

## Original Paper

Transient Receptor Potential Vanilloid 4  
Activation-Induced Increase in Glycine-  
Activated Current in Mouse Hippocampal  
Pyramidal NeuronsMengwen Qi<sup>a</sup> Chunfeng Wu<sup>c</sup> Zhouqing Wang<sup>a</sup> Li Zhou<sup>a</sup> Chen Men<sup>d</sup>  
Yimei Du<sup>e</sup> Songming Huang<sup>c</sup> Lei Chen<sup>a, b</sup> Ling Chen<sup>a</sup><sup>a</sup>Department of Physiology, Nanjing Medical University, Nanjing, <sup>b</sup>Neuroprotective Drug Discovery Key Laboratory of Nanjing Medical University, Nanjing, <sup>c</sup>Department of Neurology, Children's Hospital of Nanjing Medical University, Nanjing, <sup>d</sup>Department of Geriatrics, The First Affiliated Hospital of Nanjing Medical University, Nanjing, <sup>e</sup>Research Center of Ion Channelopathy, Institute of Cardiology, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, P.R. China

## Key Words

Transient receptor potential vanilloid 4 • Glycine receptor • Phosphorylation • Protein kinase C • Calcium/calmodulin-dependent protein kinase II • Glycine receptor subunit expression

## Abstract

**Background/Aims:** Glycine plays an important role in regulating hippocampal inhibitory/excitatory neurotransmission through activating glycine receptors (GlyRs) and acting as a co-agonist of N-methyl-D-aspartate-type glutamate receptors. Activation of transient receptor potential vanilloid 4 (TRPV4) is reported to inhibit hippocampal A-type  $\gamma$ -aminobutyric acid receptor, a ligand-gated chloride ion channel. GlyRs are also ligand-gated chloride ion channels and this paper aimed to explore whether activation of TRPV4 could modulate GlyRs.**Methods:** Whole-cell patch clamp recording was employed to record glycine-activated current ( $I_{Gly}$ ) and Western blot was conducted to assess GlyRs subunits protein expression. **Results:** Application of TRPV4 agonist (GSK1016790A or 5,6-EET) increased  $I_{Gly}$  in mouse hippocampal CA1 pyramidal neurons. This action was blocked by specific antagonists of TRPV4 (RN-1734 or HC-067047) and GlyR (strychnine), indicating that activation of TRPV4 increases strychnine-sensitive GlyR function in mouse hippocampal pyramidal neurons. GSK1016790A-induced increase in  $I_{Gly}$  was significantly attenuated by protein kinase C (PKC) (BIM II or D-sphingosine) or calcium/calmodulin-dependent protein kinase II (CaMKII) (KN-62 or KN-93) antagonists but was unaffected by protein kinase A or protein tyrosine kinase antagonists. Finally, hippocampal protein levels of GlyR  $\alpha 1$ ,  $\alpha 2$ ,  $\alpha 3$  and  $\beta$  subunits were not changed by treatment with GSK1016790A for 30 min or 1 h, but GlyR  $\alpha 2$ ,  $\alpha 3$  and  $\beta$  subunits protein levels increased in mice that were intracerebroventricularly (icv.) injected with GSK1016790A for 5 d. **Conclusion:**

M. Qi and C. Wu contributed equally to this work.

Songming Huang  
and Lei ChenDepartment of Nephrology, Children's Hospital of Nanjing Medical University,  
Department of Physiology, Nanjing Medical University, Nanjing (P.R. China)  
E-Mail smhuang@njmu.edu.cn, chenl@njmu.edu.cn

Activation of TRPV4 increases GlyR function and expression, and PKC and CaMKII signaling pathways are involved in TRPV4 activation-induced increase in  $I_{\text{Gly}}$ . This study indicates that GlyRs may be effective targets for TRPV4-induced modulation of hippocampal inhibitory neurotransmission.

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## Introduction

Transient receptor potential vanilloid 4 (TRPV4) is a member of the transient receptor potential superfamily [1]. TRPV4 displays a widespread expression in the central nervous system (CNS), including in the cortex, thalamus, hippocampus and cerebellum [2]. Activation of TRPV4 induces calcium ( $\text{Ca}^{2+}$ ) influx and depolarizes the cell membrane [3]. In addition, activation of TRPV4 can modulate voltage-gated ion channels (such as voltage-gated sodium, potassium and calcium channels) and ligand-gated ion channels (such as N-methyl-D-aspartate (NMDA)- and  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-type glutamate receptors, transient receptor potential vanilloid 1 (TRPV1) receptors and A-type  $\gamma$ -aminobutyric acid ( $\text{GABA}_A$ ) receptors) [4-9]. These reports indicate that TRPV4 is an important target for controlling cellular excitability.

Accumulating evidence has shown that activation of TRPV4 leads to an increase in neuronal excitability [10, 11]. In the CNS, the balance between excitatory and inhibitory neurotransmitter systems plays an important role in modulating neuronal excitability. Activation of TRPV4 may promote pre-synaptic glutamate release and increase post-synaptic glutamate receptors to enhance glutamatergic transmission in the hippocampus [7, 8, 12]. TRPV4 expressed in the astrocytes has been identified to be responsible for initiating excitatory gliotransmitter release to enhance hippocampal synaptic transmission in mouse [13]. Therefore, activation of TRPV4 may facilitate the excitatory neurotransmitter system function. GABA and glycine are two main inhibitory neurotransmitters that act through ligand-gated chloride ion ( $\text{Cl}^-$ ) channels and mediate inhibitory neurotransmission [14, 15]. GABAergic neurotransmission predominates in all brain regions through activation of A-type ionotropic GABA receptors ( $\text{GABA}_A$ Rs). Glycine also mediates fast inhibitory transmission via ionotropic glycine receptors (GlyRs), which predominates in the spinal cord and brainstem [14, 16]. Furthermore, GlyRs have been proven to be functionally expressed in the hippocampus of rodents [17]. Activation of GlyRs limits activity in the synaptic network by depressing suprathreshold excitatory postsynaptic potentials to subthreshold events in recordings from both CA1 pyramidal cells and interneurons [18]. GlyRs are also involved in glycine-induced long-term depression of excitatory postsynaptic currents in hippocampal CA1 pyramidal neurons [19]. In addition to the inhibition mediated through GlyRs *per se*, there is evidence about the interactions between GABAergic and glycinergic neurotransmission. The cross-inhibition between GlyRs and  $\text{GABA}_A$ Rs has been revealed in olfactory bulb cells, spinal dorsal horn neurons and hippocampal pyramidal neurons [17, 20, 21]. Therefore, GlyRs may play an important role in modulating hippocampal synaptic and network plasticity.

In our recent study, GABA-induced current recorded in hippocampal neurons was inhibited by application of TRPV4 agonists, indicative of down-regulation of  $\text{GABA}_A$ Rs [6]. GlyRs are also ligand-gated chloride ion channels, but whether they can be modulated by TRPV4 activation remains unknown. Studies have proven that GlyRs can be phosphorylated by various protein kinases such as protein kinase A (PKA), protein kinase C (PKC), calcium/calmodulin-dependent protein kinase II (CaMKII) and protein tyrosine kinase (PTK) [22-24]. In our previous studies, activation of TRPV4 modulated voltage- and ligand-gated ion channels via intracellular signaling (PKA, PKC, CaMKII, etc.) [4-9]. In this study, we examined whether glycine-activated current ( $I_{\text{Gly}}$ ) in mouse hippocampal neurons and GlyR subunit expression in hippocampi could be modulated by TRPV4 activation and further explored the possible mechanisms underlying TRPV4 action.

## Materials and Methods

### *Experimental animals*

Mice (ICR, Oriental Bio Service Inc., Nanjing, China) were used in this study. All animal experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals of Nanjing Medical University and were approved by the Ethics Committee of Nanjing Medical University (No. IACUC-1601090). All efforts were made to minimize animals suffering and to reduce the number of animals used.

### *Slice preparation*

After the mice (2–3 weeks old) were anesthetized with diethyl ether, they were decapitated and the brains were rapidly removed. 400  $\mu\text{m}$ -thick coronal brain slices were cut using a vibrating microtome (Microslicer DTK 1500, Dousaka EM Co., Kyoto, Japan) in ice-cold modified artificial cerebrospinal fluid (ACSF) as previous reported [6]. The ACSF was composed of (in mM) NaCl 126, KCl 2.5,  $\text{CaCl}_2$  1,  $\text{MgCl}_2$  1,  $\text{NaHCO}_3$  26,  $\text{KH}_2\text{PO}_4$  1.25, and D-glucose 20, oxygenated with a gas mixture of 95%  $\text{O}_2$ /5%  $\text{CO}_2$ . The hippocampal slices were transferred to a recording chamber after being incubated in ACSF for one hour at 32°C for recovery.

### *Whole-cell patch clamp recording*

Hippocampal CA1 pyramidal neurons were perfused continually with oxygenated ACSF at room temperature (22–23°C). An EPC-10 amplifier (HEKA Elektronik, Lambrecht/Pfalz, Germany) was used to record and amplify  $I_{\text{gly}}$ . The sampling rate was 10 kHz and filtered (Bessel) at 2.9 kHz. The capacitance and series resistance were compensated to 90%. 0.3  $\mu\text{M}$  tetrodotoxin (TTX) was added to the ACSF to block TTX-sensitive voltage-gated sodium current. Glycine was dissolved in the ACSF and was focally applied using a rapid drug delivery system directed toward the soma of the recorded neurons. The holding potential was –60 mV and the glass pipettes resistance was 1–3 M $\Omega$ . The pipette solution was composed of (in mM) CsCl 140,  $\text{CaCl}_2$  0.5,  $\text{MgCl}_2$  3, EGTA 5, HEPES 10, and Tris-ATP 5 at pH 7.25. The expression of TRPV4 in hippocampal CA1 pyramidal neurons was functionally verified if TRPV4 agonist (1  $\mu\text{M}$  GSK1016790A or 500 nM 5, 6-EET)-induced current was recorded.

### *Drug treatment*

In the previous studies, TRPV4 agonist was intracerebroventricularly (icv.) injected to activate TRPV4 *in vivo* [25, 26]. Here, the TRPV4 agonist GSK1016790A was injected (icv.) as previously reported [6]. After the mice (male, 9–10 weeks old) were anesthetized with 2% chloral hydrate (20 ml/kg), they were placed in a stereotactic device (Kopf Instruments, Tujunga, CA). With the help of a stepper motor-controlled microsyringe (Stoelting, Wood Dale, IL, USA), GSK1016790A (1  $\mu\text{M}$ /2  $\mu\text{l}$ /mouse) was injected into the right lateral ventricle (0.3 mm posterior, 1.0 mm lateral, and 2.5 mm ventral to the bregma) at a rate of 0.2  $\mu\text{l}$ /min. GSK1016790A was injected once daily for 5 consecutive days (GSK1016790A-injected mice). Control mice were administered an equal volume of vehicle.

### *Western blot*

Hippocampi were quickly collected after the slices were perfused with GSK1016790A or 12 h after the last icv. injection of GSK1016790A. Then hippocampi were homogenized in a lysis buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 10 mM NaF, 1 mM sodium orthovanadate, 1% Triton X-100, 0.5% sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride and a protease inhibitor cocktail (Complete; Roche, Mannheim, Germany). Protein concentrations were determined using a bicinchoninic acid (BCA) Protein Assay Kit (Pierce, Rochford, IL, USA). Total proteins (40  $\mu\text{g}$ ) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and were then transferred to a polyvinylidene difluoride (PVDF) membrane. The membrane was incubated with 5% nonfat dry milk in Tris-buffered saline/0.1% Tween 20 (TBST) for 1 h at room temperature and was then incubated with an anti- $\alpha 1$  glycine receptor (Cat: ab166935, 1:1000, Abcam, Cambridge, UK), anti- $\alpha 2$  glycine receptor (Cat: ab97628, 1:1000, Abcam, Cambridge, UK), anti- $\alpha 3$  glycine receptor (Cat: ab118924, 1:1000, Abcam, Cambridge, UK), anti- $\beta$  glycine receptor (Cat: AGR-014, 1:200, Alomone Labs Ltd, Jerusalem, Israel) or anti-glyceraldehyde 3-phosphate dehydrogenase (anti-GAPDH) antibody (Cat: ab181602, 1:5000; Abcam, Cambridge, UK) at 4°C overnight. After being washed with TBST for three times, the membrane was incubated with a horseradish peroxidase

(HRP)-labeled secondary antibody and then developed using an ECL detection Kit (Amersham Biosciences, Piscataway, NJ). Western blot bands were scanned and analyzed with ImageJ software (National Institutes of Health). Each experimental group contained 9 mice. Hippocampal samples were obtained from nine mice as a set for the western blot analysis.

#### Data analysis

All  $I_{\text{Gly}}$  data were acquired from neurons in which both  $I_{\text{Gly}}$  and TRPV4 agonist-evoked current could be recorded. To examine the concentration-dependent response of GlyRs,  $I_{\text{Gly}}$  induced by 10, 30, 50, 100 and 300  $\mu\text{M}$  glycine was normalized to the current induced by 300  $\mu\text{M}$  glycine in the same neuron. The data were fitted to the logistic equation  $I = I_{\text{max}} / [1 + (\text{EC}_{50}/C)^n]$ , where  $n$  is the Hill coefficient and  $\text{EC}_{50}$  value is the concentration of glycine required for a half-maximal response. To examine the current-voltage relationship ( $I$ - $V$  curve) of  $I_{\text{Gly}}$ ,  $I_{\text{Gly}}$  induced at different holding potentials ( $-80$ ,  $-40$ ,  $-20$ ,  $0$ ,  $+20$ ,  $+40$  and  $+60$  mV) was normalized to the current induced at a holding potential of  $-60$  mV in the same neuron. Data were expressed as means  $\pm$  S.E.M. and analyzed using PulseFit (HEKA Elektronik) and Stata 7.0 software (STATA Corporation, USA). Statistical analysis was performed using paired or unpaired  $t$  test. Differences at  $P < 0.05$  or  $P < 0.01$  were considered statistically significant.

#### Chemicals

TTX was obtained from Enzo Life Science (Ann Arbor, MI, USA). PK1 and 5(6)-epoxy-8Z,11Z,14Z-eicosatrienoic acid (5, 6-EET) were obtained from Cayman Chemical (Ann Arbor, MI, USA). All other chemicals were obtained from Sigma Chemical Company. GSK1016790A, RN1734, HC-067047, D-sphingosine, bisindolylmaleimide II (BIM II), phorbol 12-myristate 13-acetate (PMA), H-89, PK1, 8-bromoadenosine 3',5'-cyclic monophosphate sodium salt (8-Br-cAMP), lavendustin A, genistein, KN62 and KN93 were prepared as stock solutions in dimethyl sulfoxide (DMSO). The final concentration of DMSO in the bath solution or pipette solution was  $< 0.1\%$ . GSK1016790A, 5, 6-EET, RN1734, HC-067047, BIM II, PMA, 8-Br-cAMP, lavendustin A, genistein, picrotoxin and strychnine were extracellularly applied by being added to the bath solution and to the rapid drug delivery system. The slices were pre-incubated with these chemicals for at least 5-8 min before recording  $I_{\text{Gly}}$ . D-sphingosine, H-89, PK1, KN62 and KN93 were present in the pipette solution and pre-applied by dialysis into the neurons through the pipette. The concentrations of these drugs were chosen according to previous reports [6, 27-30].

## Results

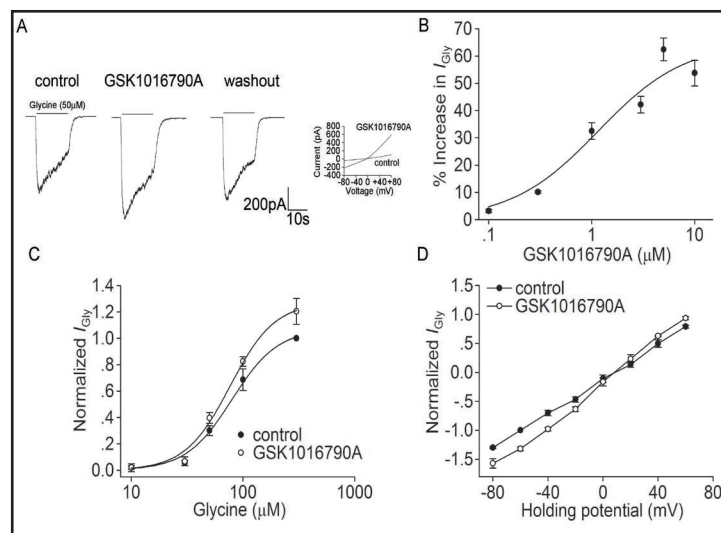
### Effect of TRPV4 agonists on $I_{\text{Gly}}$ in mouse hippocampal CA1 pyramidal neurons

GSK1016790A is a commonly used synthetic TRPV4 agonist [31]. In this study, GSK1016790A was first used to evaluate the effect of TRPV4 activation on  $I_{\text{Gly}}$ . As shown in Fig. 1A,  $I_{\text{Gly}}$  (activated by 50  $\mu\text{M}$  glycine) was reversibly increased by GSK1016790A (1  $\mu\text{M}$ ) and this effect was unlike the inhibitory effect of TRPV4 activation on GABA<sub>A</sub>Rs [6]. The average  $I_{\text{Gly}}$  was  $-14.35 \pm 1.27$  pA/pF,  $-21.57 \pm 0.98$  pA/pF and  $-15.75 \pm 1.76$  pA/pF before, during and after GSK1016790A treatment, respectively. GSK1016790A-induced increase in  $I_{\text{Gly}}$  was concentration-dependent when GSK1016790A concentrations ranging from 0.1 to 10  $\mu\text{M}$ , with an  $\text{EC}_{50}$  value of  $1.15 \pm 0.46$   $\mu\text{M}$  (Fig. 1B). Here,  $I_{\text{Gly}}$  was increased  $32.48 \pm 2.08\%$  by 1  $\mu\text{M}$  GSK1016790A ( $n = 35$ , paired  $t$  test,  $P < 0.01$ ) (Fig. 1B), and this concentration was used in the subsequent experiments.

The effect of GSK1016790A on the concentration-response curve for  $I_{\text{Gly}}$  is shown in Fig. 1C. The  $\text{EC}_{50}$  and  $n$  values of the concentration-response curve in the control group were  $79.15 \pm 6.13$   $\mu\text{M}$  and 2.11, respectively. The maximal response to 300  $\mu\text{M}$  glycine was enhanced by GSK1016790A ( $n = 9$ , paired  $t$  test,  $P < 0.01$ ), but  $\text{EC}_{50}$  ( $76.55 \pm 5.88$   $\mu\text{M}$ ) and  $n$  (2.16) values were unaffected (unpaired  $t$  test,  $P > 0.05$  in each case). Based on the concentration-response curve, 50  $\mu\text{M}$  glycine was used to activate  $I_{\text{Gly}}$  in the following experiments. The effect of GSK1016790A on the  $I$ - $V$  curve of  $I_{\text{Gly}}$  is shown in Fig. 1D. When the holding potential ranged from  $-80$  mV to  $+60$  mV,  $I_{\text{Gly}}$  was increased by the application of GSK1016790A. The reversal potential was  $8.16 \pm 0.34$  mV and  $8.38 \pm 0.40$  mV, and the ratio of current at  $+60/-80$

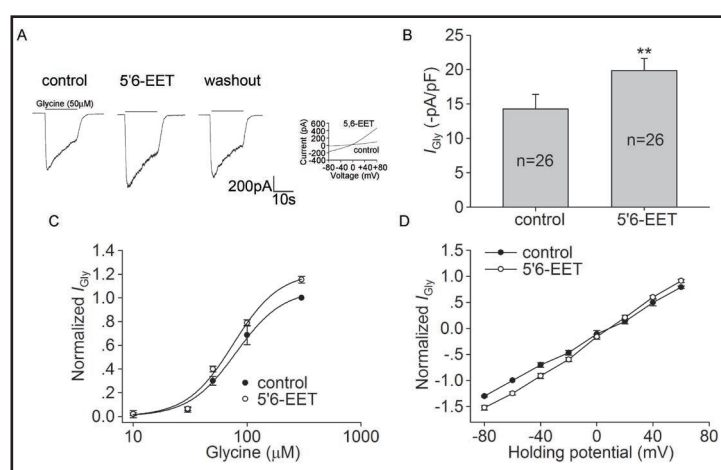


**Fig. 1.** Effect of TRPV4 agonist GSK1016790A on  $I_{Gly}$  in hippocampal CA1 pyramidal neurons. A.  $I_{Gly}$  (activated by 50  $\mu$ M glycine) was increased from -655.12 pA to -855.74 pA by 1  $\mu$ M GSK1016790A; the current recovered to -701.63 pA after washout. After recording  $I_{Gly}$ , 1  $\mu$ M GSK1016790A and 0.3  $\mu$ M TTX were added to the ACSF and a ramp protocol that depolarized from -80 mV to +80 mV over 700 ms was applied to the same neuron to test whether GSK1016790A-evoked current could be recorded. Representative recordings showing  $I_{Gly}$  and GSK1016790A-evoked current recorded in the same neuron.



B. The plot shows the increases in  $I_{Gly}$  by GSK1016790A at concentrations of 0.1, 0.3, 1, 3, 5 and 10  $\mu$ M. The concentration-response curve fits the Hill equation, with  $EC_{50}$  value of  $1.15 \pm 0.46$   $\mu$ M and  $n$  of 1.15. C. Concentration-response curves for  $I_{Gly}$  before and during GSK1016790A (1  $\mu$ M) treatment. Each point represents the normalized current from 9-12 neurons. D. I-V curves for  $I_{Gly}$  before and during GSK1016790A (1  $\mu$ M) treatment. Each point represents the normalized current from 8-10 neurons.

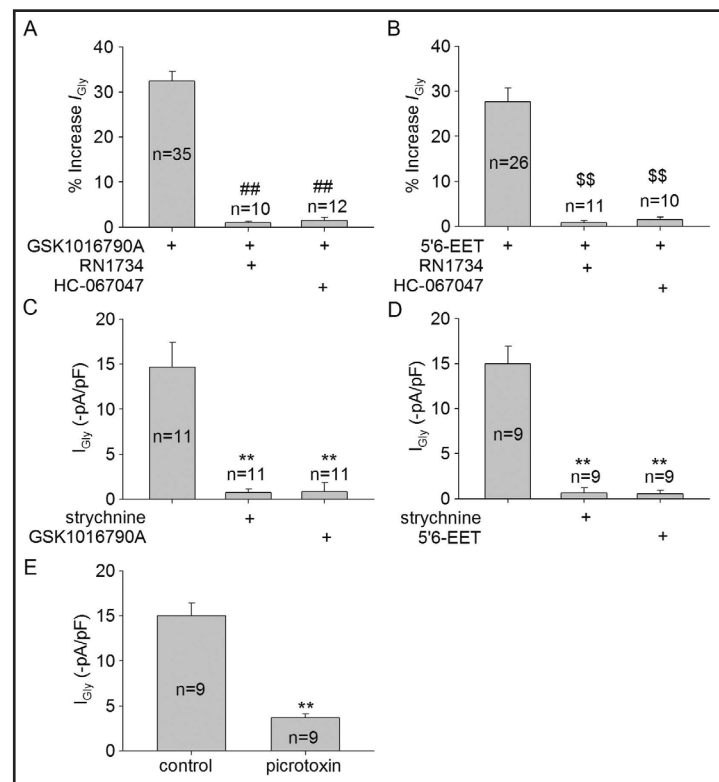
**Fig. 2.** Effect of TRPV4 agonist 5,6-EET on  $I_{Gly}$  in hippocampal CA1 pyramidal neurons. A.  $I_{Gly}$  was increased by 500 nM 5,6-EET.  $I_{Gly}$  was -709.33 pA, -921.57 pA and -811.65 pA before, during and after 5,6-EET treatment, respectively. Representative recordings showing  $I_{Gly}$  and 5,6-EET-evoked current recorded in the same neuron. B. Bar graph showing that  $I_{Gly}$  was -14.27  $\pm$  2.12 pA/pF and -19.85  $\pm$  1.78 pA/pF before and during 5,6-EET treatment. Paired t test,  $**P < 0.01$  vs. control. C. Concentration-response curves for  $I_{Gly}$  before and during 5,6-EET treatment. Each point represents the normalized current from 8-13 neurons. D. I-V curves for  $I_{Gly}$  before and during 5,6-EET treatment. Each point represents the normalized current from 8-12 neurons. Each point represents the normalized current from 7-12 neurons.



mV ( $I_{+60mV}/I_{-80mV}$ ) was  $-0.60 \pm 0.01$  and  $-0.59 \pm 0.03$  in the control and GSK1016790A-treated groups, respectively ( $n=10$ , paired t test,  $P > 0.05$  in each case; Fig. 1d).

5, 6-EET, a metabolite of arachidonate, has been reported as a natural TRPV4 agonist [31]. The effect of 5, 6-EET on  $I_{Gly}$  is shown in Fig. 2.  $I_{Gly}$  was increased from  $-14.27 \pm 2.12$  pA/pF to  $-19.85 \pm 1.78$  pA/pF by 500 nM 5, 6-EET ( $n=26$ , paired t test,  $P < 0.01$ ) and recovered to  $-15.51 \pm 2.47$  after 5, 6-EET was washed out (Fig. 2A and 2B). In the concentration-response curve for  $I_{Gly}$ , 5, 6-EET increased the maximal current activated by 300  $\mu$ M glycine ( $n=9$ , paired t test,  $P < 0.01$ ), but did not change the  $EC_{50}$  value ( $77.47 \pm 5.92$   $\mu$ M) or  $n$  (2.14) value (unpaired t test,  $p > 0.05$  in each case) (Fig. 2C). Application of 5, 6-EET treatment did not

**Fig. 3.** Effect of TRPV4 and GlyR antagonists on TRPV4 agonist-induced increase in  $I_{Gly}$ . A. In the presence of TRPV4 antagonist RN1734 or HC-067047, GSK1016790A-induced increase in  $I_{Gly}$  was decreased from  $34.48 \pm 2.08\%$  ( $n=35$ ) to  $0.97 \pm 0.28\%$  ( $n=10$ ) and to  $1.51 \pm 0.67\%$  ( $n=12$ ). Unpaired t test,  $##P < 0.01$  vs. GSK1016790A. B. 5,6-EET-induced increase in  $I_{Gly}$  ( $27.71 \pm 3.10\%$ ,  $n=26$ ) was attenuated by pre-application of RN1734 ( $0.83 \pm 0.45\%$ ,  $n=11$ ) or HC-067047 ( $1.52 \pm 0.54\%$ ,  $n=10$ ). Unpaired t test,  $##P < 0.01$  vs. 5,6-EET. C.  $I_{Gly}$  was inhibited from  $-14.70 \pm 2.71$  pA/pF to  $-0.75 \pm 0.45$  pA/pF by the application of strychnine, and the current was  $-0.85 \pm 0.98$  pA/pF in the presence of strychnine and GSK1016790A. Paired t test,  $**P < 0.01$  vs. control. D. In the presence of strychnine, the current was  $-0.65 \pm 0.55$  pA/pF and  $0.55 \pm 0.38$  pA/pF before and during 5,6-EET treatment, respectively. Paired t test,  $**P < 0.01$  vs. control. E. After application of 0.1 mM picrotoxin,  $I_{Gly}$  was reduced by  $76.04 \pm 1.76\%$  from  $-15.01 \pm 1.41$  pA/pF to  $-3.68 \pm 0.41$  pA/pF (paired t test,  $n=9$ ). Paired t test,  $**P < 0.01$  vs. control.



affect the reversal potential ( $8.34 \pm 0.31$  mV,  $n=9$ ) or  $I_{+60mV}/I_{-80mV}$  ratio ( $-0.59 \pm 0.02$ ,  $n=9$ ) of I-V curve (unpaired t test,  $P > 0.05$  in each case) (Fig. 2D). The above results imply that  $I_{Gly}$  is increased by TRPV4 agonists.

#### Effects of RN1734 and HC-067047 on TRPV4 agonists-induced increase of $I_{Gly}$

To further determine whether TRPV4 is involved in GSK1016790A- and 5,6-EET-induced increases in  $I_{Gly}$ , two specific TRPV4 antagonists, RN1734 (10  $\mu$ M) and HC-067047 (1  $\mu$ M), were used.  $I_{Gly}$  was not affected by either RN1734 (control:  $-14.82 \pm 1.16$  pA/pF, RN1734:  $-13.79 \pm 1.55$  pA/pF,  $n=13$ , paired t test,  $P > 0.05$ ) or HC-067047 (control:  $-14.62 \pm 2.99$  pA/pF, HC-067047:  $-14.50 \pm 2.71$  pA/pF,  $n=8$ , paired t test,  $P > 0.05$ ). Furthermore, GSK1016790A- and 5,6-EET-induced increases in  $I_{Gly}$  were blocked by RN1734 or HC-067047 (unpaired t test,  $P < 0.01$  in each case) (Fig. 3A and 3B). Combined with the above result, our data indicate that  $I_{Gly}$  is increased by activation of TRPV4.

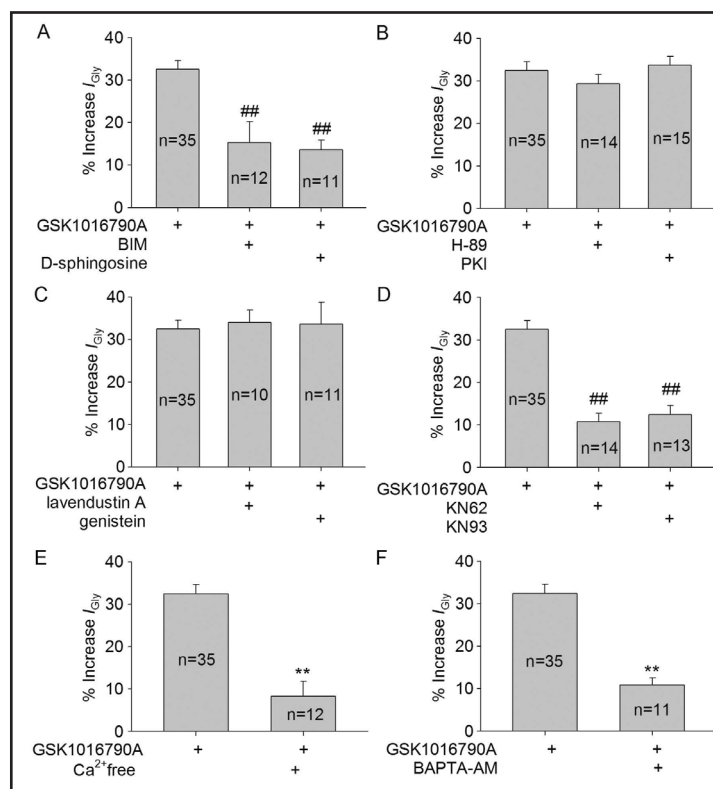
#### Effects of strychnine on TRPV4 agonist-induced increase in $I_{Gly}$

$I_{Gly}$  was inhibited  $95.78 \pm 0.98\%$  by strychnine (10  $\mu$ M), indicating that the present  $I_{Gly}$  was mediated by strychnine-sensitive GlyRs. When the neurons were pre-applied with strychnine, the current was unchanged by either GSK1016790A or 5,6-EET (Fig. 3C and 3D). In this study, we also found that  $I_{Gly}$  was not completely blocked by 0.1 mM picrotoxin, indicating that the GlyRs contained  $\beta$  subunits (Fig. 3E). Collectively, these results imply that strychnine-sensitive GlyR function is increased by activation of TRPV4.

#### Intracellular signaling pathways involved in GSK1016790A-induced increase in $I_{Gly}$

Studies have proven that  $\alpha$  and  $\beta$  subunits of GlyRs contain sequences for phosphorylation by PKA, PKC and PTK [22-24]. We next tested whether these protein kinases were responsible

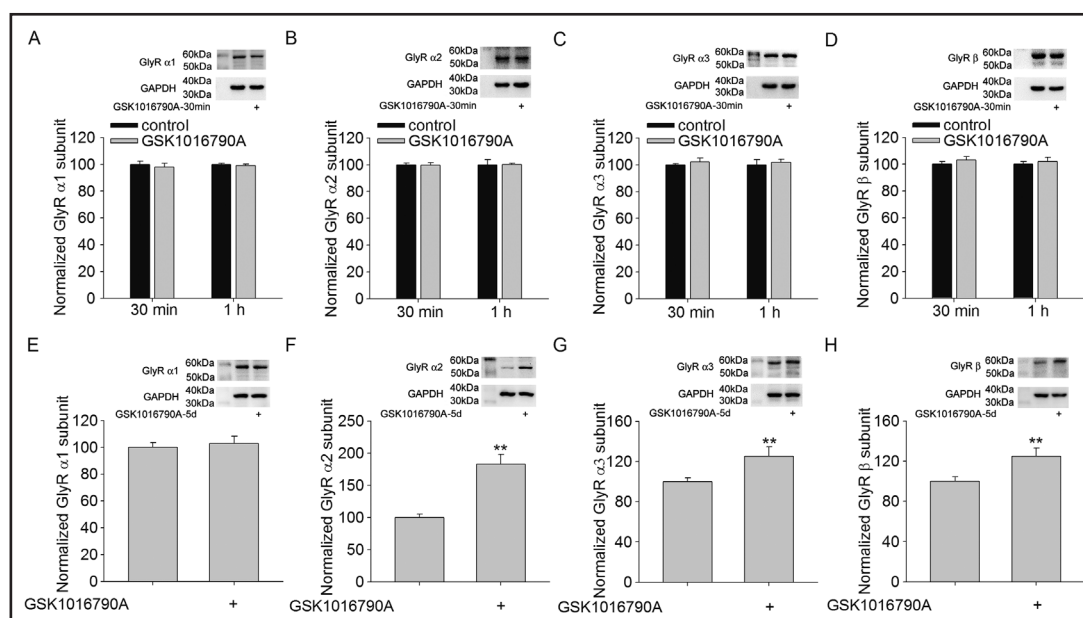
**Fig. 4.** PKC and CaMKII signaling pathways are involved in GSK1016790A-induced increase in  $I_{Gly}$ . A. GSK1016790A-induced increase in  $I_{Gly}$  was significantly attenuated by pre-application of PKC antagonists BIM or D-sphingosine. Unpaired t test,  $##P < 0.01$  vs. GSK1016790A. B. In the presence of the PKA antagonist H-89 or PKI, GSK1016790A-induced increase in  $I_{Gly}$  was  $29.31 \pm 2.17\%$  ( $n=14$ ) and  $33.73 \pm 1.98\%$  ( $n=15$ ), respectively. Unpaired t test,  $P > 0.05$ . C. After pre-application with the PTK antagonists lavendustin A or genistein, GSK1016790A-induced increase in  $I_{Gly}$  was  $34.00 \pm 2.91\%$  ( $n=10$ ) or  $33.64 \pm 5.17\%$  ( $n=11$ ), respectively. Unpaired t test,  $P > 0.05$ . D. GSK1016790A-induced increase in  $I_{Gly}$  was significantly attenuated by pre-application of CaMKII antagonists KN62 ( $n=14$ ) or KN93 ( $n=13$ ). Unpaired t test,  $##P < 0.01$  vs. GSK1016790A. E and F. GSK1016790A-induced increase in  $I_{Gly}$  was significantly attenuated when the slices were perfused with  $Ca^{2+}$  free ACSF (E) or pre-treated with BAPTA-AM (F). Unpaired t test,  $##P < 0.01$  vs. GSK1016790A.



for GSK1016790A-induced increase in  $I_{Gly}$ .  $I_{Gly}$  was increased from  $-15.13 \pm 0.91$  pA/pF to  $-18.98 \pm 1.24$  pA/pF by the PKC agonist PMA ( $1 \mu M$ ) ( $n=8$ , paired t test,  $P < 0.05$ ), whereas treatment with PKC antagonist BIM II ( $1 \mu M$ ) or D-sphingosine ( $20 \mu M$ ) reduced  $I_{Gly}$  from  $-15.03 \pm 1.15$  pA/pF to  $-12.46 \pm 1.41$  pA/pF ( $n=8$ , paired t test,  $P < 0.05$ ) and from  $-14.93 \pm 2.00$  pA/pF to  $-11.73 \pm 0.89$  pA/pF ( $n=9$ , paired t test,  $P < 0.05$ ), respectively. The present result was consistent with previous reports that activation of PKC enhanced GlyR function in hippocampal neurons [32]. As shown in Fig. 4A,  $I_{Gly}$  was increased  $15.30 \pm 4.91\%$  and  $13.64 \pm 2.17\%$  by GSK1016790A in the presence of BIM or D-sphingosine, both of which were significantly different from GSK1016790A-induced increase in  $I_{Gly}$  (unpaired t test,  $P < 0.01$ ).

Phosphorylation by PKA has been shown to both enhance and inhibit GlyR function in different neuronal preparations [33, 34]. In this study,  $I_{Gly}$  decreased from  $-15.00 \pm 1.07$  pA/pF to  $-12.40 \pm 0.32$  pA/pF when the PKA agonist 8-Br-cAMP ( $1$  mM) was extracellularly applied ( $n=8$ , paired t test,  $P < 0.01$ ).  $I_{Gly}$  increased from  $-14.73 \pm 1.61$  pA/pF to  $-18.01 \pm 1.12$  pA/pF ( $n=10$ , paired t test,  $P < 0.01$ ) and from  $-15.76 \pm 1.09$  pA/pF to  $-19.74 \pm 0.37$  pA/pF ( $n=12$ , paired t test,  $P < 0.01$ ) when the slices were exposed to PKA antagonist H-89 ( $10 \mu M$ ) or PKI ( $10 \mu M$ ). As shown in Fig. 4B, GSK1016790A-induced increases in  $I_{Gly}$  were  $29.31 \pm 2.17\%$  and  $33.73 \pm 1.98\%$  after pre-application of H-89 or PKI, respectively. These increase levels were not statistically different from the increase caused by GSK1016790A alone (unpaired t test,  $P > 0.05$ ).

PTK has been proven to enhance GlyR function in rat hippocampal neurons [22]. Here, in the presence of PTK antagonists lavendustin A ( $5 \mu M$ ) or genistein ( $50 \mu M$ ),  $I_{Gly}$  was decreased by  $17.55 \pm 3.06\%$  ( $n=8$ , paired t test,  $P < 0.05$ ) and  $20.01 \pm 2.89\%$  ( $n=9$ , paired t test,  $P < 0.01$ ), respectively. As shown in Fig. 4C, GSK1016790A-induced increase in  $I_{Gly}$  was not affected by pre-application of lavendustin A or genistein (unpaired t test,  $P > 0.05$ ). These



**Fig. 5.** Effect of chronic TRPV4 activation on GlyR subunits protein levels. A–D. The hippocampal protein levels of GlyR  $\alpha 1$  (A),  $\alpha 2$  (B),  $\alpha 3$  (C) and  $\beta$  (D) subunits did not change when the slices were treated with GSK1016790A for 30 min or 1 h. The protein levels of  $\alpha 1$ ,  $\alpha 2$ ,  $\alpha 3$  and  $\beta$  GlyR subunits were first normalized to the protein levels of GAPDH, respectively and then expressed as the percentage of control values. E–H. In GSK1016790A-injected mice, the hippocampal protein levels of GlyR  $\alpha 2$  (F),  $\alpha 3$  (G) and  $\beta$  (H) subunits increased, and the protein level of  $\alpha 1$  subunits (E) remained unchanged. The protein levels of  $\alpha 1$ ,  $\alpha 2$ ,  $\alpha 3$  and  $\beta$  GlyR subunits were first normalized to the protein levels of GAPDH, respectively. The protein levels in GSK1016790A-injected mice were expressed as the percentage of those in vehicle-injected mice (control mice). Unpaired t test,  $**P < 0.01$  vs. control.

results indicate that the PTK signaling pathway was not responsible for GSK1016790A-induced increase in  $I_{Gly}$ .

The modulation of GlyR function by CaMKII has been described in acutely isolated rat spinal neurons [35]. As a  $Ca^{2+}$  permeable channel, activation of TRPV4 induces  $Ca^{2+}$  influx. In our previous study, we found that CaMKII is involved in TRPV4-induced enhancement of NMDA receptors [7]. Here, we also tested whether CaMKII signaling was responsible for the TRPV4-induced increase in  $I_{Gly}$ . After the application of CaMKII antagonist KN62 (5  $\mu M$ ) or KN93 (5  $\mu M$ ),  $I_{Gly}$  was reduced from  $-16.10 \pm 1.03$  pA/pF to  $-11.28 \pm 0.43$  pA/pF ( $n=8$ , paired t test,  $P < 0.05$ ) and from  $-15.93 \pm 1.91$  pA/pF to  $-10.77 \pm 0.71$  pA/pF ( $n=7$ , paired t test,  $P < 0.05$ ), respectively. As shown in Fig. 4D, with KN62 or KN93 in the pipette solution, GSK1016790A treatment increased  $I_{Gly}$  by  $10.77 \pm 1.94\%$  or  $12.41 \pm 2.94\%$ , respectively. Both increase levels were significantly different from the increase caused by GSK1016790A alone (unpaired t test,  $P < 0.01$ ). These results indicate that, in addition to the PKC signaling pathway, the CaMKII signaling pathway is also involved in GSK1016790A-induced increase in  $I_{Gly}$ .

Here, if the slices were perfused with  $Ca^{2+}$ -free ACSF or pre-incubated with 10  $\mu M$  BAPTA-AM, a  $Ca^{2+}$  chelator, GSK1016790A treatment increased  $I_{Gly}$  by  $8.31 \pm 3.47\%$  ( $n=12$ ) and  $10.91 \pm 1.65\%$  ( $n=11$ ), respectively (Fig. 4E and 4F). These two increase levels were significantly different from GSK1016790A-induced increase in  $I_{Gly}$  (unpaired t test,  $P < 0.01$ ).

In previous studies, TRPV4 function can be modulated by phosphorylation by PKC [36]. The present study found that application of PKC antagonists BIM II and D-sphingosine reduced GSK1016790A-activated current (membrane potential =  $-60$  mV) by  $10.07 \pm 2.71\%$  ( $n=8$ , paired t test,  $P < 0.05$ ) and  $11.34 \pm 1.17\%$  ( $n=8$ , paired t test,  $P < 0.05$ ), respectively. The current was not affected by CaMKII antagonist KN62 (control:  $-1.24 \pm 0.09$  pA/pF, KN62:  $-1.20 \pm 0.07$



pA/pF,  $n=7$ , paired  $t$  test,  $P>0.05$ ) or KN93 (control:  $1.17\pm0.05$  pA/pF, KN93:  $-1.19\pm0.11$  pA/pF,  $n=7$ , paired  $t$  test,  $P>0.05$ ).

#### Effect of TRPV4 activation on GlyR subunits expression

Here, we examined the protein levels of GlyR  $\alpha 1$ ,  $\alpha 2$ ,  $\alpha 3$  and  $\beta$  subunits after the hippocampal slices were treated with GSK1016790A for 30 min and 1 h. These two time points were chosen based on the acute effect of TRPV4 activation in the electrophysiology recordings. As shown in Fig. 5A–5D, the protein levels of  $\alpha 1$ ,  $\alpha 2$ ,  $\alpha 3$  and  $\beta$  subunits in hippocampi did not change at 30 min or 1 h after treatment with GSK1016790A (unpaired  $t$  test,  $P>0.05$  in each case). Therefore, acute activation of TRPV4 did not affect GlyR expression.

In our previous studies, chronic activation of TRPV4 may change the expression of voltage-gated sodium channels and glutamate receptors [5, 37]. Here, hippocampal protein levels of GlyR  $\alpha 1$ ,  $\alpha 2$ ,  $\alpha 3$  and  $\beta$  subunits were examined in GSK1016790A-injected mice. Compared to the values in vehicle-injected mice, the protein levels of  $\alpha 2$ ,  $\alpha 3$  and  $\beta$  subunits increased in GSK1016790A-injected mice (unpaired  $t$  test,  $P<0.01$ ), while that of  $\alpha 1$  subunit remained unchanged (Fig. 5E–5H). This result indicates that chronic activation of TRPV4 may enhance GlyR expression.

## Discussion

GABA and glycine are two major inhibitory neurotransmitters in the mammalian brain. Glycine-induced inhibitory synaptic transmission mediated by strychnine-sensitive GlyRs is well known in the spinal cord and brain stem [14, 16]. In the hippocampus, GlyRs seem not as important as GABA<sub>A</sub>Rs, for GABA<sub>A</sub> antagonists completely abolish inhibitory postsynaptic currents. However, accumulating evidence has revealed the involvement of glycine and GlyRs in the regulation of hippocampal synapses and networks [18, 19]. Strychnine-sensitive GlyRs have been proven to be expressed in CA1 pyramidal cells in rat hippocampal slices and cultured hippocampal neurons where glycine-activated concentration-dependent currents can be recorded [38, 39]. Both  $\alpha$  homomeric and  $\alpha\beta$  heteromeric GlyRs are present in rat hippocampal pyramidal neurons [38]. In this study, glycine-activated current was recorded in CA1 pyramidal neurons in mouse hippocampal slices and  $I_{\text{Gly}}$  was completely blocked by strychnine, indicating that functionally strychnine-sensitive GlyRs were expressed in the mouse hippocampal pyramidal neurons. The GlyR  $\alpha$  subunit is the obligatory subunit, which forms functional homomeric channels or heteromeric channels together with the modulatory  $\beta$  subunit. Picrotoxin is known to selectively block  $\alpha$  homomeric GlyRs [29]. Here, about 20% of  $I_{\text{Gly}}$  remained in the presence of low dose of picrotoxin (0.1 mM), indicating that GlyRs contain  $\beta$  subunits (Fig. 3E). There were some differences in the concentration-response curve and I–V curve of  $I_{\text{Gly}}$  between the previous studies and the present data, which may be due, at least in part, to the differences of recording condition (such as the pipette solution and the extracellular solution). In our recent study, hippocampal GABA<sub>A</sub>R-mediated current was blocked by activation of TRPV4 [6]. GlyR is also a chloride channel, and  $I_{\text{Gly}}$  recorded in mouse hippocampal CA1 pyramidal neurons was unexpectedly increased by TRPV4 agonists (GSK1016790A and 5, 6-EET) (Figs. 1 and 2). We found that TRPV4 agonist action was blocked by the TRPV4 specific antagonists RN-1734 and HC-067047, further confirming the role of TRPV4 in the increased  $I_{\text{Gly}}$  (Fig. 3A and 3B). In subsequent experiments, we found that the EC<sub>50</sub> values in the concentration-response curves for  $I_{\text{Gly}}$  were unchanged by GSK1016790A or 5, 6-EET (Fig. 1C and 2C), suggesting that the TRPV4-induced increase in  $I_{\text{Gly}}$  is likely not due to increasing the ligand-binding affinity. The I–V curves showed that the reversal voltage and  $I_{+60\text{ mV}}/I_{-80\text{ mV}}$  ratio were unaltered by TRPV4 agonists, indicating that the TRPV4-induced increase in  $I_{\text{Gly}}$  is independent of the membrane voltage (Fig. 1D and 2D).

In this study, the protein levels of GlyR subunits did not change when the slices were perfused with GSK1016790A for 30 min to 1 h (Fig. 5A–5D), indicating that the increase in  $I_{\text{Gly}}$  by acute TRPV4 activation is likely due to the increase in GlyR function. Here, application of

PKC antagonist BIM II or D-sphingosine reduced GSK1016790A-activated current by about 10% and 11%, respectively, but in the presence of BIM II or D-sphingosine, GSK1016790A-induced increase in  $I_{\text{Gly}}$  was attenuated by about 53% and 59%, respectively (Fig. 4 A and 4D). Therefore, the blocking effect of PKC antagonists on GSK1016790A-induced increase was largely due to blocking the modulation on GlyR. As application of CaMKII antagonists didn't affect GSK1016790A-activated current, KN62 and KN 93 mainly acted on GlyR. It is known that activation of PKC or CaMKII depends on increases in  $[\text{Ca}^{2+}]_i$ . As TRPV4 is a calcium-permeable channel, TRPV4-mediated  $\text{Ca}^{2+}$  influx helps to increase  $[\text{Ca}^{2+}]_i$ . In addition, activation of TRPV4 enhances NMDA receptors (NMDARs) function, which facilitates increases in  $[\text{Ca}^{2+}]_i$  [7]. Here it was noted that GSK1016790A-induced increase in  $I_{\text{Gly}}$  was significantly attenuated if extracellular  $\text{Ca}^{2+}$  was removed or by BAPTA-AM (Fig. 4E and 4F), indicating that TRPV4-mediated increase in  $[\text{Ca}^{2+}]_i$  is likely responsible for the increase in GlyR function caused by TRPV4 activation. At present, we did not provide the direct evidence that the enhanced PKC or CaMKII activation may be related to the TRPV4-mediated increase in  $[\text{Ca}^{2+}]_i$  and more experiments are needed to clarify this possibility. The exact sequence for CaMKII phosphorylation has not been identified in GlyRs, but activation of CaMKII can increase glycine-induced currents in acutely isolated rat spinal neurons [35]. In this study, blockage of CaMKII by KN62 or KN93 decreased  $I_{\text{Gly}}$ , also indicating that activation of CaMKII may increase  $I_{\text{Gly}}$  in mouse hippocampal neurons. However, regarding the conditions of TRPV4 activation, it remains unclear whether activation of CaMKII phosphorylates GlyRs directly or indirectly modulates GlyRs by catalyzing its substrates.

In the hippocampus, glycine and GlyRs have been shown to mediate tonic inhibition, while GABA and GABA<sub>A</sub>Rs are responsible for both synaptic and tonic inhibition [40]. There may be a self-homeostatic regulation of hippocampal inhibition based on the crosstalk between GlyRs and GABA<sub>A</sub>Rs [17]. Activation of both GABA<sub>A</sub>Rs and GlyRs hyperpolarizes the neuronal membrane in the adult CNS through mediating  $\text{Cl}^-$  influx. When GABAergic inhibition weakens, GlyR-induced inhibition increases and thereby compensates for defects in GABAergic inhibition to a certain degree. But this compensation may be limited because of the low concentration of glycine in the cerebrospinal fluid and hippocampus [41]. On the other hand, the cross-inhibition between GlyRs and GABA<sub>A</sub>Rs attracts more researchers' attention. Activation of GlyRs can suppress the GABAergic inhibitory postsynaptic currents in the hippocampus by changing the cell input resistance or  $\text{Cl}^-$  driving force [42]. A direct receptor-receptor interaction is suggested to be responsible for a state-dependent cross-inhibition between GABA<sub>A</sub>Rs and GlyRs in rat hippocampal CA1 neurons [43]. In rat sacral dorsal commissural nucleus neurons, GlyRs-induced asymmetric inhibition of GABA<sub>A</sub>Rs is dependent on protein dephosphorylation caused by phosphatase 2B [21]. Activation of TRPV4 inhibited GABA<sub>A</sub>R-mediated current and the present data showed that activation of TRPV4 increased GlyRs function [6]. It is likely that the increased GlyR function revealed here may help to account for TRPV4-induced inhibition of GABA<sub>A</sub>R.

Although GlyR expression was not affected by acute TRPV4 activation, the expression of  $\alpha 2$ ,  $\alpha 3$  and  $\beta$  subunits were increased by sustained TRPV4 activation with the most significant increase observed for the protein level of  $\alpha 2$  subunits (Fig. 5F, 5G and 5H). The mature hippocampus displays relatively low expression levels of synaptic  $\alpha 1\beta$ -containing GlyRs, but high expression levels of extrasynaptic  $\alpha 2/\alpha 3$ -containing GlyRs [40, 44]. Therefore, it is possible that the extrasynaptic GlyR-induced tonic inhibition may be prominently affected when TRPV4 is chronically or sustainedly activated. Functional GlyRs have been detected in the spinal cord glial cells and cultured oligodendrocytes progenitors derived from cerebral cortex [45-47]. In this study, the protein levels of GlyR  $\alpha 2$ ,  $\alpha 3$  and  $\beta$  subunits of hippocampi were increased by GSK1016790A, but at present we could not determine the changes occurred in which type of cells (i.e. pyramidal neurons, interneurons or glial cells). More experiments are needed to clarify whether the pyramidal neuronal GlyR expression in hippocampi is specifically sensitive to chronic or sustained TRPV4 activation.

Glycine plays an important role in regulating hippocampal inhibitory/excitatory neurotransmission through activating GlyRs and acting as a co-agonist of NMDARs [17, 40].

There is no evidence about the interaction between GlyRs and NMDARs and at present we did not know whether activation of TRPV4 would affect the release of Gly in hippocampi, thereby modulating NMDARs. More experiments are needed to clarify this. GlyRs participate in self-homeostatic regulation of hippocampal inhibition through the cross-inhibition between GlyRs and GABA<sub>A</sub>Rs [17]. In this study, hippocampal GlyR function and expression were increased by TRPV4 activation. Therefore, GlyRs are effective targets for TRPV4-induced modulation of hippocampal inhibitory neurotransmission and even the neuronal excitability.

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## Disclosure Statement

The authors declare no conflict of interest.

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