

The kappa opioid receptor mediates the antinociceptive effect of nitrous oxide in mice

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Summary

Background: Our previous reports demonstrated that genetic deletion of μ -opioid receptor (MOP) has no influence on the anaesthetic and antinociceptive effects of N₂O in mice, and that an antagonist selective for κ -opioid receptor (KOP), but not that selective for δ -opioid receptor (DOP), suppresses the antinociceptive effect of N₂O. However, it is not known whether genetic deletion of κ -opioid receptor (KOP) affects the N₂O actions.

Methods: We measured the minimum alveolar concentration (MAC) of volatile anaesthetics in the absence and presence of N₂O. The antinociceptive action of N₂O was tested by an acetic acid-writhing test and a hot-plate test. The number of c-Fos-immunopositive cells in sections from the lumbar spinal cord was counted to test whether the descending inhibitory system participates in the pharmacological action of N₂O. The hypnotic action of N₂O was assessed by measuring the N₂O-induced decrease in the EC₅₀ for loss of righting reflex (EC₅₀-LORR) of sevoflurane.

Results: Sevoflurane MAC was not significantly reduced by N₂O and its antinociceptive action was almost completely abolished in KOP-knockout (KO) mice. The N₂O-induced increase of c-Fos-immunopositive cells in laminae III-IV of the lumbar spinal cord was significant in wild-type (WT), but not in KOP-KO mice. In contrast, sevoflurane EC₅₀-LORR was similarly reduced by N₂O in WT and KOP-KO mice.

Conclusion: Our study suggests that N₂O demonstrates its antinociceptive action and reduces sevoflurane MAC in mice through KOP activation, whereas its hypnotic potency is not dependent on KOP activation.

Key Words: Anaesthetics gases, nitrous oxide; Anaesthetics volatile; Mice, knockout; Potency, analgesic; Receptors, Opioid, kappa

Nitrous oxide (N₂O) has been clinically used as long as 170 years and exerts an antinociceptive action without major side effects. At the molecular level, N₂O inhibits the N-methyl-D-aspartate (NMDA)-type of the ionotropic glutamate receptors,¹ but controversies still remain with respect to the mechanisms of its pharmacological actions.² Naloxone, an opioid receptor antagonist, reverses the antinociceptive effect of N₂O, which suggests that opioid peptide release and opioid receptor activation are involved in this action of N₂O.³ However, it is unclear which of the naloxone-sensitive opioid receptors, including the μ -, κ - and δ -opioid receptors (MOP, KOP and DOP, respectively),⁴ is the most important in the pharmacological action of N₂O. Our previous report demonstrated that the antinociceptive effect of N₂O is not influenced by genetic deletion of MOP⁵ and is significantly reduced by a KOP-selective antagonist, but unaffected by a DOP-selective antagonist,⁶ suggesting that KOP is important in the antinociceptive action of N₂O.

The mechanism for the hypnotic action of N₂O is also unknown. Based on gene knockout experiments, it was shown that the NMDA-type of the glutamate receptor does not participate in the hypnotic action of inhaled anaesthetics, including isoflurane, sevoflurane and N₂O.⁷ However, it has not been investigated whether the opioid receptor mediates the hypnotic action of N₂O by the use of the knockout mice.

The minimum alveolar concentration (MAC), EC₅₀ value of inhaled anaesthetics for immobilizing effect to noxious stimulus, has been frequently used to compare anesthetic potency of different inhaled anaesthetics. It remains unclear whether the opioid receptor is involved in the pharmacological action of volatile anaesthetics which determines MAC. Dahan et al. showed that naloxone induces elevation of the sevoflurane MAC in mice, and the sevoflurane MAC in MOP knockout (KO) mice is 20% higher than that in wild-type

(WT) mice.⁸ Contrarily, Liao et al. reported that the sevoflurane MAC was not significantly affected by naloxone.⁹ We have shown that MAC of sevoflurane, isoflurane and halothane is not significantly affected by MOP deletion, which suggests that the pharmacological action of volatile anaesthetics which determines MAC is not mediated by MOP.⁵ However, involvement of opioid receptors other than MOP in this action has not been examined using KO mice.

Thus, using KOP-KO mice, we aimed to clarify whether KOP mediates the antinociceptive and hypnotic effects of N₂O and whether MAC of N₂O and volatile anaesthetics is affected by KOP activation.

Methods

Animals

WT C57BL/6 mice and KOP-KO mice [strain: B6.129S2-Oprk1(tm1Kff)/J, stock number: 007558] aged 6-10 weeks were obtained from Japan SLC Inc. (Shizuoka, Japan) and Jackson Laboratory (Bar Harbor, ME, USA), respectively. The total number of mice used in this study was 121 for WT and KOP-KO mice, respectively. Animals were housed in a temperature-controlled environment (22-26°C, 50% relative humidity) with lights on from 7 a.m. to 9 p.m. during an acclimatization period of at least 1 week and could freely access to food and water. After this period, all tests were performed between 9 a.m. and 3 p.m. All experiments were approved by the Animal Research Committee of Kyoto University, and were performed according to Guidelines for Proper Conduct of Animal Experiments established by Science Council of Japan. In each experiment, treatment order of wild-type and knock-out mice was randomized, and mice were randomly allocated to the treatment groups in each genotype. Every effort was made to minimize animal suffering and reduce the number of animals used. In the c-Fos immunohistochemical study, the mice were sacrificed by high dose of sodium pentobarbital as described below. In the other experiments, the mice were humanely killed by CO₂ inhalation followed by cervical dislocation after measurements. The investigator who performed the measurements (H.F.) was blinded to both the mouse type and the treatment.

Drugs and chemicals

The drugs and chemicals used in the experiments were U-50,488 (Sigma Chemical, St. Louis, MO, USA), an agonist selective for KOP¹⁰; acetic acid (Nacalai Tesque, Kyoto,

Japan); N₂O (Wakayama Sanso, Wakayama, Japan); oxygen (O₂) and nitrogen (N₂) (Taiyo Nippon Sanso Co., Tokyo, Japan); and sevoflurane and isoflurane (Abbott Japan Co., Tokyo, Japan).

Measurement of MAC

The MAC values of sevoflurane and isoflurane were estimated as described previously.^{8 9}
^{11 12} Briefly, mice were individually placed in a transparent plastic cylinder (15 cm in length, 5 cm in diameter), and allowed to breath spontaneously while inhaling sevoflurane or isoflurane at a total gas flow of 2 L min⁻¹ (100% O₂). To keep the rectal temperature between 36.5 and 37.5°C, mice were warmed with a heat lamp and a warm blanket (Animal Blanket Controller ATB-1100, Nihon Kohden, Tokyo, Japan). The concentration of sevoflurane or isoflurane was monitored by an infrared gas analyzer (Capnomac Ultima, Datex-Ohmeda, Helsinki, Finland). An anaesthetic vaporizer (Aika, Ichikawa Shiseido, Tokyo, Japan) was used to vaporize the volatile anaesthetic.

Mice inhaled each anaesthetic gas for 20 min and then a 1-min noxious stimulus was applied to the tail with an alligator clip. The stimulation site on the tail was always selected proximal to the previous test site. A gross movement of the head, trunk or extremities was judged as a positive response. The concentrations of sevoflurane and isoflurane were raised in increments of 0.3% and 0.1%, respectively, and the MAC was calculated as the mean of the highest concentration allowing a positive response and the lowest concentration preventing this response. The sevoflurane and isoflurane MAC was determined for WT and KOP-KO mice (n = 6, respectively).

The influence of N₂O on sevoflurane MAC was analyzed in WT and KOP-KO mice (n =

18, respectively). The mice were allocated to control, N₂O, and KOP agonist groups (n = 6, respectively). At the beginning of the equilibration period, mice of the KOP agonist group received 10 mg kg⁻¹ of U-50,488 subcutaneously (s.c.), while animals of the control and N₂O groups were injected with vehicle (saline). After this treatment, the MAC of sevoflurane vaporized in 70% N₂/30% O₂ (control and KOP agonist groups) or 70% N₂O/30% O₂ (N₂O group) was determined as described above. Total gas flow was 2 L min⁻¹.

Measurement of antinociceptive effects

KOP-KO and WT mice were randomly divided into control, N₂O and KOP agonist groups. Mice of the control and KOP agonist groups inhaled 30% O₂/70% N₂ for 30 min, and those of the N₂O group inhaled 30% O₂/70% N₂O for 30 min. At 30 min before antinociceptive evaluation, mice of the control and N₂O groups were injected with vehicle (saline) s.c. and those of the KOP agonist group were injected with 10 mg kg⁻¹ of U-50,488 s.c.

The antinociceptive effect against visceral pain in WT and KOP-KO mice was evaluated using an acetic acid writhing test (n = 24, respectively).¹³ Each mouse was placed in a transparent plastic chamber (12 cm in height, 22 cm in diameter) and allowed a 1-h acclimation period. Mice were pretreated as described above, inhaled the gas mixture for 30 min at a total gas flow rate of 5 L min⁻¹, and were then injected with 0.7% acetic acid (0.1 mL per 10 g of body weight) intraperitoneally (i.p.). Five minutes after acetic acid injection, the writhing reflex was counted for 10 min. The writhing reflex was defined as a lengthwise stretch of the trunk with concave arching of the back. The gas mixture was continuously delivered into the chamber during the writhing test.

Evaluation of the antinociceptive effect against somatic pain in WT and KOP-KO mice

was performed using a hot-plate test ($n = 30$, respectively).¹⁴ The results from 2 KOP-KO mice were not included in the analysis, because pretreatment drug solution was partially leaked at s.c. administration. After pretreatment, mice inhaled the gas for 30 min, and were then put on a hot plate (Muromachi Kikai, Tokyo, Japan) at 55°C. Latency was defined as the time to lick the hind legs or jump with a cutoff time of 30 s to avoid tissue damage.

Measurement of sevoflurane EC₅₀-LORR

The hypnotic action of N₂O was estimated by assessing the effect of N₂O on the EC₅₀ of sevoflurane for loss of the righting reflex (EC₅₀-LORR) in WT and KOP-KO mice ($n = 27$, respectively).¹⁵ Mice of the control and N₂O groups were injected with vehicle (saline) while those of the KOP agonist group were injected with 10 mg kg⁻¹ U-50,488 s.c. The mice then inhaled the gas mixture (flow rate, 2 L min⁻¹) in the cylinder (30% O₂/70% N₂ for the control and KOP agonist groups; 30% O₂/70% N₂O for the N₂O group). The initial sevoflurane concentration was 0.8% atm. After equilibration for 20min, the chamber was gently rotated to place the mouse on its back and the righting response was observed for 10 s. The sevoflurane concentration was increased in 0.3% increments and the response was reexamined after equilibration for 20 min. EC₅₀-LORR was defined as the midpoint between the highest concentration at which the mouse showed a righting response and the lowest concentration at which the mouse did not respond.

c-Fos immunohistochemical study

Immunohistochemical study was performed in WT and KOP-KO mice ($n = 10$, respectively), by the method described by Toda and others.¹⁶ After 90-min exposure to 30%

O₂ + 70% N₂ (n = 5) or 30% O₂ + 70% N₂O (n = 5), mice were sacrificed with sodium pentobarbital (100 mg kg⁻¹, i.p.) injection, followed by transcardial perfusion with 20 mL of 0.1 M phosphate-buffered saline (pH 7.2) (PBS) and subsequently 20 mL of the fixative solution (4% paraformaldehyde in 0.1 M PBS). The lumbar spinal cord (approximately L4/L5 level) was obtained by rapid PBS injection into the spinal canal and post-fixed in the fixative solution for 6 h. The samples were incubated in 20% sucrose in 0.1 M PBS for 24-36 h, embedded in optimal cutting temperature compound (OTC) (Tissue-Tek; Sakura Finetechnical, Tokyo, Japan), and frozen at -80°C. Sections (5-μm thick) were prepared by the use of a cryostat at -22°C and mounted on slide glasses.

After rinsing with PBS (5 min, 6 times), the sections were treated with absolute methanol containing 0.3% H₂O₂ for 30 min at room temperature to quench endogenous peroxidase activity, and then were treated with 1.3% goat serum (Vector Laboratories, Burlingame, CA, USA) for 30 min to block nonspecific binding of the antibody. Thereafter, the slides were incubated overnight at 4°C with primary rabbit anti-c-Fos antibody (1:200, sc-52, Santa Cruz Biotechnology, Santa Cruz, CA, USA), incubated with biotinylated anti-rabbit IgG antibody (Vector Laboratories) for 40 min at room temperature, and then incubated with avidin-biotin-peroxidase complex (Vector Laboratories) for 50 min at room temperature. Color development was performed with 0.03% 3,3'-diaminobenzidine tetrahydrochloride (Dojindo Laboratories, Tokyo, Japan). Slides were counterstained using 0.1% Mayer's hematoxylin.

The sections were then dehydrated using an alcohol gradient, cleared using xylene, and coverslipped. Brown nuclear staining was regarded as a c-Fos-immunopositive cell, and the number of these cells in each sections from the lumbar spinal cord was estimated using an

Olympus DP73 microscope. The distribution of c-Fos-immunopositive cells was examined in four regions: the superficial dorsal horn (laminae I-II), nucleus proprius (laminae III-IV), neck of the dorsal horn (laminae V-VI), and ventral gray (laminae VII-X),¹⁷ in three randomly selected sections in each mouse. The results are expressed as the mean number of c-Fos-immunopositive cells per section.

Statistical analysis

The sample size in each experiment was predetermined using power analysis by the use of G Power software based on previous reports to provide the appropriate power ($1-\beta = 0.8$). MAC measurement was powered to detect a 20% change in MAC by opioid receptor knockout⁸ with a variance of 0.3% for sevoflurane and 0.1% for isoflurane⁵. For assessment of the N₂O effect on sevoflurane MAC, measurement of antinociceptive effects and c-Fos immunohistochemical study, power analysis was performed by assuming differences and variances similar to those in our previous study⁵. For the effect of N₂O on sevoflurane EC₅₀-LORR, sample size calculation was performed by assuming that N₂O induces ~50% reduction in EC₅₀-LORR of volatile anaesthetics⁷. A larger number of mice were enrolled in the study to allow for possible incomplete data collection.

Data were analyzed by unpaired t-test for pairwise comparisons or by one-way analysis of variance (ANOVA) followed by Bonferroni correction as a post hoc test using Prism 5 software (GraphPad Software, Inc., San Diego, CA, USA). The results are expressed as mean (SD). In all analyses, a P value <0.05 was considered significant.

Results

MAC of volatile anaesthetics and N₂O

First, we tested whether KOP is involved in the action of N₂O and volatile anaesthetics that is estimated by MAC, because MAC has been frequently used to compare anesthetic potency of different inhaled anaesthetics. No significant difference in the MAC for isoflurane or sevoflurane was found between WT and KOP-KO mice (Table 1), suggesting that KOP does not significantly contribute to the pharmacological effect of isoflurane and sevoflurane estimated by MAC. Next, we indirectly examined whether N₂O MAC is affected by KOP by measuring the sevoflurane MAC in the absence and presence of N₂O in WT and KOP-KO mice, because there was no significant difference in sevoflurane MAC between these mice (Fig. 1). The sevoflurane MAC was significantly reduced by N₂O in WT mice ($P < 0.05$), whereas no significant decrease in sevoflurane MAC was produced by N₂O in KOP-KO mice. These results show that the sevoflurane MAC-reducing effect of N₂O in WT mice is significantly stronger than that in KOP-KO mice, in which this effect of N₂O is almost negligible. We also tested the effect of U-50,488, an agonist selective for KOP,¹⁰ on sevoflurane MAC (Fig. 1). Significant reduction of the sevoflurane MAC was induced by U-50,488 in WT mice ($P < 0.01$), but not in KOP-KO mice.

Antinociceptive action of N₂O

Figure 2 depicts the writhing responses of mice to i.p. injection of acetic acid. The writhing count was significantly decreased by N₂O and U-50,488 in WT mice ($P < 0.05$), but neither N₂O nor U-50,488 decreased the writhing count in KOP-KO mice. Figure 3 demonstrates the result of the hot-plate test comparing the antinociceptive action of N₂O

against somatic noxious stimulus. In WT mice, the latency was prolonged by N₂O and U-50,488 compared with control ($P < 0.05$). In contrast, in KOP-KO mice, no significant prolongation of the latency was produced by N₂O or U-50,488. These results show that the antinociceptive action of N₂O and U-50,488 are almost completely eliminated in KOP-KO mice.

N₂O-induced c-Fos expression in the lumbar spinal cord

Activation of γ -aminobutyric acid (GABA)-producing interneurons by N₂O through the action of the spinal descending inhibitory system was evaluated by immunohistochemical assessment of c-Fos protein expression in the lumbar spinal cord of WT and KOP-KO mice (Fig. 4). Under control conditions, there was no difference in the number of c-Fos-immunopositive cells between WT and KOP-KO mice. The number of c-Fos-immunopositive cells in laminae III-IV was significantly increased by inhalation of 70% N₂O in WT mice ($P < 0.05$). However, in KOP-KO mice, no significant increase in c-Fos-immunopositive cells was induced by N₂O in any investigated regions.

Hypnotic action of sevoflurane and N₂O

Sevoflurane EC₅₀-LORR was not significantly different between WT and KOP-KO mice under control condition, suggesting that KOP is not involved in the hypnotic action of sevoflurane (Fig. 5). We next examined whether KOP is involved in the hypnotic action of N₂O by measuring sevoflurane EC₅₀-LORR in the presence of N₂O in WT and KOP-KO mice. Inhalation of 70% N₂O induced decrease in sevoflurane EC₅₀-LORR of 35±11% and 32±10% in WT and KOP-KO mice, respectively (Fig. 5). These results demonstrate that the

hypnotic action of sevoflurane and N₂O is not significantly affected by genetic deletion of KOP. U-50,488, a KOP agonist, did not influence the sevoflurane EC₅₀-LORR, confirming that the KOP-mediated antinociceptive effect does not potentiate the hypnotic action of sevoflurane.

Discussion

The results of this study using KOP-KO mice provide insights into the mechanisms of the pharmacological actions of N₂O and volatile anaesthetics. KOP gene deletion almost completely abolished the antinociceptive effect and sevoflurane MAC-reducing effect of N₂O, but did not significantly affect the hypnotic action of N₂O. Furthermore, KOP gene deletion did not significantly affect MAC and hypnotic action of the volatile anaesthetics.

The mechanisms of general anaesthesia by volatile anaesthetics have been explored, and some researchers have reported that MOP is involved in its action.⁸ However, our previous study showed that the MAC values, which reflect the potency of inhaled anaesthetics for immobilizing action to noxious stimulation, of sevoflurane, isoflurane and halothane in MOP-deleted mice was not significantly different from those in WT mice.⁵ This suggests that MOP does not contribute to the immobilizing action to noxious stimulation of volatile anaesthetics. However, contribution of KOP to the pharmacological action of volatile anaesthetics has not been previously examined. The current study showed that MAC of volatile anaesthetics, isoflurane and sevoflurane, was not influenced by genetic deletion of KOP, which suggests that the immobilizing action in response to noxious stimulation of these anaesthetics is not mediated by KOP. Collectively, these findings suggest that opioid receptors do not play essential roles in the action of volatile anaesthetics, and thus we decided to compare N₂O-induced decrease in MAC of volatile anaesthetics between WT and KOP-KO mice in order to indirectly assess whether the MAC of N₂O is affected by KOP activity. Because essentially identical results were expected for sevoflurane and isoflurane, we used sevoflurane as a representative of volatile anaesthetics.

The MAC of N₂O is higher than 100%, which prevents direct measurement of the MAC at

atmospheric pressure. However, the MAC of N₂O can be estimated indirectly by measuring MAC for volatile anaesthetics with and without N₂O, because MAC values are additive.¹⁸ In the current study, co-administration of 70% N₂O decreased the sevoflurane MAC by about 40% in WT mice, which indicates that 70% N₂O corresponds to 40% of the MAC, giving a MAC for N₂O of nearly 175%. This value is similar to the results of direct determination of N₂O MAC values.¹⁹ Injection of a KOP-selective agonist, U-50,488, also reduced the sevoflurane MAC in WT mice, indicating that KOP-mediated analgesia can reduce MAC and that the immobilizing action estimated by MAC includes the antinociceptive component. In contrast, in KOP-KO mice, N₂O did not significantly influence the sevoflurane MAC, suggesting that the immobilizing action to noxious stimulation of N₂O, which might be based on the antinociceptive action of N₂O, is negligible in these mice. Collectively, these results show that KOP activation potentiates the immobilizing action of volatile anaesthetics and is crucial for reduction of sevoflurane MAC by N₂O, suggesting that the MAC of N₂O is affected by KOP activity.

The count of writhing responses caused by i.p. acetic acid in KOP-KO mice is significantly higher than that in WT mice,²⁰ which implicates KOP in the perception of visceral chemical pain. Consistent with this finding, the current study showed that the acetic acid-induced writhing count in KOP-KO mice is larger than that in WT mice, although the difference was not statistically significant. The antinociceptive action of the KOP-selective agonist U-50,488 was almost completely eliminated in the acetic acid-writhing test and hot-plate test, as also reported previously²⁰, which confirms the absence of functional KOP in these mice. The antinociceptive action of N₂O was also almost completely abolished in KOP-KO mice, consistent with our previous reports showing that the antinociceptive action

of N₂O was not influenced by genetic deletion of MOP in mice⁵ and that a KOP-selective antagonist suppressed the antinociceptive effect of N₂O.⁶ These results together indicate that KOP performs an important function in the antinociceptive action of N₂O.

N₂O activates the noradrenergic descending inhibitory pathway and GABAergic interneurons in the spinal cord in rat.²¹ We have shown that inhalation of 70% N₂O significantly increases the number of c-Fos-immunopositive cells in laminae III-IV in WT and MOP-KO mice, which reflects activation of GABAergic interneurons by the noradrenergic descending inhibitory pathway in the mouse spinal cord.⁵ The current study showed that the number of c-Fos-immunopositive cells was not increased in laminae III-IV of the lumbar spinal cord by inhalation of 70% N₂O in KOP-KO mice in contrast to WT mice. This finding agrees with our result showing that the antinociceptive action of N₂O was almost completely abolished by genetic deletion of KOP, and suggests that N₂O produces antinociceptive action through KOP-mediated activation of the descending inhibitory pathway.

General anaesthetics produce various pharmacological effects, including antinociception, muscle relaxation, and hypnosis. We next aimed to investigate whether the hypnotic and antinociceptive components of N₂O action are mediated by different mechanisms. Because the hypnotic action of N₂O is relatively small and it is difficult to estimate EC₅₀-LORR of N₂O directly, we assessed the hypnotic action of N₂O by measuring the N₂O-induced reduction of EC₅₀-LORR of sevoflurane.⁷ There was no significant difference in the sevoflurane EC₅₀-LORR between WT and KOP-KO mice, and this value was reduced by addition of 70% N₂O to essentially identical extent in both genotypes, suggesting that the hypnotic effect of N₂O occurs mainly through a mechanism that does not involve KOP.

Consistent with this, KOP activation by a KOP-selective agonist, U-50,488, did not affect sevoflurane EC₅₀-LORR.

There have been many reports on the mechanisms of the antinociceptive action of N₂O. We have shown the involvement of nociceptin receptors in this action^{22 23} and inhibition of glutamatergic transmission by N₂O in the spinal cord was also reported.²⁴ Sawamura and others found that the antinociceptive action of N₂O in alpha 2B adrenergic receptor knockout mice is significantly lower than that in WT mice²⁵. Corticotropin releasing factor-producing neurons and noradrenergic neurons present in the locus coeruleus have also been linked to the N₂O action.²⁶ Nitric oxide produced at the supraspinal level²⁷ and spinal level²⁸ may also participate in the antinociceptive action of N₂O in mouse and rat. Further studies are obligatory to solve how these various mechanisms and the mechanism mediated by KOP, which was demonstrated to play a central role in the antinociceptive action of N₂O in the current study, are related.

There are several limitations in this study. First, we have no direct evidence that KOP is activated by administration of N₂O, although KOP was shown to be indispensable for the antinociceptive action of N₂O. Demonstration of activation of KOP-expressing neurons or increased production or secretion of dynorphin, an endogenous peptide selectively activating KOP, by N₂O is required to show N₂O-induced KOP activation. Second, a compensatory physiological change activated by deletion of KOP might affect the mechanism of action of N₂O. We cannot completely rule out this possibility, but it is unlikely that MOP or DOP, expression of which might be increased by KOP deletion, affects N₂O action, because we have previously shown that MOP and DOP do not significantly contribute to N₂O action.^{5 6} Third, although the sevoflurane EC₅₀-LORR in WT and KOP-KO mice did not significantly

differ, it remains possible that the slope of the dose-response curve for sevoflurane-induced LORR and the effect N₂O on the slope is different between these mice. Thus, the possibility that KOP is involved in the hypnotic action of N₂O is not completely excluded.

In conclusion, we demonstrated that N₂O exerts its antinociceptive action and reduces MAC of volatile anaesthetics in mice through activation of KOP and the descending inhibitory pathway in the spinal cord, while its hypnotic potency is not dependent on KOP activation.

Declaration of interest

None declared.

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Author Contributions

H.F. performed all the experiments and wrote the first draft of the paper.

T.K. helped conduct the experiments.

K.F. contributed to the design of the experiments and supervised the experiments and writing of the manuscript.

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Table 1. Minimum alveolar concentrations of volatile anaesthetics.

Mice	Sevoflurane	Isoflurane
KOP-KO	2.70 (0.31)	1.27 (0.08)
WT	2.57 (0.04)	1.25 (0.13)

There was no significant difference in the minimum alveolar concentrations of sevoflurane and isoflurane between κ -opioid receptor knockout (KOP-KO) and wild-type (WT) mice (unpaired *t*-test). Values are shown as mean (SD), n = 6.

Figure Legends

Fig. 1. Minimum alveolar concentration (MAC) of sevoflurane in the presence and absence of nitrous oxide (N₂O) or U-50,488. The MAC of sevoflurane was significantly reduced by 70% N₂O in wild-type (WT) mice, but was not significantly affected by N₂O in κ -opioid receptor knockout (KOP-KO) mice. U-50,488, a KOP-selective agonist, at 10 mg kg⁻¹ significantly decreased the sevoflurane MAC in WT mice, but had no significant effect on the MAC in KOP-KO mice. Data are shown as mean (SD), n = 6. **P* < 0.05; ***P* < 0.01; ****P* < 0.001; NS not significantly different (one-way ANOVA followed by Bonferroni correction as a post hoc test).

Fig. 2. Antinociceptive effect of nitrous oxide (N₂O) and U-50,488 in the acetic acid-writhing test. In wild-type (WT) mice, 70% N₂O and 10 mg kg⁻¹ U-50,488 almost completely suppressed writhing responses, whereas there were no significant antinociceptive effects in κ -opioid receptor knockout (KOP-KO) mice. Data are shown as mean (SD), n = 8. **P* < 0.05; ***P* < 0.01; ****P* < 0.001; NS not significantly different (one-way ANOVA followed by Bonferroni correction as a post hoc test).

Fig. 3. Antinociceptive effect of nitrous oxide (N₂O) and U-50,488 in the hot plate test. In wild-type (WT) mice, 70% N₂O and 10 mg kg⁻¹ U-50,488 significantly prolonged the latency, whereas there were no significant antinociceptive effects in κ -opioid receptor knockout (KOP-KO) mice. Data are shown as mean (SD), n = 9 for control and N₂O groups from KOP-KO mice, and n = 10 for the other groups. The results from 2 KOP-KO mice were not included in the analysis, because pretreatment drug solution was partially leaked at s.c.

administration. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; *NS* not significantly different (one-way ANOVA followed by Bonferroni correction as a post hoc test).

Fig. 4. Effects of nitrous oxide (N_2O) on the number of c-Fos-positive cells in the lumbar spinal cord. Exposure to 70% N_2O increased the number of c-Fos-positive cells in laminae III-IV in wild-type (WT) mice. In κ -opioid receptor knockout (KOP-KO) mice, there was no significant difference in the number of c-Fos positive cells with or without N_2O . Data are shown as mean (SD), $n = 5$. * $P < 0.05$ vs. control in WT mice (one-way ANOVA followed by Bonferroni correction as a post hoc test). We do not present the photograph, because the difference between WT and KOP-KO mice is small and difficult to be noticed at a glance in a photograph.

Fig. 5. Effect of nitrous oxide (N_2O) on the concentration of sevoflurane required to cause loss of the righting reflex in half of the mice (EC_{50} -LORR). Exposure to 70% N_2O decreased the sevoflurane EC_{50} -LORR similarly in wild-type (WT) and κ -opioid receptor knockout (KOP-KO) mice. Injection of 10 mg kg^{-1} U-50,488 had no significant effect on the sevoflurane EC_{50} -LORR in both genotypes. Data are shown as mean (SD), $n = 9$. *** $P < 0.001$; *NS* not significantly different (one-way ANOVA followed by Bonferroni correction as a post-hoc test).

Figure 1

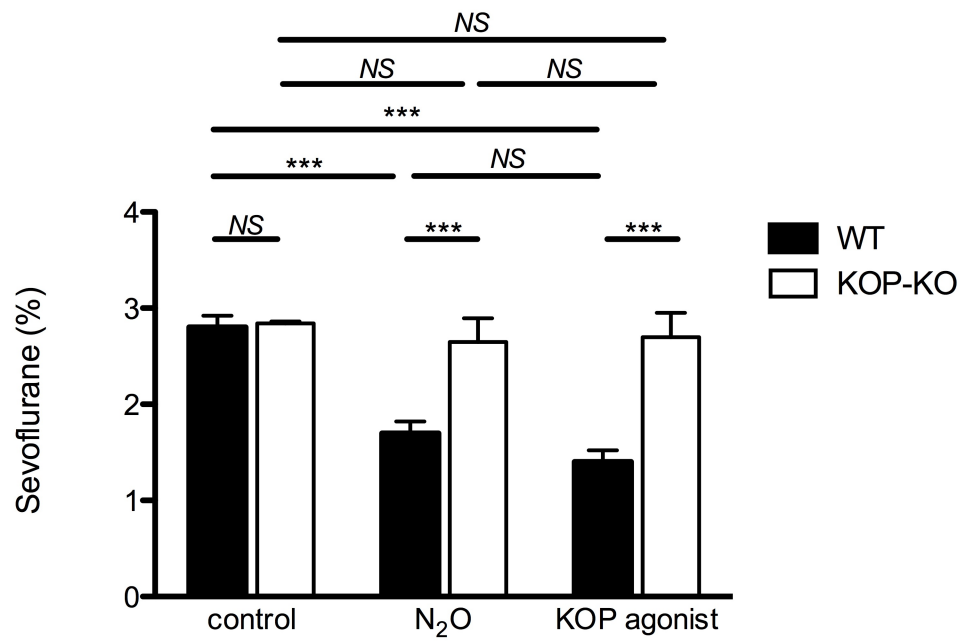


Figure 2

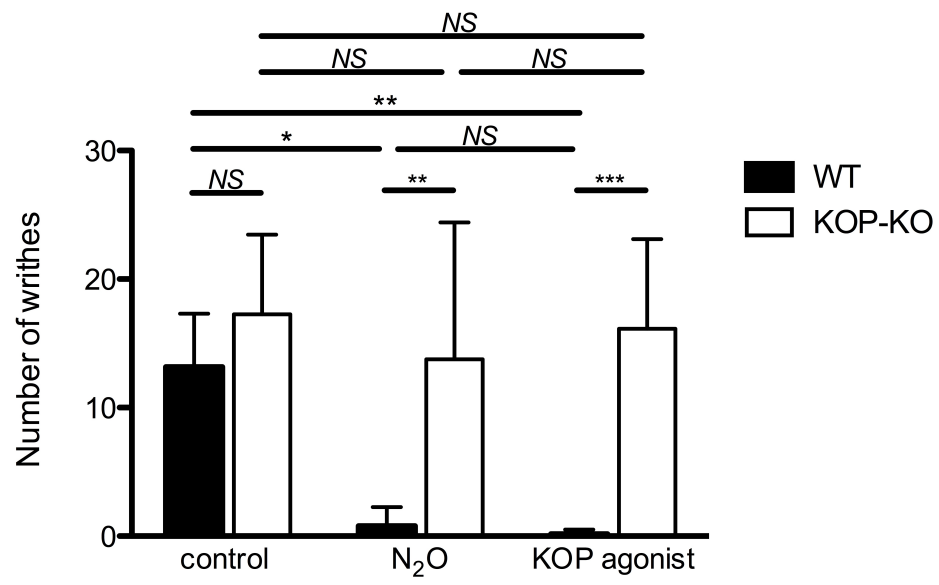


Figure 3

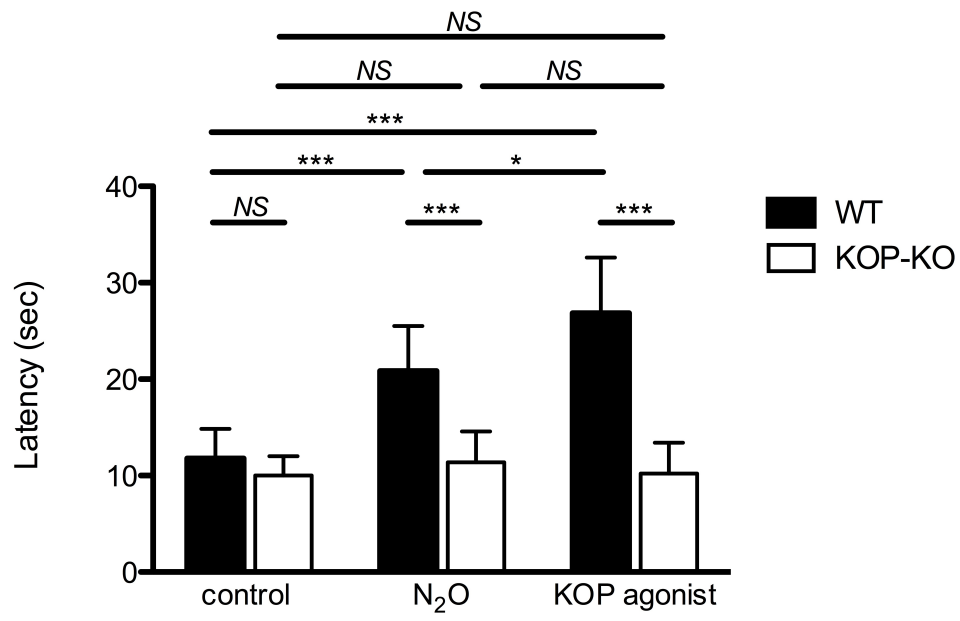


Figure 4

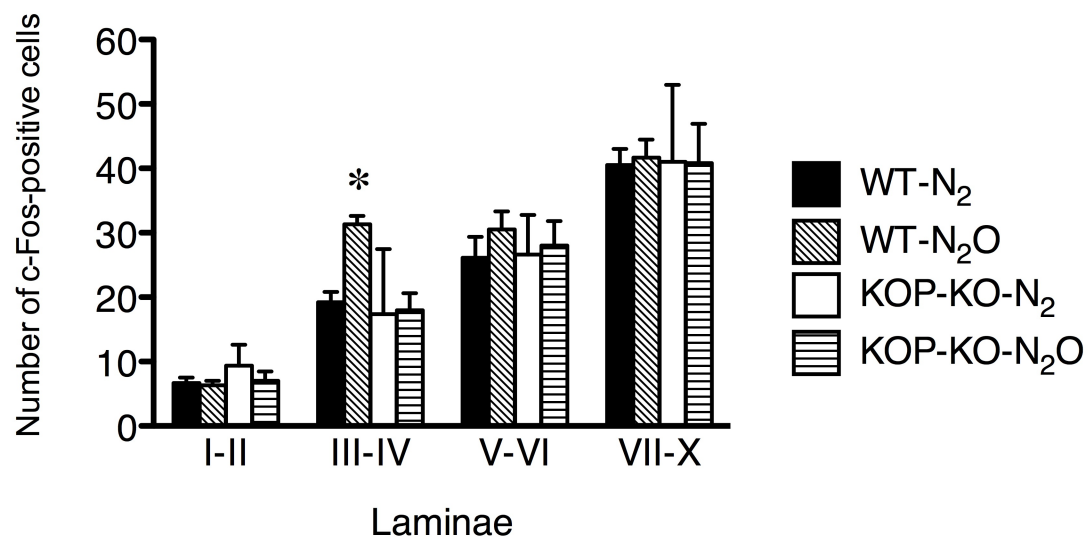


Figure 5

