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Inhibitory Effects of Honokiol on Substantia Gelatinosa Neurons of the Trigeminal Subnucleus Caudalis in Juvenile Mice

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Abstract—Inhibitory neurotransmitters such as gamma-aminobutyric acid (GABA) and glycine are known to be abundant in the substantia gelatinosa (SG) of the trigeminal subnucleus caudalis (Vc). Thus, it has been recognized as an initial synaptic site for regulating orofacial nociceptive stimuli. Honokiol, a principal active ingredient derived from the bark of Magnolia officinalis, has been exploited in traditional remedies with multiple biological effects, including anti-nociception on humans. However, the anti-nociceptive mechanism of honokiol on SG neurons of the Vc remains fully elusive. In this study, effects of honokiol on SG neurons of the Vc in mice were investigated using the whole-cell patch-clamp method. In a concentration-dependent manner, honokiol significantly enhanced frequencies of spontaneous postsynaptic currents (sPSCs) that were independent of action potential generation. Notably, honokiol-induced increase in the frequency of sPSCs was attributed to the release of inhibitory neurotransmitters through both glycinergic and GABAergic pre-synaptic terminals. Furthermore, higher concentration of honokiol induced inward currents that were noticeably attenuated in the presence of picrotoxin (a $GABA_A$ receptor antagonist) or strychnine (a glycine receptor antagonist). Honokiol also exhibited potentiation effect on glycine- and $GABA_A$ receptor-mediated responses. In inflammatory pain model, the increase in frequency of spontaneous firing on SG neurons induced by formalin was significantly inhibited by the application of honokiol. Altogether, these findings indicate that honokiol might directly affect SG neurons of the Vc to facilitate glycinergic and GABAergic neurotransmissions and modulate nociceptive synaptic transmission against pain. Consequently, the inhibitory effect of honokiol in the central nociceptive system contributes to orofacial pain management.

Key words: honokiol, substantia gelatinosa, patch-clamp method, spontaneous postsynaptic current, glycine receptors, $GABA_A$ receptors.

INTRODUCTION

For neuropathic pain, peripheral sensory inputs are relayed toward the trigeminal tract and primarily processed at the spinal trigeminal nucleus, in which subnucleus caudalis (Vc; also called the medullary dorsal horn) is responsible for pain and temperature information of dental and craniofacial regions (Tsai et al., 1999; Ren and Dubner, 2011). Additionally, the lamina II or substantia gelatinosa (SG) of the spinal dorsal horn plays a fundamental role in receiving and transmitting painful impulses from the periphery to the central nociceptive system (Cervero, 1982). Collectively, the SG of the Vc is admitted as an initial synaptic site for the regulation of orofacial nociception through small-diameter primary afferents, including myelinated Aδ- and unmyelinated C-fibers (Sessle, 2000; Santos et al., 2007). More importantly, the majority of SG neurons are inhibitory with

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Abbreviations: ACSF, artificial cerebrospinal fluid; CNS, central nervous system; GABA, gamma-aminobutyric acid; K-S, Kolmogorov-Smirnov; mIPSCs, miniature inhibitory postsynaptic currents; mPSCs, miniature postsynaptic currents; PBS, phosphate buffered saline; PND, postnatal day; SG, substantia gelatinosa; sPSCs, spontaneous postsynaptic currents; Vc, subnucleus caudalis.

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the abundant existence of gamma-aminobutyric acid (GABA) and glycine (Todd et al., 1996). In a series of experiments about the distribution, morphological characterization, and synaptic relations of glycinergic neurons in superficial spinal dorsal horn, glycine-only neurons, known as glycinergic neurons that lack GABA expression, are distributed substantially in laminae I-II and predominantly in laminae III-IV. Despite the limited number of glycine-only neurons in laminae I-II, it is believed that the majority of these glycine-only synapses are also created by axon terminals of glycinergic inhibitory neurons whose cell bodies are located in laminae III-IV. As a result, the nature of glycinergic inhibitory neurotransmission in the central nervous system (CNS) is determined by pre-synaptic mechanisms (Miranda et al., 2022).

Modern medicine uses various potential drugs extracted from natural sources to treat multiple human diseases effectively. For example, honokiol (3,5′-diallyl-4 ,2′-dihydroxybiphenyl), a major bioactive component of Magnolia officinalis bark (Fujita et al., 1973), has been identified as a promising herbal compound with numerous properties, including anti-oxidant (Dikalo et al., 2008), anti-microbial (Chang et al., 1998; Ho et al., 2001; Kim et al., 2010), anti-thrombotic (Hu et al., 2005), anti-allergic (Han et al., 2007), anti-angiogenic, and anti-tumor effects (Fried and Arbiser, 2009). Previous reports have described the ability of honokiol to cross the blood–brain barrier and to inhibit the growth of brain tumor *in vitro* and *in vivo* (Wang et al., 2011; Jun-Jun et al., 2015). To be specific, honokiol could cause apoptosis in a variety of tumors, including human colon cancer cell lines (Wang et al., 2004) and chronic lymphocytic leukemia cells, via activating caspase 8, 9, and 3 (Battle et al., 2005). Similarly, it could also protect the myocardium from ischemic injury (Tsai et al., 1996) and hepatocytes from peroxidative damage (Chiu et al., 1997).

Most interestingly, study suggests that honokiol possesses neuronal depressant properties, including anxiolytics, sedation, anti-convulsion, and neuroprotective effects (Fujita et al., 1973). Such anxiolytic, sedative, and anti-convulsant actions of honokiol involve increased phasic and tonic GABAergic neurotransmission in hippocampal dentate granule neurons (Alexeev et al., 2012). Besides, several studies have described the ability of honokiol to protect neurons mainly via mediating N-methyl-D-aspartate (NMDA) signal pathway and inhibiting inflammatory pain mediators (Lin et al., 2005, 2009; Cui et al., 2007). For example, honokiol can block glutamate, NMDA, and K^+-induced cationic signals causing repeated firing as well as inhibit NMDA receptor-induced nociception and mGluR5-induced reaction (Lin et al., 2005, 2009). Similarly, honokiol can reduce NMDA-evoked brain damage via its anti-oxidant actions in brain tissues (Cui et al., 2007).

Honokiol is also reported to exert potent neuroprotective, anti-nociceptive, and other neurological effects in the CNS via different mechanisms (Sheng et al., 2017; Chan et al., 2020). Firstly, this compound preferentially interacts with active states of voltage-gated potassium channel. Blockade of K^+ channels might contribute to anti-inflammatory or anti-nociceptive actions (Sheng et al., 2017). Secondly, neurological effects of honokiol could be partly originated from immunological and oxidative stress suppression. Two specific instances are: 1) the capacity of honokiol to retain Na^+/K^+-ATPase activity and enzymatic mitochondrial function (Chen et al., 2007); 2) the ability of honokiol to inhibit cerebral ischemic injury through blocking neutrophil infiltration and reactive oxygen species production (Liou et al., 2003).

Notably, previous studies have revealed the anti-nociceptive effect of honokiol in formalin-induced inflammatory pain states (Lin et al., 2007; Woodbury et al., 2015). Thus, the orofacial formalin test has been successfully applied for investigating the mechanisms of trigeminal pain and for evaluating analgesic responses (Clavelou et al., 1995). To be particular, in formalin-induced chronic pain model, honokiol attenuated the inflammatory phase of paw-licking response and reduced c-Fos protein expression through the blockade of receptors of excitatory amino acid in laminae I-II of the dorsal horn (Lin et al., 2007). In an effort to delve deeper into the anti-nociceptive and analgesic activities of honokiol, Woodbury et al. used a formalin injection model to provoke severe neonatal pain and found that honokiol markedly suppressed both acute and chronic pain-induced deteriorations in newborn rats (Woodbury et al., 2015). Therefore, although honokiol possesses anti-nociceptive properties and is frequently used in traditional Asian remedies, there are few scientific findings about its effects on orofacial pain. With this background information, the objective of this study was to investigate the effect of honokiol on the key site of orofacial nociceptive impulse regulation by recording synaptic events on SG neurons of the Vc using the whole-cell patch-clamp approach.

EXPERIMENTAL PROCEDURES

Animals

Electrophysiological experiments were carried out using brain slices prepared from immature male and female ICR mice (postnatal day (PND) 15–23) caged with a 12-hour light–dark cycle (light on at 07:00 am) and properly supplied with food and water. All experimental procedures involving living animals were approved by the Institutional Animal Care and Use Committee of Jeonbuk National University (CBNU 2020-0131).

Formalin-induced orofacial pain test

The orofacial formalin test was performed as previously reported (Luccarini et al., 2006). Injected formalin was prepared from commercially available stock formalin (aqueous solution of 3.7% formaldehyde; Dana Korea, Korea) and further diluted in 10% phosphate buffered saline (PBS; pH 7.4). Thus, final solution contained 0.37% of formaldehyde. Mice were randomly assigned to two groups (five per group) and subcutaneously injected with 10 μL of 0.37% formalin or PBS using a 31-gauge needle into the right or left upper lip (just lateral to nose) sequentially every morning. Two days after injection, mice were sacrificed and brain slices were prepared for electrophysiological recordings as inflammatory pain models.

Brain slice preparation

The experimental process has been thoroughly described in a previous investigation (Nguyen et al., 2021). In brief, mice were decapitated between 10:00 and 12:00 AM UTC + 9:00 (Universal Time Coordinate). Their brains were quickly excised and submerged in ice-cold, oxygenated (95% O_2 and 5% CO_2) artificial cerebrospinal fluid (ACSF) containing following chemical compounds: 126 mM NaCl, 2.5 mM KCl, 2.4 mM $CaCl_2$, 1.2 mM $MgCl_2$, 11 mM D-glucose, 1.4 mM NaH_2PO_4, and 25 mM $NaHCO_3$ (pH 7.3–7.4). The trigeminal subnucleus caudalis segment was cyanoacrylate-fixed with a 4%-agar block and placed in a cold ACSF-fulfilled tray of vibratome (VT1200S; Leica biosystem, Wetzlar, Germany). Coronal brain slices (190–220 μm in thickness) including the rostral section of Vc were prepared and allowed to recover in oxygenated ACSF at room temperature for one hour before being transferred to the recording chamber.

Electrophysiological experiments

Slices were shifted into an ACSF-immersed recording chamber and consistently perfused at a flow rate of 4–5 mL/min. Each slice was optically analyzed under an upright microscope (BX51W1; Olympus, Tokyo, Japan) using infrared-differential interference contrast optics. The SG (lamina II) of the Vc was detected as a translucent band along the lateral edge of the coronal slice and merely medial to the spinal trigeminal tract.

We performed electrophysiological experiments with two kinds of internal solutions. Firstly, a high chloride pipette solution containing 140 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, 4 mM Mg-ATP, and 10 mM EGTA (adjusted to pH = 7.3 with KOH) was used to record spontaneous postsynaptic currents (sPSCs) at a holding potential of −60 mV. Secondly, a low chloride pipette solution containing 130 mM potassium gluconate, 10 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, 4 mM Mg-ATP, and 10 mM EGTA (adjusted to pH = 7.3 with KOH) was used to record spontaneous firing under the whole-cell current-clamp mode.

Patch pipettes were fabricated from borosilicate glass capillaries (PG 52151-4; WPI, Sarasota, FL, USA) with a Flaming Brown puller (P-97; Sutter Instruments Co., Novato, CA, USA). Tip resistances of recording electrodes ranged from 4 to 6 MΩ. After achieving a gigaohm seal on SG neuron, the whole-cell recording was performed by applying a small negative pressure to rupture cell membrane patch. Signals were amplified using an Axopatch 200B (Molecular Devices, San Jose, CA, USA), then filtered at 1 kHz, and digitized at 1 kHz with an Axon Digidata 1550B (Molecular Devices, San Jose, CA, USA). A Clampex 10.6 software (Molecular Devices, CA, USA), a Mini-Analysis software (ver. 6.0.7; Synaptosoft Inc., Decatur, GA, USA), and an Origin 8 software (OriginLab Corp., Northampton, MA, USA) were used to analyze data. All experiments were performed at room temperature.

Chemicals

Honokiol, tetrodotoxin citrate (TTX), 6-cyano-7-nitro-quinoxaline-2,3-dione (CNQX), and D-2-amino-5-phosphonopentanoic acid (DL-AP5) were purchased from Tocris Bioscience (Avonmouth, Bristol, UK). The remaining chemicals, such as picrotoxin, strychnine hydrochloride (strychnine), glycine, GABA, muscimol, and chemical ingredients of ACSF were bought from Sigma-Aldrich (St. Louis, MO, USA). Stock concentration of 100 mM honokiol was prepared in dimethyl sulfoxide (DMSO) and further, mini-stocks of 1, 10 mM honokiol was made by diluting in deionized water. The maximal concentration of DMSO in the final drug concentration was normally less than 0.33%, which had no effect on membrane currents of SG neurons. Before bath perfusion, we diluted these stock solutions in ACSF to the desired working concentrations.

Data and Statistical Analysis

A Mini-Analysis software was used to analyze synaptic events. Peak detection criteria for sPSCs were set at > 10 pA amplitude threshold and > 5 ms decay time constant, described similarly in previous studies (Jang et al., 2018; Rijal et al., 2021). All missed synaptic currents were manually detected. Synaptic events were analyzed for three-minute time period for both control and honokiol treatment. Since bath application of honokiol exerted slow and long-lasting responses, synaptic events for honokiol treatment were analyzed after 3 min of its application.

The sPSC frequency and amplitude of each individual neuron and cumulative distributions of sPSC parameters were compared using the Kolmogorov-Smirnov (K-S) test. All values were presented as mean ± standard error of the mean (SEM). The relative percentage was calculated by dividing the target response by its control response and multiplying by 100. Next, any SG neurons that displayed > 20% change in the control response by honokiol were considered to have been affected and the remaining as unaffected. Statistical analysis included paired or unpaired Student's *t*-test to evaluate the difference between two groups and one-way ANOVA post-hoc Scheffe test comparing means of multiple groups. Statistical significance was considered as *p*-values less than 0.05 and the levels of significance were defined by asterisks ($^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$).

RESULTS

Eighty SG neurons from 52 juvenile mice were recorded under whole-cell voltage-clamp and current-clamp mode to assess the influence of honokiol on SG neurons of the Vc.

Honokiol increases synaptic activities on SG neurons

Perfusion of honokiol (100 μM) for five minutes significantly enhanced the frequency of sPSCs in all tested SG neurons under high chloride pipette solution, as shown in Fig. 1(**A**) (Control: 0.73 ± 0.11 Hz;

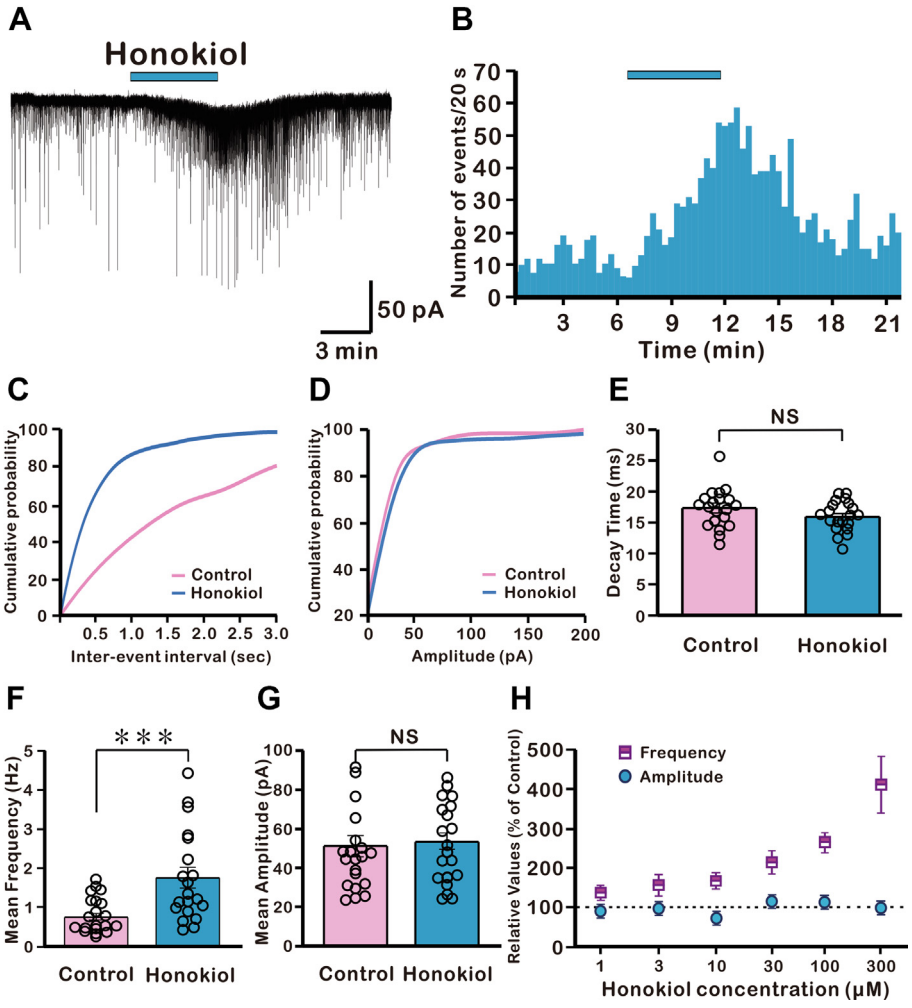

Fig. 1. Pre-synaptic effects of honokiol on SG neurons of the Vc. (**A**) A representative current trace indicating an increase in the frequency of spontaneous postsynaptic currents (sPSCs) in the presence of honokiol (100 μM). (**B**) A time–frequency histogram (bin size 20 s) of current trace in A. (**C**, **D**) Cumulative probability plots of sPSC inter-event interval (IEI) and amplitude in the absence and presence of honokiol, respectively. Take note that honokiol shifted the cumulative probability curve of IEI to the left, showing an enhancement of sPSC frequency ($P < 0.001$, Kolmogorov-Smirnov test). (**E**) The application of honokiol did not change mean decay time of sPSCs compared to control ($n = 20$; NS implicates not significant, Student's paired *t*-test). (**F**, **G**) Bar diagrams indicating mean frequency and amplitude of sPSCs in the presence of honokiol compared to control, respectively ($n = 20$; ***$P < 0.001$, NS implicates not significant, Student's paired *t*-test). (**H**) A histogram showing a concentration-dependent effect of honokiol on the frequency but not the amplitude of sPSCs ($n = 6$ for each concentration except for 100 μM honokiol, $n = 20$; ***$P < 0.001$, One-way ANOVA, followed by post-hoc Scheffe test).

Honokiol: 1.75 ± 0.26 Hz, 266 ± 24.9% of control; $n = 20$; $P < 0.001$, Student's paired *t*-test; Fig. 1(**F**)), while the amplitude of sPSCs was not affected by honokiol (Control: 49.9 ± 5.26 pA; Honokiol: 52.3 ± 4. 56 pA, 110 ± 6.80% of control; $n = 20$; $P > 0.05$, Student's paired *t*-test; Fig. 1(**G**)). The time–frequency histogram of sPSCs also revealed a remarkable enhancement in the sPSC frequency under honokiol exposure (Fig. 1(**B**)). Therefore, honokiol shifted the cumulative frequency curve of the inter-event interval (IEI) to the left compared to the control ($P < 0.001$, K-S test; Fig. 1(**C**)). Meanwhile, the cumulative amplitude curve showed no deviation by honokiol ($P > 0.05$, K-S test; Fig. 1(**D**)). Moreover, Fig. 1(**E**) shows that the application of honokiol did not alter mean decay time of sPSCs (Control: 17.4 ± 0.72 ms; Honokiol: 16.2 ± 0.57 ms, 93.5 ± 2.30% of control; $n = 20$; $P > 0.05$, Student's paired *t*-test; Fig. 1(**E**)). In addition, the relationship between various concentrations of honokiol and the frequency of sPSCs was established and plotted in Fig. 1 (**H**). Perfusion of honokiol facilitated the frequency of sPSCs in a concentration-dependent manner ($n = 6$ for each concentration except for 100 μM honokiol, $n = 20$; $P < 0.001$, One-way ANOVA post-hoc Scheffe test; Fig. 1(**H**)) without influencing their amplitude.

TTX, a voltage-sensitive Na^+ channel blocker, can inhibit the transmission of nociceptive inputs in sensory neurons. Consequently, to confirm whether the increase in the frequency of sPSCs by honokiol was dependent of action potential generation, the recording was carried out in the presence of TTX (0.5 μM). In the same neuron, the frequency of miniature postsynaptic currents (mPSCs) was strikingly boosted when the neuron was exposed to honokiol, as presented in Fig. 2(**A**) (Control: 0.63 ± 0.10 Hz; Honokiol: 1.65 ± 0.33 Hz, 265 ± 26.8% of control; $n = 12$; $P < 0.001$, Student's paired *t*-test; Fig. 2(**C**)). However, honokiol did not exert any influence on the amplitude of mPSCs (Control: 60.5 ± 7.70 pA; Honokiol: 56.6 ± 7.08 pA, 96.6 ± 7.06% of control; $n = 12$; $P > 0.05$, Student's paired *t*-test; Fig. 2(**D**)). Thus, honokiol increased the events of mPSCs, as shown by the time–frequency histogram (Fig. 2(**B**)). Furthermore, honokiol-induced change in the decay time constant of mPSCs was not observed in this study (Control: 18.8 ± 0.82 ms; Honokiol: 17.8 ± 0.80 ms, 94.7 ± 1.56% of control; $n = 12$; $P > 0.05$, Student's paired *t*-test; Fig. 2(**E**)).

To further clarify whether honokiol-mediated increase in the frequency of mPSCs depended on release of inhibitory neurotransmitters from presynaptic axon terminal, we co-applied honokiol with a mixture of TTX (0.5 μM), CNQX (10 μM; a non-NMDA glutamate receptor antagonist), and DL-AP5 (20 μM; an NMDA receptor antagonist). As a result, bath application of honokiol remarkably increased the frequency of mIPSCs

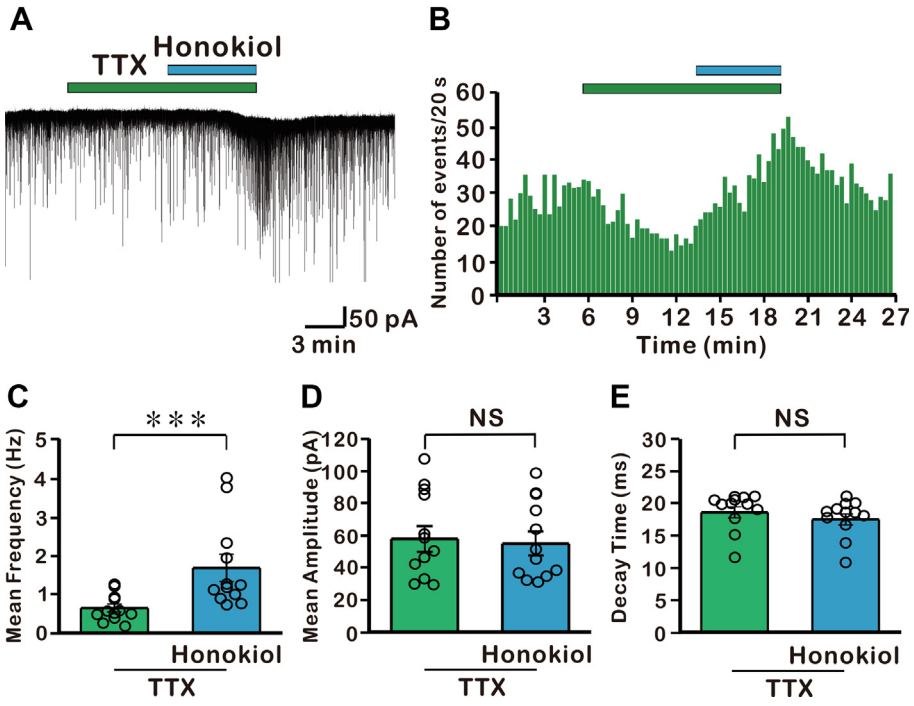

Fig. 2. Effect of honokiol on miniature postsynaptic currents (mPSCs) on SG neurons. (**A**) A representative current trace showing an increase in the frequency of mPSCs in the presence of honokiol (100 μM). (**B**) A time–frequency histograms (bin size 20 s) of current trace in A. (**C**, **D**) Bar diagrams indicating mean frequency and amplitude of mPSCs in the presence of honokiol compared to control, respectively (n = 12; ***P < 0.001, NS implicates not significant, Student's paired t-test). (**E**) A bar diagram showing mean decay time of mPSCs in control and honokiol (n = 12; NS implicates not significant, Student's paired t-test).

in all examined SG neurons, as shown in Fig. 3(**A**) (Control: 0.91 ± 0.12 Hz; Honokiol: 2.17 ± 0.29 Hz, 250 ± 28.3% of control; n = 14; P < 0.001, Student's paired t-test; Fig. 3(**C**)). Meanwhile, no significant change was observed in the amplitude of mIPSCs by honokiol application (Control: 58.6 ± 5.06 pA; Honokiol: 70.5 ± 12.7 pA, 119 ± 13.6% of control; n = 14; P > 0.05, Student's paired t-test; Fig. 3(**D**)). The time–frequency histogram also showed an enhancement in the frequency of mIPSCs on honokiol perfusion (Fig. 3 (**B**)). Additionally, the decay time constant of mIPSCs was not affected by honokiol (Control: 19.8 ± 0.21 ms; Honokiol: 18.8 ± 0.31 ms, 95.0 ± 1.41% of control; n = 14; P > 0.05, Student's paired t-test; Fig. 3(**E**)). These results indicate that honokiol enhances frequencies of inhibitory synaptic activities without any influence on their amplitudes on SG neurons.

Honokiol increases the frequency of glycinergic and GABAergic mIPSCs on SG neurons

The functioning of neuronal system involves interplay between excitatory and inhibitory transmitters, with GABA and glycine serving as principal inhibitory factors in neuronal modulation. Since the majority of SG neurons are inhibitory with the abundant existence of GABAergic and glycinergic interneurons as well as inputs from different modulatory networks (Todd et al., 1996; Yasaka et al., 2007), we performed a statistical comparison between the TTX and TTX + CNQX/AP5 conditions to quantify the contribution of glutamatergic PSCs on SG neurons of the Vc. There was no significant difference between the frequencies of mPSCs and mIPSCs on all tested neurons and further, recorded synaptic events were completely blocked by the application of TTX, picrotoxin (a non-competitive $GABA_A$ receptor antagonist) and strychnine (a glycine receptor antagonist) under the high chloride internal solution (figure not shown). As a result, to examine if the release of glycine and/or GABA is responsible for the increased mIPSC frequency, picrotoxin or strychnine were added to the superfusing solution before applying honokiol.

When neurons were exposed to TTX and picrotoxin, synaptic events were preserved and a significant increase in the frequency was observed on co-application of honokiol (Fig. 4(**A**)). As neurons were bathed in the mixture of TTX (0.5 μM) and picrotoxin (50 μM), mean frequency and mean amplitude of glycinergic mIPSCs were 0.68 ± 0. 18 Hz (n = 9) and 44.5 ± 6.15 pA (n = 9), respectively. The frequency of glycinergic mIPSCs shown here differs from other reported findings (Rhee et al., 2000; Munoz et al., 2018; Yamada et al., 2018). Thus, this variation might depend on experimental animals and neuronal populations. However, in the presence of honokiol, the mean frequency of glycinergic mIPSCs was significantly enhanced to 1.68 ± 0.41 Hz (n = 9), without remarkable change in the mean amplitude (48.0 ± 6.03 pA; n = 9; Fig. 4(**A**)). The time–frequency histogram also displayed a marked increase in the frequency of glycinergic mIPSCs by honokiol (Fig. 4 (**B**)). In the cumulative probability plot, the amplitude curve of glycinergic mIPSCs revealed no significant deflection by honokiol (P > 0.05, K-S test; Fig. 4(**C**)). Altogether, honokiol remarkably augmented the frequency of glycinergic mIPSCs in the presence of honokiol by 312 ± 74.5% compared to the control (n = 9; P < 0.05, Student's paired t-test; Fig. 4(**D**)) but had no effect on the amplitude of glycinergic mIPSCs (mean relative percentage of amplitude: 112 ± 10.1% compared to control; n = 9; P > 0.05, Student's paired t-test; Fig. 4 (**E**)). In addition, honokiol did not exert any influence on the decay time constant in the presence of TTX and picrotoxin (Control: 16.1 ± 1.23 ms; Honokiol: 16.4 ± 1.11 m s, 98.3 ± 1.06% of control; n = 9; P > 0.05, Student's paired t-test; Fig. 4(**F**)).

Conversely, Fig. 5(**A**) shows a representative trace illustrating almost blockade of synaptic events after application of strychnine on SG neurons. In the

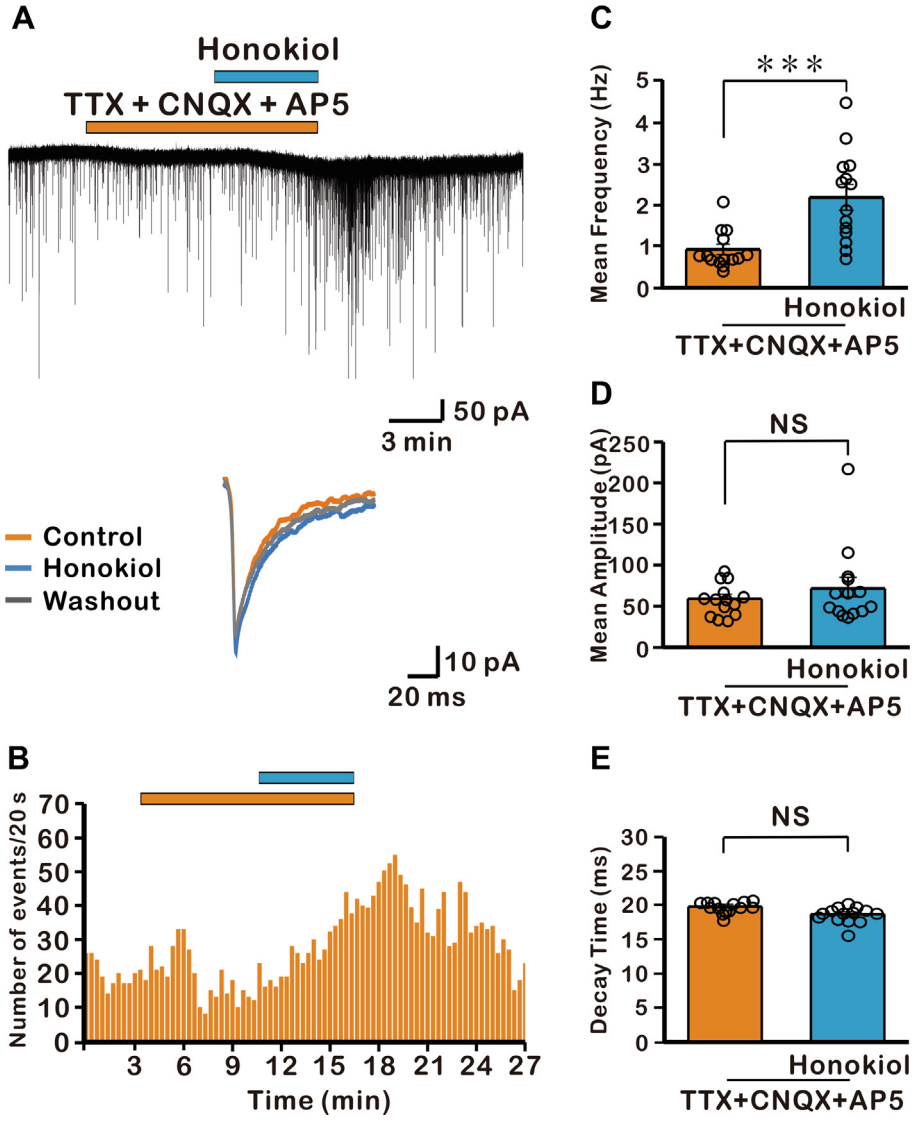

Fig. 3. Effect of honokiol on miniature inhibitory postsynaptic currents (mIPSCs) on SG neurons. (**A**) A representative current trace indicating an increase in the frequency of mIPSCs in the presence of honokiol (100 μM). Low panel average events. (**B**) A time–frequency histograms (bin size 20 s) of current trace in A. (**C**, **D**) Bar diagrams showing mean frequency and amplitude of mIPSCs in the presence of honokiol compared to control, respectively (n = 14; ***P < 0.001, NS implicates not significant, Student's paired t-test). (**E**) A bar diagram showing mean decay time of mIPSCs in control and honokiol (n = 14; NS implicates not significant, Student's paired t-test).

presence of TTX (0.5 μM) and strychnine (2 μM), mean frequency of GABAergic mIPSCs was significantly suppressed compared to control, as revealed in Fig. 5 (**A**) (Control: 0.95 ± 0.14 Hz; Strychnine alone: 0.19 ± 0.03, 22.7 ± 3.47% of control; n = 10; P < 0.001, Student's paired t-test; Fig. 5(**D**)). Similarly, mean amplitudes of mIPSCs in the absence and presence of strychnine were decreased from 41 ± 4.55 pA to 18.2 ± 1.72 pA, respectively (49.7 ± 7.98% of control; n = 10; P < 0.01, Student's paired t-test; Fig. 5(**E**)). However, when honokiol was bath applied, the frequency of GABAergic mIPSCs was increased in seven out of 10 tested neurons (70%) (P < 0.05, K-S test; Fig. 5(**C**)) and unaffected in 3 (30%) SG neurons. Therefore, Fig. 5(**B**) shows the time–frequency histogram of GABAergic mIPSCs indicating an increase in their frequency by the application of honokiol. In particular, in 70% neurons examined, honokiol enhanced the frequencies of GABAergic mIPSCs (Strychnine alone: 0.18 ± 0.03 Hz; Honokiol: 0.34 ± 0.06 Hz, 188 ± 28.3% compared to strychnine; n = 7; P < 0.05, Student's paired t-test; Fig. 5(**D**)) without altering their amplitudes (Strychnine alone: 17.0 ± 1.35 pA; Honokiol: 15.7 ± 1.04 pA, 98.4 ± 3.11% compared to strychnine; n = 7; P > 0.05, Student's paired t-test; Fig. 5(**E**)). Besides, no remarkable change was observed in the decay time constant by honokiol in the presence of TTX and strychnine (Strychnine: 15.0 ± 0.86 ms; Honokiol: 14.8 ± 0.51 ms, 99.5 ± 2.87% of control; n = 7; P > 0.05, Student's paired t-test). These findings suggest that honokiol enhances both glycinergic and GABAergic synaptic transmission on SG neurons by action potential-independent mechanisms.

Honokiol provokes inward currents on SG neurons

In addition to the effect of 100 μM honokiol in synaptic activities, 50% (10/19) of the tested SG neurons exhibited mean inward currents of −22.7 ± 3.84 pA (n = 10). However, bath application of 300 μM honokiol induced mean inward currents of −67.3 ± 10.9 p A (n = 10) in all examined neurons. The inward currents mediated by 300 μM honokiol was significantly reduced in the co-application of picrotoxin or strychnine (Fig. 6(**A,B**)). The mean relative percentage of inward currents induced by honokiol (300 μM) in the presence of picrotoxin or strychnine were 19.3 ± 7.58% (n = 5) and 53.1 ± 15.5% (n = 5) compared to honokiol alone, respectively (P < 0.05, Student's paired t-test; Fig. 6(**C**)). Altogether, these results suggest that perfusion with honokiol might have evoked direct responses on membrane potential of SG neurons by activating $GABA_A$ and glycine receptors.

Honokiol enhances glycine- and $GABA_A$-mediated responses of SG neurons

In the next set of recordings, we attempted to find out whether the presence of honokiol could evoke any

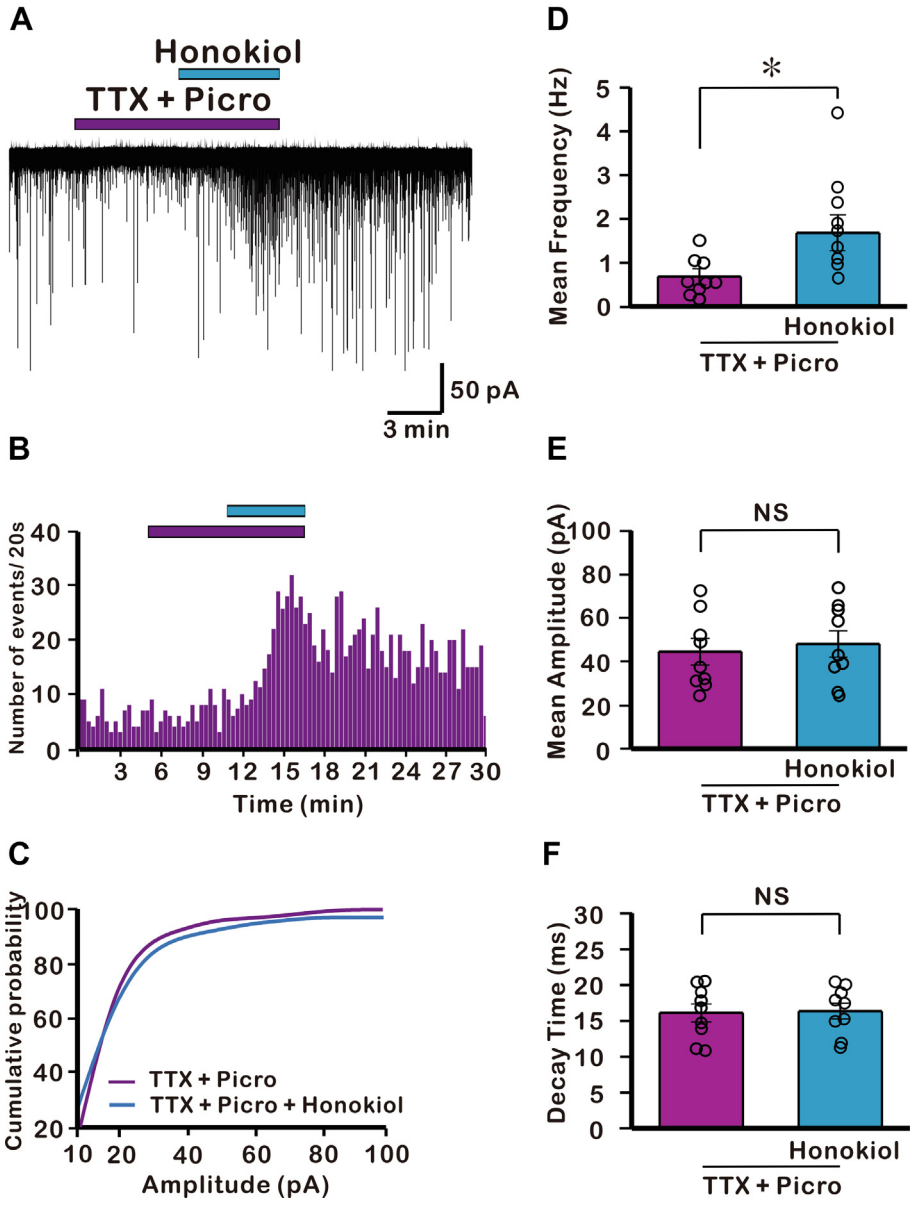

Fig. 4. Effect of honokiol on glycinergic mIPSCs of SG neurons of the Vc. (**A**) A representative current trace of glycinergic mIPSCs recorded in the presence of honokiol (100 μM) and picrotoxin (Picro, 50 μM). (**B**) A time–frequency histogram (bin size 20 s) of the trace in A displays a notable enhancement in the frequency of glycinergic mIPSCs by honokiol. (**C**) A cumulative probability plot of glycinergic mIPSC amplitude in the absence and presence of honokiol ($P > 0.05$, Kolmogorov-Smirnov test). (**D**, **E**) Bar diagrams indicating mean frequency and amplitude of glycinergic mIPSCs in the presence of honokiol compared to control ($n = 9$; $^*P < 0.05$, NS implicates not significant, Student's paired *t*-test). (**F**) A bar diagram showing mean decay time of glycinergic mIPSCs in control and honokiol ($n = 9$; NS implicates not significant, Student's paired *t*-test).

effects on glycine- and GABA-induced actions of SG neurons. We exploited a lower concentration of honokiol (100 μM) that generated small inward currents in 50% neurons tested in order to detect the correlation of honokiol with glycine and/or GABA. Fig. 7(**A**) illustrates a representative trace showing a potentiation effect of honokiol on glycine-mediated inward currents. Mean amplitudes of currents provoked by glycine (30 μM) were enhanced from −65.8 ± 9.74 pA to −110 ± 12.2 pA in the presence of honokiol (mean relative percentage of inward current: 179 ± 24.3% of control; $n = 7$; $P < 0.05$, Student's paired *t*-test; Fig. 7(**B**)).

Next, we bathed neurons in 100 μM honokiol before GABA (30 μM) or muscimol (50 nM) treatment to check how honokiol might modulate actions of GABA and muscimol. The enhancing action of honokiol on GABA response on the SG of the Vc is plotted in Fig. 8(**A**). Potentiation of GABA-mediated inward currents by honokiol was recorded in 8 (61.5%) out of 13 tested neurons (Fig. 8(**C**)). In these neurons, the mean amplitude of GABA-mediated currents was increased from −76.9 ± 16.8 pA to −131 ± 29.1 pA by honokiol (mean relative percentage of GABA mediated-inward current: 180 ± 16.3% of control; $n = 8$; $P < 0.01$, Student's paired *t*-test; Fig. 8(**D**)). On contrary, 4 (30.8%) SG neurons displayed decreased GABA response (mean relative percentage of GABA mediated-inward current: 62.2 ± 4.96% of control; $n = 4$; $P < 0.01$, Student's paired *t*-test; Fig. 8(**D**)) and one neuron remained unaffected in the presence of honokiol (Fig. 8(**C**)).

Moreover, the propensity of honokiol in enhancing inhibitory neurotransmission via $GABA_A$ receptors has been previously demonstrated (Squires et al., 1999; Ai et al., 2001; Alexeev et al., 2012). Finally, we co-applied honokiol and muscimol (a $GABA_A$ receptor agonist) to check the interaction between honokiol and $GABA_A$ receptor-induced responses. Fig. 8(**B**) exhibits a representative trace showing the enhancing effect of 100 μM honokiol on muscimol-induced response. Honokiol potentiated muscimol-mediated currents in six neurons tested whereas two remaining neurons showed no response (Muscimol alone: −63.4 ± 6.07 pA; Honokiol: −129 ± 16.1 pA, 209 ± 33.6% of control; $n = 6$; $P < 0.05$, Student's paired *t*-test; Fig. 8(**C,D**)). These results indicate that honokiol has potentiation effects on both $GABA_A$- and glycine receptor-mediated responses on SG neurons.

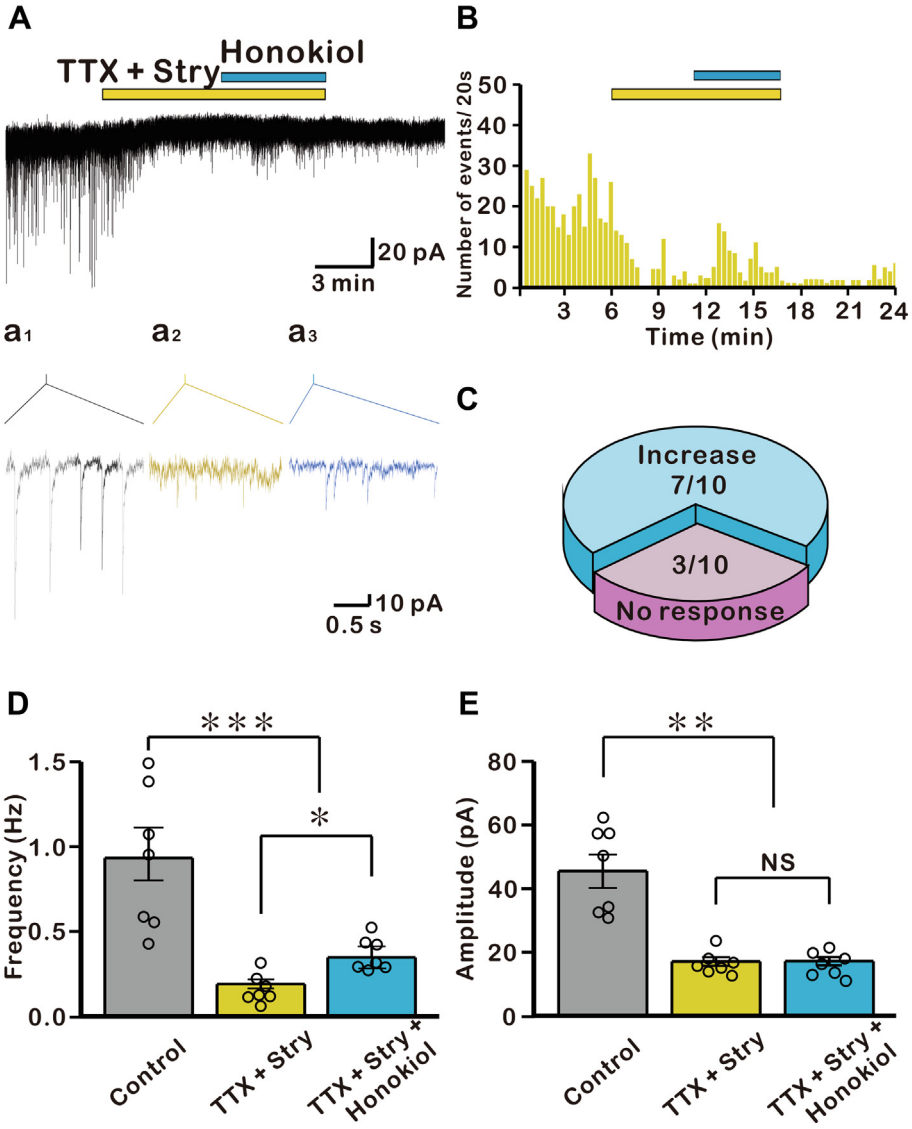

Fig. 5. Effect of honokiol on GABAergic mIPSCs of SG neurons of the Vc. (**A**) A representative current trace of GABAergic mIPSCs recorded in the presence of honokiol (100 μM) and strychnine (Stry, 2 μM). (a_1-a_3) Sections of the current trace in A show synaptic events before, during application of strychnine and during perfusion of honokiol at 2-s intervals, respectively. (**B**) A time–frequency histogram (bin size 20 s) of the trace in A displays an increase in the frequency of GABAergic mIPSCs by honokiol. (**C**) A pie chart indicating response rate on GABAergic mIPSC frequency by honokiol (**D**, **E**) Bar diagrams showing mean frequency and amplitude of GABAergic mIPSCs in the presence of honokiol compared to control, respectively (n = 7; ***P < 0.001, **P < 0.01, *P < 0.05, NS implicates not significant, Student's paired t-test).

Honokiol inhibits spontaneous firing on SG neurons in inflammatory pain model

To evaluate whether honokiol suppresses neuronal firing on SG neurons in inflammatory pain model, we recorded spontaneous firings under the whole-cell current-clamp mode.

The frequency of spontaneous neuronal firing on SG neurons in formalin-injected pain models was markedly increased compared to that in vehicle mice (Vehicle: 0.08 ± 0.03 Hz; n = 5; Formalin-injected group: 1.66 ± 0.49 Hz; n = 7; P < 0.05, Student's unpaired t-test; Fig. 9(**A**,**B**)). Bath application of honokiol decreased the frequency of spontaneous neuronal firings in examined SG neurons of the vehicle group, as shown in Fig. 9(**A**) (Honokiol: 0.02 ± 0.009 Hz, 25.1 ± 5.61% of control; n = 5; P < 0.05, Student's paired t-test; Fig. 9(**C**)). Similarly, in formalin-injected mice, honokiol significantly suppressed spontaneous neuronal firings on SG neurons (Honokiol: 0.48 ± 0.19 Hz, 25.0 ± 4.29% of control; n = 7; P < 0.01, Student's paired t-test; Fig. 9(**C**)). These results suggest that honokiol attenuates neuronal excitabilities of SG neurons in both vehicle and inflammatory pain model, which may contribute to anti-nociceptive effect.

DISCUSSION

In this investigation, we found that the honokiol-induced increase in sPSCs frequency was independent of action potential generation because TTX did not affect this frequency increase. Notably, the present study reported the inhibitory effect of honokiol on SG neurons of the Vc via both glycinergic and GABAergic presynaptic mechanisms. In addition, higher-concentration of honokiol induced inward currents that were noticeably inhibited by pretreatment with picrotoxin or strychnine. It was also recognized that the addition of honokiol enhanced glycine- and $GABA_A$ receptor-mediated responses. In inflammatory pain model, the increase in the frequency of spontaneous neuronal firing on SG neurons induced by formalin was remarkably inhibited by the application of honokiol.

The balanced interplay between excitatory and inhibitory transmissions is one of the pivotal operating mechanisms in neuronal networks. While glutamate is a major excitatory factor in the brain and spinal cord, the majority of inhibitory neurons utilize either GABA or glycine as the neurotransmitters to convey information from presynaptic to postsynaptic neurons (Dutertre et al., 2012; Leite et al., 2017). It is noteworthy that glycine receptors are used by approximately half of inhibitory synapses in the spinal cord and dorsal horn. In contrast, the remaining inhibitory ones are GABAergic (Purves et al., 2004).

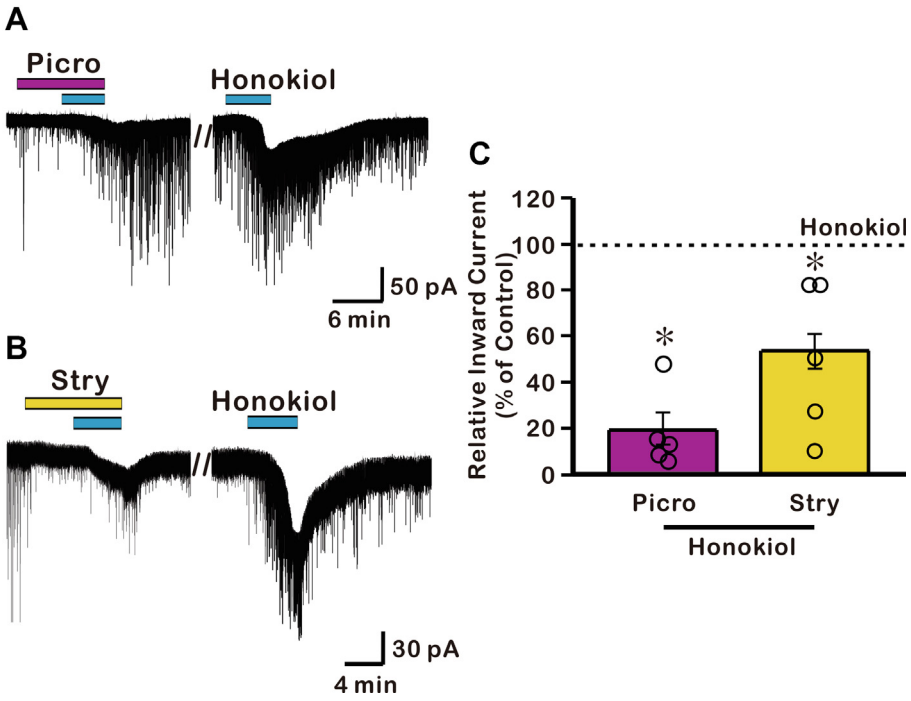

Fig. 6. Post-synaptic effect of honokiol is mediated via $GABA_A$ and glycine receptors. (**A**, **B**) Representative current traces indicating partial blockade of 300 μM honokiol-evoked inward current by picrotoxin (Picro, 50 μM) and strychnine (Stry, 2 μM), respectively. (**C**) There was a remarkable inhibition in mean relative amplitude of inward currents mediated by honokiol in the presence of picrotoxin and strychnine ($n = 5$ for each group; $^*P < 0.05$, Student's paired *t*-test).

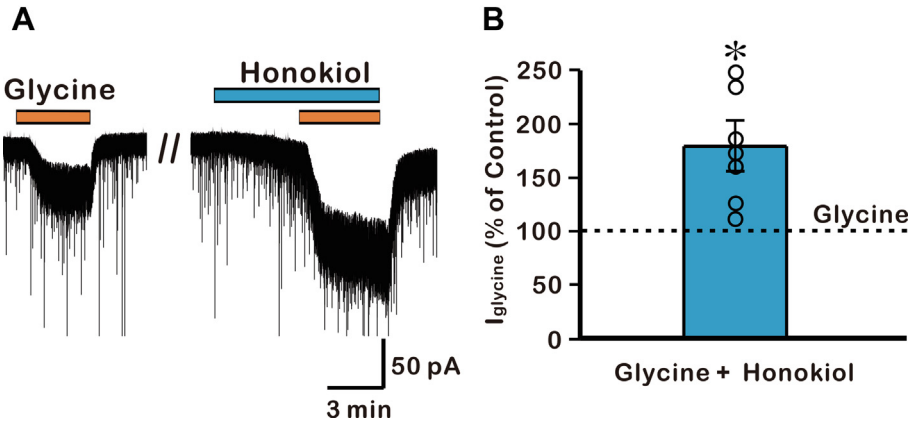

Fig. 7. Stimulatory effect of honokiol on glycine-induced responses on SG neurons. (**A**) A representative current trace demonstrating the potentiation action between honokiol (100 μM) and glycine (30 μM). (**B**) A bar diagram showing a meaningful enhancement in the mean relative amplitude of glycine-mediated inward currents during perfusion of honokiol ($n = 7$; $^*P < 0.05$, Student's paired *t*-test).

Glycinergic synapses function as rapid inhibitory neurotransmitters in processing visual and auditory inputs as well as modulating several motor and nociceptive activities (Dutertre et al., 2012). However, glycinergic inhibitory interneurons might be necrotic and/or apoptotic due to the excessive release of glutamate receptors during prolonged inflammatory pain, leading to the absence of glycinergic transmission in spinal lamina I neurons (Müller et al., 2003). Moreover, neuropathic pain resulted from certain neurological disorders is associated with the alteration of chloride homeostasis, including a decrease in K^+-Cl^- cotransporter-2 (KCC2) activity or an increase in Na^+-K^+-$2Cl^-$ cotransporter-1 (NKCC1) activity, which can dramatically affect the strength and the polarity of GABA/glycine-mediated transmission (Li et al., 2016). It has been noted that KCC2 expression changes in neuropathic pain cause the chloride ionic gradient to collapse and the glycine receptor reversal potential to shift to higher depolarized states (Mariqueo, 2020). On the other hand, iontophoretic application of glycine could reduce spontaneous and acquired responses of nociceptive spinothalamic tract cells induced by activation of receptive positions (Willcockson et al., 1984). Unlike $GABA_A$ receptors, effective therapeutic ligands based on glycine have not been currently identified despite its potentially inhibitory effects in the CNS. The present investigation reveals evidence that honokiol has the propensity to enhance significantly frequencies of glycinergic mIPSCs without affecting their amplitudes.

GABA is admitted as another predominant inhibitory neurotransmitter in the CNS by activating ionotropic chloride channel receptors. It functions with two main subtypes of receptors: ionotropic $GABA_A$ and metabotropic $GABA_B$ (Bowery and Smart, 2006). $GABA_A$ receptor, a ligand-gated chloride channel, can induce membrane hyperpolarization and decrease the excitability of neurons through rapid opening of integral ion channels (Bowery and Smart, 2006). Meanwhile, $GABA_B$ receptors are majorly linked to calcium and potassium channels by G-protein (Enna and McCarson, 2006). Here, in this study, honokiol also increased GABAergic synaptic activities shown in most SG neurons tested with smaller efficacy than glycinergic mIPSCs.

These main findings of our investigation appear paradoxical because it has been previously reported that glycine and GABA frequently colocalize at the same inhibitory synapse in spinal laminae I-II (Todd et al., 1996). There are, however, contradictory results about inhibitory neurotransmission induced by either GABA-only or glycine-only terminals along with mixed GABA-

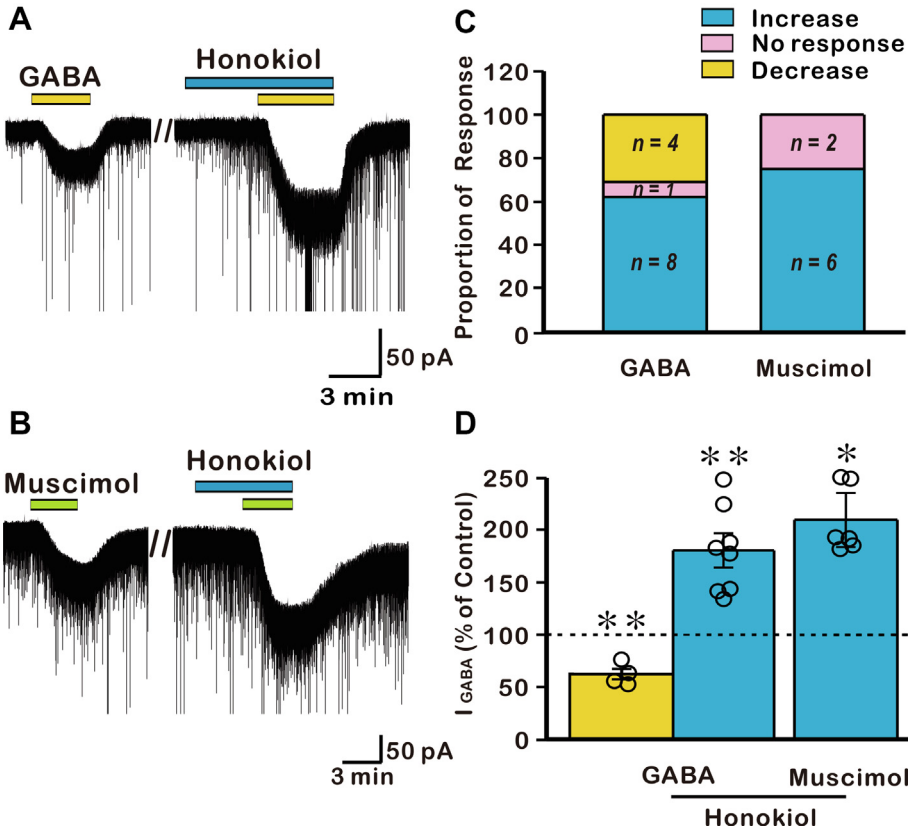

Fig. 8. Potentiation effect of honokiol on $GABA_A$-evoked responses of SG neurons. (**A**, **B**) Representative current traces showing an increase in GABA- and muscimol-mediated inward currents by honokiol (100 μM), respectively. (**C**) A stack column graph demonstrating the proportion of inward current change induced by GABA (30 μM) and muscimol (50 nM) combined with honokiol. (**D**) A bar diagram depicting change in the mean relative amplitude of GABA and muscimol currents in the presence of honokiol compared to control (n = 4–8 for GABA, n = 6 for muscimol; $^{**}P < 0.01$, $^{*}P < 0.05$, Student's paired t-test).

glycine synaptic currents (Keller et al., 2001; Miranda et al., 2022). Glycine-only-mediated mIPSCs and the significance of glycine-only synaptic inhibition in neuropathic modulation have been substantiated by numerous reports (Keller et al., 2001; Miranda et al., 2022). Therefore, the corelease of glycine and GABA is only detected during early developmental stages and ceases at lamina I-II adult synapses after 3–4 weeks postnatal. The maturation of inhibitory synapses in the spinal cord, including the affinity of receptors, their expression, or subsynaptic distribution, might play a pivotal role in the loss of coreleased GABA-glycine codetection and increased efficacy of glycinergic synapses (Keller et al., 2001).

In this study, we used juvenile ICR mice whose PND ranged from 15 to 23. Previous research found that in the PND of 8 and 23, $GABA_A$ receptor-only mIPSCs displayed a linear fourfold reduction of decay kinetics in contrast to glycine receptor-only mIPSCs that showed a lighter decrease and mainly were completed by the beginning of the second postnatal week (Keller et al., 2001). Interestingly, several systems have been also shown a developmental switch from GABAergic to glycinergic transmission for the first two postnatal weeks with a decrease in GABA-positive neurons and an increase in glycine-positive neurons (Kotak et al., 1998; Gao et al., 2001). In a similar aspect, this study also indicated that honokiol increased glycinergic synaptic events with greater efficacy than GABAergic events on SG neurons of the Vc through an action potential-independent presynaptic mechanism. However, it is necessary to conduct further research to identify effects of honokiol on neuronal regulation in age-dependent and region-specific manners.

Previous studies have revealed the correlation between the generation of neurotransmitters from presynaptic nerve terminals and concentration of presynaptic Ca^{2+} (Augustine et al., 2003). The Ca^{2+} influx can arise from entry via voltage-gated calcium channels in the plasma membrane and release from the endoplasmic reticulum, together with internal mitochondrial stores (Catterall and Few, 2008). Thus, the intracellular Ca^{2+} stores play a crucial role in synaptic transmission, which commonly occurs in the spinal SG region (Rhee et al., 2000; Yasui et al., 2011). Notably, honokiol has been reported to facilitate cytoplasmic free Ca^{2+} mobilization through the activation of phospholipase C and inositol 1,4,5-triphosphate receptors in primary cultured rat cortical neurons and neuroblastoma cells (Zhai et al., 2003). As a result, the release of Ca^{2+} from intracellular stores might contribute to the underlying mechanism of inhibitory transmitter release by honokiol.

Being coincided with the presynaptic mechanism, we also found that honokiol could perform neuronal modulation based on postsynaptic actions. Findings from this work indicate that high-concentration of honokiol apparently can induce inward currents which were suppressed in the co-application of picrotoxin or strychnine. Many previous studies have described that honokiol can interact with voltage-gated ionic channels in different cellular groups. For example, honokiol was noted to block voltage-gated K^+ channels in freshly isolated mice dorsal root ganglion neurons (Sheng et al., 2017). It also inhibited hyperpolarization-activated cation current and delayed-rectifier K^+ current in pituitary tumor (GH_3) cells and Rolf B1.T olfactory neurons (Chan et al., 2020). It is believed that the presence of honokiol could modulate functional activities in sensory neurons with a gating mechanism to open these ionic channels and shift steady-state activation curve toward negative voltage (Chan et al., 2020).

In this study, honokiol exhibited GABA- and/or glycine-mimetic responses as well as potentiation

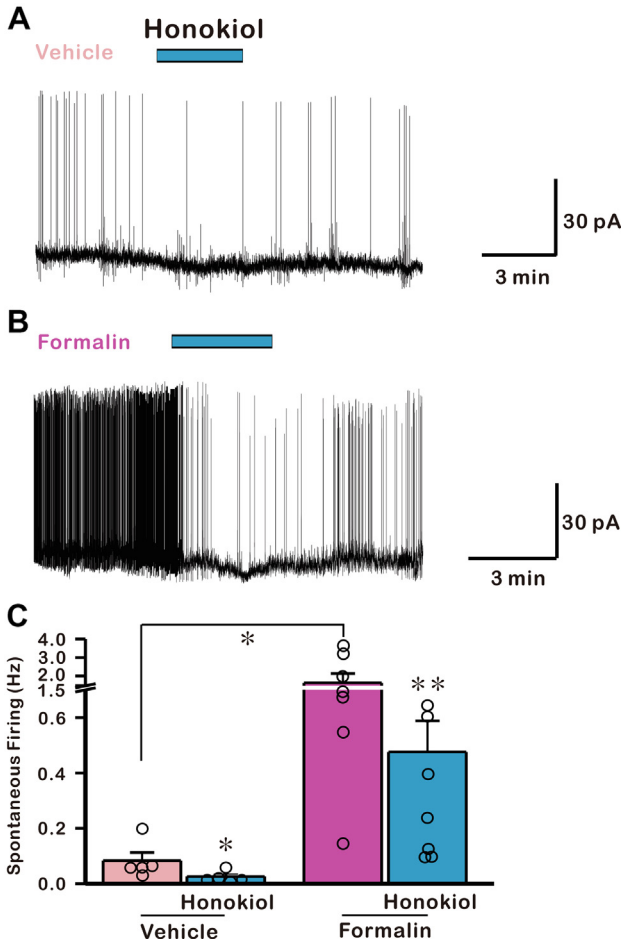

Fig. 9. Suppressive effect of honokiol on spontaneous neuronal firing of SG neurons in inflammatory pain model mice. (**A**, **B**) Representative current traces displaying action potential firing patterns of vehicle and formalin-injected groups under the whole-cell current-clamp mode, respectively. (**C**) A bar diagram showing a significant decrease in the frequency of spontaneous firing by honokiol application in both vehicle and formalin-injected groups (n = 5 for vehicle, n = 7 for formalin; $^{**}P < 0.01$, $^{*}P < 0.05$, Student's paired t-test; $^{*}P < 0.05$ for comparison between two mouse groups, Student's unpaired t-test).

effects on both GABA- and glycine-mediated responses on SG neurons of the Vc. Previous studies have also revealed the ability of natural compounds, such as citral, borneol, linalool, and resveratrol to exert inhibitory effects on SG neurons through GABA- and/or glycine receptors (Nguyen et al., 2019, 2020, 2021; Jang et al., 2022). However, the enhancing effect of honokiol on GABA was just recorded in the majority of tested SG neurons. To clarify whether this phenomenon was involved in the heterogeneous property of GABA, we subsequently, assessed the effect of honokiol on muscimol, a $GABA_A$ receptor agonist.

In agreement with previous observations, honokiol has been identified as a modulator of $GABA_A$ receptors (Squires et al., 1999; Ai et al., 2001; Alexeev et al., 2012). Because $GABA_A$ receptors have subunit heterogeneity that influences their function, a previous research explored the activity of honokiol on neuronal and recombinant $GABA_A$ receptors and concluded that this compound could enhance both phasic and tonic GABAergic neurotransmission in hippocampal dentate granule neurons (Alexeev et al., 2012). Additionally, honokiol was noted to have possible selectivity on different $GABA_A$ receptor subtypes, such as enhancing actions of ^{3}H-muscimol binding to rat brain membrane preparations and binding sites of $\alpha_2\beta_3\gamma_{2s}$, $\alpha_2\beta_3$, $\alpha_1\beta_2\gamma_{2s}$ and $\alpha_1\beta_2$ (Ai et al., 2001). It is noteworthy that honokiol enhanced GABA-induced chloride currents in all tested subtypes comparable to diazepam. On these bases, our electrophysiological data show that honokiol enhances postsynaptic responses mediated by glycine as well as $GABA_A$ receptors on the SG neurons, which could denote to essentially physiological effects of this compound on neuropathic modulation.

Interestingly, honokiol exerted a markedly suppressive effect on spontaneous neuronal firing on SG neurons in inflammatory pain model. In this study, we chose orofacial formalin test in mice as a pre-clinical model to study the efficacy of honokiol in the orofacial region because it appears to be a valid and reliable model of orofacial pain and thus, sensitive to analgesics (Clavelou et al., 1995; Raboisson and Dallel, 2004). This test is based on a chemical stimulus (formalin) causing tissue damage that mimics acute post-injury nociception in human. It has been reported that the subcutaneous injection of diluted formalin into mouse upper lips induces biphasic inflammation and behavioral reactions, such as vocalization, grooming, and scratching (Dallel et al., 1995). As a result, we hypothesized that formalin injection could sensitize trigeminal and spinal nociceptive afferents as well as dorsal horn neurons through orofacial nociceptive responses. More importantly, SG neurons of the Vc are recognized as a principal region for processing orofacial nociceptive information via myelinated Aδ and unmyelinated C fibers (Sessle, 2000). Here, for the first time, we demonstrated that formalin-induced inflammatory pain provoked neuronal hyperexcitability which was displayed by a significant increase in the spontaneous firing rate of SG neurons, similarly reported in previous studies (Sugimura et al., 2016; Farahani et al., 2021). Moreover, the inhibitory effect of honokiol on the transmission of excessive nociceptive stimuli evoked by formalin has statistically shown on the SG of the Vc, suggesting an analgesic property of honokiol on orofacial pain transmission.

Besides, several pharmacological investigations reported the propensity of honokiol to penetrate the blood–brain barrier and blood-cerebrospinal fluid barrier easily, thereby producing diverse neuronal functions in various CNS areas (Jun-Jun et al., 2015; Sarrica et al., 2018). For the first time, we reported the effect of honokiol on the enhancement of inhibitory neurotransmission as well as suppression of nociceptive transmission on the key anatomical relay site of orofacial nociceptive information in mice. These findings suggest that inhibitory effect of honokiol on SG neurons of the Vc might attribute to orofacial pain regulation in the CNS.

CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

ACKNOWLEDGEMENTS

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