



# Involvement of 5-hydroxytryptamine type 3 receptors in sevoflurane-induced hypnotic and analgesic effects in mice

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## Abstract:

In the present study, we investigated the role of 5-hydroxytryptamine type 3 (5-HT<sub>3</sub>) receptors in hypnosis and analgesia induced by emulsified sevoflurane. A mouse model of hypnosis and analgesia was established by an intraperitoneal or subcutaneous injection of emulsified sevoflurane. We intracerebroventricularly (*icv*) or intrathecally (*it*) administered YM-31636, a 5-HT<sub>3</sub> receptor agonist, to mice and observed sleep time during hypnosis. In addition, the tail withdrawal latency was measured using the tail withdrawal test, and the writhing time was determined using the acetic acid writhing test. In the hypnosis test, YM-31636 (5, 10 and 15 μg, *icv*) treatment significantly decreased emulsified sevoflurane-induced mouse sleep time ( $p < 0.05$  or  $p < 0.01$ ). YM-31636 (2.5, 5 and 10 μg, *it*) treatment significantly and dose-dependently decreased the tail withdrawal latency ( $p < 0.05$  or  $p < 0.01$ ) and increased the writhing time ( $p < 0.01$ ) of mice treated with emulsified sevoflurane. These results suggest that 5-HT<sub>3</sub> receptors may modulate the hypnotic and analgesic effects induced by emulsified sevoflurane.

## Key words:

sevoflurane, hypnosis, analgesia, 5-HT<sub>3</sub> receptors

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## Introduction

One hundred and sixty years after inhalation anesthetics were adopted into clinical practice, the underlying mechanism of action remains unknown. Recently, ligand-gated ion channels have emerged as the most promising molecular target for inhalation anesthetics [8, 15, 23, 30]. Various *in vitro* studies have reported that enhancement of inhibitory neurotransmitter function or inhibition of excitatory neurotransmitter function is a plausible method to induce anesthesia [1, 30, 32, 33].

5-Hydroxytryptamine (5-HT) is a biogenic amine that mediates a variety of physiological actions. 5-HT receptors are classified into seven major groups [9]. Of these subtypes, the 5-HT<sub>3</sub> receptor is a member of the superfamily of ligand-gated ion channel receptors. This family shares structural similarities with the nicotinic acetylcholine, glycine, and  $\gamma$ -aminobutyric acid type A (GABA<sub>A</sub>) receptors [3, 11, 12]. The 5-HT<sub>3</sub> receptors are widely distributed in both the central and peripheral nervous system and are involved in the physiologic and pathologic processes that mediate nausea, vomiting, peripheral nociception, and central

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antinociception [5, 20]. Previous studies have shown that inhalation anesthetics modulate 5-HT<sub>3</sub> receptors [25, 26]. Sevoflurane is a halogenated inhalation anesthetic. *In vitro* electrophysiological studies have demonstrated that sevoflurane inhibits 5-HT<sub>3</sub> receptors [16, 28]. Therefore, 5-HT<sub>3</sub> receptors may confer sevoflurane-dependent anesthesia. Inhalation anesthetics have two main effects, hypnosis and analgesia [19, 24]. However, whether the 5-HT<sub>3</sub> receptors are involved in sevoflurane-induced hypnotic or analgesic effects has not been determined in an *in vivo* behavioral study.

We hypothesized that if 5-HT<sub>3</sub> receptors contribute to the hypnotic and the analgesic effects of sevoflurane, the application of the 5-HT<sub>3</sub> receptor agonist YM-31636 (2-(1*H*-imidazol-4-ylmethyl)-8*H*-indenol[1,2-*d*]thiazole monofumarate) should decrease the hypnotic and analgesic effects of sevoflurane. We addressed this hypothesis by using the hypnosis test, tail withdrawal test and acetic acid writhing test.

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## Materials and Methods

### Animals

Kunming mice (22 ± 3 g) were obtained from the Experimental Animal Center of Jiangsu University. Mice were housed in a 12-hour light/dark cycle at room temperature (22 ± 2°C). Food and water were provided *ad libitum*. All experiments were performed between 8:00 and 12:00 to avoid diurnal variation during behavioral testing. The experimental protocols were approved by the Animal Care and Use Committee of Jiangsu University and complied with the National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No. 85-23, revised 1985).

### Formulation of emulsified sevoflurane

Sevoflurane was emulsified using the method of Chiari et al. [4]. Sevoflurane was dissolved in soy bean oil containing dispersed egg lecithin, which comprised the oil phase of the emulsion. Dissolution of egg lecithin in soy bean oil was facilitated with heat. After dissolution of lecithin, the oil phase was cooled to approximately 10°C before addition of sevoflurane.

The aqueous phase of the emulsion contained glycerin. The aqueous phase was cooled to 10°C. The oil phase was then added to the aqueous phase under vigorous stirring to form the initial emulsion. The initial emulsion was homogenized at high pressure to form the final emulsion. After homogenization, the emulsified sevoflurane was stored in glass vials. Vials were capped and refrigerated (2–5°C) until further experimentation. Vials of emulsion were warmed to 37°C for 2 h before administration. The target concentration of anesthetics in emulsion was approximately 8%. After warming the emulsion for 2 h at 37°C, the actual concentration of sevoflurane in the emulsion was 7.5%, as determined by gas chromatography.

### Intracerebroventricular injection

Intracerebroventricular (*icv*) administration was performed following the method of Laursen and Belknap [14]. Briefly, animals were injected at the bregma with a 50- $\mu$ l Hamilton syringe fitted with a 26-gauge needle, which had a tip that was adjusted for 2.4-mm penetration. The *icv* injection volume was 5  $\mu$ l, and injection sites were verified by injecting an equal volume of 1% methylene blue. Dye distribution into the ventricular space was observed. The dye seeped into the ventricular space, ventral surface of the brain, and the upper cervical portion of the spinal cord.

### Intrathecal injection

Conscious mice were injected intrathecally (*it*) using the method of Hylden and Wilcox [10]. At the 5th lumbar vertebra a 26-gauge needle fitted with a microsyringe was inserted into the spinal canal at a 30° angle. Transient tail extension indicated a successful injection. The solution was injected in a volume of 5  $\mu$ l over 5 s. Lidocaine (2%) was injected *it* into 10 mice, which immediately exhibited hindlimb paralysis that lasted for approximately 10 min in the preliminary experiments.

### Drug administration

The hypnotic dose of sevoflurane (20 ml/kg) was determined in preliminary experiments [6]. Sevoflurane was injected intraperitoneally (*ip*) to produce hypnosis in mice. Sevoflurane was injected *ip* (10 ml/kg) or subcutaneously (*sc*) (25 ml/kg) to produce analgesia in mice.

### Hypnosis test

Forty Kunming mice (male or female) were randomly divided into 4 groups (n = 10): the emulsified sevoflurane + artificial cerebral spinal fluid (aCSF) groups and the emulsified sevoflurane + YM-31636 (5, 10 and 15 µg) groups.

Every group was injected *ip* with a hypnotic dose of emulsified sevoflurane. One minute after loss of the righting reflex (animal remain on their back for at least 30 s) the mice were injected *icv* with aCSF or YM-31636. Sleep time (duration of the loss of righting reflex) was observed [2].

### Tail withdrawal test

All experiments were started at 10:00 a.m. and performed according to the method of Kayser et al. [13]. Briefly, each mouse was placed in a plastic tube (50 ml polypropylene conical tube) such that the tail protruded from an opening in the bottom of the tube. The distal half of the tail was dipped into a bath of circulating water that was thermostatically controlled at  $48.0 \pm 0.5^\circ\text{C}$ . The tail withdrawal latency (TWL) was measured by an experienced observer in a single blind manner. A cut off time of 20 s was used to avoid tissue damage.

Eighty Kunming mice (male or female) were randomly divided into 8 groups (n = 10): aCSF group; YM-31636 (2.5, 5 and 10 µg) groups; the emulsified sevoflurane + aCSF groups, and the emulsified sevoflurane + YM-31636 (2.5, 5 and 10 µg) groups. The aCSF and YM-31636 groups were injected *it* with aCSF and YM-31636, respectively. Groups that received emulsified sevoflurane were injected *ip* with an analgesic dose. After 5 min, each group was injected *it* with aCSF or YM-31636. Baseline TWL and TWL at 5, 10, 15, 20 and 25 min after injection was observed.

### Acetic acid writhing test

Eighty Kunming mice (male or female) were randomly divided into 8 groups (n = 10): aCSF group; YM-31636 (2.5, 5 and 10 µg) groups; the emulsified sevoflurane + aCSF groups, and the emulsified sevoflurane + YM-31636 (2.5, 5 and 10 µg) groups. The aCSF and YM-31636 groups were injected *it* with aCSF and YM-31636, respectively. The emulsified sevoflurane groups were injected *sc* with an analgesic

dose. After 10 min, each group was injected *it* with aCSF or YM-31636. One minute after *it* administration, mice were injected *ip* with 0.6% acetic acid (10 ml/kg). Each mouse was then placed into an individual Plexiglas observation cylinder (14 cm diameter; 30 cm in height). The writhing time (arching of the back, development of tension in the abdominal muscles, elongation of the body and extension of the forelimbs) within 15 min was recorded after the final injection [31].

### Statistics

Results are expressed as the mean  $\pm$  SEM. Data were statistically analyzed using SPSS16.0 software (SPSS Inc., Chicago, IL, USA). Multiple group comparisons were performed by one-way ANOVA followed by the Dunnett's *post-hoc* test. For the tail withdrawal test, the difference between baseline and drug treatment was analyzed using a paired Student's *t*-test;  $p < 0.05$  was considered statistically significant.

## Results

### Hypnosis test

*Icv* injection of YM-31636 (5, 10 and 15 µg) significantly decreased the sleep time of the mice treated with emulsified sevoflurane ( $p < 0.05$  and  $0.01$ ; Tab. 1).

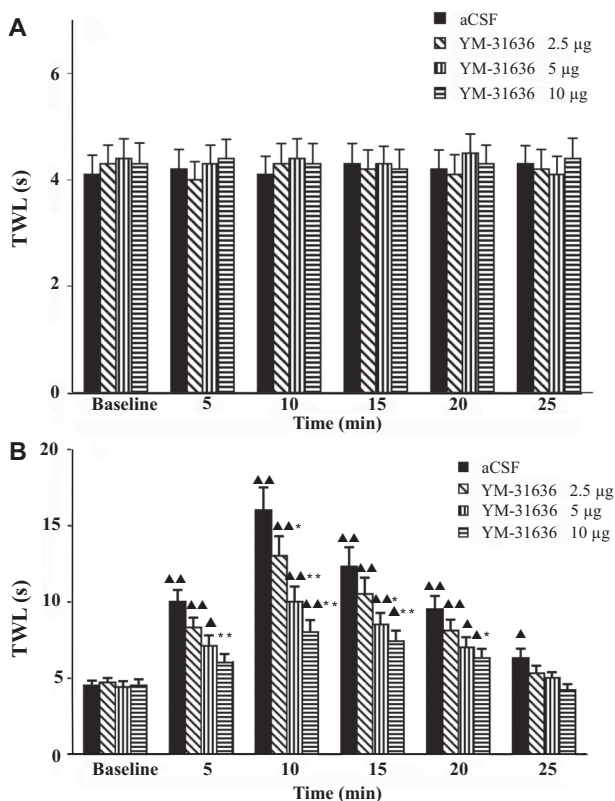
### Tail withdrawal test

The TWL of conscious mice was not affected by an *it* injection of YM-31636 (2.5, 5 and 10 µg;  $p > 0.05$

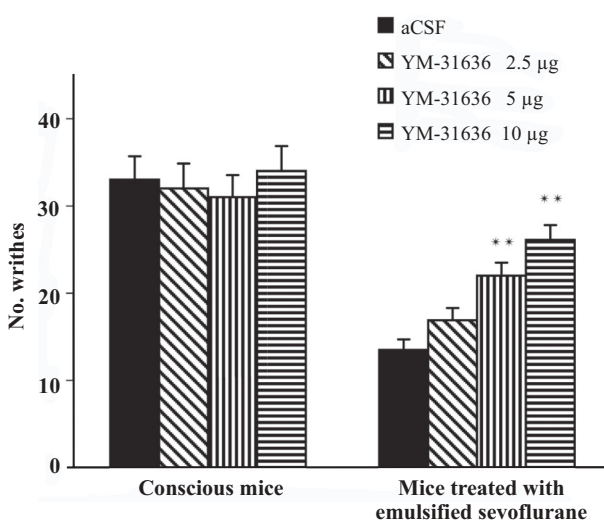
**Tab. 1.** Effects of YM-31636 treatment on the sleep time of mice treated with emulsified sevoflurane in the hypnosis test.

Group	N	Dose (µg)	Sleep time (min)
aCSF	10	—	58.7 $\pm$ 2.2
YM-31636	10	5	53.6 $\pm$ 2.3
	10	10	50.3 $\pm$ 2.4*
	10	15	43.1 $\pm$ 1.8**

Data represent the mean  $\pm$  SEM (\*  $p < 0.05$ , \*\*  $p < 0.01$  vs. aCSF group using one-way ANOVA and Dunnett's test)



**Fig. 1.** Effects of YM-31636 treatment on the tail withdrawal latency (TWL) in conscious mice (A) and mice treated with emulsified sevoflurane (B). YM-31636 (2.5, 5 and 10 µg) was injected *it*. The data represent the mean ± SEM. Ten mice were used in each group (\*  $p < 0.05$ , \*\*  $p < 0.01$  vs. aCSF group, using one-way ANOVA and Dunnett's test; ▲  $p < 0.05$ , ▲▲  $p < 0.01$  vs. baseline using paired Student's *t*-test)



**Fig. 2.** Effects of YM-31636 treatment on the acetic acid writhing times in mice. YM-31636 (2.5, 5 and 10 µg) was injected *it*. The data represent the mean ± SEM. Ten mice were used in each group (\*\*  $p < 0.01$  vs. aCSF group, using one-way ANOVA and Dunnett's test)

compared to the aCSF treatment, Fig. 1A). YM-31636 (2.5, 5 and 10 µg, *it*) treatment significantly and dose dependently decreased the TWL ( $p < 0.05$  and 0.01 compared to the aCSF treatment) of mice that received an *ip* injection of emulsified sevoflurane. YM-31636 (2.5, 5 and 10 µg, *it*) treatment decreased the TWL by 18.8 ( $p < 0.05$ ), 37.5 and 50.0% ( $p < 0.01$ ), respectively, 10 min after emulsified sevoflurane injection (Fig. 1B).

### Acetic acid writhing test

YM-31636 (2.5, 5 and 10 µg, *it*) treatment did not affect mouse behavior and the writhing time in comparison to the effects of aCSF administration in conscious mice ( $p > 0.05$ ). In the groups receiving an *sc* injection of emulsified sevoflurane, YM-31636 (2.5 µg, *it*) treatment did not affect the writhing time. However, YM-31636 (5 and 10 µg, *it*) treatment markedly increased writhing time in comparison to mice treated with aCSF ( $p < 0.01$ , Fig. 2).

### Discussion

Inhalation anesthetics are often administered through the airway. Administration needs specific types of equipment. Therefore, some inhalation experiments cannot be performed. Novel formulations of inhalation anesthetics have been developed to facilitate drug administration *in vivo*. These formulations are composed of a drug emulsification in a lipid vehicle. This preparation may be clinically useful to produce anesthesia [4]. Previous studies support the efficacy and safety of the *iv* administration of emulsified inhalation anesthetics in animals [34, 35]. According to drug pharmacokinetics and our preliminary experiments [6, 7], we confirmed that *ip* and *sc* injection of emulsified inhalation anesthetics can achieve hypnosis and analgesia similar to that produced by inhalation. In the hypnosis test, hypnotic doses of emulsified sevoflurane induced the loss of mouse righting reflex. Mouse sleep time was approximately 60 min; however, drug treatment minimally affected breathing and blood circulation. In the tail withdrawal test and the acetic acid writhing test, we used an *ip* or *sc* injection to produce analgesia. Mice not only showed the effects of analgesia but also had essentially normal be-



havior and the righting reflex was not lost. To avoid an interaction between the *ip* injection of emulsified sevoflurane and acetic acid, we chose an analgesic model that used an *sc* injection of emulsified sevoflurane. Onset velocity measures the absorption velocity from *ip* or *sc* injection. Emulsified sevoflurane is absorbed more slowly from an *sc* injection than from an *ip* injection. Therefore, the dose of emulsified sevoflurane that was applied to mice in the acetic acid writhing test was greater than that used in the hypnosis test.

General anaesthesia induces immobility, amnesia, hypnosis, and analgesia and the suppression of stress responses to noxious stimuli. To simplify our experiments, individual components of general anesthesia were observed [29]. However, there is a caveat – the effects of anesthesia are not independent. Pain increases awareness. Noxious stimuli during anesthesia increases arousal, as indicated by increased neuronal activity in the reticular formation and thalamus. The spinal cord (responsible for analgesia and anesthesia-induced immobility) interacts with the brain cortex (responsible for awareness and hypnosis).

Inhalation anesthetics supply two essential elements of anesthesia – hypnosis and analgesia. Hypnosis is defined as the lack of ‘perceptive awareness’, which is assessed by the response to non-noxious stimuli [15]. The righting reflex test determines the ability of an animal to regain an upright posture within 30 s of being placed in a supine position. In the absence of central nervous system depression or impairment, maintaining an upright posture is a natural reflex. Therefore, this assay is used to test drugs that cause significant central nervous system depression [17]. The tail withdrawal test and acetic acid writhing test are methods to measure analgesia. Analgesia is defined as the loss of pain sensation without loss of consciousness or mobility.

YM-31636 is a novel agonist of the 5-HT<sub>3</sub> receptor. Treatment with YM-31636 agitates animals and may even produce convulsions. In preliminary experiments, we observed that YM-31636 (20 µg, *icv*) treatment induced scratching and biting behavior. This suggests that the dose used in the hypnosis test was sufficient. In agreement with our hypothesis, we observed that *icv* administration of YM-31636 attenuates the hypnotic effects of emulsified sevoflurane. This is also in agreement with the findings of Mukaida et al. [16]. In this study, serotonergic activity modulated the hypnotic effects of isoflurane.

Studies in rats and goats have demonstrated that the spinal cord mediates the analgesic and immobility effects of inhalation anesthetics [21, 22]. The *it* injection in mice is an appropriate method to study spinally-mediated drug effects. According to a report by Hylden and Wilcox [10], 5 µl of methylene blue dye never diffused beyond the rostral thoracic segments. In addition, *it* injected [<sup>3</sup>H]morphine was not found in significant quantities in either the midbrain or forebrain. Therefore, we determined the behavioral effects of drugs at the spinal level. In preliminary experiments, we observed that YM-31636 (15 µg, *it*) treatment increased scratching behavior. However, in this experiment, the doses used had no effect on behavior. Many studies have demonstrated that 5-HT released from descending bulbospinal neurons exerts dual (facilitatory and inhibitory) effects on spinal nociceptive transmission – activation of 5-HT<sub>3</sub> receptors and inhibition of 5-HT<sub>1A</sub> receptors [18, 27]. Our study showed that YM-31636 (*it*) treatment significantly and dose-dependently decreased the TWL and increased the writhing time of mice treated with emulsified sevoflurane. Our data suggest that sevoflurane administration reduces current response *via* 5-HT<sub>3</sub> receptors. In support of our hypothesis, we conclude that spinal 5-HT<sub>3</sub> receptors may modulate the analgesic effects of emulsified sevoflurane.

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