The American Journal of Chinese Medicine, Vol. 49, No. 6, 1437–1448

DOI: 10.1142/S0192415X21500671

GABA- and Glycine-Mimetic Responses of Linalool on the Substantia Gelatinosa of the Trigeminal Subnucleus Caudalis in Juvenile Mice: Pain Management through Linalool-Mediated Inhibitory Neurotransmission

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Published 10 July 2021

Abstract: Linalool, a major odorous constituent in essential oils extracted from lavender, is known to have a wide range of physiological effects on humans including pain management. The substantia gelatinosa (SG) of the trigeminal subnucleus caudalis (Vc) is involved in transmission of orofacial nociceptive responses through thin myelinated Aδ and unmyelinated C primary afferent fibers. Up to date, the orofacial antinociceptive mechanism of linalool concerning SG neurons of the Vc has not been completely clarified yet. To fill this knowledge gap, whole-cell patch-clamp technique was used in this study to examine how linalool acted on SG neurons of the Vc in mice. Under a high chloride pipette solution, non-desensitizing and repeatable linalool-induced inward currents were preserved in the presence of tetrodotoxin (a voltage-gated Na^+ channel blocker), CNQX (a non-NMDA glutamate receptor antagonist), and DL-AP5 (an NMDA receptor antagonist). However,

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linalool-induced inward currents were partially suppressed by picrotoxin (a $GABA_A$ receptor antagonist) or strychnine (a glycine receptor antagonist). These responses were almost blocked in the presence of picrotoxin and strychnine. It was also found that linalool exhibited potentiation with GABA- and glycine-induced responses. Taken together, these data show that linalool has GABA- and glycine-mimetic effects, suggesting that it can be a promising target molecule for orofacial pain management by activating inhibitory neurotransmission in the SG area of the Vc.

Keywords: Substantia Gelatinosa; Linalool; Patch-Clamp Technique; Glycine Receptor; $GABA_A$ Receptor; Pain Management.

Introduction

The substantia gelatinosa (SG, lamina II of the spinal dorsal horn) is a major synaptic site for relaying peripheral nociceptive inputs to the higher brain center (Cervero, 1982). SG neurons are described as a narrow cellular band just nearby the dorsal margin of the grey matter (Zheng *et al.*, 2010; Kleiner, 2011). Orofacial sensory inputs are primarily transmitted in the spinal trigeminal subnucleus caudalis (Vc), which plays a pivotal role in processing orofacial painful stimuli (Ren and Dubner, 2011). Due to its homology with the spinal dorsal horn, the Vc is also named medullary dorsal horn (Bereiter *et al.*, 2000). SG neurons of the Vc are recognized as a key site in processing orofacial nociceptive information via thin myelinated Aδ and unmyelinated C primary afferent fibers (Sessle, 2000).

Nature has been the traditional source of various pharmaceutical agents. Terpenes are a class of secondary metabolites that are considered as important compounds in aromatic plants with medicinal use (Dorman and Deans, 2000). Linalool is one example of this class of chemical compounds found naturally in essential oils of many plant species distributed throughout the world (Sibanda *et al.*, 2004). Since ancient time, linalool-producing plants have been commonly applied in traditional medicine based on empirical evidence. With a pleasant scent like floral, lavender essential oil has been approved by the European Medicines Agency as an herbal treatment for relieving stress and anxiety (López *et al.*, 2017). It has been demonstrated that linalool inhalation with an anti-oxidative effect can reduce blood pressure and pulse rate in carpal tunnel syndrome patients (Seol *et al.*, 2016). Linalool odor-induced anxiolytic effect involves potential central neuronal mechanisms (Harada *et al.*, 2018). Linalool isolated from the essential oil of *Lippia alba* (Mill.) N. E. Brown is an effective sedating and anesthetic agent for silver catfish (Heldwein *et al.*, 2014). Linalool and lynalyl acetate-producing species also show pharmacological activities as promising anti-inflammatory agents (Peana *et al.*, 2002). Furthermore, plantar subcutaneous administration of linalool can induce an antinociception effect by activating peripheral opioid receptors (Katsuyama *et al.*, 2015). Since its widespread nature as well as its usefulness in traditional medicine with several applications, linalool was chosen to elucidate its millennial application as a herbal medicine for pain management via scientific understandings. Whole-cell patch-clamp technique was used to determine the action of linalool and its receptors on SG neurons of the Vc in mice.

Experimental Procedure

Histology

Brains were fixed in 10% neutral buffered formaldehyde, embedded in paraffin, and sectioned using a microtome (HM325; Thermo Scientific, Walldorf, Germany) to 4 μm thick sections. These tissue sections were deparaffinized and rehydrated using standard techniques and stained with hematoxylin & eosin (H&E) and cresyl violet. Stained sections were dehydrated and cleared with xylene. Mounting was performed using Canada balsam and observed with a light microscope (BX61; Olympus, Tokyo, Japan).

Animals and Brain Slice Preparation

All experiments were performed at room temperature after obtaining approval from the Institutional Animal Care and Use Committee of Jeonbuk National University (CBNU 2017-0082, CBNU 2018-072). ICR male and female mice (postnatal day 8–19) were kept in a house (light on at 07:00 and light off at 19:00) with free access to food and water. Mice were beheaded at around 10:00 and 12:00. Their brains were unfolded and dipped in ice-cold artificial cerebrospinal fluid (ACSF) containing the 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 to 7.4, bubbled with 95% O_2 and 5% CO_2). The brainstem was rapidly fixed to an agar block and immersed in ice-cold ACSF for the next excised stage. Coronal slices (180–220 μm in thickness) with the rostral part of Vc were excised using a vibratome (VT1200S; Leica biosystems, Wetzlar, Germany). These slices were stored in oxygenated ACSF for at least 1 h before electrophysiological recording.

Whole-Cell Patch-Clamp Recording

Coronal slices were transferred to the recording chamber which was ACSF submerged and constantly superfused at a rate of 4–5 ml/min. An upright microscope (BX51W1; Olympus, Tokyo, Japan) with differential interference contrast optics was used for visualization. SG neurons of the Vc were described as a translucent band, just medial to the spinal trigeminal nucleus traveling the lateral edge of coronal slices.

Patch pipettes were pulled from borosilicate glass capillary tubes (PG52151-4; WPI, Sarasota, USA) using a Flaming Brown puller (P-97; Sutter Instruments Co., Novato, CA, USA). 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 (pH 7.3 with KOH) was used to intensify the chloride current at a holding potential of −60 mV.

After filling with the pipette solution, the tip resistance of the patch pipette was between 4 MΩ and 6 MΩ. Once a gigaseal was formed with the neuronal membrane, a slight negative pressure was applied to achieve the whole-cell mode. Signals were sequentially amplified with an Axopatch 200B (Molecular Devices, San Jose, CA, USA) and digitized with an Axon Digidata 1550B (Molecular Devices). Acquired data were analyzed with a

pClamp 10.6 software (Molecular Devices) and an Origin 2018 software (OriginLab Corp., Northampton, MA, USA).

Chemicals and Statistics

Chemicals including linalool, picrotoxin, strychnine hydrochloride (strychnine), and ACSF compositions were obtained from Sigma Aldrich (USA). CNQX disodium salt (CNQX), DL-AP5 (AP5), and tetrodotoxin citrate (TTX) were purchased from Tocris Bioscience (Avonmouth, Bristol, UK). Chemicals were dissolved in ACSF and used at indicated concentrations. All data are shown as mean ± standard error of the mean (SEM). Difference in the mean between two groups was evaluated by a paired *t*-test. Statistical significance level was set at $p < 0.05$.

Results

Identification of Spinal Trigeminal Subnucleus Caudalis Site

To identify the location of Vc, the brainstem was sectioned transversely and sections were subjected to H&E staining and Nissl staining. The Vc site located in the brainstem was identified under an optical microscope (Fig. 1).

Non-Desensitizing and Repeatable Responses Induced by Linalool

The whole-cell patch-clamp technique was performed on 42 SG neurons of 30 immature mice at a voltage-clamp mode. Mean amplitude of linalool (1 mM)-induced inward currents was 33.2 ± 2.82 pA ($n = 31$).

Under a high chloride pipette solution, 1 mM linalool was successively applied into the bath to experience whether SG neurons were desensitized by repeated application of linalool. Inward currents induced by the second linalool application were similar to those of the first application using 15 neurons (Fig. 2A). No significant difference in the mean amplitude of linalool-induced inward currents was found between the first (34.3 ± 4.15 pA) and the second (34.2 ± 3.98 pA) applications ($n = 15$, $p > 0.05$; Fig. 2B). Therefore, inward currents on SG neurons of the Vc are not desensitized by consecutive administration of linalool.

Linalool-Induced Direct Postsynaptic Response is Not Mediated by the Activation of Ionotropic Glutamate Receptors

TTX, a powerful selective inhibitor of voltage-gated Na^+ channel in sensory neurons, can interfere with the generation of action potentials and depress the propagation of impulses in excitable membranes (Marcil *et al.*, 2006). In this study, TTX was co-applied with linalool to examine whether linalool could work on SG neurons via action potentials. Linalool-induced inward currents were preserved in the presence of 0.5 μM TTX using 12 SG

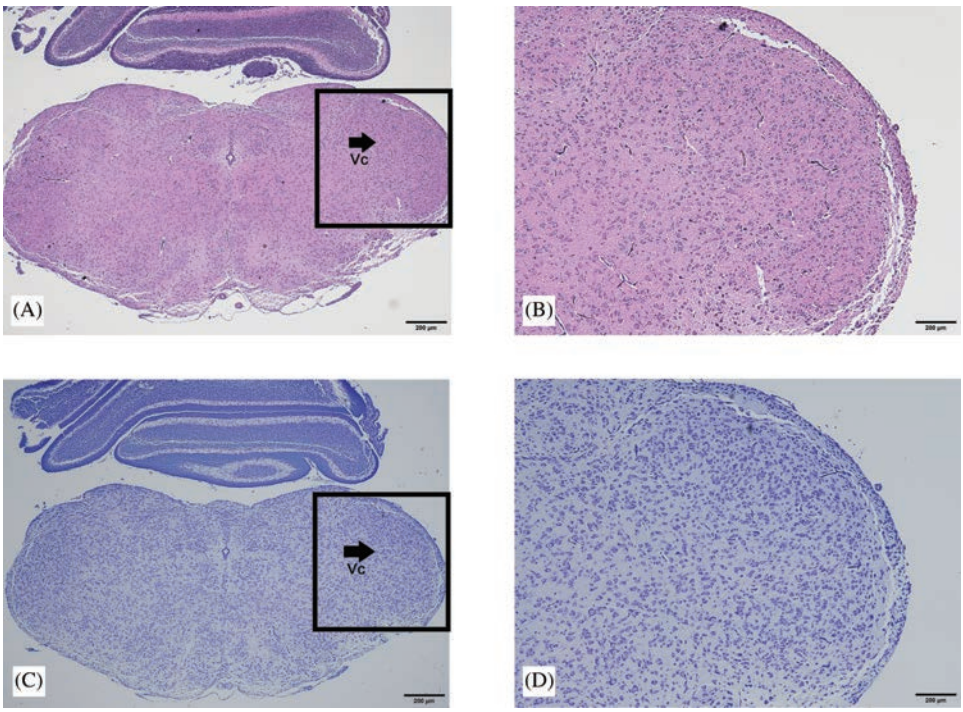


Figure 1. Micrographs of H&E-stained and cresyl violet-stained coronal brainstem sections. (A) (B) The upper micrographs are H&E-stained coronal sections of brainstem including the Vc at low magnification (40X) and high magnification (100X). (C) (D) The lower micrographs are cresyl violet-stained coronal sections of brainstem including the Vc at low magnification (40X) and high magnification (100X). All scale bars represent 200 μm.

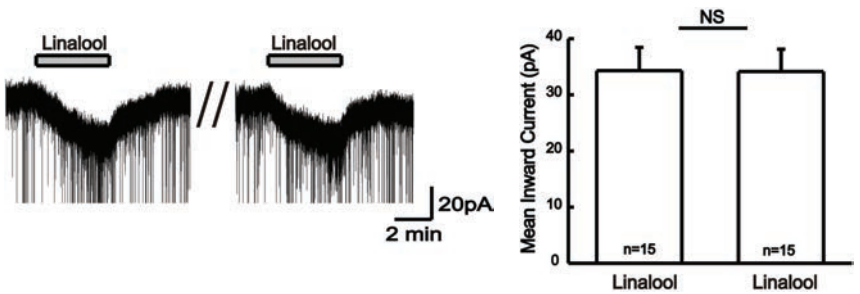
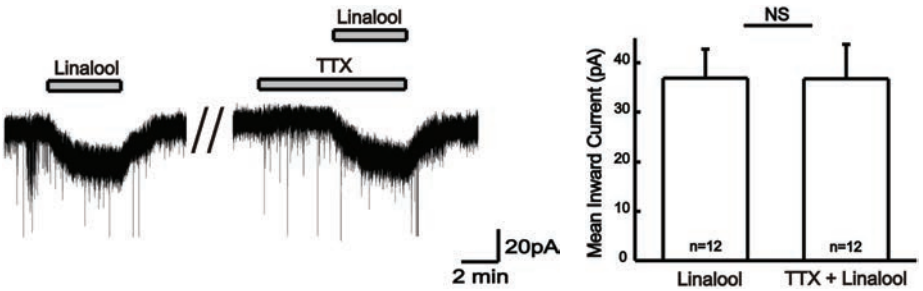


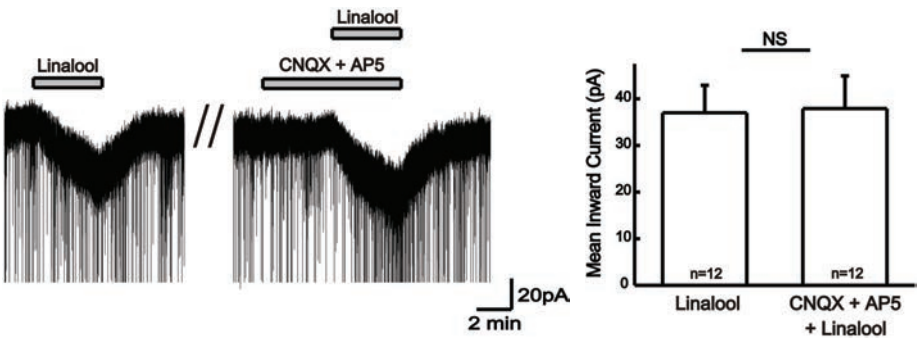
Figure 2. Non-desensitizing and repeatable inward currents induced by linalool on SG neurons of the Vc. (A) A representative trace showing repeatable responses induced by linalool (1 mM). (B) Comparison of inward currents between the first and the second applications of linalool ($n = 15$, paired t-test, $p > 0.05$).

neurons (Fig. 3A). There was no significant difference in the mean amplitude of inward currents induced by linalool between the absence (36.9 ± 5.88 pA) and the presence (36.8 ± 6.98 pA) of TTX ($n = 12$, $p > 0.05$; Fig. 3B).



(A) (B)

Figure 3. Direct postsynaptic activity of linalool on SG neurons of the Vc. (A) A representative current trace showing no effect by TTX (0.5 μM), a voltage-sensitive Na^+ channel blocker, on linalool-induced inward currents. (B) There was no significant difference in the linalool-induced inward currents between the absence and the presence of TTX ($n = 12$, paired t-test, $p > 0.05$).



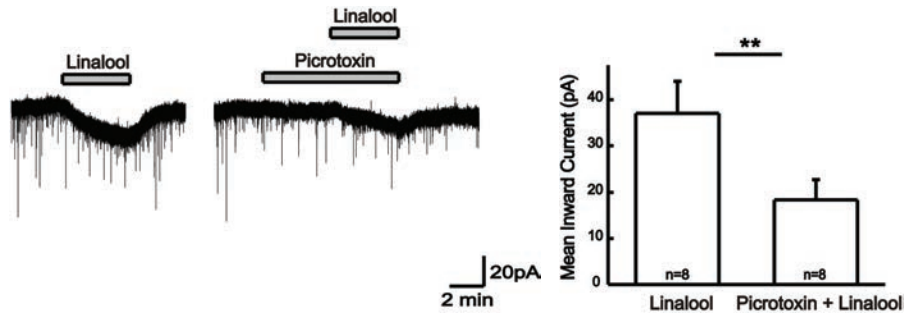
(A) (B)

Figure 4. Linalool-induced responses are not mediated by the activation of ionotropic glutamate receptors. (A) A representative current trace showing no effect of CNQX (10 μM) or AP5 (20 μM) as ionotropic glutamate receptor antagonists. (B) There was no significant difference in linalool-induced inward currents between the absence and the presence of CNQX and AP5 ($n = 12$, paired t-test, $p > 0.05$).

In the next series of experiments, considering that the effect of linalool was mediated by the activation of ionotropic glutamate receptors, linalool was examined with CNQX (a non-N-methyl-D-aspartate (NMDA) glutamate receptor antagonist) and AP5 (an NMDA receptor antagonist). The presence of CNQX and AP5 did not influence linalool-induced inward currents (Fig. 4A). For 12 neurons, inward currents induced by linalool (37.0 ± 5.91 pA) were similar to those in the presence of 20 μM AP5 and 10 μM CNQX (37.9 ± 7.02 pA, $n = 12$, $p > 0.05$; Fig. 4B). These results indicate that linalool can directly act on SG neurons and that such action is not mediated by the activation of ionotropic glutamate receptors.

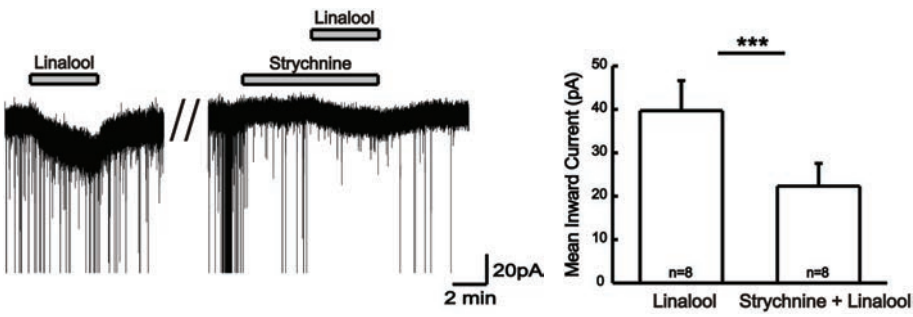
GABA- and Glycine-Mimetic Responses of Linalool

Picrotoxin, a non-competitive $GABA_A$ receptor antagonist, is commonly used for examining the function of GABA receptors via inhibition of chloride flux (Enna and McCarson, 2006).



(A) (B)

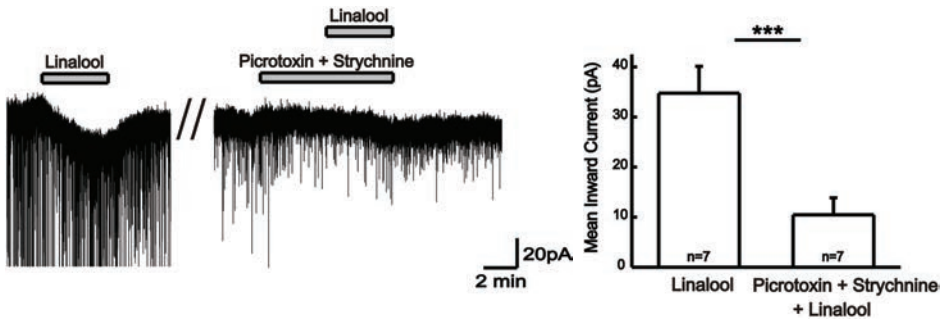
Figure 5. GABA-mimetic activity of linalool. (A) A representative current trace showing partial suppression of linalool-induced inward current by picrotoxin (50 μM), a $GABA_A$ receptor antagonist. (B) Significant suppression of mean inward currents induced by linalool in the presence of picrotoxin ($n = 8$, paired t-test, $^{**}p < 0.01$).



(A) (B)

Figure 6. Glycine-mimetic activity of linalool. (A) A representative trace showing partial suppression by strychnine (2 μM), a glycine receptor antagonist, on linalool-induced inward currents. (B) There was a significant suppression of mean inward currents by linalool in the presence of strychnine ($n = 8$, paired t-test, $^{***}p < 0.001$).

Linalool-induced currents in the presence of picrotoxin were smaller than those in the absence of picrotoxin (linalool alone) in the same neurons (Fig. 5A). The mean inward current induced by linalool was diminished from 37.0 ± 6.95 pA to 18.4 ± 4.39 pA ($n = 8$, $^{**}p < 0.05$; Fig. 5B) in the presence of 50 μM picrotoxin. Strychnine, a potent competitive glycine receptor antagonist, was then co-applied with linalool to check whether these linalool-induced currents were mediated by strychnine-sensitive glycine receptors (Dutertre *et al*., 2012). Results showed that linalool-induced currents were significantly suppressed in the presence of 2 μM strychnine (Fig. 6A). Mean amplitudes of inward currents induced by linalool in the absence and presence of strychnine were 39.7 ± 6.93 pA and 22.3 ± 5.21 pA, respectively ($n = 8$, $^{***}p < 0.001$; Fig. 6B). Furthermore, the linalool-induced inward currents were almost completely suppressed when both strychnine and picrotoxin were applied prior to the application of linalool (Fig. 7A). Mean amplitudes of inward currents induced by linalool in the absence and presence of strychnine and picrotoxin were 34.8 ± 5.37 pA and 10.5 ± 3.39 pA ($n = 7$, $^{***}p < 0.001$; Fig. 7B), respectively. Taken together, these data indicate that linalool can activate $GABA_A$ and/ or glycine receptors on SG neurons of the Vc in mice.



(A) (B)

Figure 7. GABA- and glycine-mimetic activities of linalool. (A) A representative trace showing almost complete suppression by both picrotoxin (50 μM) and strychnine (2 μM) on linalool-induced inward currents. (B) Significant inhibition of mean inward currents induced by linalool in the presence of picrotoxin and strychnine ($n = 7$, paired t-test, *** $p < 0.001$).

Effects of Linalool on GABA- and Glycine-Induced Responses

The balance of neuronal functions is critically responded by inhibitory neurotransmission, including two predominant neurotransmitters: GABA and glycine. In the voltage-clamp mode, both GABA and glycine induced inward currents under the condition of high chloride pipette solution. Lower concentration of linalool (0.3 mM) did not induce detectable membrane inward currents. It was co-applied to examine the relationship of linalool with GABA- and glycine-induced responses. GABA- and glycine-induced inward currents were enhanced by the presence of linalool (Figs. 8A and 8B). The mean amplitude of inward currents induced by GABA and linalool co-application was increased to 243 ± 33.1% ($n = 6$, ***$p < 0.001$; Fig. 8C) compared to that by GABA alone. The presence of linalool also enhanced the amplitude of glycine-induced inward currents to 217 ± 18.4% ($n = 5$, **$p < 0.01$; Fig. 8D) compared to glycine alone. These results indicate that linalool has potentiation effects on both GABA- and glycine-induced responses on SG neurons of the Vc.

Discussion

To sum up, under a high chloride pipette solution, non-desensitizing linalool-induced inward currents were not affected by the presence of TTX, CNQX, and AP5, but depressed by strychnine and picrotoxin. Furthermore, linalool demonstrated potentiation effects on both GABA- and glycine-induced responses. These results suggest that linalool possesses GABA-, glycine-mimetic responses on SG neurons of the Vc.

Various plant-derived substances have attracted attention due to their potential pharmacological properties. They could also be developed as novel therapeutic targets for orofacial antinociception. For example, inhibitory effects of natural terpenes, components prevalent in aromatic plants, on SG neurons of the Vc have been reported (Nguyen *et al.*, 2019, 2020). Linalool, a naturally floral terpene, is found in more than 200 different flowers

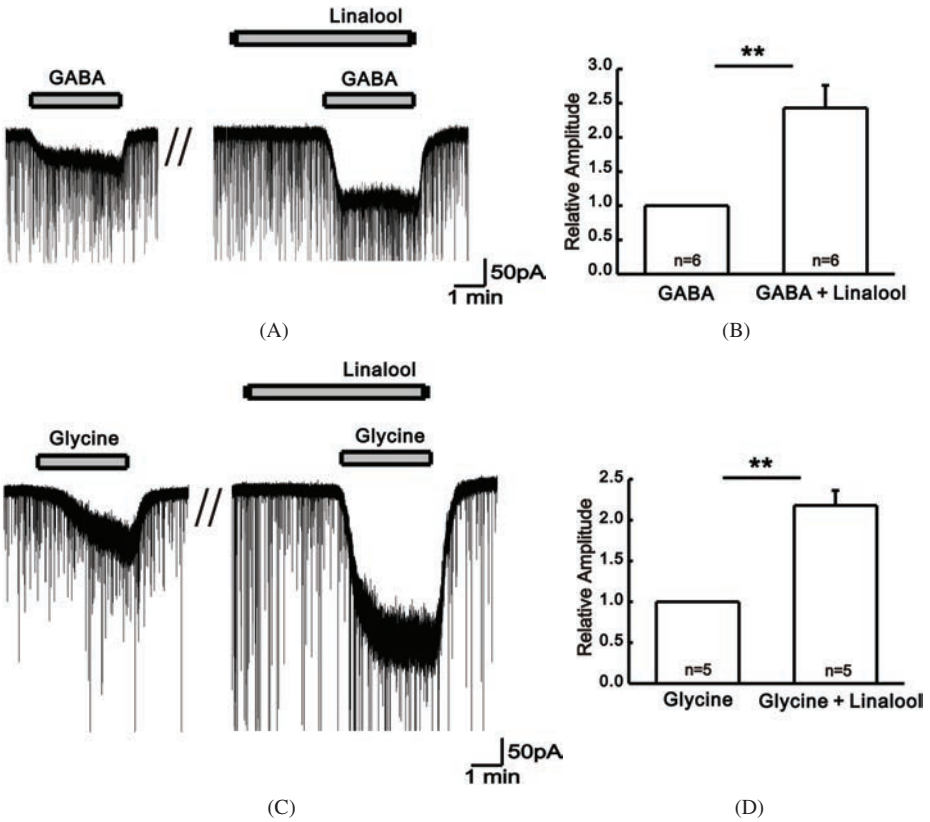


Figure 8. Increase of GABA- and glycine-induced responses by linalool. (A), (C) Representative current traces showing the potentiation effect between GABA/glycine and borneol. (B), (D) Significant enhancements in the relative amplitude of mean GABA/glycine induced inward currents in the presence of 0.3 mM linalool ($n = 6$, paired t-test, ** $p < 0.01$; $n = 5$, paired t-test, ** $p < 0.01$, respectively).

and spices of plants (National Center for Biotechnology Information, 2019). Linalool is well known as a common constituent that gives a distinctive scent of lavender. Linalool is also present in coriander, mint, cinnamon, and even in fungi. It is so outspread that human beings consume over two grams of linalool in food per year (Marnett *et al.*, 2014). It has been demonstrated that linalool can modify the nicotinic receptor-ion channel kinetics by decreasing acetylcholine release and the channel open time in the neuromuscular junction (Re *et al.*, 2000). Linalool can also lead to pain releasing by regulating adenosine A1 and A2A receptors known to induce antinociception (Peana *et al.*, 2006). These accumulated evidence, at least partially, can explain the millennial application of linalool or plant-producing linalool as an herbal medicine for antinociception.

The inhibitory transmission in the CNS is modulated by two popular neurotransmitters: GABA and glycine. Owing to its ubiquity and relatively high concentrations in mammalian CNS, GABA acts as a neurotransmitter in at least 40% of inhibitory synaptic processes

(Bowery and Smart, 2006). GABA receptors are divided into two major classes: ionotropic $GABA_A$ and metabotropic $GABA_B$. Glycine primarily regulates fast inhibitory neurotransmission in the spinal cord and brainstem via specific glycine receptors mainly involved in motor control and pain perception (Dutertre *et al.*, 2012). Once $GABA_A$ or glycine receptors are activated, neurons will hyperpolarize, thus driving the membrane potential away from the threshold for firing action potentials (Dutertre *et al.*, 2012). As mentioned in our previous studies, glycine receptors are similar to $GABA_A$ receptors on their ionotropic chloride channel receptors in the CNS (Nguyen *et al.*, 2019, 2020). Relying upon this selectively permeable characteristic, a high chloride pipette solution was used to amplify the chloride outflux at a holding potential of −60 mV. This resulted in inward currents induced by GABA, glycine, and linalool in this experiment.

As primary inhibitory neurotransmitters in the CNS, GABAergic, and glycinergic transmissions are concerned for pain mediation and perception. It is believed that loss of GABA activities might be one of the reasons for enhanced pain sensitivity in a neuropathic pain state (Yamamoto and Yaksh, 1993; Ibuki *et al.*, 1996). An experiment using normal rats indicated that spinal injection of $GABA_A$ or $GABA_B$ receptor antagonists could result in tactile allodynia and thermal hyperalgesia which could be reversed by administration of exogenous GABA agonists. Spinal $GABA_A$ agonist isoguvacine-generated specific antihyperalgesic and antiallodynic effects have been recommended as a promising therapy for neuropathic pain (Malan *et al.*, 2002). Although few studies have reported the relationship between the antinociception of linalool and GABA receptors modulation, one study using light/dark box test and elevated plus maze has indicated that linalool-induced anxiolytic effect is mediated by GABAergic transmission at benzodiazepine binding sites (Harada *et al.*, 2018). Linalool has been proven to play a role in the regulation GABAergic transmission by protecting against pentylenetetrazol and picrotoxin-induced convulsions (Elisabetsky *et al.*, 1995).

Glutamate and glutamate receptors are located in areas of the brain, spinal cord, and periphery involved in pain sensation and transmission. Psychopharmacological evaluation of linalool has revealed that this terpene has effects on modulation of glutamate activation both *in vitro* (competitive antagonism of L-[3H] glutamate binding) and *in vivo* (delayed subcutaneous *N*-methyl-D-aspartate-induced convulsions and blockade of intracerebroventricular quinolinic acid-induced convulsions) (Elisabetsky *et al.*, 1999). Linalool is effective in inhibiting homomeric $5\text{-}HT_3$ receptors known to play a substantial role in pain transmission via non-competitive mechanisms (Jarvis *et al.*, 2016). Acting as a local anesthetic treatment, linalool can depress the action potential of intact dorsal root ganglion neurons by blocking voltage-dependent Na^+ channels (Leal-Cardoso *et al.*, 2010).

In current perspectives, orofacial pain treatment is decided with a multidisciplinary management approach using drugs that could inhibit synaptic pathways (Romero-Reyes and Uyanik, 2014). Despite the ancient millennium use of linalool for medical purposes, up to now, the action of this aromatic oil on CNS has not been fully addressed. In this study, linalool exhibited GABA- and/or glycine-mimetic responses on the primary anatomical relay center for orofacial pain information. This suggests that linalool might be a promising

material for orofacial pain management at the CNS level by activating $GABA_A$ receptors and/or glycine receptors.

Acknowledgments

This research was supported by Basic Research Program through the National Research Foundation of Korea (NRF) funded by Ministry of Education (2016R1D1A3B03932241).

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