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ARTICLE

Schisandrin B, a dual positive allosteric modulator of $GABA_A$ and glycine receptors, alleviates seizures in multiple mouse models

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Epilepsy is a prevalent and severe neurological disorder and approximately 30% of patients are resistant to existing medications. It is of utmost importance to develop alternative therapies to treat epilepsy. Schisandrin B (SchB) is a major bioactive constituent of *Schisandra chinensis (Turcz.) Baill* and has multiple neuroprotective effects, sedative and hypnotic activities. In this study, we investigated the antiseizure effect of SchB in various mouse models of seizure and explored the underlying mechanisms. Pentylenetetrazole (PTZ), strychnine (STR), and pilocarpine-induced mouse seizure models were established. We showed that injection of SchB (10, 30, 60 mg/kg, i.p.) dose-dependently delayed the onset of generalized tonic-clonic seizures (GTCS), reduced the incidence of GTCS and mortality in PTZ and STR models. Meanwhile, injection of SchB (30 mg/kg, i.p.) exhibited therapeutic potential in pilocarpine-induced status epilepticus model, which was considered as a drug-resistant model. In whole-cell recording from CHO/HEK-239 cells stably expressing recombinant human $GABA_A$ receptors ($GABA_A$Rs) and glycine receptors (GlyRs) and cultured hippocampal neurons, co-application of SchB dose-dependently enhanced GABA or glycine-induced current with EC_{50} values at around 5 μM, and application of SchB (10 μM) alone did not activate the channels in the absence of GABA or glycine. Furthermore, SchB (10 μM) eliminated both PTZ-induced inhibition on GABA-induced current (I_{GABA}) and strychnine (STR)-induced inhibition on glycine-induced current ($I_{glycine}$). Moreover, SchB (10 μM) efficiently rescued the impaired $GABA_A$Rs associated with genetic epilepsies. In addition, the homologous mutants in both GlyRs-α1(S267Q) and $GABA_A$Rs-α1(S297Q)β2(N289S)γ2L receptors by site-directed mutagenesis tests abolished SchB-induced potentiation of I_{GABA} and $I_{glycine}$. In conclusion, we have identified SchB as a natural positive allosteric modulator of $GABA_A$Rs and GlyRs, supporting its potential as alternative therapies for epilepsy.

Keywords: Schisandrin B; epilepsy; $GABA_A$ receptors; glycine receptors

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INTRODUCTION

Epilepsy is a prevalent and severe neurological disorder that impacts almost 1% of the world's population. Epilepsy is a condition characterized by recurrent seizures that occur without any apparent trigger. These seizures are usually caused by abnormal activity in the neurons of central nervous system, which is often the result of an imbalance between excitatory and inhibitory neurotransmission [1]. Despite the numerous antiseizure medications (ASMs) in recent decades, around 30% of patients are still not responsive to the treatments [2]. Furthermore, the treatment of epilepsy needs a longer-term utilization of ASMs, which leads to various adverse effects such as allergic reactions, hepatotoxicity, and excessive depression of the central nervous system (CNS) [3]. These reactions impose a significant burden on both patients and society. Therefore, it is of utmost importance to develop alternative therapies to treat epilepsy.

$GABA_A$ receptors ($GABA_A$Rs), the major inhibitory receptors in the adult mammalian CNS, are important targets of epilepsy [4], and also play significant roles in the development of various neurological disorders, such as migraine, neuropathic pain, and depression [5, 6]. The majority of $GABA_A$Rs consist of two α subunits, two β subunits, and either a γ or δ subunit. Among them, γ-containing $GABA_A$Rs mainly mediate phasic inhibitory synaptic transmission, while δ-containing $GABA_A$Rs mediate tonic extrasynaptic inhibition [7]. Upon activation, the influx of chloride ions into the neuron hyperpolarizes and/or stabilizes the membrane potential and thus inhibits the neuronal excitability [8]. Glycine receptors (GlyRs), the other major inhibitory receptors in CNS, play important roles in maintaining the normal balance between excitation and inhibition in the brain [9–11]. Activation of GlyRs suppresses neuronal excitation and seizure-like events in the entorhinal cortex and hippocampus of rats [9–11]. The antagonists of these receptors, pentylenetetrazole (PTZ, blocking $GABA_A$Rs) or strychnine (STR, blocking GlyRs), have often been used to generate seizure models [12], further demonstrating the crucial role of $GABA_A$Rs and GlyRs in the development and progression of epilepsy. Mutations that cause dysfunction of $GABA_A$Rs and GlyRs also result in seizure-like symptoms [13–15].

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Schisandra chinensis fructus, the dried ripe fruit of *Schisandra chinensis (Turcz.) Baill*, is a well-known traditional Chinese medicine [16]. The extracts from *Schisandra chinensis fructus* have been reported to possess a variety of pharmacological effects on CNS disorders. These effects include neuroprotection [17], improvement of learning and memory [17], sedative-hypnotic [18], anxiolytic [19], and antidepressant effects [20]. Schisandrin B (SchB) (chemical structure was shown in Fig. 1a) is the most abundant dibenzocyclooctadiene lignan from *Schisandrae chinensis*. Due to its high availability in the brain after administration [21, 22], SchB possessed multiple neuroprotective effects [23], sedative and hypnotic properties [24]. However, there have been no reports for the effects of SchB on epilepsy. Coincidently, the present study identified SchB as a natural positive allosteric modulator of $GABA_A$Rs and GlyRs, which indicates its potential use in epilepsy. Thus, we investigated the antiseizure effects of SchB on various mouse models of seizure and explored the underlying mechanism.

MATERIALS AND METHODS

Materials

Schizandrin B was purchased from Mreda Technology Inc. (Beijing, China). Pentylenetetrazole and etomidate were purchased from Shanghai Macklin Biochemical Co., Ltd (Shanghai, China). Strychnine hydrochloride was purchased from Shanghai Aladdin Biochemical Co., Ltd (Shanghai, China). Pilocarpine hydrochloride was purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). Diazepam, GABA, sodium valproate, pentobarbital, and Poly-*D*-lysine were purchased from Sigma-Aldrich (Shanghai, China). Atropine, glycine, and NMDA were purchased from Topscience Co., Ltd. (Shanghai, China). *L*-Glutamate was purchased from Abcam (Cambridge, UK).

Animals

In total, 80 male CD-1 mice (weighing 25–30 g and aged 4–5 weeks), 24 male C57BL/6 N mice (weighing 20–22 g and aged 6–8 weeks) and 6 Sprague–Dawley rat pups (postnatal day 1, P1) were obtained from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). Animals were housed in plastic cages and given free access to standard food and water under laboratory conditions with a temperature range of 22–24 °C, humidity between 50% and 60%, and a 12 h light/dark cycle. Animal care and experimental procedures have been approved by the Institutional Animal Care and Welfare Committee of the Chinese Academy of Medical Sciences & Peking Union Medical College. Animals were randomly assigned to treatment or control groups. All the behavioral experiments were performed by experimenters who were blinded to the groups and treatments.

Construction of mutated receptor subunits

The following point mutations were introduced into human $GABA_A$ receptor cDNA α1 (gene accession number NM_000806.5): α1(H129R), α1(D219N), α1(G251S), and α1(S297Q); β2 (gene accession number NM_000813.3): β2(N289S); γ2L (gene accession number NM_198903.2): γ2L(R177G) and γ2L(P322A), and human glycine receptor cDNA α1 (gene accession number NM_000171.4): α1(S267Q), α1(S296A), α1(F380A) and α1(K385A). These mutations were generated using the Hieff Mut™ Site-Directed Mutagenesis Kit (YEASEN Biotechnology Co., Ltd., Shanghai, China). The authenticity of the DNA sequence at the mutation sites was confirmed by Sanger sequencing (Genewiz, Tianjin, China).

Cell cultures and transfections

Cell culture of cell lines. T-REx™-CHO cells stably expressing human α1β2γ2L and α2β2γ2L $GABA_A$ receptors were cultured in DMEM/F12 nutrient mixture supplemented with 10% FBS, blasticidin (10 μg·ml^{-1}), hygromycin (300 μg·ml^{-1}), zeocin (100 μg·ml^{-1}) and puromycin (1 μg·ml^{-1}). Flp-In™ T-REx™ 293 cells stably expressing human α4β3δ and α6β3δ $GABA_A$ receptors were cultured in DMEM supplemented with 10% FBS, blasticidin 10 μg·ml^{-1}, hygromycin 100 μg·ml^{-1}, zeocin 100 μg·ml^{-1} and puromycin 0.2 μg·ml^{-1}. Flp-In™-CHO cells stably expressing human α1, α2, and α3 glycine receptor subtypes were cultured in DMEM/F12 nutrient mixture supplemented with 10% FBS and 300 μg·ml^{-1} of hygromycin. T-REx™-CHO and Flp-In™-CHO cells were cultured in DMEM/F12 nutrient mixture supplemented with 10% FBS. These cells were utilized to generate recombinant mutant receptors (α1β2γ2L $GABA_A$Rs and α1 GlyRs). HEK-293 cells were cultured in DMEM supplemented with 10% FBS and were used to express human AMPA (GluA1 and GluA2) and NMDA receptors (NR1/NR2A and NR1/NR2B).

Transfections. All transfections were performed by using Lipofectamine® LTX & Plus Reagent (Invitrogen). The enhanced green fluorescent protein (EGFP) was co-transfected with the genes of interest to facilitate the visualization of the transfected cells. The cDNA combinations were prepared as follows: for AMPA receptors, the ratio of GluA1 (or GluA2) to EGFP was 1:0.1; for NMDA receptors, the ratio of NR1: NR2A (or NR2B): EGFP was 1:1:0.2; for mutant α1β2γ2L $GABA_A$ receptors, the ratio of α1: β2: γ2L: EGFP was 1:1:3:0.5; for mutant α1 glycine receptors, the ratio of α1: EGFP was 1:0.1. Transfected cells were plated on Poly-*D*-Lysine-coated glass coverslips and were used for electrophysiological recordings 24–48 h after transfection.

Primary cultures of hippocampal neurons

Hippocampal neurons were extracted from Sprague-Dawley rat pups on postnatal day 1. Briefly, the rat pups were decapitated, and their hippocampi were dissected out and minced into pieces ~1 mm^3 in size using scissors in HBSS on ice. The tissue was then digested in HBSS containing 0.125% trypsin (Gibco, USA) at 37 °C for 30 min. The cells were dissociated by undergoing three successive trituration and sedimentation steps in isolation buffer containing DNase I. The tissue debris was eliminated by filtering the cell suspension through a sterile cell strainer. The cell suspension was centrifuged at 1000 r/min for 5 min at room temperature. The pellet was then resuspended in plating medium, comprised of Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 10% horse serum (HS), 1% penicillin/streptomycin (100 units·ml^{-1} and 100 μg·ml^{-1}, respectively), and 2 mM *L*-glutamine (Sigma-Aldrich, USA). For whole-cell voltage-clamp recordings, hippocampal neurons were plated onto glass coverslips (12 mm) coated with 0.1 mg·ml^{-1} poly-*D*-lysine in 35-mm culture dishes at a density of 1×10^6 cells per dish. The cells were then incubated in a humidified 5% CO_2 incubator at 37 °C. Four hours later, the plating media was replaced with maintenance media. The maintenance media was composed of Neurobasal medium (Gibco, USA) supplemented with 2% B27 (Gibco, USA) and 2 mM *L*-glutamine (Sigma-Aldrich, USA). The culture medium was replaced with half of maintenance media every three days. All the experiments were conducted between day 10 and day 14 in vitro.

Whole-cell voltage-clamp recordings

All the whole-cell patch clamp recordings were performed at a temperature of 24 ± 2 °C using a HEKA EPC-10 amplifier (HEKA Elektronik GmbH, Germany). The currents were filtered using a low-pass filter with a cutoff frequency of 2 kHz. The standard external solution contains (in mM): 140 NaCl, 3 KCl, 1.5 $MgCl_2$, 2 $CaCl_2$, 10 HEPES, and 10 Glucose, with a pH of 7.40 adjusted with NaOH. For GABA or glycine-evoked currents in recombinant $GABA_A$ and glycine receptors, the pipette solution contained (in mM): 145 KCl, 1 $MgCl_2$, 5 EGTA, 5 Mg-ATP, 10 HEPES, adjusted to a pH of 7.3 with KOH. After filled with internal solution, the resistance of pipette was 1.5–2.5 MΩ. To measure GABA- or glycine-evoked currents in hippocampal neurons, the pipette

solution contained the following components (in mM): 145 CsCl, 1 $MgCl_2$, 5 EGTA, 5 Mg-ATP, and 10 HEPES, adjusted to a pH of 7.3 with CsOH, the resistances of pipette tip were 3.0–4.0 MΩ. For the current recording of AMPA receptors, the pipette solution contained (in mM): 140 CsF, 10 NaCl, 1 EGTA, and 10 HEPES, adjusted to a pH of 7.4 with NaOH. For the current recording of NMDA receptors, the Mg^{2+} free external solution was used (in mM): 150 NaCl, 5 KCl, 2 $CaCl_2$, 10 HEPES, and 10 glucose, adjusted to a pH of 7.4 with NaOH. The pipette solution contained (in mM): 145 KCl, 1 $MgCl_2$, 5 EGTA, 5 Mg-ATP, and 10 HEPES, adjusted to a pH of 7.3 with KOH. To record the ligand-gated ion channels, membrane potential was held at −60 mV. GABA (or glycine) currents were elicited by applying GABA (or glycine) for 5 s to cells. AMPA currents were elicited by co-applying 1 mM glutamate and 100 μM cyclothiazide for 5 s to cells. NMDA currents were elicited by applying a solution containing 100 μM NMDA and 10 μM glycine for 5 s to cells.

Pentylenetetrazole (PTZ) -induced seizures

The animal model was adapted from the method reported by Mandhane et al. [25]. Male CD-1 mice (25–30 g; $n = 8$ per group) were treated with different drugs via intraperitoneal injections (i.p.). The animals were administered SchB (10, 30, or 60 mg·kg^{-1}), sodium valproate (positive control, 300 mg·kg^{-1}), or a vehicle solution (containing 30% HP-β-CD, 5% DMSO, and 1% Tween 80), 30 min prior to the subcutaneous (s.c.) injection of PTZ (85 mg·kg^{-1}) dissolved in 0.9% sterile saline. After the injection of PTZ, the mice were closely monitored for 30 min via video recording. The latency for the onset of generalized tonic-clonic seizures (GTCS) and the mortality rate were recorded. GTCS are characterized by a rigid extension of all four limbs, exceeding a 90-degree angle with the body plane, lasting for over 10 s, followed by a loss of the righting reflex.

Strychnine (STR)-induced seizures

The animal model was adapted from the method reported by El-Mowafy et al. [26]. Male CD-1 mice (weighing 25–30 g; $n = 8$ per group) were injected intraperitoneally with SchB (10, 30, or 60 mg·kg^{-1}, i.p.), sodium valproate (positive control, 300 mg·kg^{-1}, i.p.) or vehicle control solution containing 30% HP-β-CD, 5% DMSO, and 1% Tween 80, i.p.), 30 min prior to an administration of strychnine hydrochloride (0.75 mg·kg^{-1}, s.c.) dissolved in 0.9% sterile saline. After the injection of STR, the mice were closely monitored for 30 min via video recording. The latency to the onset of GTCS and the mortality rate were recorded. GTCS are characterized as described in the PTZ model mentioned above.

Pilocarpine-induced status epilepticus

The animal model was adapted from the method by Gozzelino et al. [27]. Male C57BL/6 N mice (20–22 g; $n = 8$ per group) were administered SchB (30 mg·kg^{-1}, i.p.) or vehicle control solution containing 30% HP-β-CD, 5% DMSO, and 1% Tween 80, i.p.), 30 min prior to an administration of pilocarpine hydrochloride (360 mg·kg^{-1}, s.c.). At the time of compound injection, the animals were also administered 1 mg·kg^{-1} of atropine to block the peripheral cholinergic effects induced by pilocarpine. The animal behavior was closely monitored for 90 min through video recording and scored according to a modified Racine scale [28]: stage 0, normal activity; stage 1, freezing behavior; stage 2, tail extension, head bobbing; stage 3, continuous head bobbing and forepaw shaking; stage 4, forelimb clonus, rearing and falling; stage 5, repetitive stage 4 or big jumping; stage 6, death.

Data analysis and statistics

The behavioral data were analyzed using GraphPad Prism 8.0 software (GraphPad Software, San Diego, CA, USA). Electrophysiological data were collected and analyzed using Patchmaster and Fitmaster software (HEKA Electronics, Lambrecht, Germany), Igor Pro 6.0 (WaveMetrics, Portland, USA), GraphPad Prism 8.0 (GraphPad Software, San Diego, CA, USA), and IBM SPSS Statistics Version 26 (IBM Corp., Armonk, NY, USA). Concentration-response curves were fitted using the logistic equation with four parameters: Y = Bottom + (Top-Bottom)/(1 + 10^((LogEC$_{50}$ - X) * nH)), where Y represents the normalized peak current, X represents the concentration of compound, Bottom and Top are the minimum and maximum response, EC_{50} represents the half maximal effective concentration, and *n*H represents the Hill coefficient. All data are presented as mean ± SD, unless noted otherwise. The data were analyzed using paired or unpaired Student's *t* tests, as well as one-way ANOVA followed by Dunnett's *post-hoc* test. Statistical significance was determined at $P < 0.05$.

RESULTS

SchB potentiated recombinant glycine receptors

In an earlier screening for potentiators of α1 glycine receptors based on compounds derived from *Schisandra chinensis fructus*, SchB was identified as a positive allosteric modulator of α1 GlyRs. Therefore, we further tested the effect of SchB on the three GlyRs (α1–α3). The EC_{50} concentration for glycine was determined for each subtype (Table 1). In the presence of glycine at its EC_{10-20} concentration, SchB-induced potentiation of glycine currents ($I_{glycine}$) was studied (Fig. 1a). SchB at 30 μM showed a more dramatic potentiation on α1 GlyRs with an E_{max} value of 5.32 ± 0.27-fold. It also displayed a 3.01 ± 0.44-fold potentiation on α2 GlyRs and a 2.86 ± 0.55-fold potentiation on α3 GlyRs. (Fig. 1b; Table 1). In the meanwhile, SchB enhanced the glycine currents ($I_{glycine}$) in a concentration-dependent manner (Fig. 1c, d) with EC_{50} values of 6.11 ± 0.75 μM, 5.11 ± 1.58 μM, and 4.93 ± 1.64 μM for α1, α2, and α3 GlyRs, respectively (Table 1). However, SchB alone did not induce any activation of GlyRs, which provides solid evidence that SchB is a positive modulator of GlyRs (Fig. S1). Due to the almost saturated effects of SchB at 10 μM, the concentration was used for the subsequent studies. Next, we examined whether SchB could reverse STR-induced antagonism of α1 GlyRs. As shown in Fig. 1e, STR (30 nM, ~IC_{50}) significantly inhibited $I_{glycine}$ in CHO cells expressing α1 GlyRs. Such inhibition of $I_{glycine}$ was significantly eliminated by SchB (Fig. 1e). Meanwhile, we also investigated the impact of 10 μM SchB on the activation of glycine receptors (α1, α2, and α3 GlyRs). As shown in Fig. S2, SchB greatly enhanced the potencies of glycine but had no effects on the efficacies of glycine-induced activation on these receptors.

SchB potentiated recombinant synaptic and extrasynaptic $GABA_A$ receptors

Both GlyRs and $GABA_A$Rs belong to Cys-loop receptors that form pentameric chloride channels with greater sequence homology [29]. Therefore, we assume that SchB might be also active on $GABA_A$ receptors. We successfully constructed stable cell lines expressing both synaptic and extrasynaptic $GABA_A$ receptors (Fig. S3). Diazepam (DZP) and delta selective compound 2 (DS2) were used to verify the existence of γ2 and δ subunits, respectively. Initially the effect of SchB on α1β2γ2 $GABA_A$Rs was tested. In the presence of GABA at its EC_{10-20} concentration, the effect of SchB on GABA-elicited current (I_{GABA}) was examined (Fig. 2a). Interestingly, SchB at 30 μM also showed potentiation on α1β2γ2 $GABA_A$ receptors with an E_{max} of 2.43 ± 0.36-fold (Fig. 2b). Then we further evaluated its action on the other subtypes, including α2β2γ2, α4β3δ, and α6β3δ $GABA_A$Rs (Fig. 2b). These receptors represent the major synaptic and extrasynaptic $GABA_A$Rs, respectively [7]. As shown in Fig. 2b, SchB also exhibited potentiation effects on the tested $GABA_A$Rs. SchB at 30 μM enhanced GABA currents to 2.24 ± 0.26-fold, 3.55 ± 0.17-fold, and 2.43 ± 0.24-fold for α2β2γ2, α4β3δ, and α6β3δ $GABA_A$Rs, respectively (Fig. 2b; Table 2). In the meanwhile, SchB potentiated I_{GABA} of α1β2γ2, α2β2γ2, α4β3δ, and α6β3δ $GABA_A$Rs in a

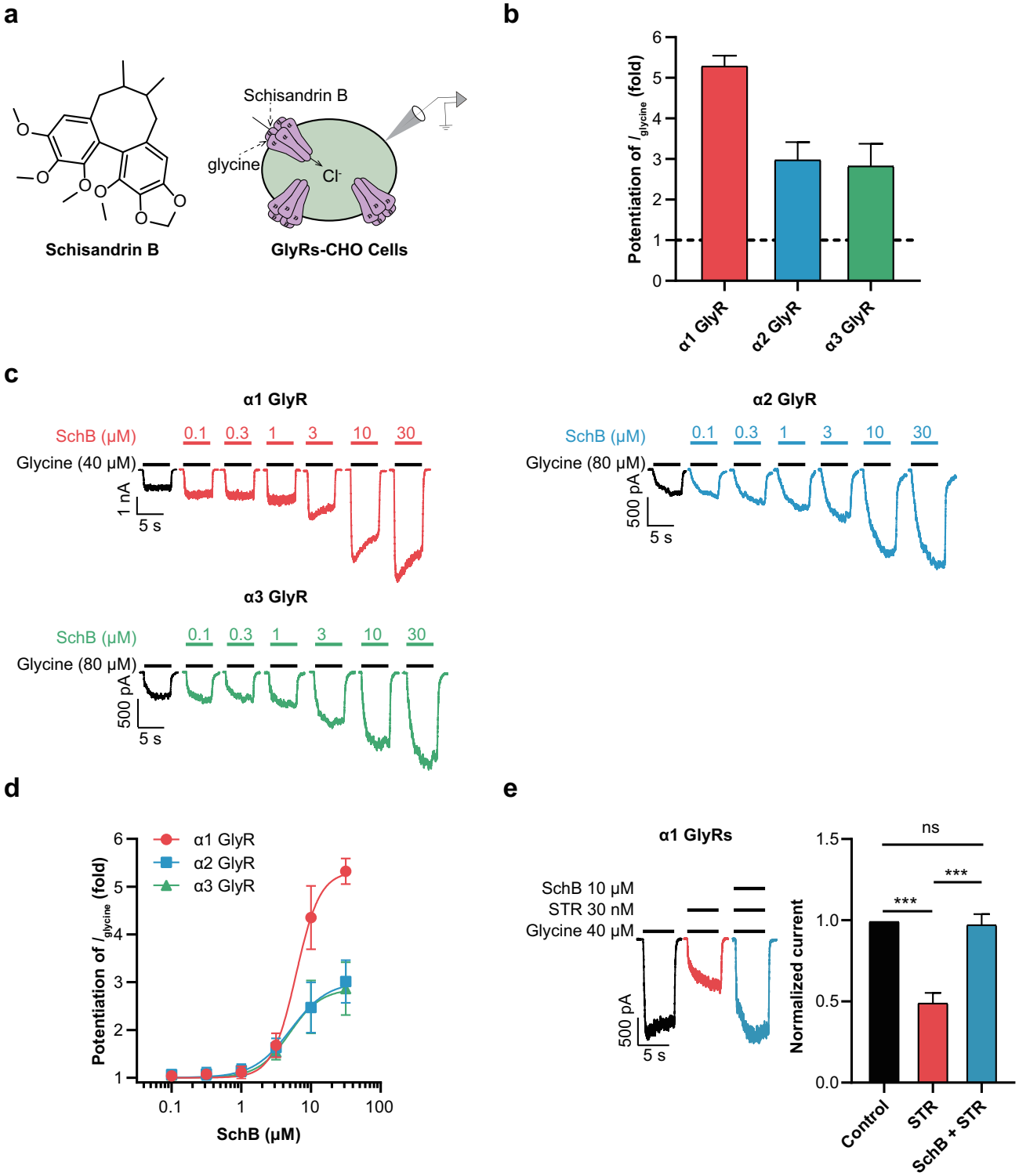

Fig. 1 SchB induced potentiation of glycine-elicited currents ($I_{glycine}$) in the recombinant α1, α2, and α3 glycine receptors. a Left, chemical structure of SchB; right, schematic diagram for patch-clamp recordings in GlyR-CHO cells. **b** Bar graphs illustrating SchB-induced potentiating effect on $I_{glycine}$. **c** Representative current traces for SchB-induced potentiation of $I_{glycine}$ activated by $EC_{10\text{-}20}$ concentrations of glycine (40 μM for α1 receptor, 80 μM for α2 receptor, and 80 μM for α3 receptor). **d** The concentration-response curves for SchB-induced potentiation of $I_{glycine}$ in cells expressing different α subunits. **e** Strychnine (STR)-induced inhibition of $I_{glycine}$ was reversed by SchB 10 μM in recombinant α1 glycine receptors.***$P < 0.001$, ns not significant ($P > 0.05$), Student's paired *t*-test. All data are expressed as the mean ± SD, $n = 5$-6.

concentration-dependent manner (Fig. 2c, d). The EC_{50} values were 4.03 ± 1.16 μM, 4.84 ± 0.95 μM, 4.93 ± 0.71 μM, and 1.51 ± 0.34 μM for α1β2γ2, α2β2γ2, α4β3δ, and α6β3δ $GABA_A$Rs in the presence of $EC_{10\text{-}20}$ concentrations of GABA, respectively. Based on the EC_{50} values and efficacies, apparently α4β3δ and α6β3δ $GABA_A$Rs were more sensitive to SchB. However, SchB alone could not activate the channels in the absence of GABA, which further convinced that SchB is a positive modulator of

Table 1. Functional parameters for the potentiation effect of SchB on the wild-type and mutant glycine receptors.

Receptor	Glycine			Glycine (in the presence of 10 μM SchB)			SchB (in the presence of $EC_{10\sim20}$ Glycine)			
	EC_{50}, μM	n_H	*n*	EC_{50}, μM	n_H	*n*	EC_{50}, μM	n_H	E_{max}, fold	*n*
α1	102.0 ± 9.4	2.45 ± 0.76	6	41.2 ± 6.0$^{\#\#\#}$	1.26 ± 0.25	5	6.11 ± 0.75	2.54 ± 0.53	5.32 ± 0.27[a]	5
α2	229.5 ± 24.0	2.13 ± 0.41	6	133.3 ± 8.9$^{\#\#\#}$	2.28 ± 0.30	5	5.11 ± 1.58	1.62 ± 0.67	3.01 ± 0.44[a]	5
α3	246.5 ± 15.4	2.25 ± 0.28	6	117.2 ± 11.3$^{\#\#\#}$	2.47 ± 0.63	5	4.93 ± 1.64	1.92 ± 0.95	2.86 ± 0.55[a]	5
α1(S267Q)	207.3 ± 18.8***	1.77 ± 0.24	5	N.D.	N.D.		N.D.	N.D.	1.32 ± 0.04***,[b]	5
α1(S296A)	81.7 ± 12.7	2.27 ± 0.78	5	N.D.	N.D.		N.D.	N.D.	3.44 ± 0.48[b]	5
α1(F380A)	75.1 ± 9.4*	2.30 ± 0.57	5	N.D.	N.D.		N.D.	N.D.	3.32 ± 0.39[b]	5
α1(K385A)	70.0 ± 12.1**	2.38 ± 0.53	5	N.D.	N.D.		N.D.	N.D.	2.79 ± 0.59[b]	5

The maximal efficacies (E_{max}, fold), EC_{50} concentrations (μM), and Hill coefficients (nH) for each receptor are presented as mean ± SD for the number (*n*) of cells tested.
N.D., not-determined.
*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, one-way ANOVA followed by Dunnett's test, compared to wild type. $^{\#\#\#}P < 0.001$, Student's unpaired *t*-test.
[a]Indicates SchB at 30 μM.
[b]Indicates SchB at 10 μM.

$GABA_A$Rs (Fig. S1). Then we also tested the effect of SchB on PTZ (0.5 mM, ~IC_{50})-induced antagonism of GABA currents (Fig. 2e). PTZ-induced inhibition of I_{GABA} was significantly reversed by SchB (Fig. 2e). Next, we investigated the impact of SchB (10 μM) on GABA-induced activation of α1β2γ2L $GABA_A$Rs. SchB not only enhanced the affinity of GABA, but also increased its efficacies (Fig. S4; Table 2).

SchB potentiated $GABA_A$ and glycine receptors in cultured hippocampal neurons

Both GlyRs and $GABA_A$Rs are highly expressed in the central nervous system. Hippocampal neurons are responsible for seizure generation and propagation [30]. Thus, we further examined the effects of SchB on GABA- and glycine-induced currents in cultured hippocampal neurons (Fig. 3a). Consistent with the data from the recombinant cells, SchB also potentiated $GABA_A$Rs and GlyRs in hippocampal neurons in a dose-dependent manner (Fig. 3b–e). The EC_{50} values were 4.47 ± 0.92 μM and 0.99 ± 0.12 μM for $GABA_A$Rs and GlyRs, respectively. The E_{max} values were 2.43 ± 0.24-fold and 5.06 ± 0.43-fold for $GABA_A$Rs and GlyRs, respectively. Meanwhile, SchB also potentiated the $GABA_A$Rs-mediated tonic currents in cultured hippocampal neurons (Fig. S5). In addition, consistent with the results observed in the recombinant cells, after applying PTZ and STR at relatively higher doses (PTZ: 0.5 mM, ~IC_{50}; STR: 30 nM, ~IC_{50}), SchB was still capable of eliminating the antagonists-induced suppressions (Fig. 3f, g). These findings provide strong evidence that SchB is an efficacious positive allosteric modulator of $GABA_A$Rs and GlyRs.

SchB alleviated PTZ-, STR-, and pilocarpine-induced seizures in mice

SchB-induced dual potentiation of both $GABA_A$Rs and GlyRs prompted us to test the effect of this natural compound on preclinical seizure models. PTZ is a non-competitive antagonist of $GABA_A$Rs [31]. It induces acute GTCS behaviors by reducing inhibitory synaptic transmission and enhancing neuron excitability. And PTZ-induced seizure model is a commonly used method for identifying potential antiseizure medications (ASMs) in preclinical studies. Therefore, the PTZ model was applied to assess the anticonvulsant activity of SchB. SchB was administered intraperitoneally at doses of 10, 30, or 60 mg·kg^{-1}, 30 min prior to the PTZ injection. After administering PTZ (85 mg·kg^{-1}, s.c.), the following parameters were measured for 30 min: the latency to the onset of GTCS, the incidence of GTCS, and mortality (Fig. 4a). As shown in Fig. 4b, SchB at doses of 30 and 60 mg·kg^{-1} significantly increased the latency to the onset of GTCS. However, no significant effect was observed at the lower dose of 10 mg·kg^{-1}. Sodium valproate (VPA, 300 mg·kg^{-1}, i.p.) was used as a positive control to verify the feasibility of the model, and significantly increased the latency to the onset of GTCS. What's more, compared to the vehicle control group, SchB reduced the incidence of GTCS (vehicle group: 100%) and mortality (vehicle group: 50%) (Fig. 4c). After the treatment of SchB at 10, 30, and 60 mg·kg^{-1}, the incidence of GTCS was 75%, 25%, and 37.5%, respectively, and the mortality rates were 25%, 12.5%, and 0%, respectively. There was no occurrence of GTCS or death in the VPA group (300 mg·kg^{-1}, i.p.) (Fig. 4c).

Strychnine (STR), the antagonist of GlyRs, could induce acute GTCS behaviors by reducing inhibitory synaptic transmission and further enhancing neuronal excitability. Since SchB potentiated glycine receptors, we wondered whether SchB was also effective in the STR-induced seizure model. To examine the antiseizure effects of SchB on STR model, we measured the latency to the onset of GTCS and mortality for 30 min after administering STR (0.75 mg·kg^{-1}, s.c.). SchB was administered intraperitoneally at doses of 10, 30, and 60 mg·kg^{-1}, 30 min prior to the STR injection (Fig. 4d). As expected, SchB at doses of 30 and 60 mg·kg^{-1} significantly increased the latency to the onset of GTCS (Fig. 4e). However, no significant effect was observed at the lower dose of 10 mg·kg^{-1}. Compared to the vehicle control group, SchB decreased the mortality rate (vehicle group: 100%) to 75%, 62.5%, and 50% at doses of 10, 30, and 60 mg·kg^{-1}, respectively. The positive control, sodium valproate (VPA, 300 mg·kg^{-1}, i.p.), significantly increased the latency to the onset of GTCS and reduced the mortality rate to 12.5% (Fig. 4e, f).

Finally, we investigated the antiseizure effects of SchB on the pilocarpine-induced status epilepticus (SE) model, which closely resembles clinical temporal lobe epilepsy. This model often results in resistance to various ASMs in patients [32]. To evaluate the antiseizure effects of SchB on pilocarpine-induced seizures, we measured the seizure scores within 90 min after administering pilocarpine (360 mg·kg^{-1}, s.c.). SchB has shown almost equal activity at the dosage 30 and 60 mg·kg^{-1} in PTZ and STR models. Therefore, SchB was administered at 30 mg·kg^{-1} (Fig. 4g). As shown in Fig. 4h, SchB resulted in a significant decrease in seizure scores. The analysis of stage distribution (Fig. 4i) revealed that all of the mice treated with the vehicle developed stage 0–4, characterized by turning to a side position, while 87.5% of the mice reached stages 5–6. Among the mice treated with SchB, 62.5% exhibited stage 0–4, and 37.5% progressed to stages 5–6.

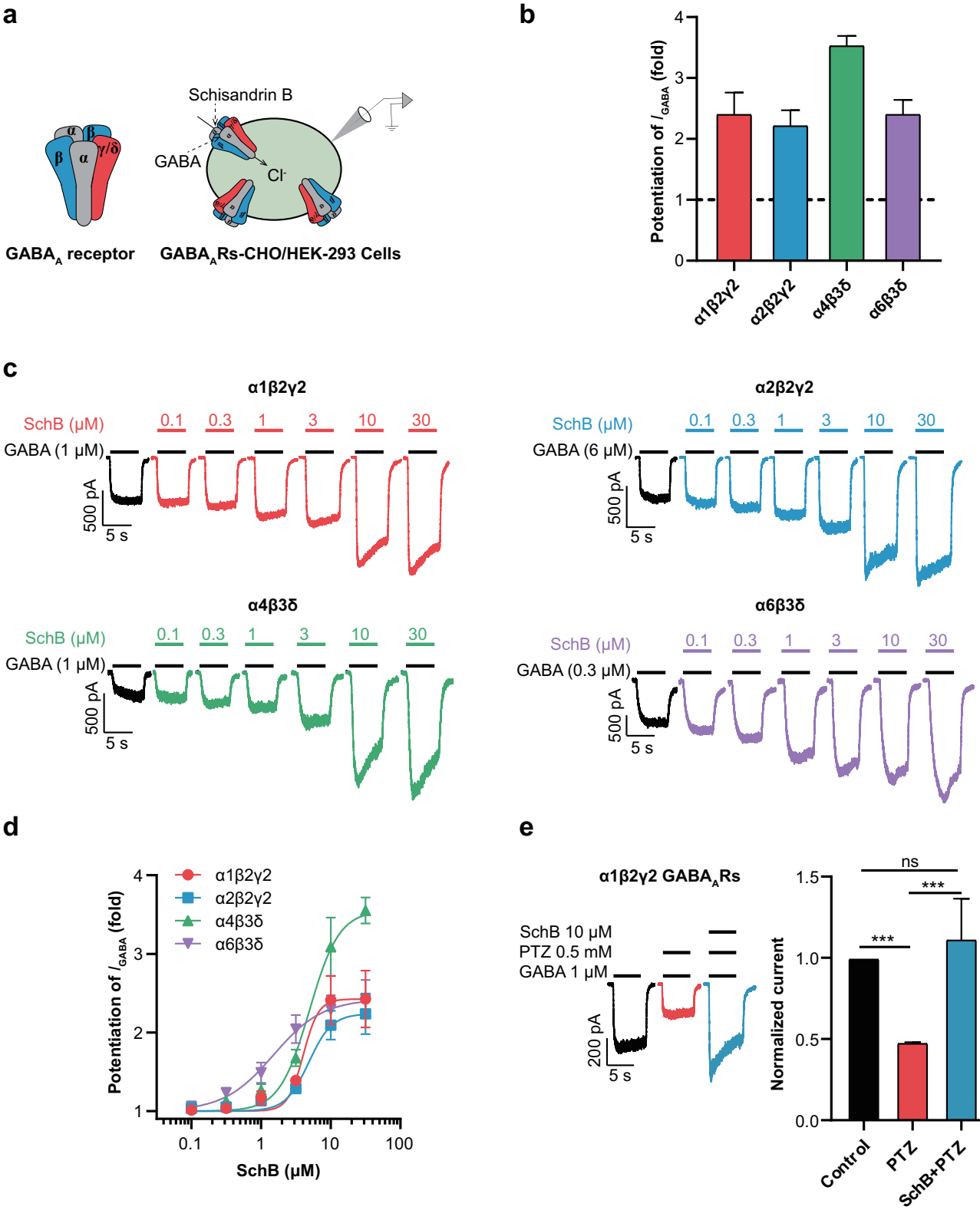

Fig. 2 SchB induced potentiation of GABA-elicited currents (I_{GABA}) in recombinant $GABA_A$ receptors (α1β2γ2L, α2β2γ2L, α4β3δ, and α6β3δ). a Schematic diagram for patch-clamp recordings in $GABA_A$R-CHO/HEK-293 cells. **b** Bar graphs depicting the potentiating effect of SchB on I_{GABA}. **c** Representative current traces for SchB-induced potentiation of GABA receptors. **d** Concentration-response curves illustrating the enhancement of I_{GABA} by SchB on α1β2γ2L, α2β2γ2L, α4β3δ, and α6β3δ $GABA_A$Rs. **e** SchB (10 μM) eliminated the pentylenetetrazole (PTZ)-induced inhibition of I_{GABA} in recombinant α1β2γ2L $GABA_A$Rs. ***$P < 0.001$, ns not significant ($P > 0.05$), Student's paired *t*-test. All data are expressed as the mean ± SD, $n = 5–6$.

Table 2. Functional parameters for the potentiation effect of SchB on the wild-type and mutant $GABA_A$ receptors.

Receptor	GABA		GABA (in the presence of 10 μM SchB)				SchB (in the presence of $EC_{10\sim20}$ GABA)			
	EC_{50}, μM	n_H	*n*	EC_{50}, μM	n_H	*n*	EC_{50}, μM	n_H	E_{max}, fold	*n*
α1β2γ2L	3.52 ± 0.49	1.41 ± 0.27	6	2.00 ± 0.36$^{###}$	1.26 ± 0.25	5	4.03 ± 1.16	3.95 ± 1.89	2.43 ± 0.36[a]	6
α2β2γ2L	22.84 ± 1.90	1.56 ± 0.16	5	15.02 ± 1.42$^{###}$	1.39 ± 0.18	5	4.84 ± 0.95	2.63 ± 0.96	2.24 ± 0.26[a]	5
α4β3δ	3.33 ± 0.51	1.00 ± 0.14	5	N.D.	N.D.		4.93 ± 0.71	2.23 ± 0.47	3.55 ± 0.17[a]	5
α6β3δ	1.70 ± 0.51	0.82 ± 0.18	5	N.D.	N.D.		1.51 ± 0.34	1.23 ± 0.31	2.43 ± 0.24[a]	5
α1(H129R)β2γ2L	7.22 ± 1.10*	1.06 ± 0.15	5	N.D.	N.D.		N.D.	N.D.	1.81 ± 0.14[b]	5
α1(D219N)β2γ2L	8.74 ± 1.63**	1.06 ± 0.13	5	N.D.	N.D.		N.D.	N.D.	2.31 ± 0.41[b]	5
α1(G251S)β2γ2L	20.80 ± 3.36***	1.30 ± 0.24	6	N.D.	N.D.		N.D.	N.D.	2.98 ± 0.84[b]	5
α1(S297Q)β2γ2L	3.55 ± 0.40	1.15 ± 0.15	5	N.D.	N.D.		N.D.	N.D.	1.45 ± 0.06**,[b]	5
α1β2(N289S)γ2L	8.74 ± 1.03***	1.40 ± 0.22	6	N.D.	N.D.		N.D.	N.D.	2.17 ± 0.34[b]	5
α1(S297Q)β2(N289S)γ2L	2.55 ± 0.40	1.28 ± 0.24	5	N.D.	N.D.		N.D.	N.D.	1.07 ± 0.10***,[b]	5
α1β2γ2L(R177G)	9.04 ± 1.13***	1.22 ± 0.18	6	N.D.	N.D.		N.D.	N.D.	3.48 ± 0.39**,[b]	5
α1β2γ2L(P322A)	6.60 ± 0.63***	1.37 ± 0.16	7	N.D.	N.D.		N.D.	N.D.	2.62 ± 0.57[b]	6

The maximal efficacies (E_{max}, fold), EC_{50} concentrations (μM), and Hill coefficients (nH) for each receptor are presented as mean ± SD for the number (*n*) of cells tested.
N.D. not-determined.
*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, one-way ANOVA followed by Dunnett's test, compared to wild type. $^{###}P < 0.001$, Student's unpaired t-test.
[a]Indicates SchB at 30 μM.
[b]Indicates SchB at 10 μM.

Overall, these results indicated that SchB could alleviate acute tonic-clonic seizures and status epilepticus.

SchB efficiently rescued the impaired $GABA_A$ receptors associated with genetic epilepsies

$GABA_A$Rs are the primary inhibitory neurotransmitter-gated ion channels in the mammalian central nervous system. They play a crucial role in providing inhibitory tone to balance the tendency of hyperexcitability in excitatory neural circuits [33]. Several point mutations of $GABA_A$Rs have been reported to be linked to various genetic epilepsies [13]. We selected four representative mutations of $GABA_A$Rs, associated with severe genetic epilepsies, to investigate the modulation of SchB on these mutant $GABA_A$Rs and explore its potential therapeutic value. These four mutations are α1(D219N, c.655G>A), α1(G251S, c.751G>A), γ2L(R177G, c.529C>G), and γ2L(P322A, c.964C>G) (Fig. 5a), closely associated with idiopathic generalized epilepsy [34], Dravet syndrome [35], febrile seizures [36], and early infantile epileptic encephalopathy, as well as the development of pharmacoresistance [37], respectively. Firstly, we evaluated the electrophysiological characteristics of these mutations. Notably, these mutations significantly decrease the sensitivity to GABA, suggesting a loss-of-function (LOF) effect for these mutations. The EC_{50} values of GABA for α1(D219N), α1(G251S), γ2L(R177G), and γ2L(P322A) were 8.74 ± 1.63 μM, 20.80 ± 3.36 μM, 9.04 ± 1.13 μM, and 6.60 ± 0.63 μM, respectively. The current densities of these mutations [α1(D219N), γ2L(R177G), and γ2L(P322A)] were significantly suppressed by ~60%–70% compared to the wild type of α1β2γ2L (144.2 ± 44.7 pA·pF^{-1}), with values of 56.7 ± 22.8 pA·pF^{-1}, 51.3 ± 28.1 pA·pF^{-1}, and 33.8 ± 16.5 pA·pF^{-1}, respectively. Additionally, the current density of α1(G251S) (10.9 ± 5.9 pA·pF^{-1}) was suppressed by ~90% (Fig. 5b, d; Table 2). Despite the decreased expression level, SchB (10 μM) still showed greater potentiation of GABA-induced current in all the mutant receptors. The efficacies reached 2.31 ± 0.41-fold, 2.98 ± 0.84-fold and 3.48 ± 0.89-fold, and 2.62 ± 0.57-fold at 10 μM for α1(D219N), α1(G251S), γ2L(R177G), and γ2L(P322A), respectively (Fig. 5e, f; Table 2).

Collectively, our findings suggest that SchB shows great potential for treating severe genetic epilepsies caused by LOF effects of $GABA_A$Rs.

Identification of amino acid residues critical for the actions of SchB in glycine receptors

A number of drugs have been reported as positive modulators of GlyRs, such as volatile anesthetics enflurane [38, 39], CBD/Δ^9-THC [40], propofol [41], and endocannabinoids [42], respectively. Possible binding sites for these compounds to the GlyRs have been demonstrated. Therefore, to clarify the possible interaction sites of SchB in glycine receptors, we successfully constructed these reported mutations in mature GlyR α1, including α1S267Q (premature: S295Q, c.883A>C_884G>A_885C>G), α1S296A (premature: S324Q, c.970T>G), α1F380A (premature: F408A, c.1222 T > G_1223T > C), and α1K385A (premature: K413A, c.1237A>G_1238A>C) (diagram shown in Fig. 6a). These mutants were expressed in CHO cells and all showed similar functional activation to glycine except α1S267Q with a weaker potency (Fig. 6b; Table 1).

As shown in Fig. 6c, d, SchB-induced potentiation (10 μM) was unaffected by the mutations S296A, F380A, and K385A. However, the mutation S267Q located in transmembrane domain 2 (TM2) almost completely abolished SchB-mediated potentiation (Fig. 6c, d). These results suggest that Ser267 in TM2 of GlyR α1 is critical for the actions of SchB.

SchB potentiated GABA-elicited currents not through diazepam- or etomidate-binding sites

To gain a deeper understanding of the molecular mechanisms underlying the modulation of $GABA_A$Rs by SchB, we conducted a series of experiments to differentiate the action site of SchB from those of DZP and etomidate (ETO). DZP and ETO are classified as classical benzenediazenes (BDZs) and general anesthetics, respectively, and both act as positive modulators of $GABA_A$Rs. To investigate whether SchB binds to the DZP-binding site, we firstly examined whether the activity of SchB could be eliminated by flumazenil, an antagonist that binds to the high-affinity site of BDZs [43]. In the presence of flumazenil (10 μM), SchB still kept its potentiation effect on α1β2γ2L (Fig. 7a). It was known that a histidine at position 129 (human) or 101 (murine) in α1 subunit is essential for the binding of classical BDZs [44]. To confirm the observation from the flumazenil test, we introduced the H129R (c.386A>G) mutation to the α1 subunit. As anticipated, it did not exhibit any response to 1 μM DZP (Fig. 7b). By contrast, SchB

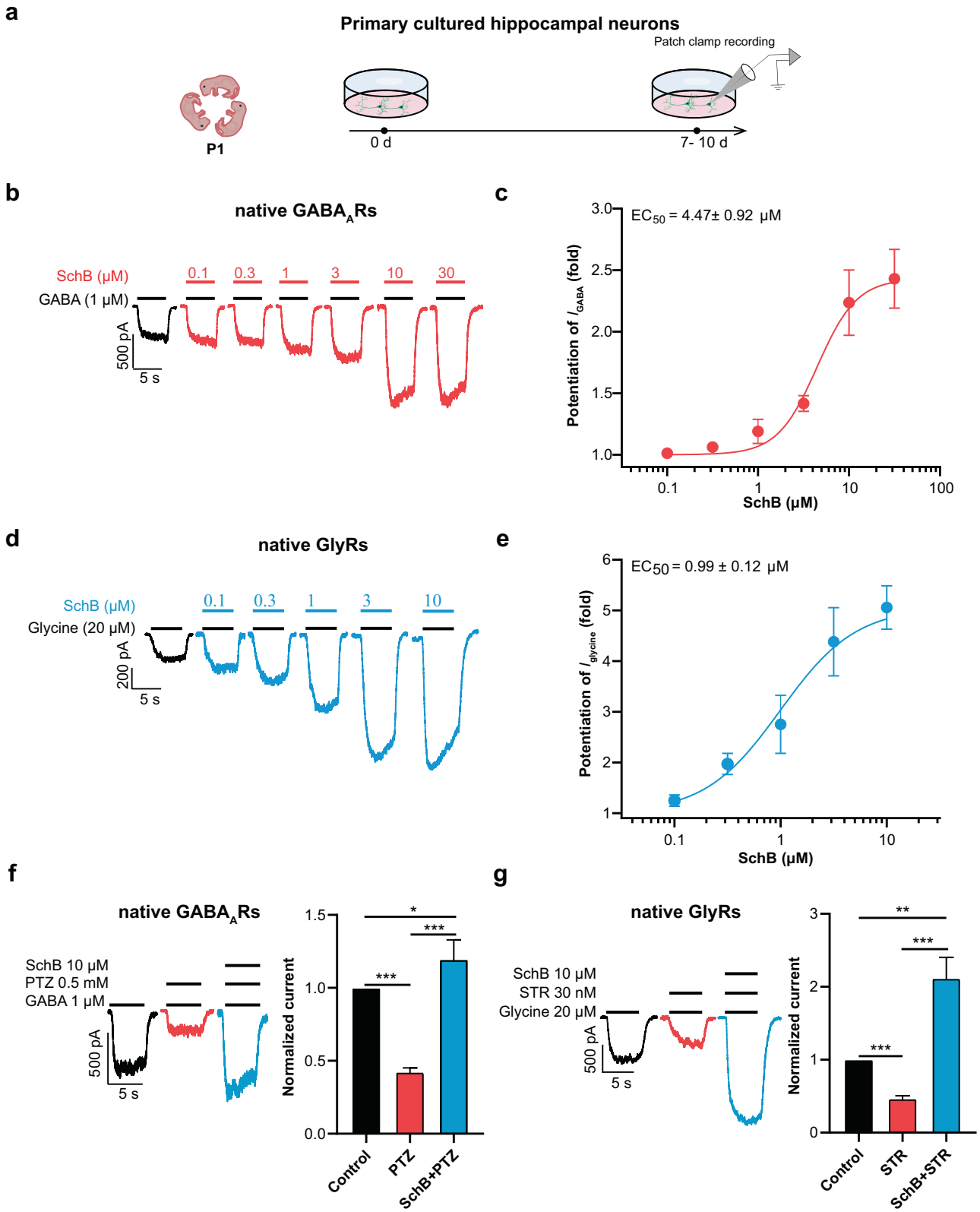

Fig. 3 Effects of SchB on GABA- and glycine-evoked currents in cultured hippocampal neurons. a The schematic diagram depicts the process of primary culture and patch-clamp recordings in hippocampal neurons. **b**, **d** Representative current traces for $GABA_A$ receptors ($GABA_A$Rs) and glycine receptors (GlyRs) recorded in cultured hippocampal neurons in the absence and presence of SchB (pre-incubated for 1 min). **c**, **e** Concentration–response curves of SchB for $GABA_A$ and glycine receptors. Each data point represents the mean ± SD, $n = 5$. **f** SchB 10 μM reversed the inhibition effect of pentylenetetrazole (PTZ) (0.5 mM) on GABA-induced currents, and (**g**) of strychnine (STR) (30 nM) on glycine-induced currents. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, Student's paired *t* test. Data are represented as mean ± SD, $n = 5$.

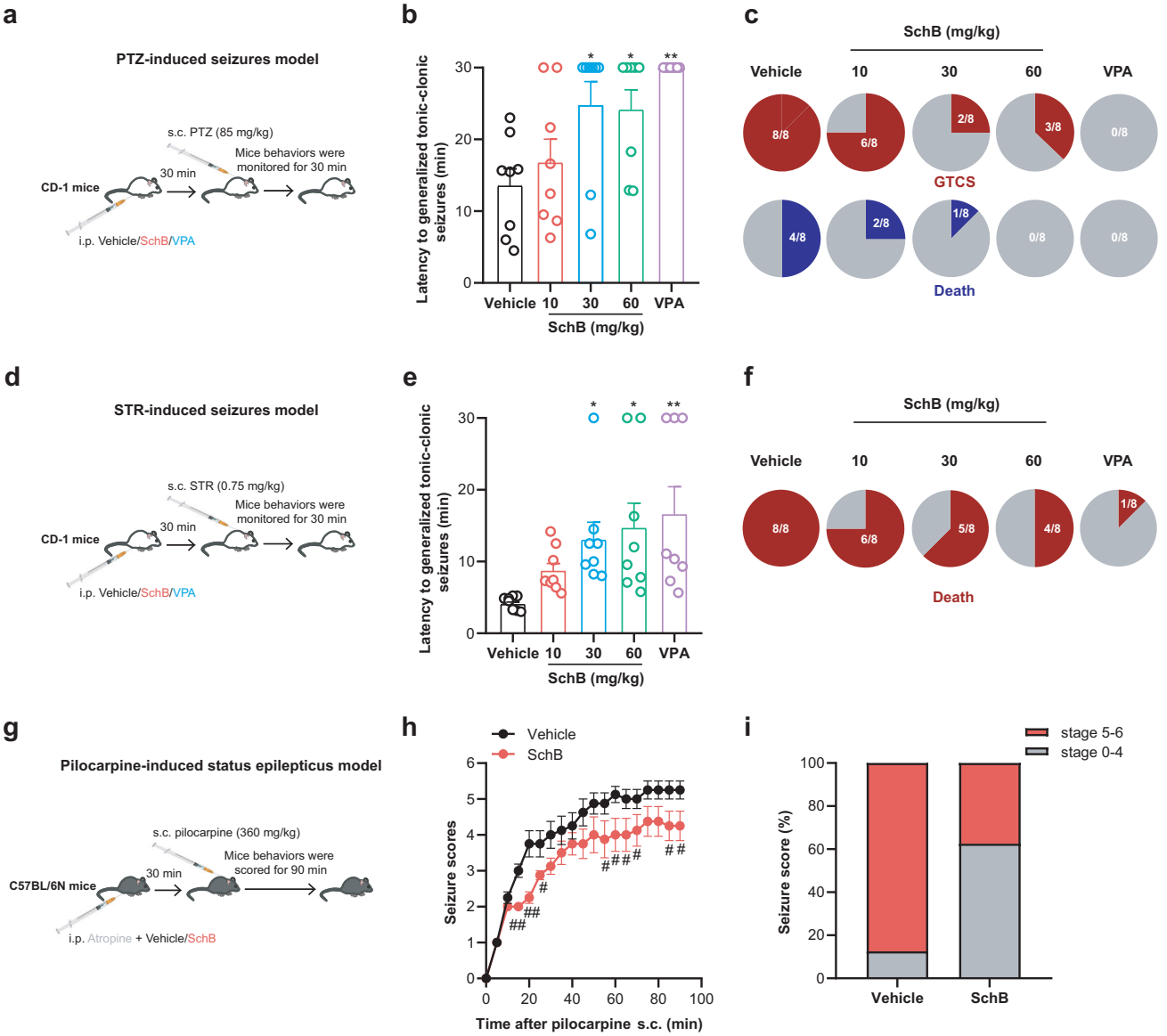

Fig. 4 Effects of SchB on seizure models in mice. a Schematic diagram illustrating PTZ-induced seizures in mice. **b** The statistical results for latency to the onset of generalized tonic-clonic seizures (GTCS) in the PTZ-induced seizure model as a result of SchB (30 and 60 mg·kg^{-1}, i.p.) and positive drug sodium valproate (VPA, 300 mg·kg^{-1}, i.p.) treatment. **c** Pie charts for the reduction in incidence of GTCS and mortality rates in the PTZ-induced seizure model after the treatment of SchB and VPA. **d** Schematic diagram illustrating STR-induced seizures in mice. **e** The statistical results for latency to onset of GTCS in the STR (0.75 mg·kg^{-1}, s.c.)-induced seizure model in the absence and presence of SchB (30, 60 mg·kg^{-1}, i.p.) and VPA (300 mg·kg^{-1}, i.p.). **f** Pie charts for the reduction in mortality rates in the STR-induced seizure model in the absence and presence of SchB (10, 30, and 60 mg·kg^{-1}, i.p.) and VPA (300 mg·kg^{-1}, i.p.). **g** Schematic diagram for pilocarpine-induced seizures in mice. **h** Effects of SchB (30 mg·kg^{-1}, i.p.) on Racine score after pilocarpine administration (360 mg·kg^{-1}, s.c.). **i** Effects of SchB (30 mg·kg^{-1}, i.p.) on the intensity of the seizure attack in mice. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with the vehicle control group using one-way ANOVA followed by Dunnett's multiple comparisons test. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with the vehicle control group using Wilcoxon rank-sum test. All data are expressed as the mean ± SEM, $n = 8$.

(10 μM) still potentiated GABA-elicted currents in this mutant (Fig. 7b; Table 2). These results indicated that the pontentiation mechanism of SchB on α1β2γ2L is different from that of BDZs.

To investigate whether SchB binds to the ETO-binding site, we conducted the following experiments. Firstly, we did a co-application test to see whether SchB and ETO show any competitive effects on GABA-elicited currents. As shown in Fig. 7c, d, the co-stimulation by both SchB (10 μM) and ETO (10 μM) resulted in a supra-additive effect, rather than competitive effects, suggesting that the action site for SchB in $GABA_ARs$ is different from that of ETO. To confirm this, we constructed the mutation β2N289S (c.866A>G), which is known to be able to abolish the potentiation of ETO [45]. The mutant channel α1β2(N289S)γ2L was functionally expressed in CHO cells (Table 2). As expected, the potentiation induced by SchB (10 μM) was not affected, but ETO (10 μM)-induced potentiation was significantly reduced (Fig. 7d).

Taken together, these results demonstrate that SchB might potentiate $GABA_ARs$ through a site distinct from the classical DZP- and ETO-binding sites.

The residues α1S297 and β2N289 located in the β+/α- subunit interfaces of TM2 in $GABA_A$ (α1β2γ2L), homologous to Ser267 in GlyR α1, are crucial for SchB-induced potentiation of GABA-elicited currents

$GABA_ARs$ and GlyRs belong to Cys-loop receptors that form pentameric chloride channels with greater sequence homology [29].

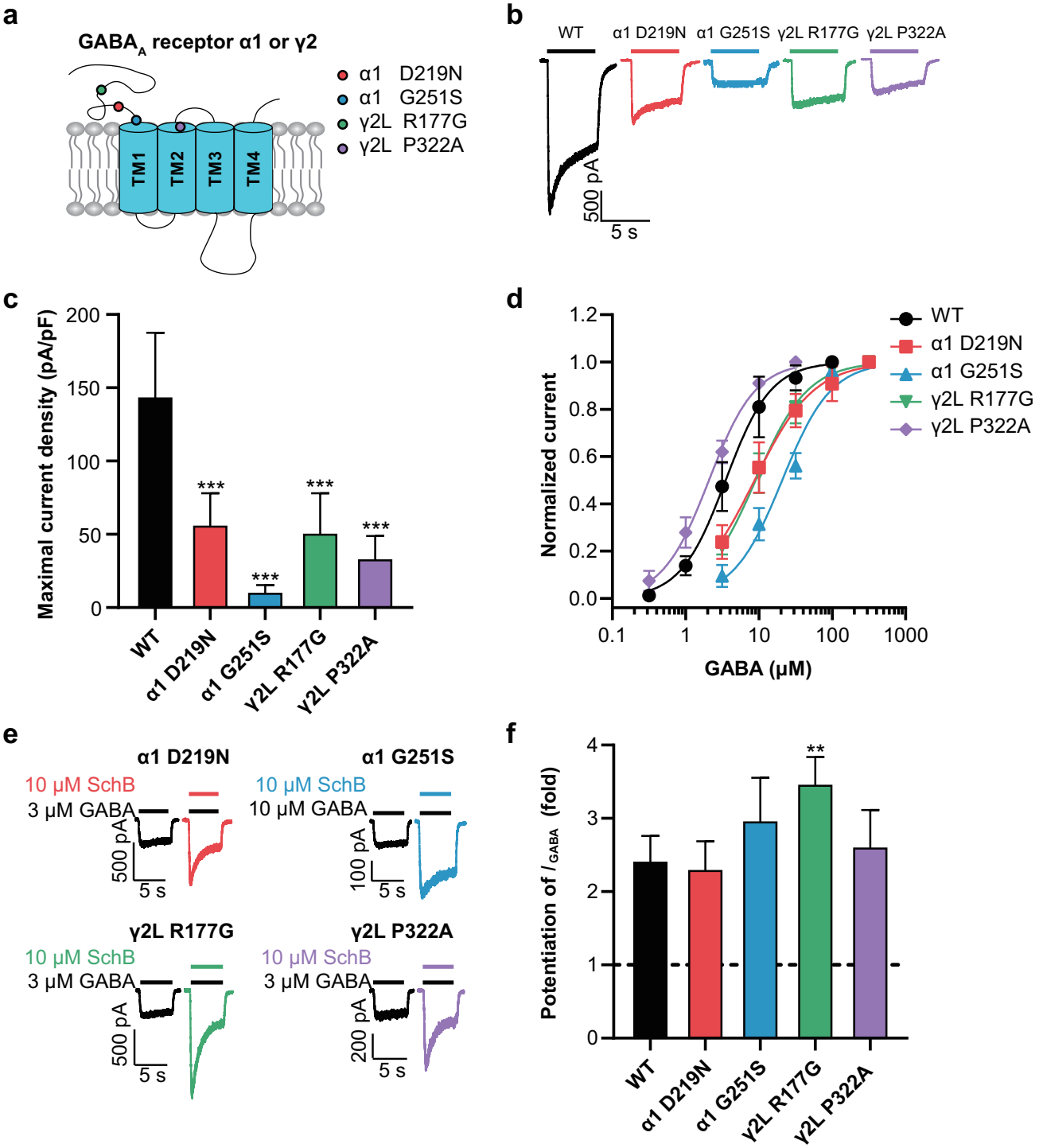

Fig. 5 Effects of SchB on mutations in $GABA_A$ receptors associated with genetic epilepsies. a Topology of $GABA_A$R α1 and γ2. This diagram displays the four transmembrane segments, as well as the N-terminal and C-terminal domains. The solid circles highlighted in various colors indicate the locations of the mutations under investigation. **b** Representative traces for the responses to the application of 1 mM GABA were recorded from CHO cells expressing mutant α1β2γ2L $GABA_A$ receptors (α1: p.D219N, p.G251S; γ2L: p.R177G, p.P322A). **c** The average current density was obtained from CHO cells expressing wild-type α1β2γ2L ($n = 8$), p.D219N ($n = 7$), p.G251S ($n = 7$), p.R177G ($n = 6$), and p.P322A ($n = 6$) mutations. $^{***}P < 0.001$, one-way ANOVA followed by Dunnett's multiple comparisons test, compared to the wild type. The data are expressed as mean ± SD. **d** The dose-response curve for GABA in wild-type α1β2γ2L ($n = 8$), p.D219N ($n = 7$), p.G251S ($n = 7$), p.R177G ($n = 6$), and p.P322A ($n = 6$) receptors was recorded. Statistically significant differences in the EC_{50} values were verified by ANOVA followed by Dunnett's multiple comparisons test. $^{**}P < 0.01$ for p.D219N, $^{***}P < 0.001$ for p.G251S, p.R177G, and p.P322A (in Table 1). **e** Representative current traces for GABA ($EC_{10\text{-}20}$)-induced GABA currents in mutant α1β2γ2L $GABA_A$ receptors in the presence and absence of 10 μM SchB (pre-incubation for 1 min). **f** Summary data for the effects of SchB on wild-type and mutant α1β2γ2 $GABA_A$ receptors. All data are expressed as the mean ± SD, $n = 5$–6.

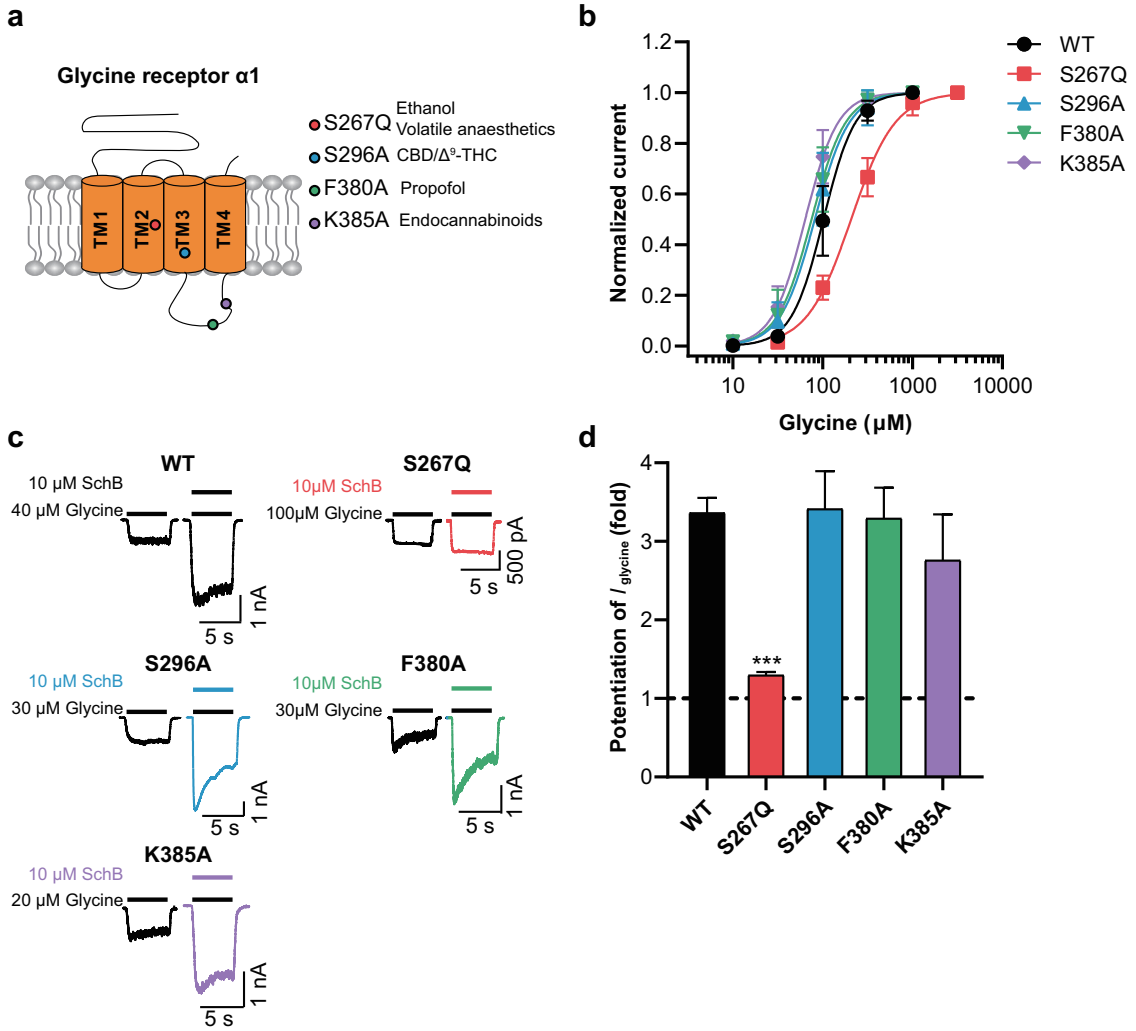

Fig. 6 Effects of mutations at sites important for other positive allosteric modulators of α1 GlyR on the activity of SchB. a Topology of GlyR α1 subunit showing the four transmembrane segments, N-terminal domain, and C-terminal domain. The solid circles highlighted in different colors indicate the location of the investigated mutations. **b** Concentration-response curves for glycine in wild-type and mutant α1 GlyRs. Responses at indicated concentrations in each cell were normalized to the maximum glycine-evoked peak current. Each data point represents the mean ± SD, $n = 5$–6. **c** Representative current traces activated by $EC_{10\text{-}20}$ glycine of wild type and mutant α1 GlyRs before and after application of 10 μM SchB (pre-incubation 1 min). **d** Summary data for the effects of SchB on wild-type and mutant α1 GlyRs. ***$P < 0.001$, one-way ANOVA followed by Dunnett's multiple comparisons test, compared to wild type. All data are expressed as the mean ± SD, $n = 5$.

Now that the mutation S267Q in GlyRs significantly reduced the activity of SchB, we would expect that the mutations from the homologous site in $GABA_AR$s might exhibit a similar effect as GlyRs. Thus, we generated mutants in the homologous positions in the $GABA_AR$ α1 and β2 subunits. By aligning the amino acid sequences between GlyR α1, $GABA_AR$ α1, and β2 subunits, Ser297 in $GABA_AR$ α1 and Asn289 in $GABA_AR$ β2 subunits were identified as the homologous sites as Ser267 in GlyR α1 (Fig. 8a). Previous studies have found that the residues α1S297 and β2N289, located in the β+/α- subunit interfaces, are critical for the potentiation of GABA-elicited currents by pentobarbital, ethanol, and volatile anesthetic enflurane in recombinant α1β2γ2L receptors [38, 46]. Before making these mutations, we did the co-application test to see whether SchB and pentobarbital show any competitive or additive effects on $GABA_{A1}$ receptors. As shown in Fig. 8b, c, the co-application of SchB (10 μM) and PB (100 μM) did not produce any additive effects on α1β2γ2L $GABA_AR$s instead of keeping the potentiating effect at the similar level as pentobarbital. These data mostly indicated a competitive effect, which suggested that the two compounds may share same or similar sites in $GABA_AR$s. We then constructed mutations α1S297Q (c.889A>C_890G>A_891C>G) and β2N289S (c.866A>G). These mutant $GABA_AR$s, including α1(S297Q)β2γ2L, α1β2(N289S)γ2L, and α1(S297Q)β2(N289S)γ2L, were functionally expressed in CHO cells (Table 2). As shown in Fig. 8b, c, the mutation S297Q in the α1 subunit significantly decreased SchB- and PB-induced potentiation in α1(S297Q)β2γ2L $GABA_AR$s. However, SchB- and PB-induced potentiation remained unaffected in mutant α1β2(N289S)γ2L $GABA_AR$s. By contrast, when using the combined mutant channels (α1(S297Q)β2(N289S)γ2L $GABA_AR$s), SchB- and PB-induced potentiation was almost completely lost. Overall, similar to PB, the results indicated that the residues α1S297 and β2N289, located in the interfaces of the β+/α- subunits, are also crucial for SchB to potentiate the activity of $GABA_A$ receptors in recombinant α1β2γ2L $GABA_AR$s. The conservation of the sequence between GlyRs (Ser267) and $GABA_AR$s (Ser297) is very critical for the action of SchB (Fig. 8d). This finding provides a solid explanation for the reason that SchB affects both $GABA_AR$s and GlyRs.

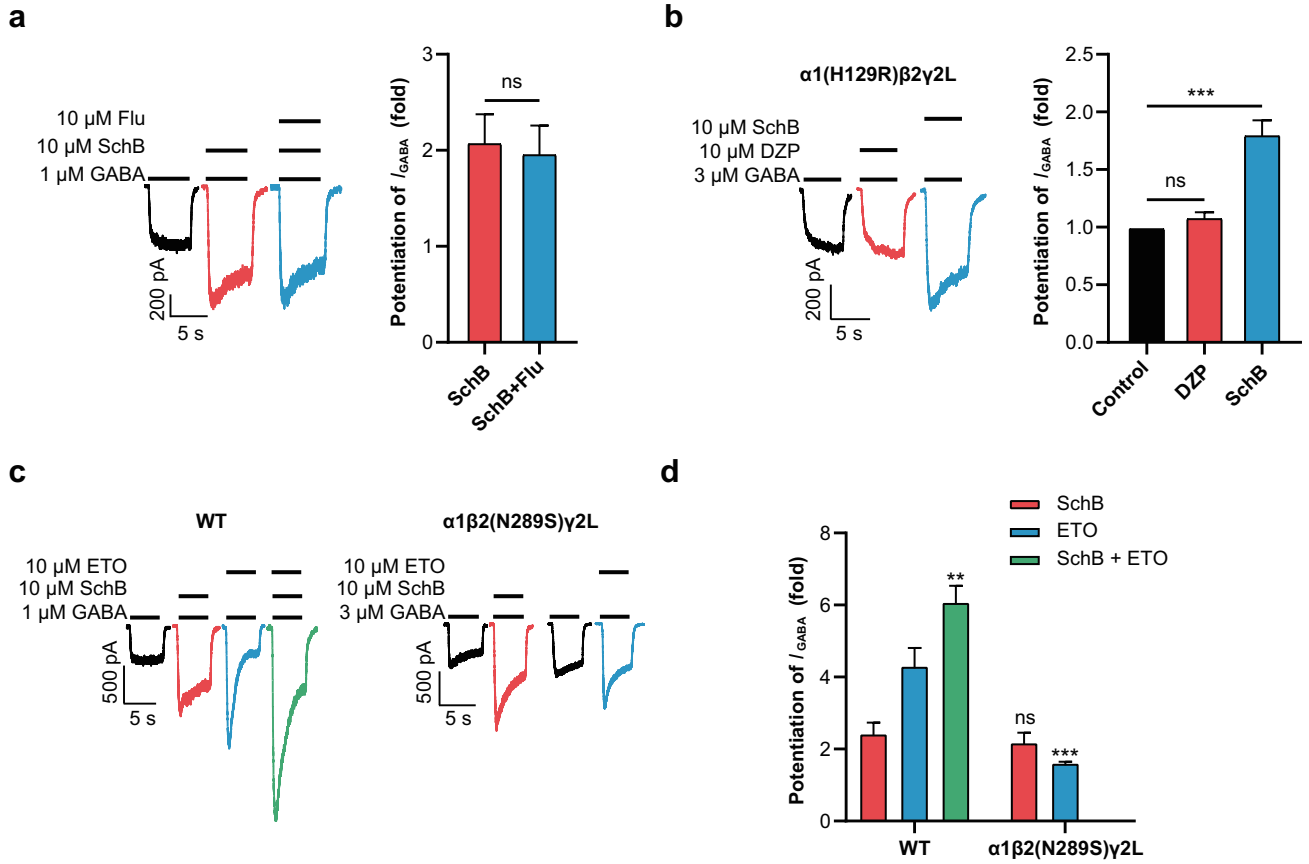

Fig. 7 SchB-induced potentiation of GABA-evoked currents (I_{GABA}) in recombinant α1β2γ2L is mediated through sites independent of those of diazepam (DZP) and etomidate (ETO). a Left, representative current traces for the potentiation of I_{GABA} by SchB in the absence and presence of flumazenil (Flu) (10 μM) in α1β2γ2L. Right, summary for SchB-induced potentiation in the absence and presence of Flu; ns not significant ($P > 0.05$), Student's paired *t*-test. **b** Left, examples of current traces for the potentiation of I_{GABA} by SchB (10 μM) and DZP (1 μM) in the mutant channel α1(H129R)β2γ2L. Right, summary for the potentiation induced by SchB and DZP. ***$P < 0.001$, ns = not significant ($P > 0.05$), Student's paired *t*-test. **c** Current traces for the potentiation of EC_{10-20} GABA-elicited currents by SchB (10 μM) and ETO (10 μM) in the wild-type (WT) and mutant channel α1β2(N289S)γ2L. **d** Summary data for the potentiation induced by SchB (10 μM) and ETO (10 μM) in the WT and mutant channel α1β2(N289S)γ2L; ***$P < 0.001$, ns not significant ($P > 0.05$), Student's unpaired *t*-test, compared to WT; **$P < 0.01$, Student's paired *t*-test, compared to WT. All data are expressed as the mean ± SD, $n = 5$.

DISCUSSION

Both GlyRs and $GABA_A$Rs are the major inhibitory receptors in the central nervous system and play important roles in maintaining the excitatory-inhibitory balance. Clinical and genetic evidences have demonstrated their advantages and potentials as targets for epilepsy treatment. Currently in drug discovery field, the strategy "one gene, one disease" for drug development has become increasingly inefficient. The situation also occurs in the field of epilepsy. A drug by acting on multiple targets within the disease network would be more preferable.

In this study, we found that SchB, a major bioactive component of *Schisandra chinensis (Turcz.) Baill*, significantly potentiated GABA- and glycine-induced currents in a dose-dependent manner in both neuronal and recombinant receptors. Importantly, SchB showed anti-seizure effects in various experimental seizure models. The primary mechanism of action for SchB in seizures appears to be related to its potentiating effect on $GABA_A$ and glycine receptors.

SchB exerted significant potentiating effects on recombinant (α1–α3) and neuronal GlyRs. Additionally, SchB effectively eliminated STR-mediated inhibition of GlyRs. These findings suggest that SchB-induced enhancement of GlyRs may contribute to its anti-seizure effect in STR-induced seizures. Compared to the potentiating effects on recombinant homologous α1-α3 GlyRs, SchB was found to be more effective on neuronal GlyRs. It is very possible that the compositions of native GlyRs receptors, especially the presence of some other auxiliary subunits, such as β-subunit, may enhance the activity of SchB.

$GABA_A$Rs play significant roles in the development of epilepsy. Recent studies have found that the surface expression of the $GABA_A$Rs β2/3 and γ2 subunits was frequently reduced during status epilepticus (SE) and temporal lobe epilepsy. However, the surface expression of the δ subunit remained unchanged [47, 48]. BZDs only affect the activity of γ-containing $GABA_A$Rs, but have no effect on δ-containing $GABA_A$Rs [49]. This may partially explain the pharmacoresistance to BZDs during prolonged SE [50]. Therefore, δ-containing $GABA_A$Rs become attractive targets for anti-seizure therapies. Encouragingly, SchB significantly potentiated both γ- and δ-containing $GABA_A$Rs, with higher activity on the latter. SchB could also decrease the severity of seizures in pilocarpine-induced SE. Thus, these results indicate that compounds like SchB might have significant advantages in the treatment of epilepsy, especially a potential as an adjunctive agent with DZP for the treatment of the later stages of SE. Mutations in genes encoding subunits of $GABA_A$Rs (GABRA1, GABRB2/B3, GABRG2, and GABRD) have been linked to several types of genetic epilepsy [13]. Our results demonstrated that SchB effectively restored the function of those mutated $GABA_A$Rs. This suggests that SchB has a therapeutic potential for severe genetic epilepsies caused by the loss-of-function mutations of $GABA_A$Rs. However, in vivo experiments are

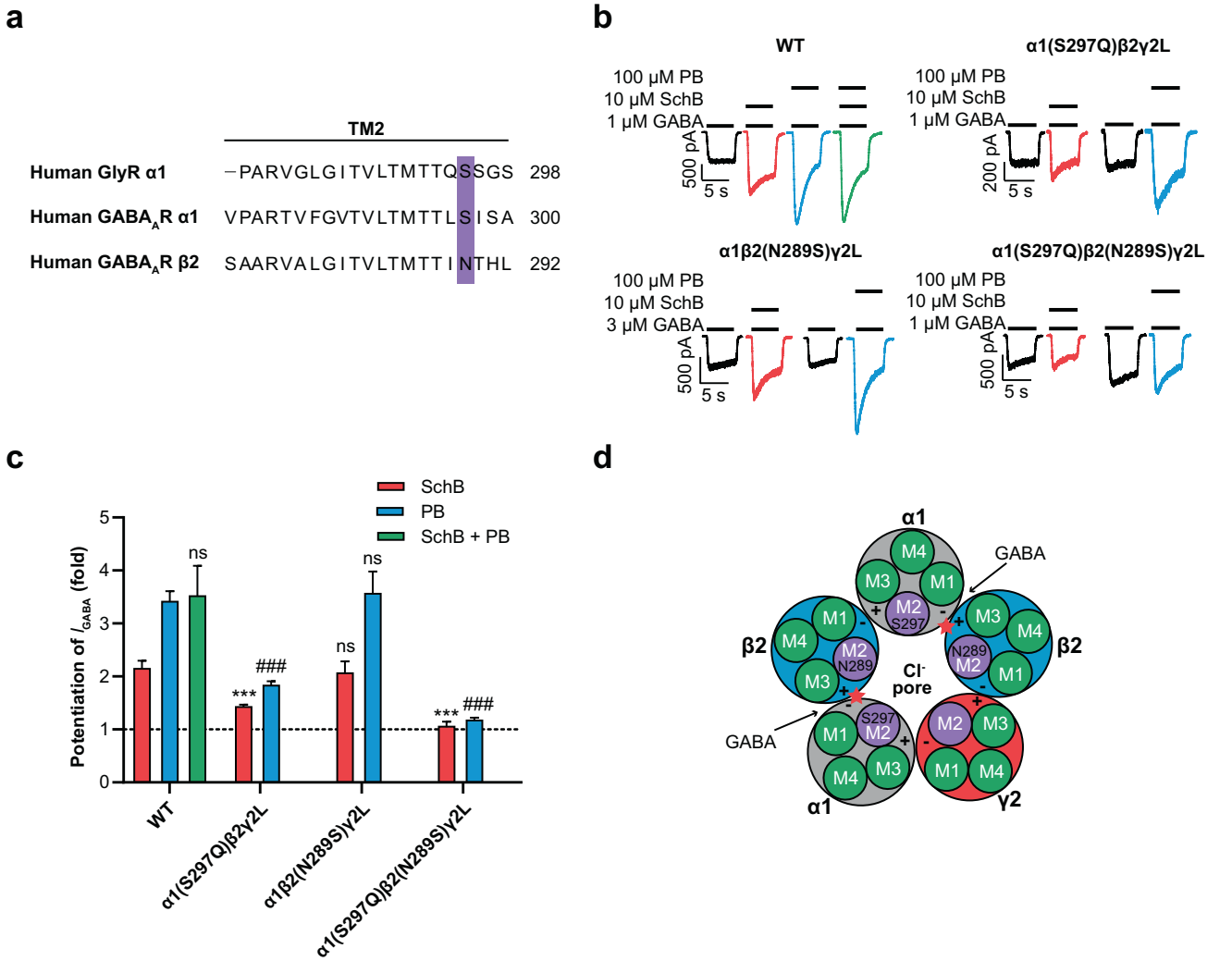

Fig. 8 The TM2 residues α1S297 and β2N289, located in the β+/α- subunit interfaces, are crucial for SchB-induced potentiation of GABA-elicited currents in recombinant α1β2γ2L. a Sequence alignment of the transmembrane domains 2 (TM2) of human GlyR α1, $GABA_AR$ α1, and β2 subunits. **b** Representative traces depicting $EC_{10\text{-}20}$-induced GABA currents in the absence and presence of 10 μM SchB, 100 μM pentobarbital (PB) or the co-application of the two compounds in wild type (WT), α1(S297Q)β2γ2L, α1β2(N289S)γ2L, and α1(S297Q)β2(N289S) γ2L $GABA_ARs$. **c** Summary data for the effects of SchB and PB on wild-type and mutant α1β2γ2 $GABA_ARs$. $^{***}P < 0.001$, $^{###}P < 0.001$, ns = not significant ($P > 0.05$), one-way ANOVA followed by Dunnett's multiple comparisons test, compared to the wild type. All the data are expressed as the mean ± SD, $n = 5$. **d** α1β2γ2L $GABA_A$ receptor is composed of two α, two β and one γ2L subunit. Two GABA-binding sites are located at the interfaces of the two β+/α- subunits. The scheme illustrates the localization of the point mutations α1 S297 and β2 N289 in TM2. "+" and "−" indicate the positive and negative sides of the subunits. The red stars represent possible binding sites for SchB and PB.

necessary to validate this effect in the future. Previous studies showed that α1-containing $GABA_ARs$ are associated with sedation [51]. α2-containing $GABA_ARs$ have been linked to anxiolysis [52]. Antidepressant effects of neuroactive steroids are mainly dependent on δ-containing $GABA_ARs$ in the postpartum period [53], such as the recent approved drugs, brexanolone injection (ZULRESSO™), a mixture of allopregnanolone, an endogenous inhibitory pregnane neurosteroid, and sulfobutylether-beta-cyclodextrin [54]. In the present study, we found SchB significantly potentiated both α1β2γ2L, α2β2γ2L, α4β3δ, and α6β3δ $GABA_ARs$, which may account for those reported effects, such as sedative-hypnotic [18], anxiolytic [19], and antidepressant effects [20].

Finally, we thoroughly investigated the potentiation mechanism of SchB on $GABA_ARs$ and GlyRs. The data suggest that SchB potentiated glycine-elicited currents through the sites different from the classical positive allosteric modulators such as CBD/Δ^9-THC-, propofol-, and endocannabinoid-binding sites. Nevertheless, the mutation S267Q in TM2 of GlyR α1 almost completely eliminated SchB-mediated potentiation. Coincidentally, the mutation has also been found to eliminate ethanol- (or anesthetics such as enflurane)-induced function enhancement of GlyRs [38].

Meanwhile, the subunit dependence of SchB in $GABA_ARs$ is distinct from that of DZP, indicating that SchB might function at a different site from DZP. We showed that the potentiation effect of SchB on αβ2γ2L is not interfered by flumazenil or mutant α1(H129R)β2γ2L $GABA_ARs$. Furthermore, the co-stimulation of SchB and ETO resulted in a synergistic effect. The mutation β2(N289S), which eliminated ETO-induced potentiation, did not affect SchB-induced potentiation. These results suggest that the action site of SchB is independent of those of DZP and ETO. Based on the fact that $GABA_ARs$ and GlyRs belong to Cys-loop receptors that form pentameric ion channels [29], we investigated whether the residues in $GABA_ARs$ homologous to Ser267 (premature: Ser295) in GlyR α1 subunit were crucial for the effect of SchB on $GABA_ARs$. Notably, SchB-induced potentiation was significantly

reduced on α1(S297Q)β2γ2L, but not changed on α1β2(N289S)γ2L. Furthermore, the combined mutant constructs of α1(S297Q)β2(N289S)γ2L almost abolished SchB-induced potentiation. Coincidently, the sites happened to be able to diminish pentobarbital (PB)-induced potentiation of α1β2γ2L $GABA_A$Rs [46]. The ability of PB to directly activate $GABA_A$ receptor may contribute to its anesthetic and sedative actions at higher doses [55, 56]. However, unlike barbiturates, SchB did not directly activate $GABA_A$Rs. Barbiturates and SchB also differ in their activity on other targets. For example, barbiturates have been shown to block AMPA receptors [57], whereas our findings indicated that SchB did not show any effects on either AMPA or NMDA receptors (Fig. S6). These effects distinguished SchB from classical positive allosteric modulators of $GABA_A$Rs and GlyRs, thereby demonstrating the unique pharmacological profile of SchB.

In conclusion, our results demonstrated, for the first time, that SchB is a dual positive allosteric modulator of $GABA_A$Rs and GlyRs, alleviates the seizures in multiple mouse models. However, it cannot be excluded that other mechanisms might be involved, and additional studies will be necessary to be explored for the actions of SchB in CNS. Meanwhile, for the goal of creating innovative candidates with enhanced bioavailability and permeability through the blood–brain barrier, this study may provide a valuable scaffold for further structural design.

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AUTHOR CONTRIBUTIONS

JW: Investigation, Methodology, Formal analysis, and Writing-original draft; MZ, YCJ, ML, and KXY: Investigation, Methodology, and contributing reagents. HBY: Investigation, Methodology, Writing-review & editing, Funding acquisition, Supervision, and Project administration. All authors have approved the final version of the manuscript.

ADDITIONAL INFORMATION

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