mutations, that result in a reduction in or the loss of channel activity cause human epilepsy [34]. Activators capable of augmenting these K_V functions have been indicated to be effective in the treatment of human epilepsy [36, 43]. However, the inhibition of K_V channels, particularly neuronal I_K, has also been thought to contribute to the antiepileptic effects of AEDs. For example, LEV, a first-line AED, reduced the I_K but not the I_A in hippocampal neurons of rats and guinea pigs, resulting in a prolonged single action potential duration and the reduced generation of the repeated action potentials, which is thought to be an important mechanism underlying the antiepileptic effects of LEV [44]. Interestingly, similar to LEV, GM also preferentially inhibited I_K rather than I_A. However, whether and how the GM inhibition of K_V channels indeed contributes to its antiepileptic effects requires further investigation. It is worth noting that GM did not affect Kir2.1 channels (Fig. 4c, d), another major type of potassium channel expressed in neurons. Unlike I_K and I_A channels, both of which contain four VSD surrounding a central pore domain, Kir2.1 channels lack a VSD [45], highlighting the importance of the VSD in the effects of GM on K_V channels.

In terms of ligand-gated ion channels associated with epilepsy, GM potently inhibited ACh-induced currents, with an IC_{50} of 1.3 (1.1–1.6) μmol/L, but did not affect GABA, AMPA, NMDA, or KA-induced currents (Fig. 5b–f), which distinguishes GM from AEDs targeting either GABA or glutamate receptors, such as diazepam, phenobarbital, and perampanel [19]. nACh receptors, which are broadly expressed in the neuron soma, dendrites, preterminal axon regions, and axon terminals, play an important role in modulating neuron excitability and neurotransmission [25, 46]. It has been reported that nACh receptors can modulate the release of neurotransmitters such as glutamate, GABA, dopamine and serotonin [46–48]. The reduction in neuron excitability and neurotransmission through the inhibition of nACh receptors is thought to be beneficial in the treatment of epilepsy [19]. Numerous preclinical studies have revealed that antagonists of nACh receptors can relieve seizures in various models [28]. At relatively low concentrations, some AEDs, such as carbamazepine, oxcarbazepine and lamotrigine, inhibit nACh receptors [28]. Among these AEDs, carbamazepine has been used to treat human ADNFLE, a genetic form of epilepsy caused by mutations in nACh receptors [28]. Collectively, we speculate that the inhibition of nACh receptors may contribute to the antiepileptic effects of GM.

It is common that one AED will modulate multiple channels. For example, the potential targets of topiramate, a first-line AED, include Na_V and Ca_V channels and ligand-gated GABA and glutamate channels; the targets of lamotrigine and zonisamide include Na_V, Ca_V and GABA channels [19]. Considering that GM may act on multiple targets, we speculated that GM may also exhibit a broad antiepileptic treatment spectrum, similar to lamotrigine and zonisamide. Indeed, in addition to the glutamate-induced seizure model, GM also showed excellent antiepileptic activity in both MES and 6-Hz seizure models (Tables 1 and 2). Rhynchophylline, another major component isolated from UR, also exhibited antiepileptic activity. However, in contrast to GM, rhynchophylline showed inhibitory effects on NMDA-induced currents [49]. Depressed locomotor activity is a potential side effect of UR extractions, such as isorhynchophylline and perhaps rhynchophylline [50]. However, no significant decrease in locomotor activity was observed after the administration of GM at concentrations as high as 60 mg/kg, suggesting the high tolerance of GM. Finally, it is interesting to note that of the two active antiepileptic components of UR, the blood–brain barrier permeability rate of GM is nearly five fold higher than that of rhynchophylline [51]. Therefore, further structure-activity relationship studies of GM-based derivatives might be considered as an appropriate approach for novel antiepileptic drug development.

AUTHOR CONTRIBUTIONS
ZBG, CGH and XMX designed and conceived the experiments; ZQX and YMZ performed the electrophysiological experiments; LZ performed the animal experiments; XQC provided guidance on electrophysiological techniques; XTT isolated GM for the electrophysiological experiments; all authors analyzed the data; and ZQX, YMZ, and ZBG wrote the paper.

ADDITIONAL INFORMATION
Competing interests: The authors declare no competing interests.

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Geissoschizine methyl ether is a channel inhibitor
ZQ Xie et al.

637

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