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Original article

# Behavioural effects of inhalation exposure to dizocilpine (MK-801) in mice

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ABSTRACT

The complex pathophysiology of brain disorders and the difficulty of delivering therapeutic agents to the brain remain major obstacles in the research and development of new therapeutic methods for brain disorders. Therefore, delivering existing therapeutic agents to the central nervous system is expected to provide benefits in various diseases. In this study, we investigated whether inhaled central nervous system drugs reached the brain and affected mouse behaviour. Dizocilpine (MK-801), which increases locomotor activity in mice, was mainly used to study this hypothesis. First, we administered MK-801, an *N*-methyl-D-aspartate receptor antagonist, to mice via inhalation and examined whether it induced excessive activity similar to that observed after intraperitoneal administration. We also examined the time- and dose-dependency of drug induced changes in mouse behaviour after MK-801 inhalation. Next, we investigated whether inhalation of scopolamine, pentobarbital, and imipramine also affected mouse behaviour. Mice that inhaled MK-801 showed MK-801–induced hyperactivity similar to that observed following intraperitoneal administration. Furthermore, the extent of activity changed in a time- and dose-dependent manner after MK-801 inhalation. Inhalation of pentobarbital, scopolamine, and imipramine also changed mouse behaviour. These results demonstrate that inhalation of MK-801 exerts effects similar to those achieved with intraperitoneal and oral administration in mice. Thus, central nervous system agonists can reach the brain efficiently via inhalation. This finding may facilitate the development of improved therapies for brain disorders.

## 1. Introduction

Disorders of the central nervous system (CNS) represent a substantial emotional, economic, and social burden for patients, their families, and society. Despite intensive research efforts, there remains a great unmet need for therapeutic agents in many such disorders. The major obstacles in research and development of new therapeutic agents for the brain are related to the complex pathophysiology of brain damage, the difficulty of brain entry for small and large drugs, and the risk, complexity, and cost of the clinical trials required for regulatory approval [1]. Therefore, delivery of existing therapeutic agents to the CNS is expected be more beneficial for various CNS disorders [2].

Under normal circumstances, the blood-brain barrier (BBB) plays an important role in protecting the delicate environment of the brain. However, during attempts to introduce exogenous therapeutic agents into the CNS, the BBB prevents 98% of small and large molecules from reaching their intended targets. This lack of access to the brain is a major barrier to drug development for CNS disorders [3]. Even if some drugs can penetrate the BBB, additional challenges remain. Many drugs undergo degradation in the gastrointestinal tract and metabolisation in the liver limit the levels of numerous drugs in the blood. Increasing oral doses to compensate for limited brain access can cause unacceptable gastrointestinal or systemic adverse events. Although injections may appear to be an obvious alternative route for introducing drugs into the blood, they can cause pain and scar tissue in cases requiring frequent dosing and needle phobia in children. Thus, injections remain an

*Abbreviations:* ANOVA, analysis of variance; BBB, blood-brain barrier; CNS, central nervous system; i.p., intraperitoneal; NIH, National Institutes of Health; NMDA, *N*-methyl-D-aspartate

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impractical and unattractive delivery route in many respects. Therefore, the demand for needle-free drug delivery technology is increasing.

One novel approach to addressing this problem is to non-invasively bypass the BBB. Intranasal administration allows drugs to bypass systemic circulation and has the potential to deliver drugs directly to the brain [4,5]. Direct delivery in this manner reduces the potential for side effects and can increase the efficacy of neurotherapeutics [6]. Thus, nasal drug delivery is currently recognised as a very promising route for the delivery of therapeutic compounds, including biopharmaceuticals.

The existing methods for nasal administration require the use of a dedicated device to administer the liquid containing the drug [7,8]. In this study, we examined whether it is possible to deliver a drug to the brain in a manner similar to inhalation of essential oils in aromatherapy without using specialised equipment. Inhalation is the most common method of using substances and is the shortest path to the brain, with almost instantaneous effects. Smoking is a good example of the effects of inhalation. The smoke enters the lungs where it is immediately absorbed into the bloodstream and travels to the brain. Because breathing is an intrinsic function, inhaled delivery of drugs offers an intriguing advantage over intravenous injection. In healthy adults, more than 12,000 L of air pass through the nose daily [9]. In fact, inhalation is also the classic method for administering both therapeutic agents and toxic narcotics [10]. However, despite the perceived usefulness of rapid-acting delivery, delivery by inhalation is limited to pulmonary delivery for the treatment of lung-related diseases such as asthma, chronic obstructive pulmonary disease, cystic fibrosis, and respiratory infections.

Dizocilpine (MK-801) is a non-competitive *N*-methyl-D-aspartate (NMDA) receptor antagonist with a low molecular weight, and blockade of glutamatergic transmission by MK-801 induces robust and dose-dependent increases in locomotor activity [11]. Intraperitoneal administration of MK-801 causes schizophrenia-like behavioural and metabolic changes in the animal's brain [12,13]. Therefore, intraperitoneal administration of MK-801 has been used in many experiments to produce an animal model of schizophrenia [14–17]. However, none of the previous studies have investigated whether inhaled MK-801 reaches the brain and exerts its effects.

We started our experiments by using MK-801 to confirm the effects of inhalation of locomotor stimulant. In this study, we used a nebuliser for inhalational administration of MK-801 to the mice. Inhalation using a nebuliser has long been used to deliver therapeutic agents to the airways and lungs. The nebulisation process is identical to the process by which essential oils are converted into fine air particles and inhaled in aromatherapy. Inhalation of essential oils is said to stimulate the brain to transmit signals through the olfactory system and release neurotransmitters (e.g. serotonin, noradrenaline, dopamine, or endocannabinoids) that modulate nociceptive transmission. However, studies on the cellular and molecular mechanisms underlying these effects are lacking. It is expected that this research will also provide an opportunity to elucidate the mechanism of action of essential oils by inhalation.

The purpose of this study was to clarify whether administration of the CNS agonist MK-801 via inhalation affects the CNS in mice and influences mouse behaviour in the same way as other administration methods. The results of this study will indicate the potential of inhalation as a new method for CNS agonists to access the brain and elucidate the mechanism by which nasally inhaled molecules affect the CNS.

## 2. Materials and methods

### 2.1. Animals

We used 15-week-old male mice (C57BL/6) for the experiments. 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-h light/dark cycle at 23 °C – 26 °C. We made every effort to minimise the number of animals used and promote their comfort and well-being. The 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 the Kawasaki Medical School's Advanced Research Center. Each animal was subjected to experimental manipulations only once (n = 7–10 animals per group).

### 2.2. Reagents

(+)-MK-801 (130-17381, FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) was diluted in saline to a concentration of 0.1 mg/mL and administered intraperitoneally at a dose of 0.2 mg/kg. This dose was selected on the basis of previous studies showing schizophrenia-like behaviours in mice following intraperitoneal injections of MK-801 at 0.2 mg/kg [18–20]. Pentobarbital (Somnopentyl, Kyoritsu Seiyaku, Tokyo, Japan) was diluted in saline to a concentration of 15 mg/mL and administered intraperitoneally at a dose of 30 mg/kg. Scopolamine (S0021, Tokyo Chemical Industry Company, Tokyo, Japan) was diluted in saline to a concentration of 1 mg/mL and administered intraperitoneally at a dose of 2 mg/kg. Imipramine (097-06491, FUJIFILM Wako Pure Chemical Corporation) was diluted in saline to a concentration of 5 mg/mL and administered intraperitoneally at a dose of 20 mg/kg. Although these drugs are not structural analogues, they all act on the CNS. However, no previous study has investigated whether these drugs can reach the brain and exert their effects after inhalational administration.

### 2.3. Inhalation of reagents

The inhalation apparatus was similar to that used in a previous study [21]. Mice were exposed to each reagent with a mesh nebuliser (NEB-01, CUSTOM Corporation, Tokyo, Japan). Inhalation of the reagent was carried out in a sealed container. The nebuliser was placed on a stainless steel wire lid on a new breeding cage (235 mm × 325 mm × 170 mm) surrounded by an outer container made with parts from larger cages (292 mm × 440 mm × 200 mm) (Fig. 1A). The mice were unable to lick the reagents. Approximately 5 min after nebuliser placement, the mice were placed in the internal cage for 30 min.

### 2.4. Behavioural tests

All behavioural experiments were performed during the light phase (9:00–16:00). We tested the mice in random order. After testing, the apparatus was cleaned with 70% ethanol and with water containing super-oxidised hypochlorous acid to prevent any bias due to olfactory cues.

### 2.5. Locomotor activity test

For measurements of locomotor activity, the mice were acclimated to the single housing environment. Locomotor activity data were measured using a photobeam activity system (ACTIMO-100, BRC Company, Nagoya, Japan), and activity counts were recorded at 10-min intervals.

### 2.6. Effects of inhaled MK-801 on mouse locomotor activity

The mice were randomly divided into three groups (n = 10 for each group) and treated with MK-801 at 0.2 mg/kg (intraperitoneal route), 0.1 mg/mL of MK-801 (inhalation), or saline (inhalation) 30 min before the behavioural test. Before measurement of locomotor activity, the mice were acclimated to the single housing environment for 120 min.

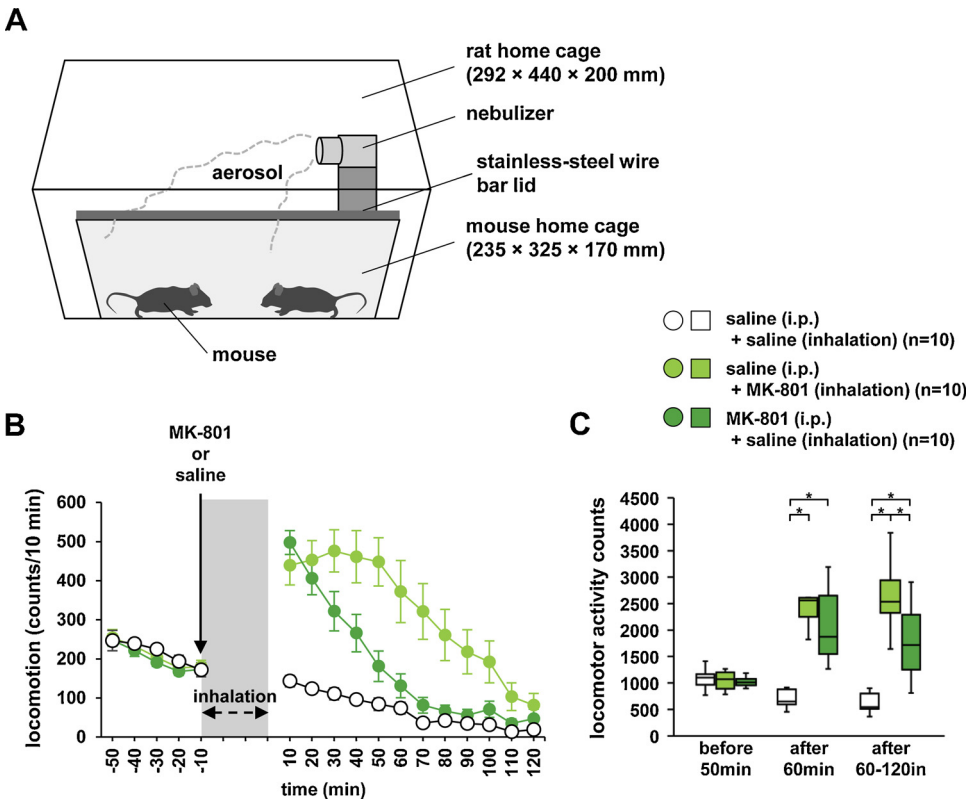


**Fig. 1.** Inhalation apparatus and the MK-801–induced effects on locomotion.
(A) The apparatus consisted of a mouse home cage and an outer container consisting of two rat cage bases forming a shell. The nebuliser was placed on a stainless steel wire bar lid on the home cage. The mouse home cage was placed inside two larger rat cages. (B) Spontaneous locomotor activity in each 10-min period. After 60 min, animals were injected with MK-801 or saline and inhaled MK-801 or saline. Next, locomotor activity was assessed for 120 min. (C) Number of times a photobeam was broken during a 50-min period before the administration of MK-801 or saline and during the two consecutive 60-min periods immediately after the administration of MK-801 or saline. The "saline (i.p.) + saline (inhalation)" group was treated with saline via the intraperitoneal and inhalation routes. The "saline (i.p.) + MK-801 (inhalation)" group was treated with saline via intraperitoneal administration and with MK-801 via inhalation. The "MK-801 (i.p.) + saline (inhalation)" group was treated with MK-801 via intraperitoneal administration and with saline via inhalation. Data are presented as means ± standard errors in B and in box plots in C. *, significant difference between groups ($p < 0.05$). The p values were calculated using two-way repeated-measures analysis of variance in B and two-way analysis of variance in C. Abbreviation: i.p., intraperitoneal

### 2.7. Effects of MK-801 on mouse locomotor activity following administration via different routes

The mice were randomly divided into four groups and treated with 0.4 mg/kg of MK-801 (oral; n = 9), 0.1 mg/mL of MK-801 (inhalation; n = 6), 0.2 mg/kg of MK-801 (intraperitoneal route; n = 9), or saline (inhalation; n = 7) at the start of the behavioural test. Before measurement of locomotor activity, the mice were acclimated to the single housing environment for 180 min.

### 2.8. Time-dependent effect of MK-801 inhalation on mouse locomotor activity

The mice were randomly divided into four groups and inhaled 0.1 mg/mL of MK-801 for 0 min (n = 7), 10 min (n = 10), 30 min (n = 10), or 60 min (n = 10) before the behavioural test. Before measurement of locomotor activity, the mice were acclimated to the single housing environment for 180 min.

### 2.9. Dose-dependent effect of MK-801 inhalation on mouse locomotor activity

The mice were randomly divided into four groups and inhaled MK-801 at 0.05 mg/mL (n = 8), 0.10 mg/mL (n = 8), or 0.30 mg/kg (n = 8) or saline (n = 8) 30 min before the behavioural test. Before measurement of locomotor activity, the mice were acclimated to the single housing environment for 210 min.

### 2.10. Effects of pentobarbital inhalation on mouse locomotor activity

The mice were randomly divided into three groups (n = 10 for each group) and treated with 30 mg/kg of pentobarbital (intraperitoneal route), 15 mg/mL of pentobarbital (inhalation), or saline (inhalation) 30 min before the behavioural test. Before measurement of locomotor activity, the mice were acclimated to the single housing environment for 120 min.

### 2.11. Effects of scopolamine inhalation on mouse locomotor activity

The mice were randomly divided into three groups (n = 8 for each group) and treated with 2 mg/kg of scopolamine (intraperitoneal route), 1 mg/mL of scopolamine (inhalation), or saline (inhalation) 30 min before the behavioural test. Before measurement of locomotor activity, the mice were acclimated to the single housing environment for 120 min.

### 2.12. Effects of imipramine inhalation on mouse depressive-like behaviour

The mice were randomly divided into three groups (n = 10 for each group) and treated with 20 mg/kg of imipramine (intraperitoneal route), 5 mg/mL of imipramine (inhalation), or saline (inhalation) 30 min before the behavioural test. Each mouse was suspended by the tail 60 cm above the floor in a white plastic chamber by using adhesive tape placed < 1 cm from the tip of the tail. Its behaviour was recorded for 6 min. Images were captured via a video camera, and immobility time was measured. In this test, immobility time was defined as the total time during which the animals stopped struggling for ≥1 s. Data acquisition and analysis were performed automatically by using video tracking software (ANY-MAZE, Stoelting Company, Wood Dale, IL, USA).

### 2.13. Statistical analyses of behavioural test results

Data were analysed with two-way analysis of variance (ANOVA) followed by Tukey's test or with two-way repeated-measures ANOVA followed by Fisher's least significant difference test. A p value of $< 0.05$ was regarded as statistically significant. Data are presented as means ± standard errors or with box plots.

**Table 1**
p values for Fig. 1.

| **A:** Fig. 1**B** | | | p value |
|---|---|---|---|
| saline (i.p.) + saline (inhalation) | vs | saline (i.p.) + MK-801 (inhalation) | 0.016 |
| saline (i.p.) + saline (inhalation) | vs | MK-801 (i.p.) + saline (inhalation) | < 0.001 |
| saline (i.p.) + MK-801 (inhalation) | vs | MK-801 (i.p.) + saline (inhalation) | 0.062 |

| **B:** Fig. 1**C** | | | p value |
|---|---|---|---|
| before 50 min | | | |
| saline (i.p.) + saline (inhalation) | vs | saline (i.p.) + MK-801 (inhalation) | 0.918 |
| saline (i.p.) + saline (inhalation) | vs | MK-801 (i.p.) + saline (inhalation) | 0.588 |
| saline (i.p.) + MK-801 (inhalation) | vs | MK-801 (i.p.) + saline (inhalation) | 0.822 |
| after 60 min | | | |
| saline (i.p.) + saline (inhalation) | vs | saline (i.p.) + MK-801 (inhalation) | < 0.001 |
| saline (i.p.) + saline (inhalation) | vs | MK-801 (i.p.) + saline (inhalation) | < 0.001 |
| saline (i.p.) + MK-801 (inhalation) | vs | MK-801 (i.p.) + saline (inhalation) | 0.259 |
| after 60-120 min | | | |
| saline (i.p.) + saline (inhalation) | vs | saline (i.p.) + MK-801 (inhalation) | < 0.001 |
| saline (i.p.) + saline (inhalation) | vs | MK-801 (i.p.) + saline (inhalation) | 0.005 |
| saline (i.p.) + MK-801 (inhalation) | vs | MK-801 (i.p.) + saline (inhalation) | 0.046 |

## 3. Results

### 3.1. Locomotor activity in mice after MK-801 inhalation

First, we tested the effects of MK-801 inhalation on mouse locomotor activity in comparison with the effects after intraperitoneal administration of MK-801. Intraperitoneal injection of MK-801 resulted in a robust increase in locomotor activity, which lasted for a further 120 min (Fig. 1B, C Table 1). Inhalation of MK-801 also markedly increased locomotor activity in mice (Fig. 1B). The increased basal activity lasted for an additional 120 min (Fig. 1B, C). Locomotor activity counts for the last 60-min period after administration of MK-801 were significantly higher in mice that inhaled MK-801 than in mice that received MK-801 injections (Fig. 1B, C).

### 3.2. Effects of MK-801 on locomotor activity in mice following administration via different routes

We examined whether MK-801 administration affects locomotor activity in an administration route–dependent manner. A single dose of MK-801 administered orally to normal mice rapidly increased the locomotor activity, and the increase lasted for a further 120 min (Fig. 2A, B Table 2A, B). Inhalation of MK-801 induced elevated levels of locomotor activity in mice. MK-801 gradually increased locomotor activity 30 min after the start of inhalation (Fig. 2A, B). MK-801 administered intraperitonially increased locomotor activity in mice, and the increase lasted for a further 120 min (Fig. 2A, B). Locomotor activity was significantly higher in mice that inhaled MK-801 than in mice that received MK-801 orally or intraperitonially or that received saline (Fig. 2A, B).

### 3.3. Inhalation time–dependency of the locomotor effects of MK-801 in mice

We examined whether MK-801 inhalation affects locomotor activity in an inhalation time–dependent manner. The examined MK-801 inhalation times ranged from 10 to 60 min. Inhalation of MK-801 for 30 or 60 min induced elevated levels of locomotor activity in mice (Fig. 3A, B Table 2C, D). Inhalation of MK-801 for 60 min markedly increased the locomotor activity counts in comparison with mice that inhaled MK-801 for 30 min. We observed no significant difference in

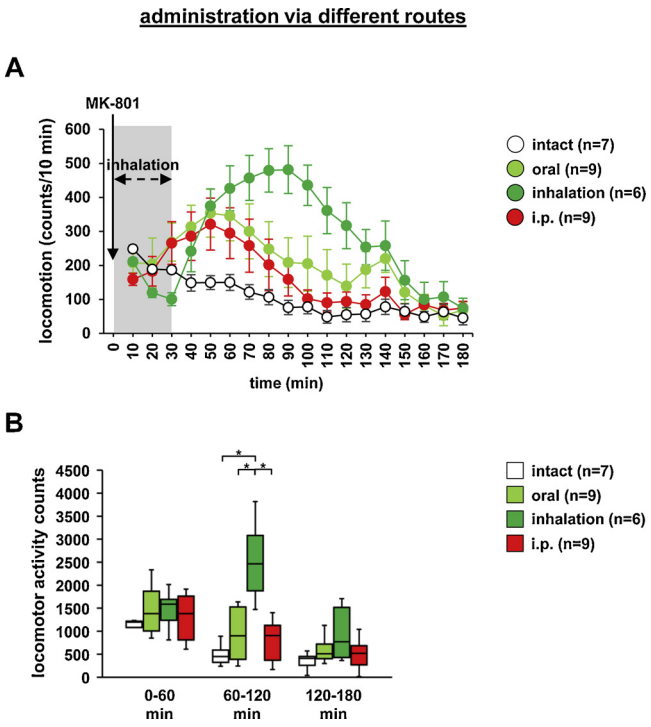


**Fig. 2.** Effects of MK-801 on locomotor activity following administration via different routes.
(A) Spontaneous locomotor activity was assessed in each 10-min period following MK-801 administration via different routes (oral, inhaled, and intraperitoneal administration of MK-801 and inhalation of saline) for 180 min in the locomotor activity test. (B) Number of times a photobeam was broken during three consecutive 60-min periods immediately after the administration of MK-801 or saline. *, significant differences among groups ($p < 0.05$). The p values were calculated by using two-way repeated-measures analysis of variance in A and two-way analysis of variance in B. Abbreviation: i.p., intraperitoneal

**Table 2**
p values for Fig. 2, 3 and 4.

| **A:** Fig. 2**A** | | | p value | **C:** Fig. 3**A** | | | p value | **E:** Fig. 4**A** | | | p value |
|---|---|---|---|---|---|---|---|---|---|---|---|
| intact | vs | oral | 1 | control | vs | 10 min | 1 | saline | vs | 0.05 mg/ml | 1 |
| intact | vs | inhalation | 1 | control | vs | 30 min | 0.228 | saline | vs | 0.10 mg/ml | 0.02 |
| intact | vs | i.p. | 0.398 | control | vs | 60 min | < 0.001 | saline | vs | 0.30 mg/ml | < 0.001 |
| oral | vs | inhalation | 0.315 | 10 min | vs | 30 min | 0.044 | 0.05 mg/ml | vs | 0.10 mg/ml | 0.082 |
| oral | vs | i.p. | 1 | 10 min | vs | 60 min | < 0.001 | 0.05 mg/ml | vs | 0.30 mg/ml | < 0.001 |
| inhalation | vs | i.p. | 0.04 | 30 min | vs | 60 min | 0.003 | 0.10 mg/ml | vs | 0.30 mg/ml | < 0.001 |
| **B:** Fig. 2**B** | | | p value | **D:** Fig. 3**B** | | | p value | **F:** Fig. 4**B** | | | p value |
| 0-60 min | | | | 0-60 min | | | | 0-60 min | | | |
| intact | vs | oral | 0.363 | control | vs | 10 min | 0.938 | saline | vs | 0.05 mg/ml | 0.884 |
| intact | vs | inhalation | 0.77 | control | vs | 30 min | 0.007 | saline | vs | 0.10 mg/ml | 0.006 |
| intact | vs | i.p. | 0.652 | control | vs | 60 min | < 0.001 | saline | vs | 0.30 mg/ml | 0.023 |
| oral | vs | inhalation | 0.948 | 10 min | vs | 30 min | 0.001 | 0.05 mg/ml | vs | 0.10 mg/ml | 0.037 |
| oral | vs | i.p. | 0.957 | 10 min | vs | 60 min | < 0.001 | 0.05 mg/ml | vs | 0.30 mg/ml | 0.113 |
| inhalation | vs | i.p. | 1 | 30 min | vs | 60 min | < 0.001 | 0.10 mg/ml | vs | 0.30 mg/ml | 0.954 |
| 60-120 min | | | | 60-120 min | | | | 60-120 min | | | |
| intact | vs | oral | 0.311 | control | vs | 10 min | 1 | saline | vs | 0.05 mg/ml | 1 |
| intact | vs | inhalation | 0.002 | control | vs | 30 min | 0.263 | saline | vs | 0.10 mg/ml | 0.679 |
| intact | vs | i.p. | 0.784 | control | vs | 60 min | < 0.001 | saline | vs | 0.30 mg/ml | < 0.001 |
| oral | vs | inhalation | 0.065 | 10 min | vs | 30 min | 0.176 | 0.05 mg/ml | vs | 0.10 mg/ml | 0.704 |
| oral | vs | i.p. | 0.83 | 10 min | vs | 60 min | < 0.001 | 0.05 mg/ml | vs | 0.30 mg/ml | < 0.001 |
| inhalation | vs | i.p. | 0.011 | 30 min | vs | 60 min | < 0.001 | 0.10 mg/ml | vs | 0.30 mg/ml | < 0.001 |
| 120-180 min | | | | 120-180 min | | | | 120-180 min | | | |
| intact | vs | oral | 0.336 | control | vs | 10 min | 0.999 | saline | vs | 0.05 mg/ml | 1 |
| intact | vs | inhalation | 0.092 | control | vs | 30 min | 0.727 | saline | vs | 0.10 mg/ml | 1 |
| intact | vs | i.p. | 0.926 | control | vs | 60 min | 0.615 | saline | vs | 0.30 mg/ml | < 0.001 |
| oral | vs | inhalation | 0.793 | 10 min | vs | 30 min | 0.63 | 0.05 mg/ml | vs | 0.10 mg/ml | 1 |
| oral | vs | i.p. | 0.674 | 10 min | vs | 60 min | 0.48 | 0.05 mg/ml | vs | 0.30 mg/ml | < 0.001 |
| inhalation | vs | i.p. | 0.237 | 30 min | vs | 60 min | 0.995 | 0.10 mg/ml | vs | 0.30 mg/ml | < 0.001 |

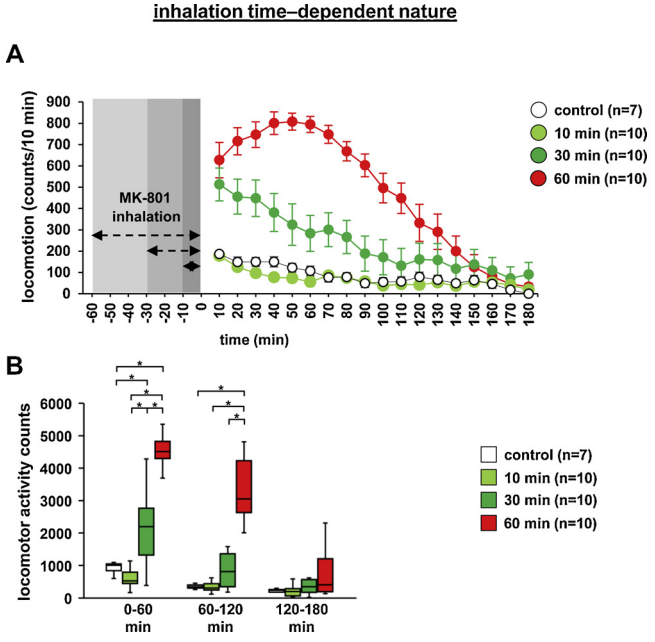


**Fig. 3.** Inhalation time–dependent nature of MK-801–induced locomotion.
(A) Spontaneous locomotor activity was assessed in each 10-min period following different durations of MK-801 inhalation (0, 10, 30, and 60 min) for 180 min in the locomotor activity test. (B) Number of times a photobeam was broken during three consecutive 60-min periods immediately after the administration of MK-801. *, significant differences among groups ($p < 0.05$). The p values were calculated using two-way repeated-measures analysis of variance in **A** and two-way analysis of variance in B.

the locomotor activity counts between mice exposed to MK-801 for 10 min and mice that were not exposed to MK-801 (Fig. 3A, B).

### *3.4. Inhalation dose–dependency of the locomotor effects of MK-801 in mice*

We examined whether MK-801 inhalation affects locomotor activity in a dose-dependent manner. The examined MK-801 doses ranged from 0.05 to 0.30 mg/kg. Mice that inhaled MK-801 at 0.05 mg/kg did not show increased locomotor activity (Fig. 4A, B Table 2E, F). Inhaling MK-801 at 10 mg/kg resulted in an increase in locomotor activity, which lasted for a further 60 min (Fig. 4A, B). Mice that inhaled MK-801 at 0.30 mg/kg showed increased locomotor activity, which lasted for a further 210 min (Fig. 4A, B).

### *3.5. Inhalation effects of pentobarbital or scopolamine on locomotor activity in mice*

Next, we examined how the choice of inhalational or intraperitoneal administration affected the influence of scopolamine and pentobarbital on locomotor activity in mice. A previous study indicated that sub-hypnotic doses of pentobarbital significantly increased locomotor activity measured for 30 min in mice [22]. Administration of scopolamine has also been shown to markedly increase locomotor activity in mice [23,24]. Intraperitoneal pentobarbital injections resulted in robust decreases in locomotor activity that were consistent with a sub-hypnotic effect (Fig. 5A**, B** Table 3A, B). Inhalation of pentobarbital produced a marked increase in locomotor activity that persisted for 20 min (Fig. 5A**, B**). Intraperitoneal injection with scopolamine did not result in an increase in locomotor activity (Fig. 5C**, D** Table 3C, D). Inhalation of scopolamine produced a marked increase in locomotor activity (Fig. 5C**, D**), which lasted for a further 120 min.

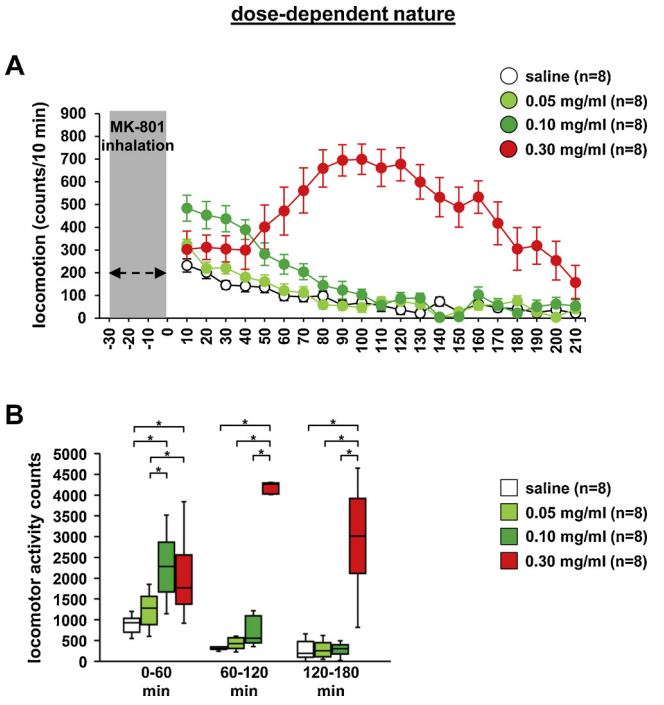


**Fig. 4.** Dose-dependent nature of MK-801–induced locomotion.
(**A**) Spontaneous locomotor activity was assessed in each 10-min period following different doses of MK-801 inhalation (0.05, 0.10, and 0.30 mg/mL) for 210 min in the locomotor activity test. (B) "Number of times a photobeam was broken during three consecutive 60-min periods immediately after the administration of MK-801 or saline. *, significant differences among groups ($p < 0.05$). The p values were calculated using two-way repeated measures analysis of variance in A and two-way analysis of variance in B.

### *3.6. Inhalation effects of imipramine on depressive-like behaviour in mice*

We also examined how the choice of inhalational or intraperitoneal administration affected the influence of the antidepressant imipramine on depressive-like behaviour in mice. In the tail-suspension test, mice intraperitoneally administered with imipramine were significantly less immobile than mice that received only saline (Fig. 5E, **F** Table 3E, F). Thus, inhalation with imipramine resulted in a reduction in immobility time (Fig. 5E**, F**). No differences were observed in immobility between mice intraperitoneally administered with imipramine and those that inhaled imipramine.

## 4. Discussion

In this study, we show that structurally diverse CNS agents can affect mouse behaviour following inhalation. In particular, the findings show that inhalation of MK-801 increased the activity levels of mice as much as intraperitoneal and oral administration did. The findings of this study will facilitate the development of new inhalation-based therapeutic methods for CNS agonists, establishment of new animal testing methods, and drug management of CNS agonists.

We evaluated the formulation of behavioural (spontaneous motor) responses via different administration routes of MK-801 to mice. We showed that mice that inhaled MK-801 showed increased activity similar to that observed with intraperitoneal and oral administration. MK-801 is a non-competitive antagonist of NMDA receptors that are abundantly distributed in the CNS [25,26]. Consistent with all other pharmacological agents that induce locomotor sensitisation, MK-801 has been reported to be associated with enhanced dopamine release in the nucleus accumbens [27]. The results show that MK-801 reached the CNS of mice following inhalation and affected the behaviour of mice. Although a mouse could lick or drink MK-801 sprayed by the nebuliser, the inhalation exposure periods were too short for a mouse to drink or lick an amount of MK-801 greater than what it would receive under oral administration conditions. Moreover, inhalation of MK-801 for 10 min did not affect the activity of mice, but inhalation for more than 30 min increased their activity levels. Inhalation of MK-801 for 60 min further increased mouse activity. This suggests that the amount of MK-801 reaching the brain increases depending on the inhalation time.

In experiments with intraperitoneal administration of MK-801, the administered dose has been known to change the behaviour of mice [28,29]. In this experimental method, mouse behaviour was affected when the dose of inhaled MK-801 was 0.1 mg/kg or greater. Furthermore, when the dose of MK-801 increased further, the activity levels of the mice increased. The results show that MK-801 acts on the mouse brain and that administration via inhalation is as effective as intraperitoneal administration. This result is also consistent with the fact that drug levels in the blood differ according to the dose and the inhalation time for inhalation anaesthetics and bronchial drugs [30,31].

We have shown similar findings for the effects of scopolamine, pentobarbital, and imipramine on mice. To the best of our knowledge, there are no reports showing the effects of inhalation of the CNS agonists used in this study. The results of these experiments show that the behavioural changes induced by MK-801 inhalation are not due to chance and that it is possible that CNS agonists are efficiently transferred to the brain following inhalation.

Further studies are needed to determine the details underlying the access to the brain observed in this study. The possible pathways include (1) a route to reach the brain from the nasal cavity via the olfactory nerve pathway and the trigeminal nerve pathway, (2) absorption into the bloodstream from the nasal cavity and permeation into the BBB, and (3) uptake from respiratory system pathways and absorption into the bloodstream. Future studies should use radiolabelling to investigate this issue by tracing the distribution of MK-801 in brain tissue following intranasal administration.

Studies have suggested that the intranasal route can deliver drugs directly from the nasal cavity along the olfactory and trigeminal nerves to the brain [32]. For example, a study in which rats received haloperidol via intranasal administration found that haloperidol was undetectable in plasma collected shortly after haloperidol dosing [33]. This finding suggests that haloperidol was transported directly from the nasal cavity to the brain without any absorption into circulating blood. In recent studies supporting nose-to-brain transport along the olfactory and trigeminal nerves, it has been shown that substances are rapidly transported into the brain and/or brainstem through associations with neurofilaments present along neuroanatomical processes [34,35].

Intranasal delivery is distinctive among the various strategies currently available for drug targeting. In fact, intranasal administration has been attempted for various therapeutic agents, including sumatriptan for migraine headaches, desmopressin for the treatment of diabetes mellitus in response to lactation, oxytocin for breast milk secretion, and the dopaminergic agonist apomorphine to accelerate childbirth [36,37]. Currently, nasal administration requires that the use of a dedicated device for administering a drug solution [38,39]. In this study, we showed that MK-801 can exert effects on the brain following aromatherapy-style inhalation without the use of a dedicated device.

Administration of a therapeutic drug via inhalation is expected to be useful for various patient groups, such as patients with dementia and patients with post-operative delirium, which can make it difficult to take medicines. The results of this study suggest that the administration of drugs by inhalation is an attractive option for delivering drugs to the brain and that delivery by inhalation is superior to other options for delivering drugs to the brain. Our findings have particularly compelling implications for experiments with mice, for which intraperitoneal injections are potentially stressful due to the invasive nature of the injections and the need for handling by the experimenter. Our results suggest that intraperitoneal injections can be replaced with drug inhalation to reduce the stress levels in mice. Our research results will

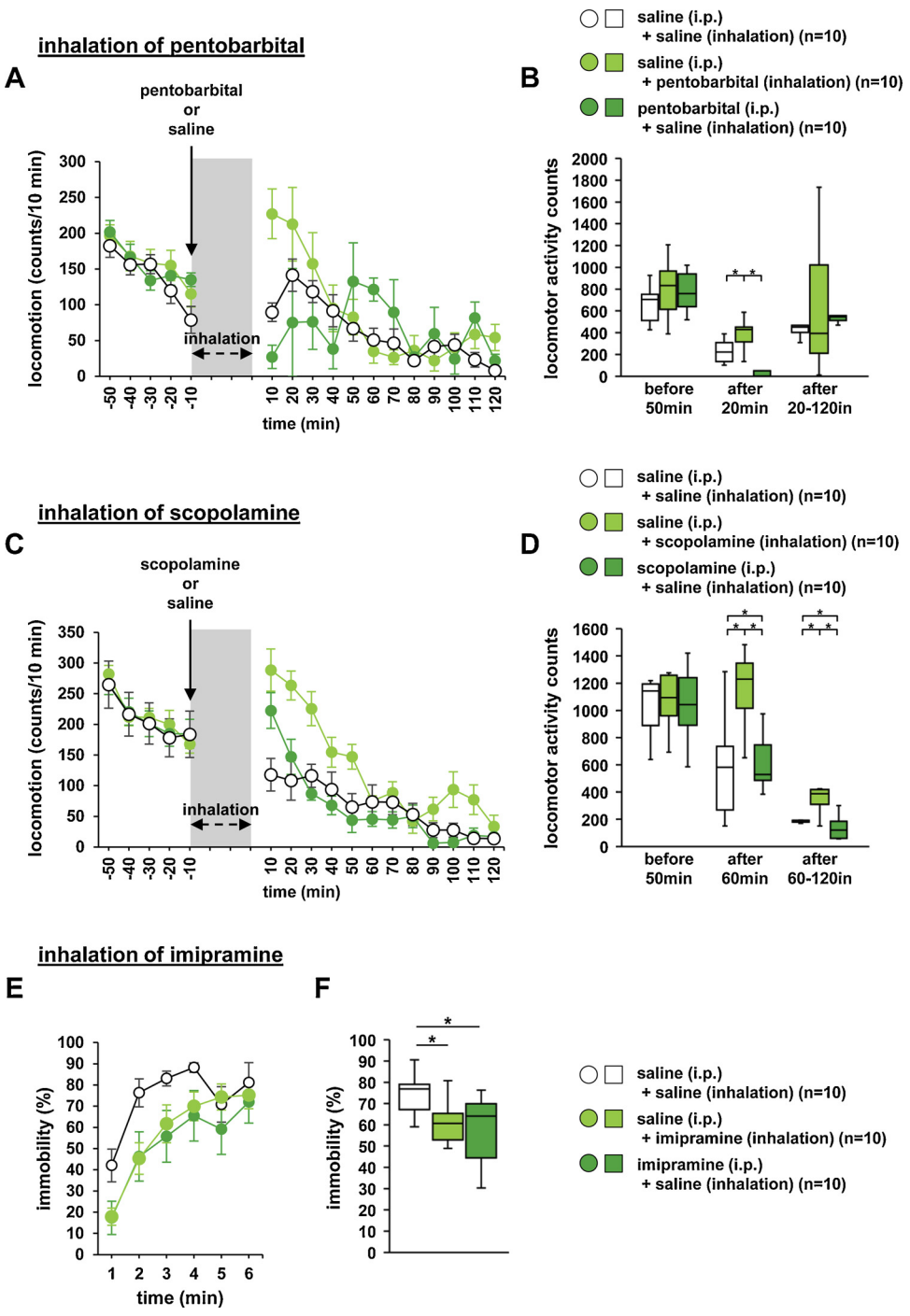


**Fig. 5.** Induction of locomotion with pentobarbital and scopolamine and depressive-like behaviours with imipramine.
(A) Spontaneous pentobarbital-induced locomotor activity was assessed in each 10-min period. After 50 min, animals were injected with pentobarbital or saline and inhaled pentobarbital or saline. Next, locomotor activity was assessed for 120 min. (B) Number of times a photobeam was broken during a 50-min period before the administration of pentobarbital or saline, during the first 20 min after the administration of pentobarbital or saline, and during the period 20–120 after the administration of pentobarbital or saline. The "saline (i.p.) + saline (inhalation)" group was treated with saline via the intraperitoneal and inhalation routes. The "saline (i.p.) + pentobarbital (inhalation)" group was treated with saline via intraperitoneal administration and with pentobarbital via inhalation. The "pentobarbital (i.p.) + saline (inhalation)" group was treated with pentobarbital via intraperitoneal administration and with saline via inhalation. (C) Spontaneous scopolamine-induced locomotor activity was assessed in each 10-min period. After 50 min, animals were injected with scopolamine or saline and inhaled scopolamine or saline. Next, locomotor activity was assessed for 120 min. (D) Number of times a photobeam was broken during a 50-min period before the administration of scopolamine or saline and during the two consecutive 60-min periods immediately after the administration of scopolamine or saline. The "saline (i.p.) + saline (inhalation)" group was treated with saline via the intraperitoneal and inhalation routes. The "saline (i.p.) + scopolamine (inhalation)" group was treated with saline via intraperitoneal administration and with scopolamine via inhalation. The "scopolamine (i.p.) + saline (inhalation)" group was treated with scopolamine via intraperitoneal administration and with saline via inhalation. (E) Mean proportion of time spent immobile in each 1-min period following imipramine vapor inhalation for 30 min in the tail-suspension test. (F) Total immobility times The "saline (i.p.) + saline (inhalation)" group was treated with saline via the intraperitoneal and inhalation routes. The "saline (i.p.) + imipramine (inhalation)" group was treated with saline via intraperitoneal administration and with imipramine via inhalation. The "imipramine (i.p.) + saline (inhalation)" group was treated with imipramine via intraperitoneal administration and with saline via inhalation. Data are presented as means ± standard errors in A, C, and E and in box plots in B, D, and F. *, significant differences among groups ($p < 0.05$). The p values were calculated using two-way repeated measures analysis of variance in A, C, and E and two-way analysis of variance in B, D, and F. Abbreviation: i.p., intraperitoneal

also facilitate the establishment of new experimental methods.

This study shows that chemical substances act on the CNS by inhalation. However, researchers and many other people may inhale volatilised chemical solutions. Thus, this study shows the necessity of properly managing chemicals to promote safe laboratory working conditions.

In many studies, fragrant compounds have been shown to be capable of crossing the BBB and interacting with CNS receptors, so fragrance inhalation has a major impact on brain function [40,41]. When essential oils are inhaled into the nasal cavity, aerosolised molecules attach to the cilia of olfactory receptors lining the olfactory epithelium [42,43]. The results of these experiments also address one aspect of the mechanisms underlying the effects of inhalation of essential oils.

## 5. Conclusions

This study showed that inhalation of the CNS agonists MK-801, pentobarbital, scopolamine, and imipramine had effects on the CNS in mice and influenced their behaviour. Although the pathway by which drugs reach the brain after inhalation remains unclear, this study's

**Table 3**
p values for Fig. 5.

| **A:** Fig. 5**A** | | | p value |
|---|---|---|---|
| saline (i.p.) + saline (inhalation) | vs | saline (i.p.) + pentobarbital (inhalation) | 1 |
| saline (i.p.) + saline (inhalation) | vs | pentobarbital (i.p.) + saline (inhalation) | 0.704 |
| saline (i.p.) + pentobarbital (inhalation) | vs | pentobarbital (i.p.) + saline (inhalation) | 1 |
| **B:** Fig. 5**B** | | | p value |
| before 50 min | | | |
| saline (i.p.) + saline (inhalation) | vs | saline (i.p.) + pentobarbital (inhalation) | 0.628 |
| saline (i.p.) + saline (inhalation) | vs | pentobarbital (i.p.) + saline (inhalation) | 0.962 |
| saline (i.p.) + pentobarbital (inhalation) | vs | pentobarbital (i.p.) + saline (inhalation) | 0.876 |
| after 20 min | | | |
| saline (i.p.) + saline (inhalation) | vs | saline (i.p.) + pentobarbital (inhalation) | 0.043 |
| saline (i.p.) + saline (inhalation) | vs | pentobarbital (i.p.) + saline (inhalation) | 0.438 |
| saline (i.p.) + pentobarbital (inhalation) | vs | pentobarbital (i.p.) + saline (inhalation) | 0.009 |
| after 20-120 min | | | |
| saline (i.p.) + saline (inhalation) | vs | saline (i.p.) + pentobarbital (inhalation) | 0.868 |
| saline (i.p.) + saline (inhalation) | vs | pentobarbital (i.p.) + saline (inhalation) | 0.767 |
| saline (i.p.) + pentobarbital (inhalation) | vs | pentobarbital (i.p.) + saline (inhalation) | 0.957 |
| **C:** Fig. 5**C** | | | p value |
| saline (i.p.) + saline (inhalation) | vs | saline (i.p.) + scopolamine (inhalation) | 0.002 |
| saline (i.p.) + saline (inhalation) | vs | scopolamine (i.p.) + saline (inhalation) | 0.05 |
| saline (i.p.) + scopolamine (inhalation) | vs | scopolamine (i.p.) + saline (inhalation) | 1 |
| **D:** Fig. 5**D** | | | p value |
| before 50 min | | | |
| saline (i.p.) + saline (inhalation) | vs | saline (i.p.) + scopolamine (inhalation) | 0.984 |
| saline (i.p.) + saline (inhalation) | vs | scopolamine (i.p.) + saline (inhalation) | 0.998 |
| saline (i.p.) + scopolamine (inhalation) | vs | scopolamine (i.p.) + saline (inhalation) | 0.993 |
| after 60 min | | | |
| saline (i.p.) + saline (inhalation) | vs | saline (i.p.) + scopolamine (inhalation) | 0.003 |
| saline (i.p.) + saline (inhalation) | vs | scopolamine (i.p.) + saline (inhalation) | 0.966 |
| saline (i.p.) + scopolamine (inhalation) | vs | scopolamine (i.p.) + saline (inhalation) | 0.005 |
| after 60-120 min | | | |
| saline (i.p.) + saline (inhalation) | vs | saline (i.p.) + scopolamine (inhalation) | 0.048 |
| saline (i.p.) + saline (inhalation) | vs | scopolamine (i.p.) + saline (inhalation) | 0.658 |
| saline (i.p.) + scopolamine (inhalation) | vs | scopolamine (i.p.) + saline (inhalation) | 0.005 |
| **E:** Fig. 5**E** | | | p value |
| saline (i.p.) + saline (inhalation) | vs | saline (i.p.) + imipramine (inhalation) | 0.042 |
| saline (i.p.) + saline (inhalation) | vs | imipramine (i.p.) + saline (inhalation) | 0.043 |
| saline (i.p.) + imipramine (inhalation) | vs | imipramine (i.p.) + saline (inhalation) | 1 |
| **F:** Fig. 5**F** | | | p value |
| saline (i.p.) + saline (inhalation) | vs | saline (i.p.) + imipramine (inhalation) | 0.038 |
| saline (i.p.) + saline (inhalation) | vs | imipramine (i.p.) + saline (inhalation) | 0.039 |
| saline (i.p.) + imipramine (inhalation) | vs | imipramine (i.p.) + saline (inhalation) | 0.639 |

findings suggest that intranasal administration techniques can facilitate the development of new therapeutic strategies for CNS disorders.

## 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. HU, MO, and TI developed the concept and design of the study. HU, SS, and YT acquired, analysed, and interpreted the data. HU and MO drafted the manuscript. SM, NK, KW, YM, and TI performed critical revisions of the manuscript for important intellectual content. MO and TI supervised the study.

## Conflicts of interest

The authors declare that there are no conflicts of interest.

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