Linalool odor reliefs oral ulcerative mucositis-induced pain via descending pain modulatory system

Masato Iida

Nihon University Graduate School of Dentistry

Major in Dysphagia Rehabilitation

(Directors: Profs. Koichiro Ueda, Masamichi Shinoda, Assist. Prof. Suzuro Hitomi)

Contents

The following article and an unpublished data (Fig. 1I) are included in this doctoral thesis.

Iida M, Hitomi S, Hayashi Y, Shibuta I, Tsuboi Y, Ueda K, Iwata K, Shinoda M (2023) Analgesic effect of linalool odor on oral ulcerative mucositis-induced pain in rats. Brain Res Bull 206, 110844.

Abstract

Oral ulcerative mucositis (OUM) induces severe pain, leading to a low quality of life. Linalool odor exposure has recently been reported to suppress inflammatory pain in the hind paws. However, the analgesic effect of linalool odor on orofacial pain remains unclear. In this study, we examined the mechanism underlying the analgesic effect of linalool odor on oral pain caused by OUM using nocifensive behavioral and immunohistochemical analyses in rats. OUM was developed by treating the labial fornix region of the inferior incisors with acetic acid. Linalool at 1% was exposed for 5 min at 30 min before nocifensive behavioral measurements. OUM induced spontaneous pain and mechanical allodynia, which were suppressed by the linalool odor. Mechanical allodynia in the hind paw following the injection of complete Freund's adjuvant was also suppressed by linalool odor. Application of lidocaine to the olfactory bulb attenuated the inhibition of spontaneous pain and hyperactivation of trigeminal spinal nucleus caudalis neurons in OUM model rats. Linalool odor exposure-induced neuronal activation in the locus coeruleus (LC) of OUM model rats was decreased by lidocaine application to the olfactory bulb. The decrease in neuronal activation in the LC was attenuated by the administration of orexin 1 receptor (OX-1) antagonist to the LC. These results suggest that linalool odor stimulation through the olfactory pathway activates LC neurons via OX-1 signaling, leading to the suppression of OUM-induced pain.

Introduction

Scents stimulate the olfactory system, affect the sensory system, and can cause sedation and analgesia. For example, aromatherapy using essential oils is used to manage various symptoms, such as anxiety, depression, pain, and insomnia (Farrar and Farrar, 2020). Recently, the inhalation of lavender, rose, and peppermint oils has been reported to reduce pain in human (Lee et al., 2018). However, the underlying analgesic mechanism has not been fully elucidated.

Linalool is an enantiomeric monoterpene found in essential oils such as lavender, bergamot, and rosewood (Becker et al., 2021). Linalool has several pharmacological properties, including antioxidant, anti-inflammatory, anxiolytic, and antinociceptive effects (de Cássia da Silveira et al., 2017; Hussain et al., 2008; Katsuyama et al., 2015). In animal studies, components of various essential oils, such as linalool, carvacrol, and p-cymene, have been reported to exert analgesic effects (de Cássia da Silveira et al., 2017). Injection of linalool into the mouse hind paw ameliorated formalin- and paclitaxel-induced nocifensive behavior via the activation of peripheral opioid signaling (Katsuyama et al., 2012; Katsuyama et al., 2015). Recently, linalool odor has been shown to have an antinociceptive effect in mice (Higa et al., 2021; Tashiro et al., 2016). During linalool exposure, noxious heat- and formalin-induced pain behaviors are inhibited via orexinergic transmission (Higa et al., 2021; Tashiro et al., 2016).

Orexin, classified as orexin A and B, is an endogenous neuropeptide produced in the lateral

hypothalamic neurons and is participated in the regulation of eating behavior, sleep patterns, and antinociceptive responses (Mohammad-Pour Kargar et al., 2015; Razavi and Hosseinzadeh, 2017; Sakurai et al., 1998; Toor et al., 2021). G-protein-coupled orexin receptors, OX-1 and OX-2, have been identified and are broadly distributed in the cerebral cortex, paraventricular thalamic nucleus, hippocampus, ventromedial hypothalamic nucleus, dorsal raphe nucleus, and locus coeruleus (LC), all of which are involved in the descending pain inhibitory system (Marcus et al., 2001; Razavi and Hosseinzadeh, 2017). Actually, microinjection of orexin A into the LC has inhibited formalin-induced pain-related behavior, and the analgesic effect has been inhibited by intra-LC microinjection of an OX-1 antagonist, suggesting that OX-1 signaling in the LC contributes to the exertion of the analgesic effect (Mohammad-Pour Kargar et al., 2015).

Oral ulcerative mucositis (OUM) is a common painful disease that affects oral functions, such as eating or speaking (Munoz-Corcuera et al., 2009). Various drugs, including anesthetics and antibiotics, are used for rinsing, coating, and patching to relieve severe OUM-induced pain (Lalla et al., 2014). However, there are significant considerations such as a short effect period, loss of touch sensation, inadequate analgesia, and pain induction caused by applying drugs to the OUM region (Saunders et al., 2013). Truly, in the previous animal studies, topical application of indomethacin ameliorated OUM-induced spontaneous pain but not mechanical allodynia, whereas lidocaine application caused hypoalgesia in the healthy mucosa, and the

analgesic effect was exerted in a short period (Hitomi et al., 2015; Hitomi et al., 2016). If linalool odor can relieve OUM-induced pain without these issues, a new pain-control therapy can be available without direct contact with the OUM region.

In the present study, I determined whether linalool odor could relieve OUM-induced pain and examined the mechanism underlying the analgesic effects of linalool odor using immunohistochemical and molecular biology methods in a rat OUM model.

Materials and Methods

Animals

Male Wistar rats (Japan SLC, Hamamatsu, Japan; n = 205) were used for all experiments. The rats were housed in clear cages in a room maintained on a light-dark cycle (L:D, 12:12 h) in a temperature- and humidity-controlled (21–23°C and 40–60%, respectively) with food pellets and water provided ad libitum under specific-pathogen-free conditions. Each cage contained 2 rats. All the experiments in the study complied with the ARRIVE guidelines and conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All the experiments (animal protocol numbers: AP20DEN008, AP21DEN015, AP22DEN027, and AP22DEN042) were approved by The Animal Experimentation Committee of Nihon University. The procedures in this study were performed according to the guidelines of the International Association for the Study of Pain (Zimmermann, 1983). The rats were randomly chosen for each experiment. All behavioral observations were performed in a manner that was blinded to the experimental conditions.

OUM model

Fifty percent acetic acid soaked in a filter paper (3 × 3 mm, Whatman, Maidstone, UK) was treated to the labial fornix region of the inferior incisors for 30 s under deep anesthesia with

intraperitoneal administration of butorphanol at 2.5 mg/kg (Meiji Seika Pharmaceutical, Tokyo, Japan), medetomidine at 0.375 mg/kg (Xenoac, Fukushima, Japan), and midazolam at 2.0 mg/kg (Sand, Tokyo, Japan). Given that acetic acid treatment induced visually apparent ulceration in the treated oral mucosal region on days 2–3 (Hitomi et al., 2015; Hitomi et al., 2017; Nodai et al., 2018), OUM model rats on day 2 after acetic acid treatment were used in the present study.

Evaluation of spontaneous pain, capsaicin-induced pain, and mechanical allodynia

To evaluate spontaneous oral pain, the duration of spontaneous mouth rubbing behavior using both forelimbs was measured in a clear plastic cage (30 × 30 × 30 cm) for 10 min after 30 min linalool exposure. In a similar manner, capsaicin-evoked intraoral pain was assessed in the same plastic cage for 3 min immediately after application of capsaicin (100 μM in 10% dimethyl sulfoxide (DMSO)-containing distilled water, Wako, Tokyo, Japan) into the labial fornix region of the inferior incisors. Prior to behavioral measurements, all rats were acclimated to a clear plastic cage at least three times.

To measure the mechanical head-withdrawal threshold, the mouth of conscious rats was opened in a handmade cylindrical shading box (6 × 6 × 13 cm) with a hole through which the nose could stick out (Hitomi et al., 2015). Firstly, the mental skin of 6-week-old rats under

anesthesia with intraperitoneal administration of butorphanol at 2.5 mg/kg, medetomidine at 0.375 mg/kg, and midazolam at 2.0 mg/kg was pierced with a magnetized ring (22-gauge-like size, Daiso Sangyo, Hiroshima, Japan). Subsequently, the rats were trained for 2–3 weeks prior to the measurements to stably expose the labial fornix region while conscious, attained by the attachment of a small neodymium magnet with a 4 g weight to the piercing. The head-withdrawal threshold in mechanical stimulation of the oral mucosa was measured using von Frey filaments (0.02, 0.04, 0.07, 0.16, 0.2, 0.3, 0.4, and 0.6 g; North Coast Medical, Morgan Hill, CA, USA). The mechanical head-withdrawal threshold was defined as the lowest intensity required to evoke an escape attempt in at least 3 of the 5 tests.

Rats received the intraplanar injection of complete Freund's adjuvant (CFA) with the same volume of saline (50 μL) into the right hind paw under deep anesthesia with intraperitoneal administration of butorphanol at 2.5 mg/kg, medetomidine at 0.375 mg/kg, and midazolam at 2.0 mg/kg. The rats were acclimated in a clear plastic cage (19 × 21 × 15 cm) on wire-netting for 3 days. The mechanical withdrawal threshold in the hind paw was measured by mechanical stimulation using a set of von Frey filaments (4, 6, 8, 10, 15, 26, 50, and 60 g; North Coast Medical) through wire-netting on awakening 30 min after linalool or vehicle exposure. The paw withdrawal threshold was defined as the lowest intensity required to evoke an escape attempt in at least 3 of the 5 tests.

Linalool application

1. Linalool odor exposure

Linalool was purchased from Tokyo Chemical Industry (Tokyo, Japan). The linalool solution used in this study was diluted with saline solution. Linalool at 3% was used as an emulsion because of the low solubility. Linalool odor was filled in a closed box (30 × 30 × 25 cm) by placing a filter paper dipped in 1 mL of the linalool solution at 0.1, 1, and 3% diluted in saline in a closed box for 10 min. Rats were placed in a closed box and exposed to linalool odor for 5 min. In some rats, the linalool odor was exposed twice before behavioral measurement or exposed for 30 min.

2. Linalool topical application

Linalool solution at 10 μg/mL diluted in the 10% tween 80 containing saline or the vehicle was applied for 5 min by placing a 30 μL-soaked cotton swab on the OUM region under 2% isoflurane anesthesia on day 2 in the OUM rats. Thirty min after the treatment, the duration of spontaneous mouth rubbing time were measured for 10 min.

Induction of olfactory bulb dysfunction

To induce olfactory bulb dysfunction, lidocaine hydrochloride monohydrate (2% dissolved in saline; 5 μL, Sigma-Aldrich, St. Louis, MO, USA) was applied to the olfactory bulb. One

week before lidocaine application, rats were placed in a stereotaxic apparatus under deep anesthesia with butorphanol at 2.5 mg/kg, medetomidine at 0.375 mg/kg, and midazolam at 2.0 mg/kg. As previously described (Kaji et al., 2016), the skull was exposed and a small hole (diameter: 1 mm) was drilled directly above the left olfactory bulb. The cannula (PE10, Nazme, Tokyo, Japan) was extended into the bulb through a hole (3 mm below the skull surface, 8 mm anterior from the bregma, and 2–3 mm lateral to the sagittal suture) and fixed to the skull with dental cement.

The component of fox feces, 2,3,5-Trimethyl-3-thiazoline (TMT, Tokyo Chemical Industry), is used as a stimulus to induce fear in predators of naive rodents (Delfino-Pereira et al., 2020; Nikaido and Nakashima, 2011). The rats were habituated to a clear plastic cage for 10 min for 3 days. TMT- or saline-applied cotton were randomly placed at the farthest two corners of the box and subsequently placed into the plastic cage 3 min after lidocaine or saline application into the olfactory bulb. The time spent in half of the TMT side was measured for 3 min.

Administration of OX-1 antagonist into the LC

The OUM model rats were placed in a stereotaxic apparatus under deep anesthesia with butorphanol at 2.5 mg/kg, medetomidine at 0.375 mg/kg, and midazolam at 2.0 mg/kg. The skull was exposed, and a small hole (diameter: 1 mm) was made at the position (10.1 mm

posterior from the bregma, and 1.31 mm left lateral to the sagittal suture). The guide cannula (diameter: 0.5 mm, length: 9 mm), which was extended in the left LC (6.5 mm below the skull surface angle 10° in the rostral direction) through the hole, was fixed to the skull with three stainless steel screws and dental cement. One week after the canulation, the awake rats were lightly held and administered 1 μL of the OX-1 antagonist SB334867 (80 nmol/μL/rat dissolved in 10% DMSO, Tocris Biotechne, Minneapolis, MN, USA) or vehicle (10% DMSO) through the canula. Ten min after administration, the rats were exposed to linalool or the vehicle for 5 min. The duration of the spontaneous mouth rubbing behavior was measured 30 min after each exposure. Two hours after injection into the LC, the brain tissue was extracted for immunohistochemistry.

Immunohistochemistry

Rats were deeply anesthetized with intraperitoneal administration of butorphanol at 2.5 mg/kg, medetomidine at 0.375 mg/kg, and midazolam at 2.0 mg/kg, and transcardially perfused with 0.9% ice-cold saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer. The brainstem segment was excised and post-fixed overnight at 4°C. For cryoprotection, the tissues were subsequently transferred to 20% sucrose (w/v) in phosphate-buffered saline (PBS) for 2 days. The tissue was sectioned at a thickness of 30 μm for the trigeminal spinal subnucleus

caudalis (Vc) and 40 μm for LC using a freezing microtome. After incubation with 0.3% H_2O_2 for 30 min and blocking with 3% normal goat serum in PBS for 1 h, the free-floating sections were incubated with rabbit anti-c-Fos polyclonal antibody (1:1000; Abcam, Cambridge, UK) or rabbit anti-OX-1 antibody (1:1000, Proteintech, Rosemont, IL, USA) for 72 h at 4°C. The sections were then incubated with biotinylated goat anti-rabbit IgG (1:600; Vector Laboratories, Burlingame, CA, USA) for 2 h and a peroxidase-conjugated avidin-biotin complex (1:100; ABC, Vector Laboratories) for 1 h at room temperature. After washing with 0.05 M Tris buffer (TB), the sections were incubated in 0.035% 3,3-diaminobenzidine-tetra HCl (Sigma–Aldrich), 0.2% nickel ammonium sulfate, and 0.05% peroxide in 0.05 M TB. Sections were gently rinsed in PBS, and mounted on MAS-coated slides (Matsunami, Tokyo, Japan). Images were captured using a fluorescence microscope (BZ-9000; Keyence, Osaka, Japan). The number of the c-Fos-immunoreactive (IR) cells in Vc was counted from every fourth section, and the mean number of c-Fos-IR cells (3 sections/rat) was obtained from each rat.

Rotarod test

The effect of linalool odor on the motor function was evaluated using a rotarod (model 47700; Ugo Basile, Gemonio, Italy). Rats were placed on a moving rotarod (diameter: 6 cm) at 4 rpm, and the speed was increased from 4 to 40 rpm over 3 min, forcing them to walk forward to

avoid falling. The latency to fall was measured (cutoff: 180 s). The rats were acclimated to the rotarod 2 days prior to the experiments. On the experimental day, the latency immediately prior to each exposure was recorded, and the effects of linalool or vehicle exposure on motor performance were assessed for 20 min.

Statistical analysis

Data are presented as the mean ± standard error of the mean, median, and interquartile range (25–75%). Dotted plots indicate individual sample sizes, where N represents the number of rats tested. Data normality was assessed using the Shapiro–Wilk test. The Kruskal–Wallis test, followed by Dunn's multiple comparisons test, was used to compare multiple groups as a non-parametric procedure for mechanical head-withdrawal threshold analysis. The Mann-Whitney U test was used to compare the 2 groups as a non-parametric procedure for mechanical head and paw withdrawal threshold analysis. Unpaired *t*-test was used to compare the 2 groups as a parametric procedure for analysis of the duration of rubbing and immunohistochemical analysis. Dunnett's *post hoc* tests were performed using a one-way analysis of variance (ANOVA) to analyze the duration of rubbing behavior. Differences were considered statistically significant at $P < 0.05$. Statistical analyses were performed using GraphPad Prism 8 software package (GraphPad Prism Software, San Diego, CA, USA).

Results

Linalool odor exposure suppresses spontaneous pain and mechanical allodynia

An oral ulcer was observed on day 2 after the acetic acid treatment of the mucosal surface (Fig. 1A). The spontaneous rubbing time was prolonged in OUM rats compared with naive rats (Fig. 1B). Exposure to linalool at 1%, but not 0.1% for 5 min, significantly inhibited the increase in rubbing time in the OUM rats. The linalool at 1% did not change the rubbing time in naive rats. The increased rubbing time in OUM rats which had been exposed to linalool odor twice before the measurement was also significantly inhibited by linalool exposure (Fig. 1B), suggesting that linalool odor can cause repeated analgesic effects on OUM-induced pain. The exposure inhibited the extension of rubbing by 1% linalool exposure but not by that of 0.1% (Fig. 1C). There was no significant difference in rubbing time between 1% linalool exposure for 30 min and that for 5 min. The result indicates that analgesic effect of linalool is independent on exposure time. The extended rubbing time following capsaicin application to the oral mucosa was significantly inhibited by linalool at 1% in naive and OUM rats (Fig. 1D). The head-withdrawal threshold in mucosal mechanical stimulation significantly decreased on day 2 after acetic acid treatment. The decrease in mechanical head-withdrawal threshold was recovered at 30 min and returned to 120 min after 1% linalool exposure (Fig. 1E). Linalool exposure did not alter the mechanical head-withdrawal threshold in naive rats (Fig. 1F). To determine whether

the linalool exposure has analgesic effect on inflammatory mechanical allodynia in the hind paw, the withdrawal threshold in mechanical stimulation of the inflamed hind paw was measured after 1% linalool odor exposure. CFA-induced mechanical allodynia was significantly inhibited by 1% linalool exposure (Fig. 1G). Exposure of linalool at 1% and 3% with the same manner did not affect the motor function (Fig. 1H). To determine whether the topical application of linalool to the OUM region also shows analgesic effect, duration of spontaneous rubbing time was measured. The rubbing time was significantly decreased by linalool application to the OUM region (Fig. 1I).

Analgesic effect of linalool exposure is inhibited by olfactory deprivation

Lidocaine was applied to the olfactory bulb to prevent olfaction and to determine the effect of linalool olfactory stimulation on OUM-induced pain. First, to confirm olfactory deprivation, the time spent in a natural predator odor TMT-rich environment was measured. The time spent in the TMT odor-rich environment was significantly increased by lidocaine application compared with vehicle injection in naive rats. (Fig. 2A). Linalool odor-induced inhibition of prolonged rubbing time in OUM rats was significantly suppressed by lidocaine application into the olfactory bulb compared to vehicle injection (Fig. 2B). c-Fos was expressed in the Vc of the naive and OUM rats (Fig. 2C). The number of c-Fos-IR neurons in the Vc was increased by

OUM and decreased by linalool exposure. Lidocaine injection into the olfactory bulbs suppressed linalool-induced inhibition of the increase in the number of c-Fos-IR neurons in the Vc of OUM rats (Fig. 2D).

Linalool exposure activates the descending inhibitory modulation via orexin signaling in the LC

To determine whether linalool exposure activates LC neurons, the expression of c-Fos-IR in neurons was analyzed in the LC following linalool exposure. The number of c-Fos-IR LC neurons was significantly higher in the OUM rats than those in the naive rats (Fig. 3B). The increase in the number of c-Fos-IR neurons was further enhanced by linalool exposure, suggesting that LC neurons were activated by linalool exposure. This enhancement was significantly inhibited by lidocaine application to the olfactory bulb (Fig. 3A and B). OX-1 was expressed in the LC region of naive rats (Fig. 3C). Compared with vehicle administration, administration of the OX-1 antagonist SB334867 to the LC significantly increased the duration of spontaneous rubbing in linalool-exposed OUM rats (Fig. 3D).

Discussion

The present study demonstrated a linalool odor-induced analgesic effect on OUM-induced pain in rats, without affecting motor function. The analgesic effect of linalool exposure was inhibited by olfaction deprivations, suggesting that linalool exposure suppresses OUM-induced pain via olfaction. Furthermore, linalool exposure activated LC neurons involved in the descending pain modulation system. These results suggest that LC neuronal activation via linalool odor activates the descending pain inhibitory system, leading to the amelioration of OUM-induced pain.

Olfactory cells in the olfactory epithelium receive various chemical substances and olfactory information is transmitted to neurons in the olfactory bulb. Further, the olfactory cortex is composed of the piriform cortex, amygdala, olfactory tubercle, and entorhinal cortex (Bhatia-Dey and Heinbockel, 2021). Subsequently, the information is transmitted directly or via the thalamus to the orbitofrontal cortex and hypothalamus, which are involved in discriminating between odors and emotional and physiological responses, respectively (Bhatia-Dey and Heinbockel, 2021). In the present study, lidocaine application to the olfactory bulbs inhibited the linalool-induced analgesic effects on OUM-induced spontaneous pain and mechanical allodynia, suggesting that the olfactory system is involved in linalool-induced analgesia. Linalool odor activates the hypothalamic orexin neurons (Tashiro et al., 2016). The orexin

neurons from the hypothalamus project widely throughout the brain, including the LC , which expresses OX-1 (Marcus et al., 2001; Trivedi et al., 1998), and contributes to pain modulation (Nakamoto et al., 2023; Razavi and Hosseinzadeh, 2017; Soya and Sakurai, 2020). The number of c-Fos-IR neurons in the LC increased following linalool exposure in OUM rats. This was suppressed by lidocaine application to the olfactory bulb, suggesting that linalool odor activates LC neurons via the olfactory bulb. In addition, pharmacological blockage of OX-1 signaling in the LC eliminated linalool-induced inhibition of OUM-induced spontaneous pain. Previous studies have reported that the microinjection of orexin-A into the LC activates OX-1 and produces the endocannabinoid 2-arachidonoylglycerol, a cannabinoid type-1 receptors (CB1Rs) agonist, leading to the suppression of formalin-induced pain via CB1R signaling (Lee et al., 2020; Mohammad-Pour Kargar et al., 2015; Scavone et al., 2010). CB1R activation by intracerebroventricular injection of a CB1 agonist increases LC neuronal activity, suppressing the activity of the descending inhibitory pathway, and inhibiting gamma-amino butyric acid-ergic neurotransmission in the periaqueductal gray (Ho et al., 2011; Mohammad-Pour Kargar et al., 2015; Muntoni et al., 2006). From these reports, CB1Rs signaling may also be involved in linalool-induced analgesia in the present study. Second possible analgesic pathway via OX-1 signaling in LC neurons is the noradrenergic pathway. Stimulation of OX-1 in the LC accelerates noradrenaline release into the spinal dorsal horn (SDH), leading to the inhibition of

SDH neuronal hyperexcitability via the activation of inhibitory α_2 adrenoceptors or excitatory α_1 adrenoreceptors in inhibitory interneurons, thereby suppressing mechanical hypersensitivity (Nakamoto et al., 2023). Third, the linalool-analgesia has driven the orexin neurons and the direct projection of orexinergic nerves from the lateral hypothalamus to the spinal cord mediate the analgesia via OX-1 (Higa et al., 2021). In addition, OX-1 in the Vc has been involved in the orofacial pain transmission (Kooshki et al., 2016). Therefore, as well as the olfactory bulb-hypothalamus-LC-Vc pathway, probably the direct projection to the Vc pathway from the lateral hypothalamus is also involved in the linalool-induced analgesia in the orofacial region. On the other hands, I cannot deny the possibility that the injected the OX-1 antagonist (SB334867) directly affect to the Vc region. However, since the Vc region and LC region are far apart (almost 5 mm distance) and 1 μl of SB334867 was administrated slowly, SB334867 would not spread to the Vc. Furthermore, although unilateral administration of SB334867 to the LC inhibited linalool-induced analgesic effect on OUM-induced pain (Fig. 3D), the contralateral orexin signaling in the LC still was presumed to drive the descending pain-control system. If bilateral OX-1 receptor in the LC would be antagonized, the endogenous descending pain-control system (not by linalool) will be also blocked. In that case, the rubbing time will prolong more than that in OUM rats without SB334867 administration and linalool exposure. In the present study, the rubbing time did not prolonged to more than the control level,

suggesting that linalool odor-induced analgesia was partially inhibited. Including these studies, the results suggest that linalool odor exposure ameliorates OUM-induced pain by activating the descending pain inhibitory system through the orexinergic, cannabinoidergic, and noradrenergic pathways.

Odors can be recognized as pleasant or unpleasant (Rolls et al., 2003). In humans, pleasant and unpleasant scents activate different brain areas, including the medial and lateral orbitofrontal cortices (Rolls et al., 2003; Tizard and Skow, 2021). Generally, because lavender- and bergamot-containing linalool are considered preferable odors, linalool odor-induced analgesia may be induced by olfactory hedonic processing, which is directly responsible for pleasure-related analgesia owing to the enhanced opioidergic neurotransmission (Kut et al., 2011). Although I did not assess its relationship to the opioidergic pain inhibitory system, pleasure-related analgesia may have been involved in the linalool-induced analgesia in the present study.

In the present study, the analgesic effect of linalool odor for 5 min on OUM-induced pain was observed 30 min after exposure. The suppression of OUM-induced mechanical allodynia vanished 120 min after linalool exposure, suggesting that the analgesic effect of linalool odor persisted for more than 30 min after exposure. Since many terpenoids have been known to enter the body easily by inhalation, penetration through the skin, or oral absorption (Zárybnický et

al., 2018), there is a possibility to exert the analgesic effect through the blood. A previous study measured the plasma concentration of volatile substances after the inhalation of essential oils containing these substances. These substances were detected 1 h after inhalation, but were not detected at 30 min (Muchtaridi, 2011). Thus, plasma linalool may not have been involved in the analgesic effect of linalool odor in the present study. However, because the concentration and inhalation methods differ, further experiments are required to examine this possibility.

Linalool has several direct effects including anti-inflammatory (Li et al., 2016), antioxidant (Celik and Ozkaya, 2002; Sabogal-Guáqueta et al., 2019), antimicrobial (Zhong et al., 2021), and analgesic effects (de Cássia da Silveira et al., 2017). In the present study, topical application of linalool to the OUM region decreased spontaneous rubbing time compared with vehicle application, suggesting that linalool can suppress the OUM-induced spontaneous pain directly. In some previous reports, the topical application of frankincense oil containing linalool inhibits inflammation via cyclooxygenase-2 overexpression induced by peripheral formalin injection (Li et al., 2016). Subcutaneous injection of linalool dose-dependently suppressed paclitaxel-induced mechanical allodynia and hyperalgesia from 15 to 60 min after injection by enhancing peripheral opioidergic signaling (Katsuyama et al., 2012). Furthermore, linalool application to the conjunctiva of rabbits inhibited electrically evoked contractions for 10 min after application, indicating that linalool has a local anesthetic activity (Ghelardini et al., 1999). Given the

suppression of OUM-induced pain from 30 min after exposure to linalool odor in this study, it is plausible that various direct effects exerted within 1 h following linalool application could be implicated in the analgesic impact of linalool odor exposure. If so, the volatile substances of linalool may stimulate not only the peripheral olfactory epithelium but also other mucosal regions, including the oral mucosa. However, since the analgesic effect in the present study was lost by lidocaine application to the olfactory bulb, the direct effect of linalool, either via the percutaneous or hematogenous route, on OUM-induced pain may be negligible. In addition, linalool is a weak agonist of the nociceptive chemo-sensor transient receptor potential ankyrin 1 (TRPA1) (Fothergill et al., 2016; Riera et al., 2009). It has the potential to induce a pungent sensation by stimulating TRPA1 expression in the olfactory epithelium during exposure to linalool odor (Nakashimo et al., 2010). However, no algesic effects of linalool were observed in previous and present studies (Kashiwadani et al., 2021), suggesting that linalool concentrations of at least 1% do not show an algesic effect via TRPA1.

Linalool odor can suppress OUM-induced pain via the olfactory system without contact with the OUM region. Thus, it is advantageous for head and neck cancer patients with intraoral pain, which is a side effect of chemoradiotherapy caused by eating, swallowing, and speaking in the OUM regions. Our data showed that the analgesia induced by linalool odor exposure persisted for over 30 min and 3rd linalool exposure also suppressed spontaneous pain. Therefore, it is

possible that exposure to linalool-containing lavender and bergamot essential oils before eating and during oral care can prevent touch-evoked pain, indicating that linalool odor exposure is the preferred therapy for chemoradiotherapy-induced intraoral pain. Importantly, chemoradiotherapy of the head and neck region has been reported to change and decrease olfaction, such as odor discrimination and identification (Bernhardson et al., 2009; Hölscher et al., 2005). Therefore, normal olfactory function is necessary to maximize the analgesic effect of the linalool odor.

There is still a question of whether other odors can exert an analgesic effect, and if the analgesic mechanism of linalool odor has been adopted for other odors. Further research is needed to determine the detailed mechanism underlying this odor-induced analgesic effect.

Conclusion

The findings indicate that exposure to linalool odor alleviates OUM-induced pain through the olfactory pathway. Since microinjection of an OX-1 antagonist into the LC alleviated linalool-induced inhibition of spontaneous pain, OX-1 signaling in the LC is likely to mediate the analgesic effect of linalool on OUM-induced pain. These results in this study provide a possible new therapy with long-lasting analgesic effects without pain induction by contact with the OUM region.

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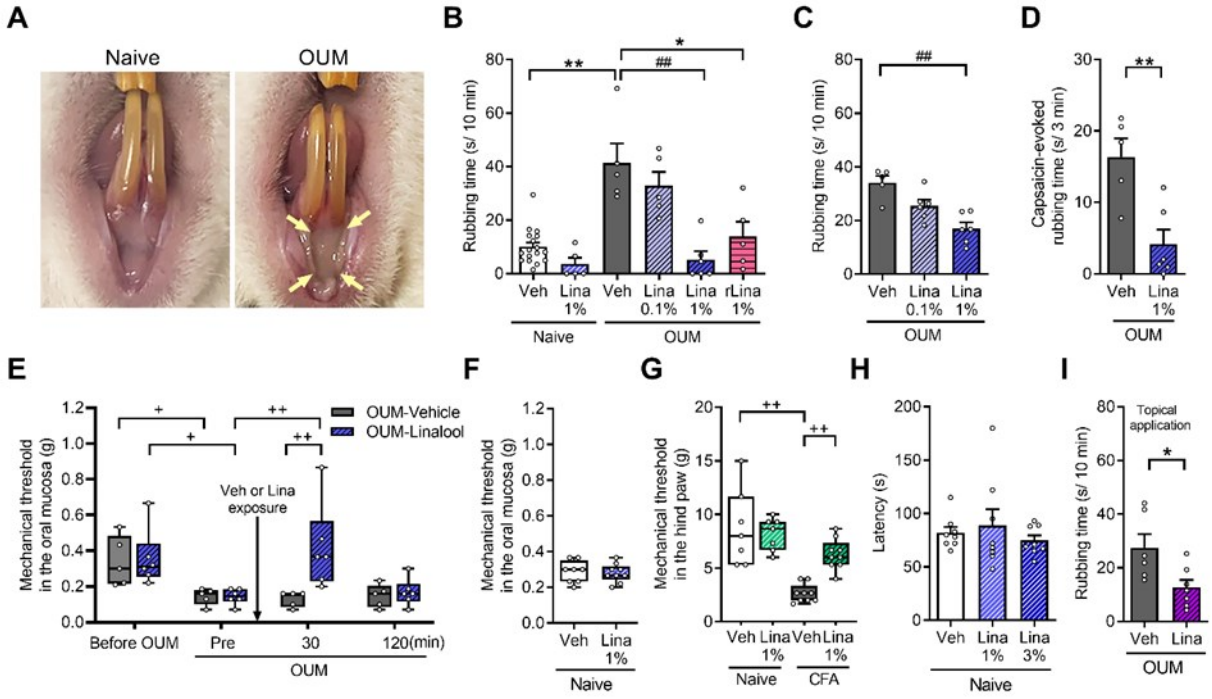

Fig. 1. Effects of linalool exposure on pain-related behaviors.

(A) Representative images of oral mucosa in naive and oral ulcerative mucositis rats. Yellow arrows indicate OUM. (B) Effects of vehicle (saline) or linalool exposure for 5 min at 0.1 and 1% on spontaneous mouth rubbing time. rLina: third exposure of 1% linalool. * $P < 0.05$, ** $P < 0.01$, unpaired *t*-test. ## $P < 0.01$, Dunnett's multiple comparisons test following one way ANOVA. (C) The spontaneous mouth rubbing time following linalool exposure at 1% for 30 min in OUM rats. ## $P < 0.01$, Dunnett's multiple comparisons test following one way ANOVA. (D) The duration of rubbing time during a 3 min period following the application of 100 μM capsaicin to the labial fornix region of the inferior incisors in naive and OUM rats. * $P < 0.05$, ** $P < 0.01$, unpaired *t*-test. (E) Head-withdrawal threshold in mechanical stimulation in the oral mucosa following vehicle or 1% linalool exposure for 5 min in OUM rats. + $P < 0.05$, ++ $P < 0.01$, Kruskal–Wallis test was followed by Dunn's multiple comparisons test. (F) Head-withdrawal threshold in mechanical stimulation in the oral mucosa following vehicle or 1% linalool exposure for 5 min in naive rats. (G) Paw withdrawal threshold in mechanical stimulation in the hind paw following vehicle or 1% linalool exposure for 5 min in naive and CFA-injected rats. ++ $P < 0.01$, Kruskal–Wallis test was followed by Dunn's multiple comparisons test. (H) Latency to fall from a rod in naive rats following vehicle or 1% and 3% linalool exposure for 5 min. (I) The spontaneous mouth rubbing time following linalool application at 10 μ g/mL for 5 min in OUM rats. * $P < 0.05$, unpaired *t*-test.

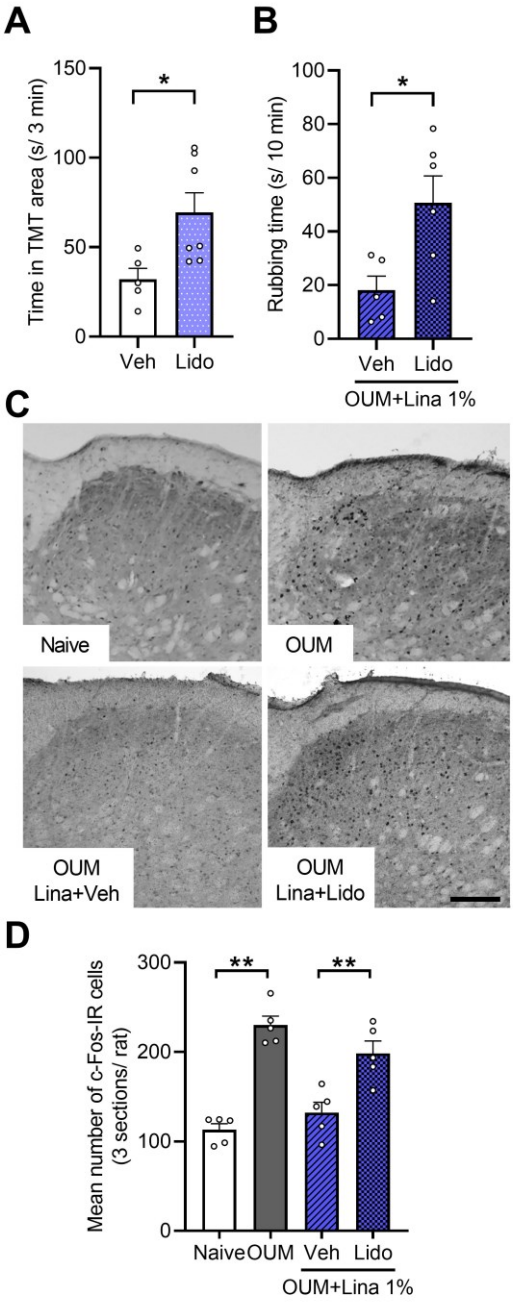

Fig. 2. Effect of olfactory deprivation on the linalool-induced analgesia.

(A) Time-spent in the 2,3,5-Trimethyl-3-thiazoline (TMT) odor-rich environment for 3 min in naive rats. * $P < 0.05$, unpaired *t*-test. (B) The duration of spontaneous rubbing time for 10 min following vehicle (saline) or lidocaine application to the olfactory bulb in the linalool-exposed OUM rats. * $P < 0.05$, unpaired *t*-test. (C) Typical images of c-Fos expressions in the Vc in naive and OUM rats. Scale bar = 200 μm. (D) Mean number of c-Fos-immunoreactive neurons in the Vc. ** $P < 0.01$, unpaired *t*-test. OUM+Lina: linalool-exposed OUM rats. OUM+Veh: Vehicle-exposed OUM rats.

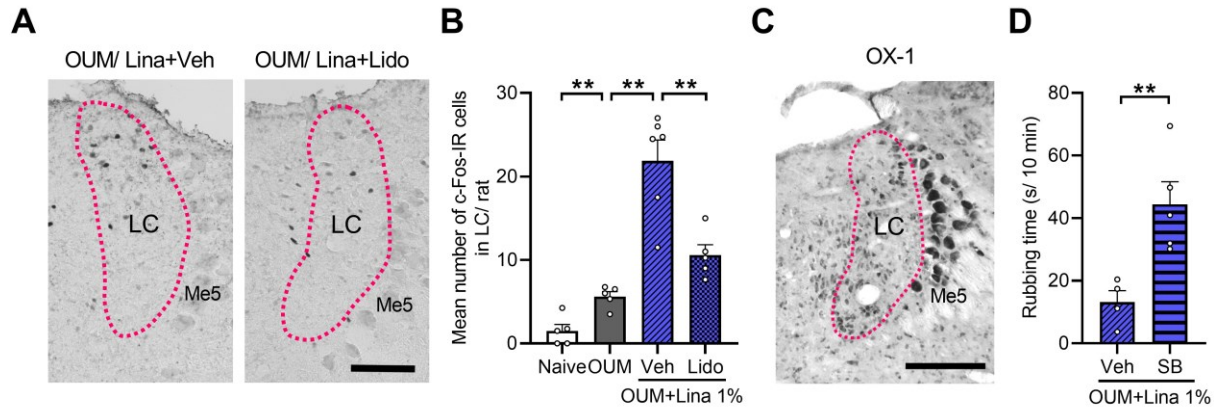

Fig. 3. Linalool exposure activates the descending inhibitory modulation via orexin neuron in the locus coeruleus (LC).

(A) Expression of c-Fos-immunoreactive (IR) neurons in the LC, 120 min after linalool exposure following vehicle (saline) or lidocaine application to the olfactory bulb in OUM rats. Scale bar = 100 μm. Me5: the mesencephalic trigeminal sensory nucleus. (B) Mean number of c-Fos-IR neurons in the LC. ** $P < 0.01$, unpaired *t*-test. (C) Orexin 1 receptor (OX-1) expression in the LC region. Scale bar = 100 μm. (D) The spontaneous rubbing time for 10 min period 20 min after linalool exposure following vehicle or the OX-1 receptor antagonist SB334867 administration to the LC in OUM rats. ** $P < 0.01$, unpaired *t*-test.