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Comprehensive behavioral study of the effects of vanillin inhalation in mice

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ABSTRACT

Vanillin is widely used in food and cosmetics, among other substances, for its sweet smell. However, the neuropsychological effects of vanillin inhalation have not been elucidated. In this study, we investigated the effect of vanillin inhalation on mouse behavior. First, we investigated whether the aroma of vanillin was attractive or repulsive for mice. Thereafter, the mice inhaled vanillin for 20 min before each test in a series of behavioral tests (elevated plus maze, open field, Y-maze, tail suspension, cotton bud biting, and Porsolt forced swim tests). In these tests, the mice showed a neutral response to vanillin. Mice that inhaled vanillin had a suppressed pain response in the hot plate test. In addition, the grip strength of the forelimbs of mice that inhaled vanillin was decreased. No significant differences were found between the mice inhaling vanillin and control mice in the open field, Y-maze, tail suspension, forced swimming, and aggression tests. These results show that vanillin inhalation has anti-nociceptive effects, similar to other routes of administration. The results also show that vanillin inhalation does not cause significant behavioral effects.

1. Introduction

In recent years, there has been increasing interest in alternative medicine, including aromatherapy. Aromatherapy is a type of complementary medicine widely used for the management of chronic pain, depression, anxiety, insomnia, and stress-related disorders [1,2]. Essential oils used in aromatherapy are believed to have a physiological effect [3–5]. Essential oils are absorbed into the olfactory and respiratory systems by inhalation or are transdermally incorporated into the body by massage [6]. Inhalation of essential oils transfers signals from the olfactory system to the brain, and the brain regulates behavior and mood by secreting neurotransmitters, such as serotonin and dopamine [1]. Therefore, various plant-derived essential oils have traditionally been used to treat psychiatric disorders, such as depression, anxiety, neurosis, attention deficit hyperactivity disorder, and bipolar disorder. However, the scientific basis for the effect of many essential oils is lacking [2,7]. Moreover, the molecular mechanisms of action of many inhaled scent molecules on the central nervous system are unknown.

Vanillin (4-hydroxy-3-methoxybenzaldehyde) is the main component of natural vanilla. Vanillin, a single molecule extracted from vanilla beans, is a common scent widely used in perfumes, foods, and pharmaceuticals [8,9]. Vanillin is also present as a major component of phenolic Styrax [10]. It has been reported that vanillin has antioxidant, anti-inflammatory, anti-apoptotic, and anti-stress actions [11–14]. However, the exact pharmacological mechanism of action of vanillin has not been elucidated. Although vanillin is included in many foods and cosmetics due to its attractive aroma, the neuropsychological effects that occur following the inhalation of vanillin have not been widely reported. Previous studies have reported that the inhalation of

Abbreviations: ANOVA, analysis of variance; NIH, National Institutes of Health; PFST, Porsolt forced swim test

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vanillin leads to increased appetite in mice [15]. This report shows that vanillin readily passes through the blood-brain barrier and induces effects at the neuronal level. Therefore, we hypothesized that inhaled vanillin may have neuropsychological effects. This study aims to clarify the behavioral changes induced by the inhalation of vanillin in mice. We also aimed to investigate whether inhaled vanillin caused an antinociceptive effect.

2. Materials and methods

2.1. Animals

We used 11-week-old male C57BL/6 mice for these experiments. The mice were randomly divided into two groups; one group was exposed to vanillin (n = 10) and the other was not (n = 10). We purchased the animals from Charles River Laboratories (Kanagawa, Japan) and housed them in cages with food and water provided ad libitum under a 12-/12-h light/dark cycle and a temperature range of 23 °C – 26 °C. We made every effort to minimize the suffering and number of animals used. These experiments complied with the U.S. National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals (NIH Publication No. 80-23, revised in 1996) and were approved by the Committee for Animal Experiments at Kawasaki Medical School Advanced Research Center.

2.2. Inhalation of vanillin

Vanillin was acquired from FUJIFILM Wako Pure Chemical Corporation (224-00682, Osaka, Japan) and was diluted in distilled water to obtain a concentration of 1% (v/v), immediately before the experiments. We used 1% vanillin, which according to convention is the concentration closest to the saturated solution [16]. Distilled water was used as a control. The inhalation apparatus consisted of a custom-made olfactometer. A piece of absorbent cotton (4 × 4 cm) impregnated with 2 mL of 1% vanillin was placed into the odor chamber (150 × 100 × 100 mm). Clean air was introduced into the odor chamber from a compressed air cylinder, and output odor air was used for ventilation in the observation chamber (150 × 100 × 100 mm) at a constant rate (5 L/min). After 10 min of pre-ventilation with odorous air, each mouse was individually placed into the observation chamber. The mice were subjected to vanillin for 20 min before each behavioral test.

2.3. Odor preference test

The apparatus was rectangular (30 × 60 × 40 cm). Two transparent cages (7.5 × 7.5 × 10 cm, with several holes 1 cm in diameter) were placed at both the ends of the apparatus (Fig. 1A). Each mouse was placed in the box for 6 min and was allowed free exploration to habituate. Exposure to olfactory stimuli was performed by gently impregnating a piece of absorbent cotton (4 × 4 cm) with 1 mL of distilled water or 1% vanillin. The absorbent cotton was placed in one of the transparent cages that were located in the corners of each lateral compartment. The subject mouse was placed at the center of the box and was allowed to explore the entire box for 6 min. One side of the rectangular area was identified as the vanillin area and the other as the control area. The amount of time spent in each cage and around each cage during the 6-min sessions was measured. The preference index for vanillin was calculated as (time around vanillin/[time around control + time around vanillin]). All the components of the apparatus were cleaned after each phase of this test. This test was conducted before the other behavioral experiments. Data were video recorded and analyzed using video tracking software (ANY-MAZE, Stoelting Co., Wood Dale, IL, USA).

2.4. Behavioral tests

All behavioral experiments were performed during the light phase (9:00–16:00). Each behavioral test was separated from the next by at least one day. We tested the mice in a random order. After completion of each test, the apparatus was cleaned with 70% ethanol and water with superoxidized hypochlorous acid to prevent any bias due to olfactory cues.

2.4.1. Hot plate test

The hot plate test was used to evaluate nociception or sensitivity to a painful stimulus [17]. Mice were placed on a hot plate at 55.0 °C ± 0.3 °C, and the latency to the first hind-paw response was recorded. The hind-paw responses counted were foot shakes or paw licks. A latency period of 30 s was defined as complete analgesia and used as the cut-off time to prevent tissue injuries.

2.4.2. Neuromuscular strength evaluation

Neuromuscular strength was examined using the grip strength test. A grip strength meter was used to assess forelimb grip strength. Mice were lifted and held by the tail such that their forepaws could grasp a wire grid; they were then pulled back gently until they released the grid. The peak force applied by the forelimbs was recorded in Newton (cN).

2.4.3. Cotton bud biting test

Aggressive behavior was examined using the cotton bud biting test based on a previous study [18]. The mice were held in an experimenter's hand, and a sterilized cotton bud was held close to their face. Biting of the cotton bud was considered aggressive behavior. Each mouse was tested 10 times. Analysis was conducted based on the total number of biting attacks.

2.4.4. Elevated plus maze test

Anxiety-like behavior was examined using the elevated plus maze test. The apparatus consisted of two open arms (8 × 25 cm) and two closed arms of the same size with 30-cm-high transparent walls. The arms were constructed using white plastic plates and were elevated to a height of 40 cm above the floor. Arms of the same type were located opposite each other. Each mouse was placed in the central square of the maze, facing one of the closed arms, and was allowed to move freely between the two arms for 10 min. The mice were video recorded and the number of arm entries, distance traveled (m), and time spent in the open arms were analyzed using the ANY-MAZE software.

2.4.5. Open field test

Exploratory behavior, anxiety-like behavior, and general activity were examined using the open field test. In the open field test, each mouse was placed in the center of the apparatus consisting of a square area surrounded by high walls (40 × 40 × 40 cm). The total distance traveled (m) and the time spent in the central area (s) were recorded. The central area was defined as the 20 × 20 cm area located at the center of the field. The test chamber was illuminated at 100 lx. Data were collected over a 30-min period. Data analysis was performed automatically using the ANY-MAZE software.

2.4.6. Y-maze test

Spatial working memory was measured using a Y-maze apparatus (arm length: 40 cm, arm bottom width: 3 cm, arm upper width: 10 cm, height of wall: 12 cm). The mice were placed at the center of the Y-maze field. Visual cues were placed around the maze in the testing room and were constant throughout the testing sessions. Mice were examined with no prior learning. The total distance traveled (m) and the number of entries and alternations were recorded and analyzed automatically using the ANY-MAZE software. Data were collected for 10 min.

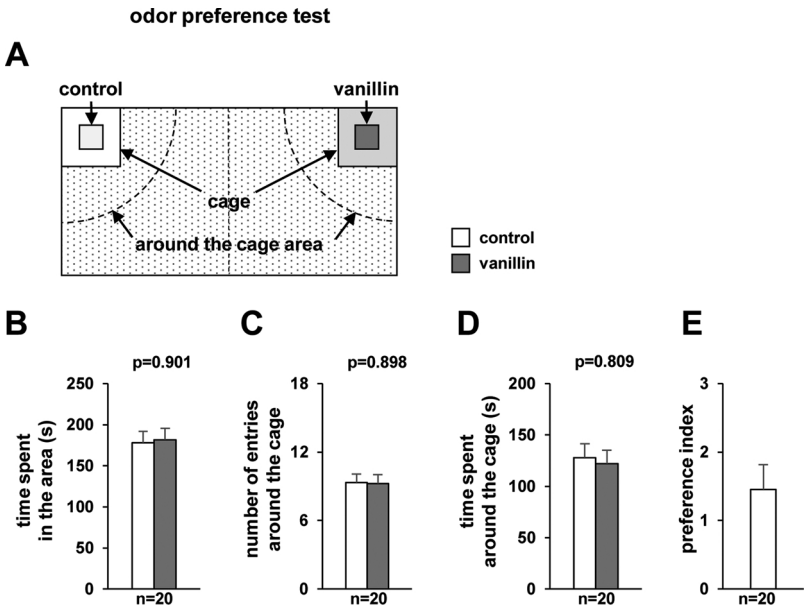

Fig. 1. Vanillin preference in mice.
(A) Schematic diagram of the odor preference test. (B–E) Graphs showing the time spent in each area (B), number of entries around the cage (C), time spent around the cage (D), and 2-PE preference index (E). All data are presented as box plots. *, significant difference compared to control ($p < 0.05$). The p-values in B, C, and D were calculated using the Student's *t*-test. (B–E) n = 20.

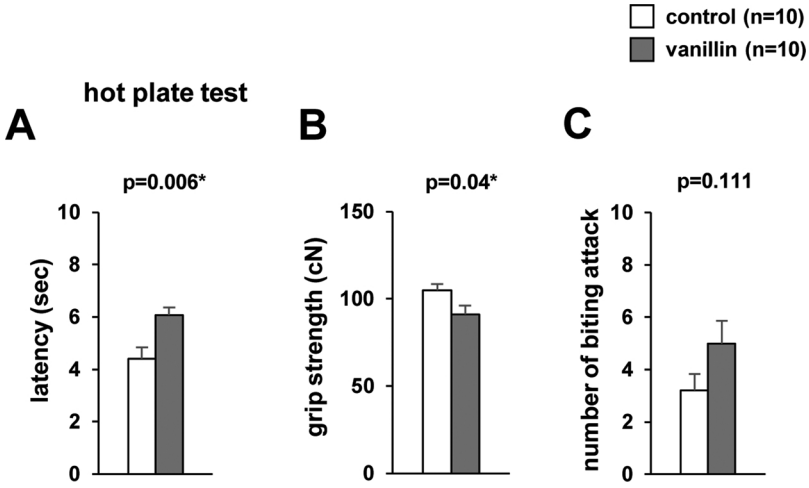

Fig. 2. Effect of vanillin on normal physical characteristics and aggressive behavior.
(A) Hot plate test. (B) Grip strength. (C) Number of times biting on the cotton bud. All data are presented as box plots. *, significant difference compared to control ($p < 0.05$). The p-values were calculated using the Student's *t*-test. (A–C) control: n = 10, vanillin: n = 10.

2.4.7. Tail suspension test

Depressive-like behavior was examined using the tail suspension test and Porsolt forced swim test (PFST). Each mouse was suspended by the tail at 60 cm above the floor in a white plastic chamber using adhesive tape placed < 1 cm from the tip of the tail. The resultant behavior was recorded for 10 min. Images were captured via a video camera, and immobility time was measured. In this test, the 'immobile period' was defined as the period when the animals stopped struggling for ≥1 s. Data acquisition and analysis were performed automatically using ANY-MAZE software.

2.4.8. Porsolt forced swim test

The apparatus for the PFST consisted of four Plexiglas cylinders (20-cm height × 10-cm diameter). The cylinders were filled with water (23 °C) up to a height of 7.5 cm, based on previous studies [19,20]. The mice were placed into the cylinders, and their behavior was recorded over a 10-min test period. As in the tail-suspension test, immobility time was evaluated using the ANY-MAZE software.

2.5. Statistical analyses

Data were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's test and two-way repeated measures ANOVA followed by Fisher's LSD test, Student's *t*-test, or paired *t*-test. Differences with a p-value < 0.05 were regarded as statistically significant. Data are presented as mean ± standard error of the mean (SEM).

3. Results

3.1. Odor preference

We examined whether mice found vanillin attractive or repulsive. No differences were observed in the time spent in the different areas of the apparatus, the number of entries around the cage, or the time spent around the cage (Fig. 1B, $t = 0.123$, $p = 0.901$; Fig. 1C, $t = 0.13$, $p = 0.898$; Fig. 1D, $t = 0.246$, $p = 0.809$). The vanillin preference index was approximately 0.5 (Fig. 1E).

3.2. Effect of vanillin on neuromuscular strength and aggressive behavior

To evaluate the acute phase response rapidly and assess acute, thermal pain in a relatively inexpensive way, we used the hot plate test. Mice were placed on a hot plate to assess nociception. Mice exposed to vanillin had a significantly higher pain threshold than did control mice (Fig. 2A, $t = 3.126$, $p = 0.006$).

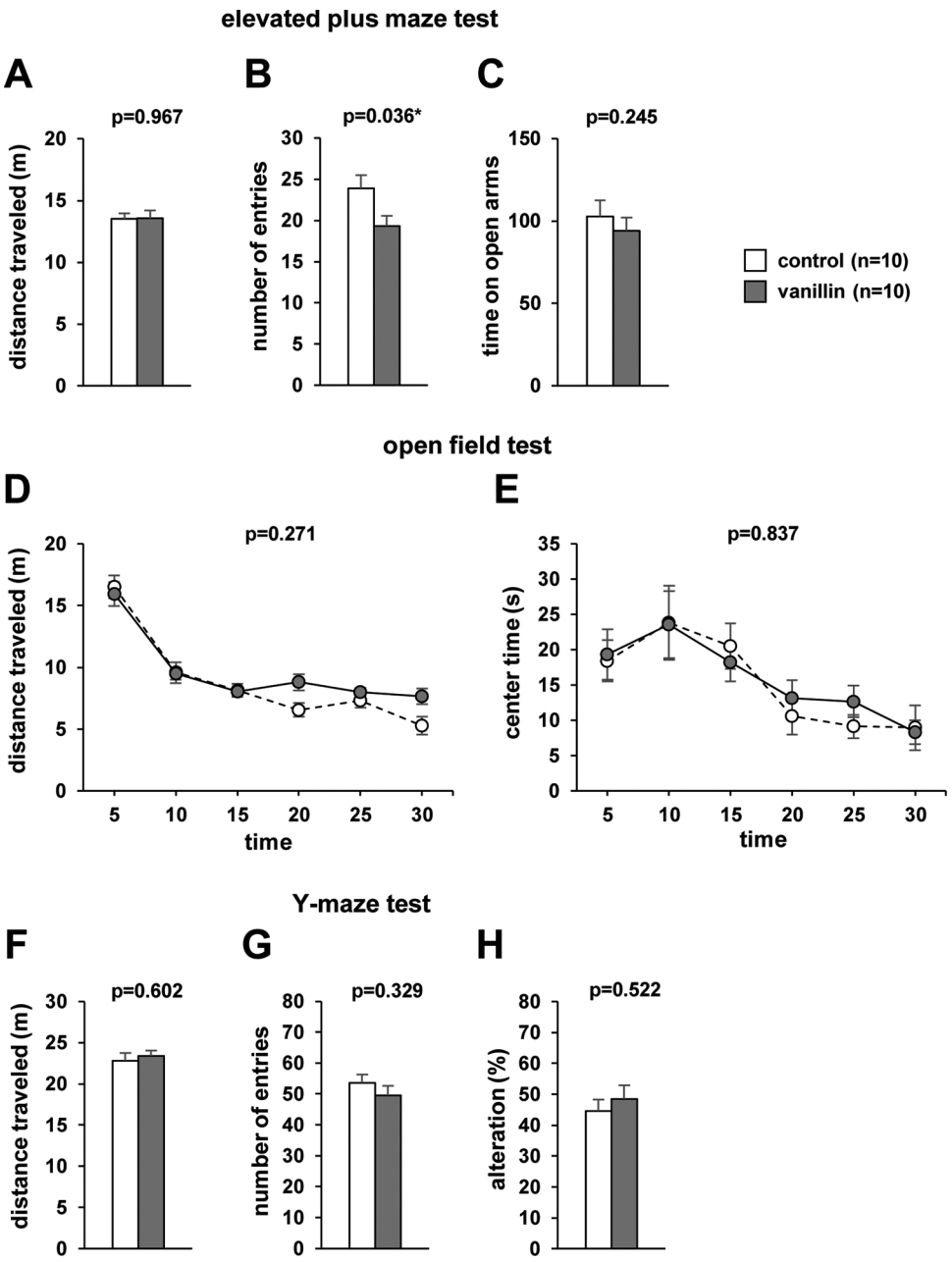

Fig. 3. Effect of vanillin on performance on the elevated plus maze, open field, and Y-maze tests.
(A–C) Elevated plus maze test. Graphs showing the total distance traveled (A), the percentage of open arm entries (B), and the time spent in the open arms (C) in the elevated plus maze test. (D–E) Open field test. Graphs showing the distance traveled (D) and time spent in the central area (E) in each 5 min-period of the open field test. (F–H) Y-maze test. Graphs showing the total distance traveled (F), total number of arm entries (G), and percentage of alternations (H). All data are presented as box plots. *, significant difference compared to controls ($p < 0.05$). The p-values were calculated using the Student's *t*-test in A–C, and F–H and two-way repeated measures analysis of variance in D and E. (A–H) control: n = 10, vanillin: n = 10.

We compared the neuromuscular strength of mice exposed to vanillin and control mice. The grip strength was significantly lower in mice exposed to vanillin (Fig. 2B, $t = 2.217$, $p = 0.04$).

We evaluated the aggressive behavior of mice after vanillin inhalation. There were no significant differences in the number of biting attacks between mice exposed to vanillin and control mice (Fig. 2C, $t = 1.678$, $p = 0.111$).

3.3. Effect of vanillin in the elevated plus maze test

In the elevated plus maze test, we evaluated anxiety-like behavior in mice after vanillin inhalation. No differences were observed in the total distance traveled (Fig. 3A, $t = 0.042$, $p = 0.967$) or the time spent in the open arms (Fig. 3C, $t = 0.706$, $p = 0.245$) between mice exposed to vanillin and control mice. The number of total entries into open arms was significantly lower in mice exposed to vanillin than in control mice (Fig. 3B, $t = 2.266$, $p = 0.036$).

3.4. Effect of vanillin in the open field test

In the open field test, we observed no significant difference in the total distance traveled (Fig. 3D, $F_{1,18} = 1.292$, $p = 0.271$) or the time spent in the central area (Fig. 3E, $F_{1,18} = 0.043$, $p = 0.837$) between the two groups.

3.5. Effect of vanillin in the Y-maze test

We examined the effect of vanillin on short-term spatial working memory by monitoring spontaneous alternation behavior in the Y-maze test. There were no significant differences between the groups in the total distance traveled (Fig. 3F, $t = 0.53$, $p = 0.602$), number of arm entries (Fig. 3G, $t = 1.0$, $p = 0.329$), or alternation percentage (Fig. 3H, $t = 0.653$, $p = 0.522$).

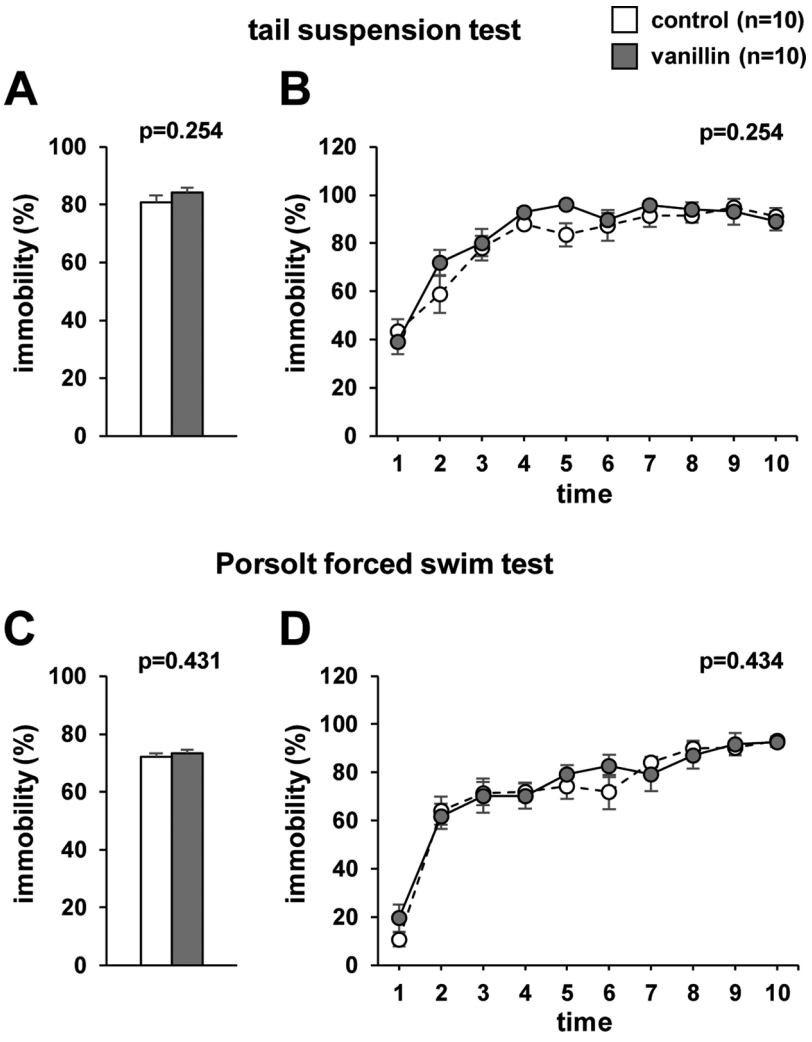

Fig. 4. Effect of vanillin on depressive-like behavior.
Graphs showing the proportion of total time spent immobile (A) and the proportion of time spent immobile in each 1-min period (B) in the tail suspension test. Graphs showing the proportion of total time spent immobile (C) and the proportion of time spent immobile in each 1-min period at day 1 (D) and in the Porsolt forced swim test. All data are presented as box plots. *, significant difference compared to controls ($p < 0.05$). The p-values were calculated using the Student's *t*-test in A and C, and two-way repeated measures analysis of variance in B and D. (A–D) control: n = 10, vanillin: n = 10.

3.6. Effect of vanillin on depressive-like behavior in mice

We evaluated depressive-like behavior in mice after vanillin inhalation. In the tail-suspension test, we found no significant differences between groups with regard to the percentage of time spent immobile (Fig. 4A, $t = 1.18$, $p = 0.254$; Fig. 4B, $F_{1,18} = 1.386$, $p = 0.254$). In the PFST, no significant differences were observed between the groups (Fig. 4C, $t = 0.806$, $p = 0.431$; Fig. 4D, $F_{1,18} = 0.641$, $p = 0.434$).

4. Discussion

In this study, we showed that the inhalation of vanillin has anti-nociceptive and muscle relaxant effects in mice. The mice that inhaled vanillin did not show significant changes compared with control mice in the other behavioral tests.

Vanillin is regarded as an attractive fragrance for people and is considered as an indispensable material for foods and perfumes. In fact, vanillin exposure is considered to be generally comfortable for humans and is said to induce a positive mood [21]. However, it is difficult to examine the physiological actions of vanillin in humans. In the present study, mice showed behavior that indicated that vanillin was neither attractive nor repellent. However, since we performed the odor preference test in an open area, it is possible that the odor of vanillin had diffused through the whole apparatus, and thus, the mice did not show a positive result for vanillin. However, it has been previously shown that mice positively approach fragrant objects [22]. In previous studies, it has been reported that mice exhibit neutral behavior against vanillin [23]. This is in line with our results that show that vanillin does not induce anxiety or aggression in mice.

Mice that had inhaled vanillin showed anti-nociceptive effects in the hot plate test. Previous studies have shown that oral administration of vanillin to mice has an anti-nociceptive effect in the acetic acid writhing test [11,24]. Furthermore, oral administration of vanillin to rats has been reported to reduce allodynia induced by chronic constriction of the sciatic nerve [25]. These reports are consistent with the results of this study, which show that vanillin has an anti-nociceptive effect.

Vanillin is the only trioxide essential oil component for which anti-nociceptive activity has been reported [11,26]. Vanillin contains three highly reactive functional groups in its structure: aldehydes, phenols, and ethers [27]. Vanillin has numerous structural similarities with other compounds like eugenol, zingerone, and capsaicin and can potentially exhibit similar pharmacological activity via the vanilloid receptor. A limited number of natural substances exhibit agonist/antagonist activity towards the vanilloid receptor. The structural features

of these molecules include a vanillyl (3-methoxy-4-hydroxyphenyl) group. Many vanilloid substances, such as capsaicin and resinifera toxin, have analgesic activity by agonistically desensitizing transient receptor potential-vanilloid 1 (TRPV1) channels found in dorsal roots and trigeminal ganglia [28–30]. Furthermore, vanillin has also been reported to exhibit potential central anti-nociceptive effects mediated through opioid receptors [31]. This anti-nociceptive action of vanillin may be mediated by the α_2-adrenoceptor and the opioid receptor, but not by the α_1-adrenoceptor and the serotonin receptor [11].

It is reported that eugenol containing a vanillyl group shows an appetite-enhancing effect like vanillin [32]. Moreover, eugenol is used as a dental analgesic [33,34], while the administration of eugenol to mice has a muscle relaxant effect [35]. As described above, vanillin may bind to the vanilloid receptor in the same manner as eugenol and thus produce an analgesic effect and a muscle relaxation effect. The muscle relaxing action of vanillin has not been reported so far. However, it has been reported that vanillin promotes arterial relaxation by blocking Ca_2 channels [36]. Vanillin also acts on ileal smooth muscle calcium channels and prevents acetylcholine-induced contraction via non-competitive inhibitory kinetics [37]. Vanillin may promote its muscle-relaxing effect by altering tissue blood flow by intramuscular vasodilation [38].

Although the mechanism of action of inhaled vanillin is unknown, it is presumed to have a similar mechanism of action to that of orally administered vanillin. However, further studies are needed to elucidate the analgesic mechanism of action of inhaled vanillin.

In this study, we showed that the anti-nociceptive effect of vanillin can be demonstrated by the inhalation of vanillin in mice. Linalool, one of the major odor components of lavender extract, is known to reduce the pain response when injected subcutaneously [39,40]. In recent years, however, inhaled linalool was also shown to alleviate pain [41]. The analgesic effects of the inhalation of fragrance molecules have not been sufficiently investigated. More importantly, it is not clear how scent molecules act on nerves following inhalation. One likely hypothesis is that the volatile compound acts by entering the bloodstream via the nasal or pulmonary mucosa. The skin permeability of a drug has a high correlation with its lipid solubility or lipophilicity [42]. Drugs with molecular weights up to 100 are easily absorbed from the nasal mucosa [43]. Since vanillin is a low-molecular-weight molecule and is a lipophilic chemical substance, it is thought to be easily absorbed from the nasal mucosa. This chemical is expected to have good permeability in brain tissue.

In this study, we used an significantly higher vanillin concentration than that used in foods. It is not clear whether a pharmacological effect is exhibited in humans due to the ingestion of vanillin from foods and cosmetics. Further studies are needed to investigate whether the effect of vanillin is dependent on the concentration of vanillin in the air. It is also necessary to investigate the concentration threshold at which vanillin exhibits its physiological actions.

This study showed that the inhalation of vanillin increased anxiety-like behavior in mice in the elevated plus maze test. However, in the open field test, no change was observed between mice inhaling vanillin and control mice with regard to anxiety-like behavior. Therefore, the vanillin-induced anxiety results were not consistent between the open field and elevated plus maze test. This difference is presumed to be due to anxiety about wide spaces and heights [44]. The influence of vanillin on anxiety-like behavior is unknown, but the mice may feel anxious owing to the muscle relaxation effect of vanillin. Administration of a muscle relaxant to mice reduces the number of entries into the open arm of the elevated plus maze test [45]. In addition, compounds that exhibit anti-anxiety effects may have side-effects such as insomnia and muscle relaxation [46]. Further studies are necessary to clarify the influence of vanillin on anxiety-like behavior. The relationship between the analgesic activity and muscle relaxation activity of vanillin has not been reported so far. However, most of the centrally acting analgesics have central nervous system depressant effects, and thus affect locomotor activity by reducing motor activity [47]. Locomotor activity is considered an indicator of arousal or alertness of mental activity, and a decrease in activity is an indicator of calmness and sedation as a result of reduced excitability of the central nervous system [48].

The rate of vanillin metabolism in the live body is unknown. Therefore, even in this study, there is a possibility that vanillin remained in the body in the days following the day of administration. As humans frequently inhale vanillin in daily life, further studies to investigate the pharmacokinetics of vanillin in the body are also necessary.

No significant differences were found between the mice inhaling vanillin and control mice in the open field, Y-maze, tail suspension, and forced swimming tests. It has been reported that vanillin may attenuate cognitive decline in a mouse model of cognitive dysfunction [13]. However, it has not been reported whether vanillin can improve the cognitive function in naïve mice. On the other hand, vanillin has been reported to have an antidepressant effect [49]. The difference between the previous report and this study may be due to the administration method and dose. Considering that there are few articles on clinical trials and animal experiments related to the effects of vanillin, the results of this study are believed to be reasonable.

This study is the first to report the effect of vanillin inhalation on behavior in mice. It is also the first to report the anti-nociceptive effect of vanillin inhalation. Our findings demonstrate a scientific basis for the effect of vanillin, which complements previous studies on vanillin aromatherapy.

5. Conclusions

Inhalation of vanillin has anti-nociceptive and muscle relaxant effects and may increase anxiety-like behavior in mice. However, the inhalation of vanillin had no effect on mouse activity or cognitive function. This study provides a scientific basis for the neuropsychological effects of essential oils containing vanillin. Inhalation of essential oils containing vanillin may lead to effective sedation.

Author contributions

All authors had full access to all study data and take full responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: H.U., A.S., and M.O. Data acquisition: H.U., S.S., Y.T., and Y.F. Data analysis and interpretation: H.U., A.S., and S.S. Drafting of the manuscript: H.U., A.S., Y.T., and M.O. Critical revision of the manuscript for important intellectual content: A.S., S.M., N.K., K.W., Y.T., Y.M., M.O., and T.I. Statistical analyses: H.U and S.S. Study supervision: M.O. and T.I.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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