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Inhibition of cAMP-phosphodiesterase 4 (PDE4) potentiates the anesthetic effects of Isoflurane in mice

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Abstract

Despite major advances, there remains a need for novel anesthetic drugs or drug combinations with improved efficacy and safety profiles. Here, we show that inhibition of cAMP-phosphodiesterase 4 (PDE4), while not inducing anesthesia by itself, potently enhances the anesthetic effects of Isoflurane in mice. Treatment with several distinct PAN-PDE4 inhibitors, including Rolipram, Piclamilast, Roflumilast, and RS25344, significantly delayed the time-to-righting after Isoflurane anesthesia. Conversely, treatment with a PDE3 inhibitor, Cilostamide, or treatment with the potent, but non-brain-penetrant PDE4 inhibitor YM976, had no effect. These findings suggest that potentiation of Isoflurane hypnosis is a class effect of brain-penetrant PDE4 inhibitors, and that they act by synergizing with Isoflurane in inhibiting neuronal activity. The PDE4 family comprises four PDE4 subtypes, PDE4A to PDE4D. Genetic deletion of any of the four PDE4 subtypes in mice did not affect Isoflurane anesthesia *per se*. However, PDE4D knockout mice are largely protected from the effect of pharmacologic PDE4 inhibition, suggesting that PDE4D is the predominant, but not the sole PDE4 subtype involved in potentiating Isoflurane anesthesia. Pretreatment with Naloxone or Propranolol alleviated the potentiating effect of PDE4 inhibition, implicating opioid- and β-adrenoceptor signaling in mediating PDE4 inhibitor-induced augmentation of Isoflurane anesthesia. Conversely, stimulation or blockade of α_1-adrenergic, α_2-adrenergic or serotonergic signaling did not affect the potentiation of Isoflurane hypnosis by PDE4 inhibition. We further show that pretreatment with a PDE4 inhibitor boosts the delivery of bacteria into the lungs of mice after intranasal infection under Isoflurane, thus providing a first example that PDE4 inhibitor-induced potentiation of Isoflurane anesthesia can critically impact animal models and must be considered as a factor in experimental design. Our findings suggest that

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Author contributions:

IVA, WR, and AB performed the time to righting assays; WR, IVA, AK, JR, and WM performed the locomotion assays; AB and WR performed the *Pseudomonas aeruginosa* infection experiments; IVA, WR and LA performed the body temperature measurements; IVA, AK, WR, AB and LA generated the animals and maintained the mouse colonies; WR designed the experiments and analyzed the data; WR wrote a first draft and all authors edited and approved the manuscript.

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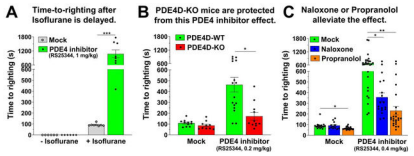
PDE4/PDE4D inhibition may serve as a tool to delineate the exact molecular mechanisms of Isoflurane anesthesia, which remain poorly understood, and may potentially be exploited to reduce the clinical doses of Isoflurane required to maintain hypnosis.

Graphical Abstract

PDE4 inhibition potently enhances the hypnotic effects of Isoflurane in mice (**A**). The effect is mediated by PDE4D (**B**) and is alleviated by blockade of opioid- or β-adrenoceptors (**C**).



Keywords

PDE4; Isoflurane anesthesia; β-adrenergic signaling; hypnosis; *Pseudomonas aeruginosa*

1. INTRODUCTION

Inhalation anesthetics, particularly the halogenated ethers Isoflurane, Sevoflurane, and Desflurane, are widely used in the clinic to induce and/or maintain general anesthesia during surgical procedures[1–3]. They are often the preferred choice as they allow for precise fine-tuning of the anesthetic state, are low cost, easy to use, and provide fast induction and short recovery times. Isoflurane, the most potent of the halogenated ethers, is also widely used in animal research; its application extending far beyond surgeries to various procedures that require immobilization of the animal, even if for short times, such as for the delivery of reagents (e.g. tail vein or intracerebroventricular injections) or *in vivo* imaging (e.g. ultrasound).

The clinical success of Isoflurane and related ethers is in part owed to the fact that they are generally well tolerated. Common adverse effects associated with these drugs are cardiac and/or respiratory depression[1, 4, 5] which, however, are characteristic of any form of systemic anesthesia. In addition, upon recovery from Isoflurane anesthesia, ~30% of patients experience post-operative nausea and vomiting (PONV)[1, 6, 7], which is commonly treated with pre- and/or post-operative antiemetics. In addition, shivering or effects resembling allergic reactions such as rash, hives, itching, swelling, trouble breathing, dizziness, or passing out have been reported[1]. These can sometimes be intense, thus limiting the utility of the halogenated ethers for susceptible patients. While very rare, fluranes may also cause severe, potentially life-threatening, side effects including malignant hyperthermia[8], liver and kidney toxicity, or arrhythmias[1].

Lab research and animal studies have indicated potential concerns regarding the long-term effects of inhalation anesthetics, such as an impairment of neurocognitive function[1, 9–11] or an increased risk of tumor recurrence/progression[12, 13]. Inhalation anesthetics, including Isoflurane, have been demonstrated to cause neuronal cell death as well as long-term

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neurocognitive dysfunction[9–11] in neonatal/juvenile rodents and juvenile non-human primates. Furthermore, Isoflurane and other halogenated ethers facilitate the formation of β-amyloid and tau oligomers which may have implications for the neuropathogenesis of Alzheimer's disease[14, 15]. However, it remains unclear if, and to which extent, any of these findings may apply to humans. This subject is complex and remains under investigation. For example, animal studies reported that fetal/neonatal or juvenile rodents are significantly more prone to neurodevelopmental changes resulting from flurane exposure compared to adult animals. Conversely, inhalation anesthetics are widely used in children, and no evidence confirming inhalation anesthesia as a risk factor for neurodevelopmental deficits has emerged[10]. In a subset of adult patients undergoing surgeries, the procedure *per se* can lead to temporary cognitive impairments, and so may general anesthesia produced by any agent. But it remains to be determined whether the specific use of Isoflurane or related ethers represents an independent risk factor for cognitive impairment[9]. Similarly, no evidence has emerged that continuous, low-level exposure to waste anesthetic gases represents a unique risk for healthcare workers[16]. Nevertheless, given the significant side effects experienced by some patients, and the potential risk of long-term effects uncovered in animal studies, any reduction in the doses required to induce or maintain anesthesia may potentially be beneficial to both patients and healthcare workers.

The second messenger cAMP has been shown to modulate many functions of the central nervous system ranging from cognition and memory formation[17, 18], mood and emotions[19], to psychosis[20] or nociception[21]. The cellular concentration of cAMP is determined by the equilibrium between the rate of its synthesis by adenylyl cyclases, and the rate of its hydrolysis and inactivation by cyclic nucleotide phosphodiesterases (PDEs)[22]. PDEs comprise a superfamily of isoenzymes that are grouped into 11 PDE families based on sequence homology as well as their substrate kinetics and pharmacologic properties[23]. The PDE4 family is the largest, comprising four genes, PDE4A-D, that together generate likely over 25 protein variants *via* use of alternate promoters and alternative splicing[24, 25]. PAN-selective PDE4 inhibition produces numerous therapeutic effects[26–29] including potent anti-inflammatory effects[30], memory and cognition improvement[17, 18], as well as cardiovascular[31], metabolic[32] and antineoplastic[33] effects. However, adverse effects, particularly nausea and emesis, have constrained their clinical utility and commercial success until now.

While exploring potential anti-inflammatory benefits of PDE4 inhibition in a mouse model of bacterial lung infection, we noticed that pre-treatment with a PDE4 inhibitor augmented the intensity and duration of Isoflurane anesthesia that we employed for the intranasal delivery of the bacteria. As a role of cAMP signaling in general, and an effect of PDE4 inhibition in particular, on Isoflurane anesthesia has not been reported before, and to delineate any potential impact of altered Isoflurane anesthesia on our animal model, we have further explored this observation.

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2. MATERIALS AND METHODS

2.1. Drugs

Piclamilast (RP73401; 3-(Cyclopentyloxy)-N-(3,5-dichloropyridin-4-yl)-4-methoxybenzamide), Rolipram (4-(3-cyclopentyloxy-4-methoxyphenyl)pyrrolidin-2-one), Roflumilast (3-(cyclopropylmethoxy)-N-(3,5-dichloropyridin-4-yl)-4-(difluoromethoxy)benzamide), Cilostamide (N-cyclohexyl-N-methyl-4-[(2-oxo-1H-quinolin-6-yl)oxy]butanamide), Prazosin ([4-(4-amino-6,7-dimethoxyquinazolin-2-yl)piperazin-1-yl]-(furan-2-yl)methanone), Clonidine (N-(2,6-dichlorophenyl)-4,5-dihydro-1H-imidazol-2-amine) and Yohimbine (methyl (1S,15R,18S,19R,20S)-18-hydroxy-1,3,11,12,14,15,16,17,18,19,20,21-dodecahydroyohimban-19-carboxylate) were from Cayman Chemical (Ann Arbor, MI, USA), Naloxone from MP Biomedicals (Irvine, CA, USA), Propranolol from Millipore Sigma (St. Louis, MO, USA), Ondansetron (9-methyl-3-[(2-methylimidazol-1-yl)methyl]-2,3-dihydro-1H-carbazol-4-one) from Acros Organics (Fair Lawn, NJ, USA), YM976 (4-(3-chlorophenyl)-1,7-diethylpyrido[2,3-d]pyrimidin-2-one) was from Tocris/Bio-Techne (Minneapolis, MN, USA) and RS25344 (1-(3-nitrophenyl)-3-(pyridin-4-ylmethyl)pyrido[2,3-d]pyrimidine-2,4-dione) was obtained from Santa Cruz Biotech (Santa Cruz, CA, USA). All drugs were initially dissolved in DMSO, subsequently diluted into phosphate-buffered saline (PBS), pH 7.4, containing final concentrations of 5% DMSO and 5% Cremophor EL (Millipore Sigma, St. Louis, MO, USA) and were applied by intraperitoneal (i.p.) injection (100 μl per 20 g body weight).

2.2. Animals

Wildtype C57BL/6 mice for experimentation were generated in-house using breeders obtained from Charles River Laboratories (Wilmington, MA). Mice deficient in PDE4A[34], PDE4B[35] and PDE4D[36] mice were generated by Drs. S.-L. Catherine Jin and Marco Conti (Stanford University, CA, USA; also see[37]) and kindly distributed *via* the Mutant Mouse Resource and Research Centers (MMRRC, http://www.mmrrc.org, PDE4A stock ID# 034793-UCD, PDE4B stock ID# 034682-UCD, PDE4D stock ID# 034588-UCD) of the University of California at Davis (CA, USA). PDE4C knockout mice ($Pde4c^{tm1.1(KOMP)Wtsi/J}$) were generated by the National Institutes of Health (NIH) Knockout Mouse Program (KOMP; www.komp.org) and kindly distributed *via* the KOMP repository at the University of California at Davis (CA, USA). Please see Supplement Material in[38] for a description of the PDE4C knockout mouse; additional details are available on the website of the Mutant Mouse Regional Resource Centers (MMRRC; http://www.mmrrc.org; Stock number 049025-UCD). All PDE4 knockout mouse colonies were maintained on a C57BL/6 background by Het/Het breeding and homozygous PDE4KO mice were compared to their respective wildtype littermates. All mice were group housed at up to four mice per cage with *ad libitum* access to food and water and were maintained in a temperature-controlled (22–23°C) vivarium with a 12-h light/dark cycle. Adult mice ≥10 weeks of age, ≥18 g of body weight, and of either sex were used for experimentation by equally and randomly dividing cage littermates into experimental groups. Experimenters were blinded to the identity of the injected drugs until data acquisition and analyses were completed. All experiments and procedures were conducted in accordance with the guidelines described in the Guide for the Care and Use of Laboratory Animals (National

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Institutes of Health, Bethesda, MD, USA) and were approved by the University of South Alabama Institutional Animal Care and Use Committee.

2.3. Measurement of Time to Righting

Thirty min after injection of test drugs or solvent control, mice were placed in an anesthesia induction chamber ventilated with 3% Isoflurane in 100% oxygen at 1 L/min for 10 min. The animals were then removed from the anesthesia chamber, placed into new cages on their backs and the time to first complete righting (when they turn on all four paws; Fig. 1A–C) was recorded.

2.4. Measurement of locomotor activity

Mice were injected (i.p.) with test drugs and immediately placed in the SmartCage™ system (AfaSci Research Laboratories, Redwood City, CA, USA) which tracks the animal's position and its movement in the cage based on the animal's disruption of an array of infrared light beams. The distance each mouse travels within consecutive 5-min periods was used as readout of locomotor activity and is plotted from the moment the mouse is placed in the system.

2.5. Measurement of core body temperature

Core body temperature was measured using a thermocouple thermometer (MicroTherma 2T) with mouse rectal probe (RET-3), both from Braintree Scientific (Braintree, MA, USA), following the manufacturer's instructions.

2.6. *Pseudomonas aeruginosa* infection

Pseudomonas aeruginosa lab strain *PA01* was grown on Luria Bertani (LB) nutrient agar overnight and subsequently suspended to 5×10^6 colony forming units (cfu) per 50 μl volume in PBS.

For intranasal infections, mice were placed in an anesthesia induction chamber ventilated with 3% Isoflurane in 100% oxygen at 1 L/min for 2–3 min until the animals lose consciousness. Mice were then removed from the chamber, a 50 μl inoculum of *Pseudomonas aeruginosa* suspended in PBS is placed on the nose of the scruffed mouse using a P200 pipet until the liquid is completely inhaled. Animals are then placed in dorsal recumbency back into their cages to recover from anesthesia. In a subset of experiments, mice were anesthetized *via* i.p. injection of Ketamine/Xylazine (80 and 10 mg/kg in PBS; i.p.), rather than using Isoflurane, to perform intranasal infections.

For intratracheal infections, anesthesia was induced by placing mice in an anesthesia induction chamber ventilated with 3% Isoflurane in 100% oxygen at 1 L/min for 2–3 min until the animals lose consciousness as determined by the absence of the toe pinch reflex. Mice were then intubated using the Mouse Endotracheal Intubation Kit (Kent Scientific, Torrington, CT, USA) following the manufacturer's instructions. In short, unconscious mice are transferred onto an intubation stand where Isoflurane anesthesia is further maintained *via* a nose cone. A fiberoptic cable illuminated from an LED light source is used as a guidewire and is funneled *via* the mouse's open mouth and through the epiglottis into the trachea. A

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20-gauge cannula is slid over the fiberoptic guidewire into the trachea and the guidewire is then retracted. The 50 μl inoculum of *Pseudomonas aeruginosa* suspended in PBS is drawn up into a 1 mL tuberculin slip tip syringe in front of a 300 μl air cushion. The syringe is placed onto the canula and the bacteria inoculum followed by the air cushion is injected into the mouse trachea in one fluent movement. The cannula is then removed, and mice are placed back in their cages to recover.

To determine bacterial load in mouse tissues, the animals were euthanized 45 min after *PA01* inoculation using EUTHASOL® Euthanasia Solution (Patterson Veterinary, Greeley, CO, USA) followed by cervical dislocation, and the lungs were then extracted, minced with scissors, and homogenized in PBS using a dounce glass homogenizer. Serial dilutions of the resulting lung homogenates were plated on LB agar plates to determine the number of live bacteria which are reported as cfu (colony forming units).

2.7. Data and Statistical Analysis

All data are expressed as the mean ± SEM and n numbers indicate the number of individual animals assessed. The GraphPad Prism 8.3 software (GraphPad Software Inc, San Diego, CA, USA) was used to perform statistical analyses. Mann-Whitney test with 95% confidence interval was used to compare two treatment groups and Kruskal-Wallis followed by Dunn's *post hoc* test was used to determine differences between more than two treatment groups. Time courses were analyzed using two-way ANOVA with Tukey's post hoc test. Statistical differences are indicated as # (not significant; $p>0.05$), * ($p<0.05$), ** ($p<0.01$), and *** ($p<0.001$).

3. RESULTS

3.1. Treatment with PAN-PDE4 inhibitors potentiates the anesthetic effects of Isoflurane in mice

While exploring the potential anti-inflammatory benefits of PDE4 inhibition in a mouse model of bacterial lung infection, we noticed that mice pretreated with the PDE4 inhibitor Piclamilast/RP73401 (5 mg/kg, i.p.; 1 h prior) appeared to lose consciousness faster upon exposure to Isoflurane anesthesia (e.g. loss of righting), and appeared deeper asleep (e.g. no muscle movement in response to moving or horizontal displacement of the anesthesia chamber). Subsequent to intranasal *Pseudomonas aeruginosa* infection, Piclamilast-treated mice also remained unconscious for longer time periods (250 ± 33 s, n=10) compared to solvent controls (59 ± 7 s, n=10), suggesting that PDE4 inhibition enhances the anesthetic effects of Isoflurane.

As this effect of PDE4 inhibition on Isoflurane anesthesia has not been reported before, and to reveal any potential impact of altered Isoflurane anesthesia on our animal model, we further explored this observation using time-to-righting assays as readouts. In short, after induction of anesthesia (3% Isoflurane in 100% oxygen for 10 min), the mouse is placed on its back into a new cage (Fig. 1A) and the time until it awakes and turns onto its abdomen/paws (Figs. 1B/C) is recorded, which is a common measure to assess the hypnotic action of anesthetics. As shown in Fig. 1D, pretreatment with several distinct, brain-penetrant PDE4

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inhibitors including Piclamilast, Rolipram, Roflumilast, and RS25344 (1 mg/kg, i.p., 30 min prior to Isoflurane anesthesia) delayed the recovery of mice from Isoflurane anesthesia, whereas treatment with YM976[39] (1 or 5 mg/kg, i.p.), a PAN-PDE4 inhibitor that exhibits similar efficacy in inhibiting cAMP hydrolysis by PDE4 compared to Piclamilast or Roflumilast, but does not efficiently cross the blood-brain barrier, had no effect. Treatment with the PDE3-selective inhibitor Cilostamide (5 mg/kg; i.p.) also did not affect Isoflurane anesthesia (Fig. 1D).

3.2. RS25344 exhibits high potency in modulating Isoflurane anesthesia

While all brain-penetrant PDE4 inhibitors tested at the dose of 1 mg/kg increased the duration of Isoflurane anesthesia, the amplitude of their effects is not the same. RS25344 seemed to be by far the most efficacious, inducing up to a 10-fold extension of anesthesia compared to solvent controls (Fig. 1D). To delineate this difference, we generated dose-response curves for RS25344 and the clinically used PDE4 inhibitor Roflumilast. As shown in Fig. 2A, RS25344 potentiates Isoflurane anesthesia at a dose as little as 0.04 mg/kg, whereas doses of ≥1 mg/kg Roflumilast are required to significantly prolong Isoflurane anesthesia (Fig 2B).

3.3. Role of PDE4D in PDE4 inhibitor-induced Isoflurane anesthesia

The PDE4 family comprises four subtypes, PDE4A, B, C and D. To determine whether one of the four PDE4 subtypes is predominantly associated with potentiation of Isoflurane anesthesia, we compared the time to righting after Isoflurane anesthesia in mice deficient in PDE4A (Fig. 3A), PDE4B (Fig. 3B), PDE4C (Fig. 3C) or PDE4D (Fig. 3D) to their respective wildtype littermates. As shown in the two left-most bars of Figs. 3A–D (Mock), genetic ablation of any of the four PDE4 subtypes *per se* did not affect the duration of Isoflurane hypnosis. To exclude the possibility that compensatory changes may have obscured the role of a specific PDE4 subtype, the effect of PDE4 inhibitor treatment on Isoflurane anesthesia was then tested in each knockout mouse line. As shown in Figs. 3A to D, treatment with RS25344 (0.2 mg/kg) increased the time to righting in PDE4A-, PDE4B-, and PDE4C-knockout mice to similar levels as their wildtype littermates. In contrast, while mice deficient in PDE4D (Fig. 3D) treated with RS25344 did sleep longer than solvent control mice, they did recover earlier from Isoflurane anesthesia compared to wildtype controls in the presence of RS25344. This pattern was mirrored in PDE4DKO mice and wildtype controls treated with the structurally distinct PDE4 inhibitor Roflumilast (Fig. 3D).

3.4. PDE4 inhibition by itself produces sedative, but not anesthetic effects

Treatment with PAN-PDE4 inhibitors has been shown to induce characteristic behavioral changes, particularly a reduction in locomotor activity (hypokinesia), that may indicate sedative effects[40]. Upon treatment with high doses (≥1 mg/kg) of PDE4 inhibitors, the mice often stop moving shortly after PDE4 inhibitor treatment, although they will walk haltingly when prompted. This is illustrated by the measurement of travel distance of mice placed in a new cage (Fig. 4A). In solvent-treated mice, locomotion is initially high as the animals explore the new environment and gradually decreases, until it reaches a steady state within ~2 h. Conversely, shortly after treatment with either Roflumilast or RS25344 (3 mg/kg), mice stop exploring the new cage. Similar to the potentiation of Isoflurane anesthesia (Figs.

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1/2), if tested at the same dose, the effect is more pronounced with RS25344 than Roflumilast, as the onset of hypokinesia is faster with RS25344, and leads to almost complete immobility for the entire duration of the test (5 h). PDE4 inhibition has also been reported to induce hypothermia in mice[40], which parallels a common side effect of general anesthesia. As shown in Fig. 4B, treatment with a high dose of RS25344 (5 mg/kg) induces a rapid and substantial decrease in body temperature that is equal to the level of hypothermia induced by Isoflurane anesthesia over the first 15 min. The time courses eventually separate as the body temperature of Isoflurane-anesthetized mice continues to decrease for the following hour, whereas the body temperature of RS25344-treated mice stabilizes at a new, reduced cold-defense set point of ~32°C.

As these observations imply that PDE4 inhibition induces sedative effects, we probed whether treatment with high doses of a PDE4 inhibitor may induce anesthetic effects even in the absence of Isoflurane. As shown in Fig. 4C, while high doses of RS25344 (5 mg/kg) significantly potentiate the duration of Isoflurane anesthesia (two right bars), treatment with RS25344 *per se* does not ablate the righting reflex in the absence of isoflurane (two left bars) as the mice immediately turn on their abdomen after being placed onto their backs.

3.5. Role of adrenergic-, serotonergic-, and opioid receptors in mediating and/or modulating the effect of PDE4 inhibitors on Isoflurane anesthesia

Prior studies have shown that treatment with PDE4 inhibitors shortens the duration of anesthesia induced by Ketamine/Xylazine anesthesia in rats, mice and ferrets[41–43], and PDE4 inhibitors are thought to produce this effect by antagonizing the activation of α_2-adenoceptors by Xylazine[42, 44]. Thus, while the effect of PDE4 inhibition on Ketamine/Xylazine anesthesia, a shortening, is the opposite of its effect on Isoflurane anesthesia, an elongation, we wished to test whether α_2-adrencoptor signaling may be involved in PDE4 inhibitor-induced potentiation of Isoflurane anesthesia. As shown in Fig. 5, treatment with the α_2-adrenoceptor agonist Clonidine potently increased the duration of Isoflurane hypnosis, producing significant effects at as a little as 0.04 mg/kg. However, the efficacy of Clonidine is limited, as even higher doses of Clonidine (e.g. 1 and 5 mg/kg) only doubled the duration of Isoflurane hypnosis. Treatment with the α_2-adrenoceptor antagonist Yohimbine (1 mg/kg; i.p.) had no effect on the duration of Isoflurane sleep, and neither α_2-adrenoceptor agonism (Clonidine), nor antagonism (Yohimbine), altered the potentiation of Isoflurane anesthesia induced by PDE4 inhibition (RS25344, 0.4 mg/kg, i.p.).

We then tested whether blockade of additional receptors that are known to modulate Isoflurane anesthesia (opioid-, 5-HT_3-, and α_1-adrenergic receptors[45–47]) and/or to be regulated by PDE4 in other physiologic paradigms (β-adrenergic receptors[23, 24, 37]), may alter PDE4 inhibitor-induced potentiation of Isoflurane anesthesia. As shown in Fig. 6, the opioid receptor blocker Naloxone had no effect on the duration of Isoflurane anesthesia *per se*, but partially reversed the potentiating effect of PDE4 inhibitor treatment. Conversely, the 5-HT_3 receptor blocker Ondansetron did not affect Isoflurane anesthesia *per se* nor protect from the potentiating effect of PDE4 inhibition. The β-adrenoceptor blocker Propranolol had the most pronounced effect on the duration of sleep in the presence of RS25344. Unexpectedly, treatment with Propranolol also shortened anesthesia induced by Isoflurane

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itself (in the absence of PDE4 inhibitor). Finally, the α_1-adrenoceptor antagonist Prazosin enhanced the duration of Isoflurane anesthesia *per se* and further increased sleep time also in the presence of PDE4 inhibitor.

3.6. In a bacterial lung infection model, PDE4 inhibition increases the effectiveness of intranasal inhalation under Isoflurane anesthesia in mice

The current study grew out of our initial observation that mice treated with PDE4 inhibitors appeared to be deeper and longer asleep after Isoflurane anesthesia employed to deliver a bacterial inoculum into the lungs of mice *via* intranasal inhalation. Intranasal infection under anesthesia is more effective than infecting an awake, scruffed mouse as several response mechanisms (e.g. altered breathing patterns, sneezing, and fast head movements) that serve to prevent inhalation of fluid in the nasal passages and airways are suppressed under anesthesia. We thus questioned whether the delay in recovery from anesthesia may affect the efficiency of intranasal delivery of bacteria into the lungs of PDE4 inhibitor-treated mice. To test this, mice were euthanized 45 minutes after bacterial infection, the lungs extracted, homogenized and the number of live bacteria in lung tissue homogenates was determined. As shown in Figure 7A, when utilizing intranasal infection under isoflurane anesthesia to deliver the bacteria, mice pretreated with the PDE4 inhibitor Piclamilast have significantly more bacteria in their lungs, compared to solvent control mice. Conversely, pre-treatment with PDE4 inhibitor had no effect on the number of bacteria delivered into the lungs after intranasal infections performed under Ketamine/Xylazine anesthesia (Fig. 7B) or if bacteria were delivered *via* intratracheal injection under Isoflurane anesthesia (Fig. 7C).

4. DISCUSSION

4.1. Potentiation of Isoflurane anesthesia is a class effect of PAN-PDE4 inhibitors in mice

We report here for the first time that inhibition of PDE4, but not inhibition of PDE3, induces a dose-dependent potentiation of Isoflurane anesthesia as detected using time-to-righting assays in mice (Figs. 1, 2). Several structurally distinct PAN-PDE4 inhibitors, including Piclamilast, Rolipram, Roflumilast and RS25344 (all 1 mg/kg, i.p.; Fig. 1), all delay recovery from Isoflurane anesthesia, suggesting this is a class effect of PDE4 family-selective inhibitors. Conversely, YM976, a PAN-PDE4 inhibitor that has a similar potency to inhibit PDE4s compared to Piclamilast and Roflumilast *in vitro* and *in vivo*[39], but is distinguished by its poor brain penetrance, had no effect on the duration of Isoflurane anesthesia, even if tested at a 5-fold higher dose. Together, these findings indicate that, in order to potentiate Isoflurane anesthesia, PDE4 inhibitors must enter the spinal cord and/or higher regions of the brain that are involved in anesthesia and are protected by the blood-brain-barrier. By the same token, this excludes the possibility that PDE4 inhibition may produce its effects in the periphery, such as on the uptake, distribution, or metabolism of Isoflurane.

Anesthesia comprises at least two main phenomena: 1. *Immobility*, which in animals is generally assessed by determining the minimum alveolar concentration (MAC) of an inhalation anesthetic required to prevent muscle movement in response to a noxious stimulus (e.g. tail clamping or electric shock); and 2. *Hypnosis*, which is assessed by measuring time

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to righting as utilized in the current study. Thus, strictly speaking, our data only confirm a potentiating effect of PDE4 inhibition on Isoflurane-induced hypnosis. Our observations suggest that PDE4 inhibition may similarly enhance Isoflurane-induced immobility, as PDE4 inhibitor-treated mice do not exhibit muscle reflex/limb movement in response to noxious stimuli (e.g. noise in the room, or movement of the anesthesia chamber) while under Isoflurane anesthesia compared to solvent-control mice. However, these subjective observations will have to be confirmed in future studies.

4.2. Potential mechanisms for the unique potency of RS25344

Intriguingly, while all brain-penetrant PDE4 inhibitors significantly potentiate Isoflurane anesthesia when tested at the same dose, RS25344 induces the most substantial delay to time-of-righting, up to 10-fold over solvent controls (Fig. 1D). Detailed dose-response curves reveal that RS25344 is considerably more potent than Roflumilast, as doses of as little as 0.04 mg/kg (Fig. 2A) induce significant effects, compared to ≥1 mg/kg for Roflumilast (Fig. 2B). A combination of unique pharmacodynamic and pharmacokinetic properties of RS25344 may provide an explanation for its unique effects. First, prior studies have shown that RS25344 exhibits some preference for PDE4D[48, 49] over other PDE4 subtypes, which might be advantageous given the predominant role of PDE4D in mediating the effect of PDE4 inhibitors on Isoflurane anesthesia (Fig. 3). Second, a PKA-mediated phosphorylation and activation of PDE4D increases the potency of RS25344 to inhibit the enzyme by ~100-fold[50], thus providing a possible explanation for the high potency of RS25344 to potentiate Isoflurane anesthesia, while also implicating PKA-phosphorylated/activated PDE4(D) as the actual target/effector to potentiate Isoflurane anesthesia. PDE4D is expressed as at least 11 known protein variants, which can be grouped into so-called “long” and “short” PDE4 variants[24, 25, 51]. Long forms contain an extended N-terminus that harbors a conserved PKA-phosphorylation motif, and phosphorylation at this site activates long forms. Conversely, so-called short forms lack these N-terminal regions and are not phosphorylated and/or activated by PKA. Thus, the effects of RS25344 also suggest that the molecular target to potentiate Isoflurane anesthesia are long-PDE4/PDE4D variants, which are abundantly expressed in all regions of the brain[52]. Third, RS25344 distinguishes two distinct conformations of PDE4s termed HARBS (high-affinity Rolipram-binding state) and LARBS (low-affinity Rolipram-binding state), exhibiting high affinity towards HARBS which is also enriched in brain compared to the periphery[53, 54]. These effects distinguish RS25344 from Piclamilast and Roflumilast, which have similar, low-nano molar potency as RS25344 (IC_{50} for Piclamilast = 0.3 to 2 nM[48, 55, 56]; IC_{50} for Roflumilast = 0.2 to 5 nM[49, 57]; IC_{50} for RS25344 = 0.3 to 19 nM[48, 49]), but do not exhibit a preference for PDE4D, and do not exhibit increased affinity towards PKA-phosphorylated PDE4 or the HARBS confirmation. However, RS25344 shares all these unique pharmacodynamic properties with Rolipram. The main reason for the differences in their ability to affect Isoflurane anesthesia may simply be due to the ~100-fold higher potency of RS25344 to inhibit cAMP hydrolysis by PDE4 compared to Rolipram (IC_{50} for RS25344 = 0.3 to 19 nM; IC_{50} for Rolipram ≥ 45 nM[48, 49]).

In addition to its unique pharmacodynamics, advantageous pharmacokinetic properties (such as a preferential distribution to and enrichment in brain regions involved in anesthesia) likely

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contribute to the unique effectiveness of RS25344 in potentiating Isoflurane anesthesia. Indeed, its faster onset/induction of hypokinesia compared to Roflumilast (Fig. 4A) is likely reflective of its rapid distribution into the brain. Moreover, RS25344 and YM976 share some structural similarities (both are pyridopyrimidine derivatives; Fig. 1D) and also inhibit PDE4 with IC_{50}s in the same, low nanomolar range in *in vitro* assays (IC_{50} for YM976=2.2 nM[56]; IC_{50} for RS25344 = 0.3 to 19 nM[39, 48, 49].Thus, differences in their tissue distribution (YM976 does not effectively cross the blood-brain barrier[39]) likely explain their disparate effects on Isoflurane anesthesia (Fig. 1D).

While theoretically possible, we consider it unlikely that an off-target effect, in addition to inhibiting PDE4, is responsible for the potent effects of RS25344 for two reasons: First, RS25344 is distinguished from other PDE4 inhibitors by higher potency, rather than higher efficacy (see Fig. 2; note that even at 5 mg/kg, the dose response curve for Roflumilast has not plateaued), which would require an off-target effector with higher affinity for RS25344, compared to PDE4. Second, ablation of PDE4D in mice causes a significant decrease in the ability of RS25344 to potentiate Isoflurane anesthesia, suggesting that PDE4(D) is the principal target whereby RS25344 exerts its effects on Isoflurane anesthesia (Fig. 3D). However, in light of the fact the Isoflurane itself does affect multiple targets in the cAMP signaling pathway, including protein kinase A (PKA) and adenylyl cyclase[58], it is possible that Isoflurane itself may affect the pharmacodynamic and/or the pharmacokinetic behavior/properties of the various PDE4 inhibitors tested in a manner favorable to RS25344.

4.3. Role of PDE4D in mediating the effect of PAN-PDE4 inhibitors on Isoflurane anesthesia

Prior studies have shown that despite exhibiting seemingly identical substrate kinetics (k_m ~ 1 to 3 μM cAMP; V_{max} ~ 1 to 10 μmol/min/mg protein), individual PDE4 subtypes and splicing variants exert distinct and non-overlapping physiological roles[23, 24, 37, 51]. Expression of individual PDE4 isoforms in distinct cells and tissues may contribute to this outcome in *in vivo* models. However, even if expressed in the same cell, distinct PDE4 variants exert unique functions and generally cannot compensate for each other. This begs the question of which PDE4 subtype(s) regulate Isoflurane anesthesia. Experiments using knockout mice for each of the four PDE4 subtypes suggest that PDE4D may be the predominant, but is likely not the sole, PDE4 subtype involved in the potentiating effect of PDE4 inhibitors on Isoflurane anesthesia. As shown in Figs. 3A–D, ablation of any individual PDE4 subtype by itself, does not delay recovery from Isoflurane anesthesia suggesting that ablation of any single PDE4 subtype is either not sufficient to produce an effect, or that compensatory mechanisms in response to the long-term, global PDE4 ablation in the knockout mice preclude the Isoflurane phenotype. However, the potentiating effect of treatment with RS25344 is significantly reduced in PDE4D knockout mice, compared to their wildtype littermates, whereas PDE4A-, PDE4B-, and PDE4C knockout mice do not behave differently from their wildtype littermates, indicating that PDE4D is the primary target of PAN-PDE4 inhibitors to potentiate Isoflurane anesthesia. However, RS25344 delays recovery from anesthesia compared to solvent controls even in PDE4D knockout mice, consistent with the idea that RS25344 inhibits one or more additional PDE4 subtypes to produce its full effect. It remains to be determined whether acute pharmacologic

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inhibition of PDE4D is sufficient to potentiate Isoflurane anesthesia because highly selective PDE4D inhibitors are currently not available. However, as discussed above, a limited selectivity for PDE4D may well be a contributing factor for the high potency of RS25344, compared to other PAN-PDE4 inhibitors.

4.4. Elucidating the molecular mechanisms of PDE4 inhibitor-induced potentiation of Isoflurane anesthesia

As shown previously[40], acute PDE4 inhibition produces effects mirroring the induction of anesthesia, such as reduced locomotion (hypokinesia; Fig. 4A), or a reduction in core body temperature (Fig. 4B). However, even after treatment with high doses of RS25344, the animals do not lose consciousness at any time, given that their righting reflex remains intact (Fig. 4C). Thus, the dramatic effect of RS25344 on the time-to-righting after Isoflurane must be described as synergistic. By definition, individual drugs must act *via* distinct molecular mechanisms to produce synergistic effects. Therefore, PDE4 inhibition provides a novel pharmacologic approach to enhance Isoflurane anesthesia and is unlikely to act *via* the same mechanisms that directly mediate Isoflurane's actions[45, 47, 59, 60].

While an effect of PDE4 inhibition on Isoflurane anesthesia has not been reported to our knowledge before, prior studies have shown that PDE4 inhibition affects the duration of Ketamine/Xylazine anesthesia. Studies have shown that treatment with PDE4 inhibitors shortens the duration of Ketamine/Xylazine anesthesia in rats, mice and ferrets[41–43], and PDE4 inhibitors are thought to produce this effect by antagonizing the activation of α_2-adenoceptors by Xylazine[42, 44]. Thus, while the effect of PDE4 inhibition on Ketamine/Xylazine anesthesia, a shortening, is the opposite of its effect on Isoflurane anesthesia, an elongation, we tested whether α_2-adrencoptor signaling may be involved in PDE4 inhibitor-induced potentiation of Isoflurane anesthesia. The α_2-adrenoceptor agonist Clonidine potently increased the duration of Isoflurane hypnosis, producing significant effects at as a little as 0.04 mg/kg (Fig. 5). This aligns with prior reports indicating that α_2-adrenoceptor agonists can lower the MAC of Isoflurane in humans[46]. However, the efficacy of Clonidine is somewhat limited, as even much higher doses induce a doubling of the duration of Isoflurane hypnosis at best. Treatment with an α_2-adrenoceptor antagonist, Yohimbine, had no effect on the duration of Isoflurane sleep, and neither agonism (Clonidine), nor antagonism (Yohimbine), altered potentiation of isoflurane sleep induced by treatment with RS25344. These data suggest that while Isoflurane anesthesia can be prolonged by activation of α_2-adrenoceptors with Clonidine, α_2-adrenoceptor activation is not directly involved in producing anesthesia by Isoflurane *per se*, nor its potentiation by PDE4 inhibition.

The complex molecular mechanisms of how Isoflurane and related ethers induce anesthesia remain poorly defined, primarily because these drugs act as low-affinity ligands on a plethora of molecular targets. Current knowledge suggests that Isoflurane induces anesthesia through direct effects on multiple sites including glycine- and N-methyl-D-aspartate (NMDA)-receptors (immobility) as well as γ-aminobutyric acid A ($GABA_A$)-receptors (hypnosis) in addition to multiple effectors that exert indirect/modulating roles including α-adrenergic-, opioid-, and 5-hydroxytryptamine 3 (5-HT_3)- or 5-HT_2-receptors[45–47, 59–63]. Further research is needed for a complete understanding of the molecular mechanism of

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these inhalation anesthetics, which in turn would facilitate rational, target-oriented development of novel anesthetics.

To begin exploring the molecular mechanism(s) of PDE4 inhibitor-mediated potentiation of Isoflurane anesthesia, we tested whether blockade of receptors that are known to modulate Isoflurane anesthesia (opioid-, 5-HT_3, and α_1-adrenergic receptors[45–47]) and/or are known to be regulated by PDE4 in other physiologic paradigms (β-adrenergic receptors[31, 64]), may alter PDE4 inhibitor-induced potentiation of Isoflurane anesthesia. As shown in Fig. 6, the 5-HT_3 receptor blocker Ondansetron did not affect Isoflurane anesthesia nor modulate the potentiating effect of PDE4 inhibition, suggesting that the duration of Isoflurane hypnosis in mice, and its potentiation by PDE4 inhibition, are both independent of baseline 5-HT_3 receptor signaling in the absence of exogenous agonist, and parallels prior reports that Isoflurane does not produce immobility by directly activating 5-HT_3 receptors[45]. The α_1-adrenoceptor antagonist Prazosin enhanced the duration of Isoflurane anesthesia and further increased sleep time in the presence of PDE4 inhibitor indicating that while blockade of α_1-adrenoceptor signaling potentiates Isoflurane anesthesia, it acts independently and is not mediating or modulating the effect of PDE4 inhibition on Isoflurane. The effects of Prazosin on the duration of Isoflurane hypnosis align with prior reports indicating that Isoflurane may induce pronociceptive effects *via* supraspinal α_1-adrenoceptors[65], hence, suppression of this mechanism by Prazosin may serve to deepen anesthesia. The opioid receptor blocker Naloxone had no effect on the duration of Isoflurane anesthesia, suggesting that in the absence of exogenous agonist, opioid receptors do not directly mediate Isoflurane hypnosis. However, Naloxone partially reversed the potentiating effect of PDE4 inhibitor treatment suggesting that PDE4 inhibition may act in part *via* inducing or amplifying opioid receptor signaling. The β-adrenoceptor blocker Propranolol had the most pronounced effect on the duration of sleep in the presence of RS25344, suggesting that PDE4 inhibition may potentiate Isoflurane anesthesia by amplifying the G_s/cAMP axis of β-adrenoceptor signaling. Unexpectedly, Propranolol also reduced the duration of Isoflurane anesthesia *per se*, indicating that Isoflurane anesthesia may be mediated in part *via* β-adrenoceptor activation, revealing a heretofore unrecognized role of β-adrenoceptor signaling in mediating Isoflurane anesthesia. Thus, the substantial reduction of anesthesia after Isoflurane/PDE4 inhibitor treatment may result from the combined ablation of the effects of PDE4 inhibitors and Isoflurane on β-adrenoceptor signaling.

4.5. Experimental and clinical relevance of PDE4 inhibitor-induced potentiation of Isoflurane anesthesia

We show here that pretreatment with a PDE4 inhibitor boosts the delivery of bacteria into the lungs of mice after intranasal infection under Isoflurane (Fig. 7A); thus providing a first example that PDE4 inhibitor-induced potentiation of Isoflurane anesthesia can critically impact animal models and must be considered as a factor in experimental design. Treatment with PDE4 inhibitors produces a range of acute effects in mice, including anti-inflammatory effects, which may theoretically affect a lung infection model. However, we consider the increased delivery of bacteria into the lungs a direct consequence of the delayed recovery of PDE4 inhibitor-treated mice from Isoflurane anesthesia, given that we do not observe the same effect when using Ketamine/Xylazine (Fig. 7B), likely because recovery from

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Ketamine/Xylazine-anesthesia is much slower (~30 min) compared to Isoflurane (~ 1 min), or upon intratracheal delivery of the bacteria (Fig. 7C), likely because this approach is independent of the animals' breathing pattern and hence consciousness. A prior report has shown that the bacterial load in mice pretreated with the PDE4 inhibitor Roflumilast (≥5 mg/kg) is elevated at 16 h after intranasal infection under Isoflurane[66]. While the authors propose that this results from the immunosuppressant effects of the PDE4 inhibitor, one may speculate that an increase in initial loading due to effects on Isoflurane anesthesia may have been a contributing factor.

Despite significant progress, there remains a need for novel anesthetics with improved efficacy and adverse profiles; particularly drugs that exploit synergism between distinct molecular mechanisms to augment anesthetic effects, without producing synergism (or additivity) of adverse effects[46]. This could principally be accomplished by development of a single molecule that acts simultaneously on distinct molecular targets, or by discovery of suitable combinations of drugs that act on individual targets to produce synergistic anesthetic effects. Having established a relevance for the PDE4 inhibitor-mediated potentiation of Isoflurane anesthesia in an animal model, begs the question of whether this observation may also be exploited for clinical applications. Clearly, the observation that inhibition of PDE4 exerts a major impact on the efficacy of Isoflurane anesthesia provides a novel pharmacologic tool to study and delineate how Isoflurane and related ethers promote anesthesia (as well as how they induce their adverse effects) at the molecular level, thus facilitating target validation and rational drug development. Whether inhibition of PDE4 *per se* could be pursued as an approach to potentiate Isoflurane's anesthetic effects, perhaps acting in a dose-sparing manner, remains to be tested. Specifically, whether our findings in mice translate to humans, and whether effects of PDE4 inhibition on Isoflurane-induced hypnosis will similarly extend to Isoflurane-induced immobility, remains to be verified. In addition, side effects, particularly nausea and emesis, have historically dampened enthusiasm for PAN-PDE4 inhibitors as therapeutics for various indications[51, 67]. Thus, the clinical utility of PDE4 inhibitors in enhancing the anesthetic effects of Isoflurane, may also depend on the interaction between the adverse effects of these drugs (e.g. negate, neutral, additive, or synergistic). If confirmed in humans, our data suggest that RS25344 may serve as a useful lead structure for the development of drugs that potentiate Isoflurane anesthesia, and that PDE4D may be the main molecular target whereby PAN-PDE4 inhibitors extend Isoflurane-induced sleep; thus, selective pharmacological inhibition of PDE4D may be sufficient to potentiate Isoflurane anesthesia.

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Abbreviations:

DMSO	Dimethyl sulfoxide

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$GABA_A$ receptors	γ-aminobutyric acid A receptors
5-HT_3 receptor	5-Hydroxytryptamine receptor 3
i.p.	intraperitoneal
KO	Knockout
NMDA receptors	N-methyl-D-aspartate receptors
PBS	Phosphate-buffered saline
PDE	cyclic nucleotide phosphodiesterase
PDE4	cAMP phosphodiesterase 4
WT	Wildtype

References

1. Brioni JD, Varughese S, Ahmed R, Bein B. A clinical review of inhalation anesthesia with sevoflurane: from early research to emerging topics. J Anesth. 2017:764–78. vol. 5.
2. Clar DT, Richards JR. Anesthetic Gases. Text. 2020/3/25 2020;doi:https://www.ncbi.nlm.nih.gov/books/NBK537013/
3. Servin FS. Update on Pharmacology of Hypnotic Drugs. Current opinion in anaesthesiology. 2008 8 2008;21(4)doi:10.1097/ACO.0b013e3283079b82
4. Preckel B, Bolten J. Pharmacology of Modern Volatile Anaesthetics. Best practice & research Clinical anaesthesiology. 2005 9 2005;19(3)doi:10.1016/j.bpa.2005.01.003
5. Patel SS, Goa KL. Sevoflurane. OriginalPaper. Drugs. 2012-10-31 2012;51(4):658–700. doi:doi:10.2165/00003495-199651040-00009
6. Apfel CC, Stoecklein K, Lipfert P. PONV: A Problem of Inhalational Anaesthesia? Best practice & research Clinical anaesthesiology. 2005 9 2005;19(3)doi:10.1016/j.bpa.2005.03.001
7. Apfel CC, Kranke P, Katz MH, et al. Volatile Anaesthetics May Be the Main Cause of Early but Not Delayed Postoperative Vomiting: A Randomized Controlled Trial of Factorial Design. British journal of anaesthesia. 2002 5 2002;88(5)doi:10.1093/bja/88.5.659
8. Mullins MF. Malignant Hyperthermia: A Review. Journal of perianesthesia nursing : official journal of the American Society of PeriAnesthesia Nurses. 2018 10 2018;33(5)doi:10.1016/j.jopan.2017.04.008
9. Belrose JC, Noppens RR. Anesthesiology and Cognitive Impairment: A Narrative Review of Current Clinical Literature. BMC anesthesiology. 12/27/2019 2019;19(1)doi:10.1186/s12871-019-0903-7
10. McCann ME, Soriano SG. Does General Anesthesia Affect Neurodevelopment in Infants and Children? BMJ (Clinical research ed). 12/09/2019 2019;367doi:10.1136/bmj.l6459
11. Stratmann G, Sall JW, May LD, Loepke AW, Lee MT. Beyond Anesthetic Properties: The Effects of Isoflurane on Brain Cell Death, Neurogenesis, and Long-Term Neurocognitive Function. Anesthesia and analgesia. 2010 2 2010;110(2)
12. Jiao B, Yang C, Huang NN, Yang N, Wei J, Xu H. Relationship Between Volatile Anesthetics and Tumor Progression: Unveiling the Mystery. Current medical science. 2018 12 2018;38(6)doi:10.1007/s11596-018-1970-6
13. Kim R Effects of surgery and anesthetic choice on immunosuppression and cancer recurrence. Journal of translational medicine. 1/18/2018 2018;16(1)doi:10.1186/s12967-018-1389-7
14. Xie Z, Xu Z. General Anesthetics and β-amyloid Protein. Progress in neuro-psychopharmacology & biological psychiatry. 12/02/2013 2013;47doi:10.1016/j.pnpbp.2012.08.002

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15. Jiang J, Jiang H. Effect of the Inhaled Anesthetics Isoflurane, Sevoflurane and Desflurane on the Neuropathogenesis of Alzheimer's Disease (Review). Molecular medicine reports. 2015 7 2015;12(1)doi:10.3892/mmr.2015.3424
16. Herzog-Niescery J, Seipp HM, Weber TP, Bellgardt M. Inhaled Anesthetic Agent Sedation in the ICU and Trace Gas Concentrations: A Review. Journal of clinical monitoring and computing. 2018 8 2018;32(4)doi:10.1007/s10877-017-0055-6
17. Richter W, Menniti FS, Zhang HT, Conti M. PDE4 as a target for cognition enhancement. Expert Opin Ther Targets. 7 2013;doi:10.1517/14728222.2013.818656
18. Reneerkens OA, Rutten K, Steinbusch HW, Blokland A, Prickaerts J. Selective phosphodiesterase inhibitors: a promising target for cognition enhancement. Psychopharmacology (Berl). 1 2009;202(1–3):419–43. doi:10.1007/s00213-008-1273-x [PubMed: 18709359]
19. Xu Y, Zhang HT, O'Donnell JM. Phosphodiesterases in the central nervous system: implications in mood and cognitive disorders. Handbook of experimental pharmacology. 2011 2011; (204)doi:10.1007/978-3-642-17969-3_19
20. Murthy VS, Mangot AG. Psychiatric aspects of phosphodiesterases: An overview. Indian J Pharmacol. 2015:594–9. vol. 6.
21. Li ZH, Cui D, Qiu CJ, Song XJ. Cyclic nucleotide signaling in sensory neuron hyperexcitability and chronic pain after nerve injury. Neurobiology of pain (Cambridge, Mass). 3/08/2019 2019;6doi:10.1016/j.ynpai.2019.100028
22. Sassone-Corsi P The cyclic AMP pathway. Cold Spring Harb Perspect Biol. 12 2012;4(12)doi:10.1101/cshperspect.a011148
23. Conti M, Beavo J. Biochemistry and physiology of cyclic nucleotide phosphodiesterases: essential components in cyclic nucleotide signaling. Annu Rev Biochem. 2007;76:481–511. doi:10.1146/annurev.biochem.76.060305.150444 [PubMed: 17376027]
24. Houslay MD, Adams DR. PDE4 cAMP phosphodiesterases: modular enzymes that orchestrate signalling cross-talk, desensitization and compartmentalization. Biochem J. 2 15 2003;370(Pt 1):1–18. doi:10.1042/bj20021698 [PubMed: 12444918]
25. Conti M, Richter W, Mehats C, Livera G, Park JY, Jin C. Cyclic AMP-specific PDE4 phosphodiesterases as critical components of cyclic AMP signaling. J Biol Chem. 2 2003;278(8):5493–6. doi:10.1074/jbc.R200029200 [PubMed: 12493749]
26. Maurice DH, Ke H, Ahmad F, Wang Y, Chung J, Manganiello VC. Advances in targeting cyclic nucleotide phosphodiesterases. Nat Rev Drug Discov. 4 2014;13(4):290–314. doi:10.1038/nrd4228 [PubMed: 24687066]
27. Baillie GS, Tejeda GS, Kelly MP. Therapeutic targeting of 3',5'-cyclic nucleotide phosphodiesterases: inhibition and beyond. Nat Rev Drug Discov. 10 2019;18(10):770–796. doi:10.1038/s41573-019-0033-4 [PubMed: 31388135]
28. Houslay MD, Schafer P, Zhang KY. Keynote review: phosphodiesterase-4 as a therapeutic target. Drug Discov Today. 11 15 2005;10(22):1503–19. doi:S1359-6446(05)03622-6 [pii] 10.1016/S1359-6446(05)03622-6 [PubMed: 16257373]
29. Zhang KY, Ibrahim PN, Gillette S, Bollag G. Phosphodiesterase-4 as a potential drug target. Expert Opin Ther Targets. 12 2005;9(6):1283–305. doi:10.1517/14728222.9.6.1283 [PubMed: 16300476]
30. Jin SL, Ding SL, Lin SC. Phosphodiesterase 4 and its inhibitors in inflammatory diseases. Chang Gung Med J. May-Jun 2012;35(3):197–210. doi:3503/350301 [pii] [PubMed: 22735051]
31. Houslay MD, Baillie GS, Maurice DH. cAMP-Specific phosphodiesterase-4 enzymes in the cardiovascular system: a molecular toolbox for generating compartmentalized cAMP signaling. Circ Res. 4 13 2007;100(7):950–66.doi:100/7/950 [pii] 10.1161/01.RES.0000261934.56938.38 [PubMed: 17431197]
32. Wu C, Rajagopalan S. Phosphodiesterase-4 inhibition as a therapeutic strategy for metabolic disorders. Obes Rev. 5 2016;17(5):429–41. doi:10.1111/obr.12385 [PubMed: 26997580]
33. Peng T, Gong J, Jin Y, et al. Inhibitors of phosphodiesterase as cancer therapeutics. Eur J Med Chem. 4 2018;150:742–756. doi:10.1016/j.ejmech.2018.03.046 [PubMed: 29574203]
34. Hansen RT, Conti M, Zhang HT. Mice deficient in phosphodiesterase-4A display anxiogenic-like behavior. Psychopharmacology (Berl). 2 2014;doi:10.1007/s00213-014-3480-y

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35. Jin SL, Conti M. Induction of the cyclic nucleotide phosphodiesterase PDE4B is essential for LPS-activated TNF-alpha responses. Proc Natl Acad Sci U S A. 5 2002;99(11):7628–33. doi:10.1073/pnas.122041599 [PubMed: 12032334]
36. Jin SL, Richard FJ, Kuo WP, D'Ercole AJ, Conti M. Impaired growth and fertility of cAMP-specific phosphodiesterase PDE4D-deficient mice. Proc Natl Acad Sci U S A. 10 1999;96(21):11998–2003. [PubMed: 10518565]
37. Jin SLC, Richter W, Conti M. Insights into the physiological functions of PDE4 derived from knockout mice. in Cyclic Nucleotide Phosphodiesterases in Health and Disease (Beavo JA, Francis SH, Houslay MD, ed), P 323–346, CRC Press, Boca Raton, FL. 2006;
38. McDonough W, Aragon IV, Rich J, et al. PAN-selective inhibition of cAMP-phosphodiesterase 4 (PDE4) induces gastroparesis in mice. FASEB journal : official publication of the Federation of American Societies for Experimental Biology. 8/01/2020 2020;doi:10.1096/fj.202001016RR
39. Aoki M, Fukunaga M, Sugimoto T, et al. Studies on mechanisms of low emetogenicity of YM976, a novel phosphodiesterase type 4 inhibitor. J Pharmacol Exp Ther. 9 2001;298(3):1142–9. [PubMed: 11504812]
40. McDonough W, Rich J, Aragon IV, et al. Inhibition of type 4 cAMP-phosphodiesterases (PDE4s) in mice induces hypothermia via effects on behavioral and central autonomous thermoregulation. Biochemical pharmacology. 7/20/2020 2020;180doi:10.1016/j.bcp.2020.114158
41. Robichaud A, Savoie C, Stamatiou PB, et al. Assessing the emetic potential of PDE4 inhibitors in rats. Br J Pharmacol. 1 2002;135(1):113–8. doi:10.1038/sj.bjp.0704457 [PubMed: 11786486]
42. Robichaud A, Stamatiou PB, Jin SL, et al. Deletion of phosphodiesterase 4D in mice shortens alpha(2)-adrenoceptor-mediated anesthesia, a behavioral correlate of emesis. J Clin Invest. 10 2002;110(7):1045–52. doi:10.1172/jci15506 [PubMed: 12370283]
43. Robichaud A, Tattersall FD, Choudhury I, Rodger IW. Emesis induced by inhibitors of type IV cyclic nucleotide phosphodiesterase (PDE IV) in the ferret. Neuropharmacology. 2 1999;38(2):289–97. doi:10.1016/s0028-3908(98)00190-7 [PubMed: 10218871]
44. Robichaud A, Savoie C, Stamatiou PB, Tattersall FD, Chan CC. PDE4 inhibitors induce emesis in ferrets via a noradrenergic pathway. Neuropharmacology. 2001;40(2):262–9. doi:10.1016/s0028-3908(00)00142-8 [PubMed: 11114405]
45. Sonner JM, Antognini JF, Dutton RC, et al. Inhaled Anesthetics and Immobility: Mechanisms, Mysteries, and Minimum Alveolar Anesthetic Concentration. Anesthesia and analgesia. 2003 9 2003;97(3)doi:10.1213/01.ane.0000081063.76651.33
46. Hendrickx JF, Eger EI, Sonner JM, Shafer SL. Is Synergy the Rule? A Review of Anesthetic Interactions Producing Hypnosis and Immobility. Anesthesia and analgesia. 2008 8 2008;107(2)doi:10.1213/ane.0b013e31817b859e
47. Eckle VS, Hucklenbruch C, Todorovic SM. [What do we know about anesthetic mechanisms?: hypnosis, unresponsiveness to surgical incision and amnesia]. Der Anaesthesist. 2009 11 2009;58(11)doi:10.1007/s00101-009-1618-9
48. Saldou N, Obernolte R, Huber A, et al. Comparison of recombinant human PDE4 isoforms: interaction with substrate and inhibitors. Cell Signal. 6 1998;10(6):427–40. doi:10.1016/s0898-6568(97)00169-1 [PubMed: 9720765]
49. Burgin AB, Magnusson OT, Singh J, et al. Design of phosphodiesterase 4D (PDE4D) allosteric modulators for enhancing cognition with improved safety. Nat Biotechnol. 1 2010;28(1):63–70. doi:nbt.1598 [pii] 10.1038/nbt.1598 [PubMed: 20037581]
50. Alvarez R, Sette C, Yang D, et al. Activation and selective inhibition of a cyclic AMP-specific phosphodiesterase, PDE4D3. MolPharmacol. 1995;48:616–622.
51. Lugnier C Cyclic nucleotide phosphodiesterase (PDE) superfamily: a new target for the development of specific therapeutic agents. Pharmacology & therapeutics. 2006 3 2006;109(3)doi:10.1016/j.pharmthera.2005.07.003
52. Richter W, Jin SL, Conti M. Splice variants of the cyclic nucleotide phosphodiesterase PDE4D are differentially expressed and regulated in rat tissue. Biochem J. 6 2005;388(Pt 3):803–11. doi:10.1042/BJ20050030 [PubMed: 15717866]
53. Souness JE, Rao S. Proposal for pharmacologically distinct conformers of PDE4 cyclic AMP phosphodiesterases. Cell Signal. 1997 May-Jun 1997;9(3–4):227–36. [PubMed: 9218122]

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54. Boomkamp SD, McGrath MA, Houslay MD, Barnett SC. Epac and the high affinity rolipram binding conformer of PDE4 modulate neurite outgrowth and myelination using an in vitro spinal cord injury model. Br J Pharmacol. 5 2014;171(9):2385–98. doi:10.1111/bph.12588 [PubMed: 24467222]
55. Richter W, Conti M. The oligomerization state determines regulatory properties and inhibitor sensitivity of type 4 cAMP-specific phosphodiesterases. J Biol Chem. 7 2004;279(29):30338–48. doi:10.1074/jbc.M312687200 [PubMed: 15131123]
56. Aoki M, Kobayashi M, Ishikawa J, et al. A novel phosphodiesterase type 4 inhibitor, YM976 (4-(3-chlorophenyl)-1,7-diethylpyrido[2,3-d]pyrimidin-2(1H)-one), with little emetogenic activity. The Journal of pharmacology and experimental therapeutics. 2000 10 2000;295(1)
57. Hatzelmann A, Morcillo EJ, Lungarella G, et al. The preclinical pharmacology of roflumilast--a selective, oral phosphodiesterase 4 inhibitor in development for chronic obstructive pulmonary disease. Pulm Pharmacol Ther. 8 2010;23(4):235–56. doi:S1094–5539(10)00045–3 [pii] 10.1016/j.pupt.2010.03.011 [PubMed: 20381629]
58. Constantinides C, Murphy K. Molecular and Integrative Physiological Effects of Isoflurane Anesthesia: The Paradigm of Cardiovascular Studies in Rodents using Magnetic Resonance Imaging. Frontiers in cardiovascular medicine. 7/29/2016 2016;3doi:10.3389/fcvm.2016.00023
59. Grasshoff C, Antkowiak B. Effects of Isoflurane and Enflurane on GABAA and Glycine Receptors Contribute Equally to Depressant Actions on Spinal Ventral Horn Neurones in Rats. British journal of anaesthesia. 2006 11 2006;97(5)doi:10.1093/bja/ael239
60. Petrenko AB, Yamakura T, Sakimura K, Baba H. Defining the role of NMDA receptors in anesthesia: are we there yet? European journal of pharmacology. 1/15/2014 2014;723doi:10.1016/j.ejphar.2013.11.039
61. Narahashi T, Aistrup GL, Lindstrom JM, et al. Ion channel modulation as the basis for general anesthesia. Toxicology letters. 11/23/1998 1998;100–101doi:10.1016/s0378-4274(98)00184-2
62. Larsen M, Langmoen IA. The effect of volatile anaesthetics on synaptic release and uptake of glutamate. Toxicology letters. 11/23/1998 1998;100–101doi:10.1016/s0378-4274(98)00165-9
63. Kotani N, Akaike N. The effects of volatile anesthetics on synaptic and extrasynaptic GABA-induced neurotransmission. Brain research bulletin. 2013 4 2013;93doi:10.1016/j.brainresbull.2012.08.001
64. Bobin P, Belacel-Ouari M, Bedioune I, et al. Cyclic nucleotide phosphodiesterases in heart and vessels: A therapeutic perspective. Arch Cardiovasc Dis. Jun-Jul 2016;109(6–7):431–43. doi:10.1016/j.acvd.2016.02.004 [PubMed: 27184830]
65. Kingery WS, Agashe GS, Guo TZ, et al. Isoflurane and nociception: spinal alpha2A adrenoceptors mediate antinociception while supraspinal alpha1 adrenoceptors mediate pronociception. Anesthesiology. 2002 2 2002;96(2)doi:10.1097/00000542-200202000-00023
66. Kasetty G, Papareddy P, Bhongir RK, Egesten A. Roflumilast Increases Bacterial Load and Dissemination in a Model of Pseudomononas Aeruginosa Airway Infection. J Pharmacol Exp Ther. 4 2016;357(1):66–72. doi:10.1124/jpet.115.229641 [PubMed: 26865680]
67. Bourguignon JJ, Désaubry L, Raboisson P, Wermuth CG, Lugnier C. 9-Benzyladenines: potent and selective cAMP phosphodiesterase inhibitors. Journal of medicinal chemistry. 6/06/1997 1997;40(12)doi:10.1021/jm960827x

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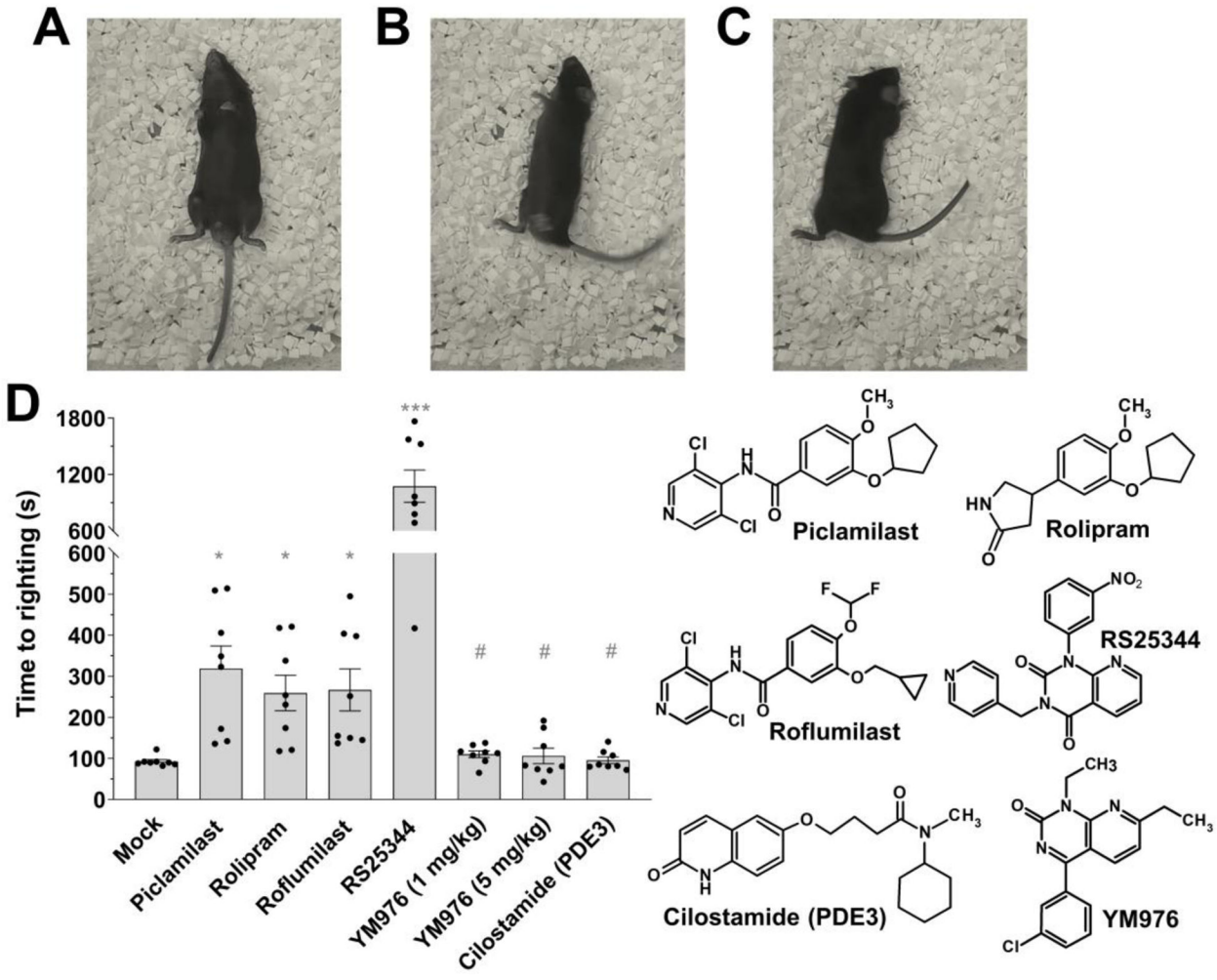

Fig. 1. Potentiation of Isoflurane anesthesia is a class effect of brain-penetrant PAN-PDE4 inhibitors.

(**A-C**) Representative images illustrating the approach to measure "Time to righting" after induction of anesthesia using Isoflurane. Upon removal from the Isoflurane induction chamber, the unconscious mice are placed on their backs (**A**). As the anesthetic effect of Isoflurane wears off and the animals awaken, the righting reflex, an automatic reaction to move the body in its normal position, kicks in (**B**) and the mice turn onto their abdomen (**C**). The time from removal from the Isoflurane induction chamber to the time to first righting is recorded. (**D**) Thirty min after i.p. injection of the brain-penetrant PAN-PDE4 inhibitors Piclamilast, Rolipram, Roflumilast, or RS25344 (each at 1 mg/kg), the poorly brain-penetrant PAN-PDE4 inhibitor YM976[39] (1 or 5 mg/kg as indicated), the PDE3 inhibitor Cilostamide (5 mg/kg) or solvent controls (Mock), mice were placed in an anesthesia induction chamber ventilated with 3% Isoflurane in 100% oxygen at 1 L/min for 10 min. The animals were then removed from the anesthesia chamber, placed into new cages on their backs and the time to first righting was recorded. Data represent the mean ± SEM. Statistical significance was determined using Kruskal-Wallis and Dunn's post hoc tests and is indicated as # (not significant; $p>0.05$), * ($p<0.05$), and *** ($p<0.001$). The chemical structures of the PDE inhibitors used are shown for comparison.

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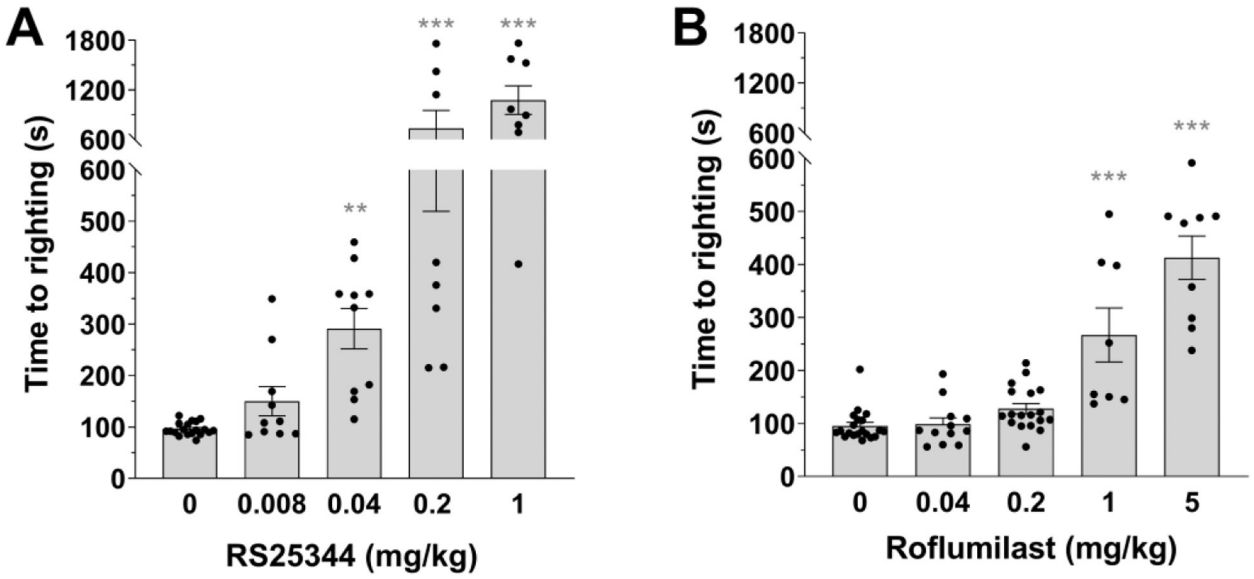

Fig. 2. Dose-dependent extension of Isoflurane anesthesia by PDE4 inhibitors RS25344 and Roflumilast.

Thirty min after injection with the indicated doses of the PAN-PDE4 inhibitors RS25344 (**A**) or Roflumilast (**B**), mice were placed in an anesthesia induction chamber ventilated with 3% Isoflurane in 100% oxygen at 1 L/min for 10 min. Mice were then removed from the anesthesia chamber, placed on their backs into new cages and the time to first righting was measured. Data represent the mean ± SEM. Statistical significance was determined using Kruskal-Wallis and Dunn's post hoc tests and is indicated as ** ($p<0.01$), and *** ($p<0.001$).

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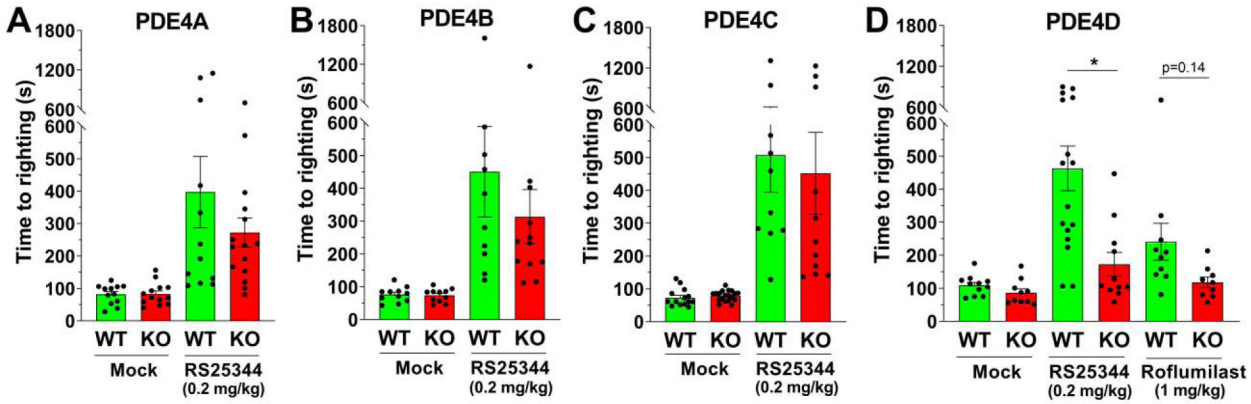

Fig. 3. Selective ablation of individual PDE4 subtypes *per se* does not potentiate Isoflurane anesthesia, but ablation of PDE4D protects from the effect of PAN-PDE4 inhibition.

Thirty min after i.p. injection with the PAN-PDE4 inhibitor RS25344 (0.2 mg/kg), Roflumilast (1 mg/kg) or solvent control (Mock), mice were placed in an anesthesia induction chamber ventilated with 3% Isoflurane in 100% oxygen at 1 L/min for 10 min. Mice were then removed from the anesthesia chamber, placed on their backs into new cages and the time to first righting was measured. Genetic ablation of any of the four PDE4 subtypes by itself (Mock; two left columns of each graph) does not delay righting after Isoflurane anesthesia compared to their respective wildtype littermates. Treatment with RS25344 produces a significant delay in time to righting in 4AWT (**), 4AKO (**), 4BWT (*), 4BKO (**), 4CWT (***), 4CKO (***),4DWT (***), and 4DKO mice (*, p=0.044). Upon treatment with RS25344, mice deficient in PDE4A (**A**), PDE4B (**B**) or PDE4C (**C**) take similar times to recover from anesthesia compared to the wildtype littermates (RS25344; two right columns of each graph) suggesting that they are not protected from the potentiating effect of PDE4 inhibitor treatment on the duration of Isoflurane anesthesia. Conversely, compared to their PDE4 inhibitor-treated wildtype littermates, mice deficient in PDE4D (**D**) recover earlier from anesthesia after treatment with either RS25344 or Roflumilast, suggesting that they are partially protected from the potentiating effect of PDE4 inhibition on the duration of Isoflurane anesthesia. Data represent the mean ± SEM. Statistical significance was determined using Kruskal-Wallis and Dunn's post hoc tests and is indicated as * ($p<0.05$).

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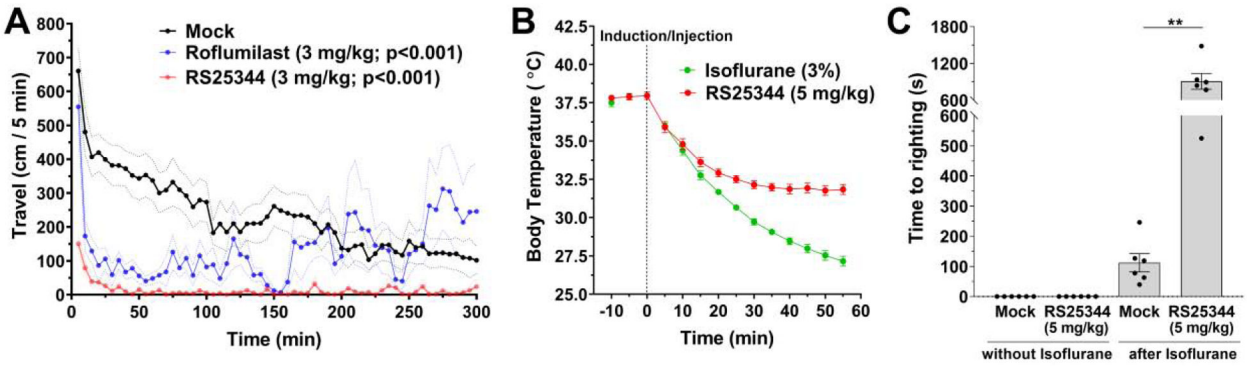

Fig. 4. PDE4 inhibition *per se* produces sedative, but not anesthetic effects.

PDE4 inhibition produces potentially sedative effects as reflected by reduced locomotion. Mice were injected (i.p.) with the PDE4 inhibitors RS25344 (3 mg/kg; n=6) or Roflumilast (3 mg/kg; n=6), or with solvent controls (Mock; n=12), placed immediately in a new cage and locomotion was assessed using SmartCageTM technology. Traces represent changes in travel distance (cm per 5 min interval) and are expressed as the mean ± SEM. Traces for RS25344 and Roflumilast were statistically different ($p<0.001$) from the solvent control as determined by two-way ANOVA and Tukey's post hoc test. (**B**) Mirroring the effect of systemic anesthesia, PDE4 inhibition induces hypothermia in mice. Shown is the time course of core body temperature, measured using a rectal probe thermometer, after treatment with the PDE4 inhibitor RS25344 (5 mg/kg; i.p.; n=8) or after induction of Isoflurane anesthesia (3% Isoflurane in 100% oxygen; n=5). The striated line indicates the time point of drug injection or induction of anesthesia. Data represent the mean ± SEM. (**C**) Thirty min after injection with 5 mg/kg RS25344 or solvent control, we attempted to place mice on their backs (two left panels; without Isoflurane). However, mice immediately return onto their abdomen suggesting that treatment with RS25344 by itself does not produce anesthetic effects. Conversely, if anesthesia is induced with Isoflurane (3% Isoflurane in 100% oxygen at 1 L/min for 10 min), treatment with RS25344 produces a significant potentiation of Isoflurane hypnosis (two right panels; after Isoflurane) suggesting that the effect of PDE4 inhibition on Isoflurane anesthesia is mechanistically synergistic. Data represent the mean ± SEM. Statistical significance was determined using Mann-Whitney test with 95% confidence interval and is indicated as

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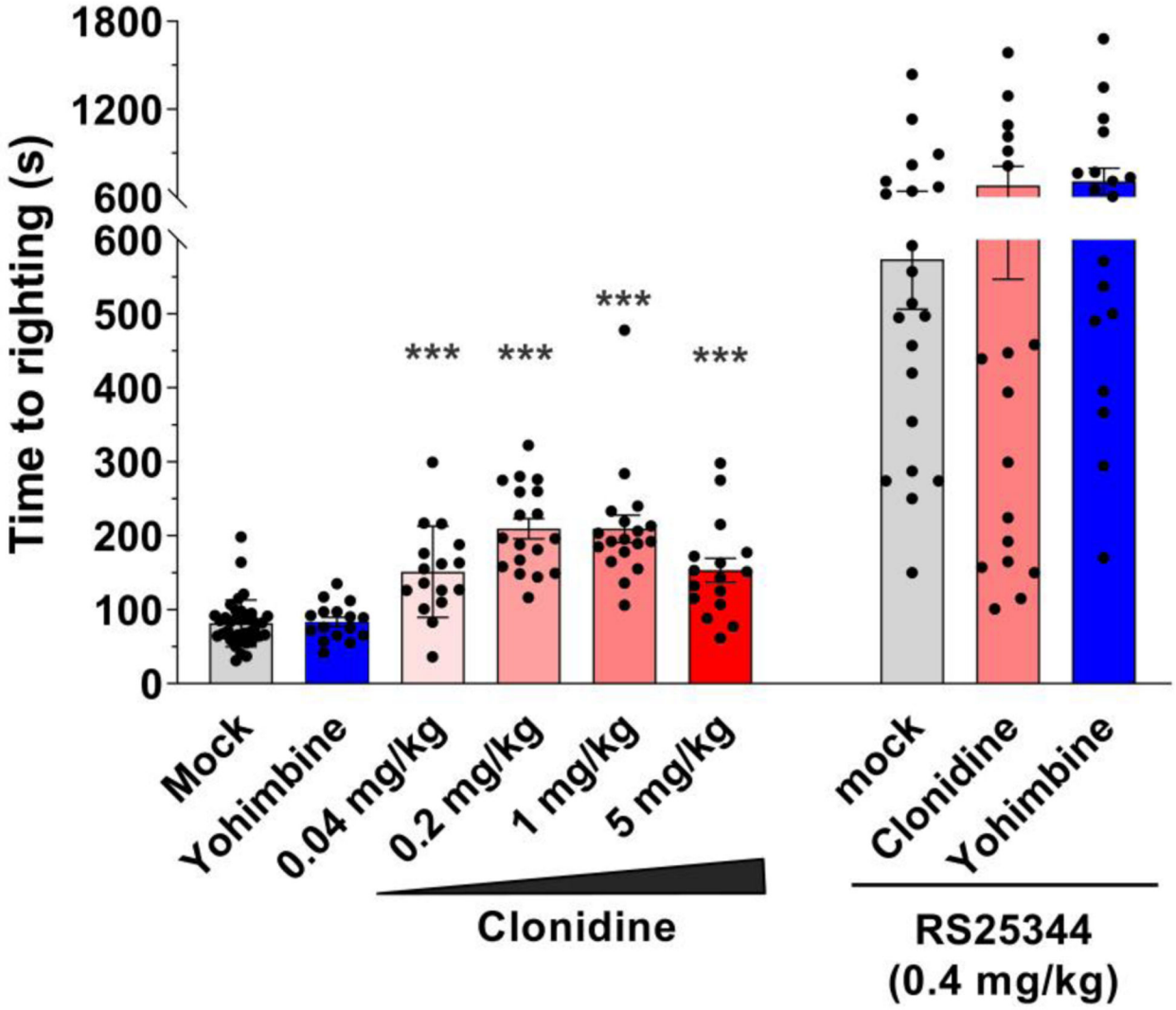

Fig. 5. Role of α_2-adrenoceptor signaling on the duration of Isoflurane anesthesia in mice.

Mice were injected with the indicated doses of the α_2-adrenoceptor agonist Clonidine, or the α_2-adrenoceptor antagonist Yohimbine (1 mg/kg, i.p.), the PDE4 inhibitor RS25344 (0.4 mg/kg, i.p.) and/or solvent controls (Mock). Thirty min after drug injection, mice were placed in an anesthesia induction chamber ventilated with 3% Isoflurane in 100% oxygen at 1 L/min for 10 min. Mice were then removed from the anesthesia chamber, placed on their backs into new cages and the time to first righting was recorded. In the absence of PDE4 inhibitor, treatment with Clonidine significantly increased the duration of Isoflurane anesthesia whereas Yohimbine (1 mg/kg) had no effect. Neither Clonidine (1 mg/kg) nor Yohimbine (1 mg/kg) significantly changed the duration of Isoflurane anesthesia in the presence of the PDE4 inhibitor RS25344 (0.4 mg/kg). Data represent the mean ± SEM. Statistical significance was determined using Kruskal-Wallis and Dunn's post hoc test and is indicated as *** ($p<0.001$).

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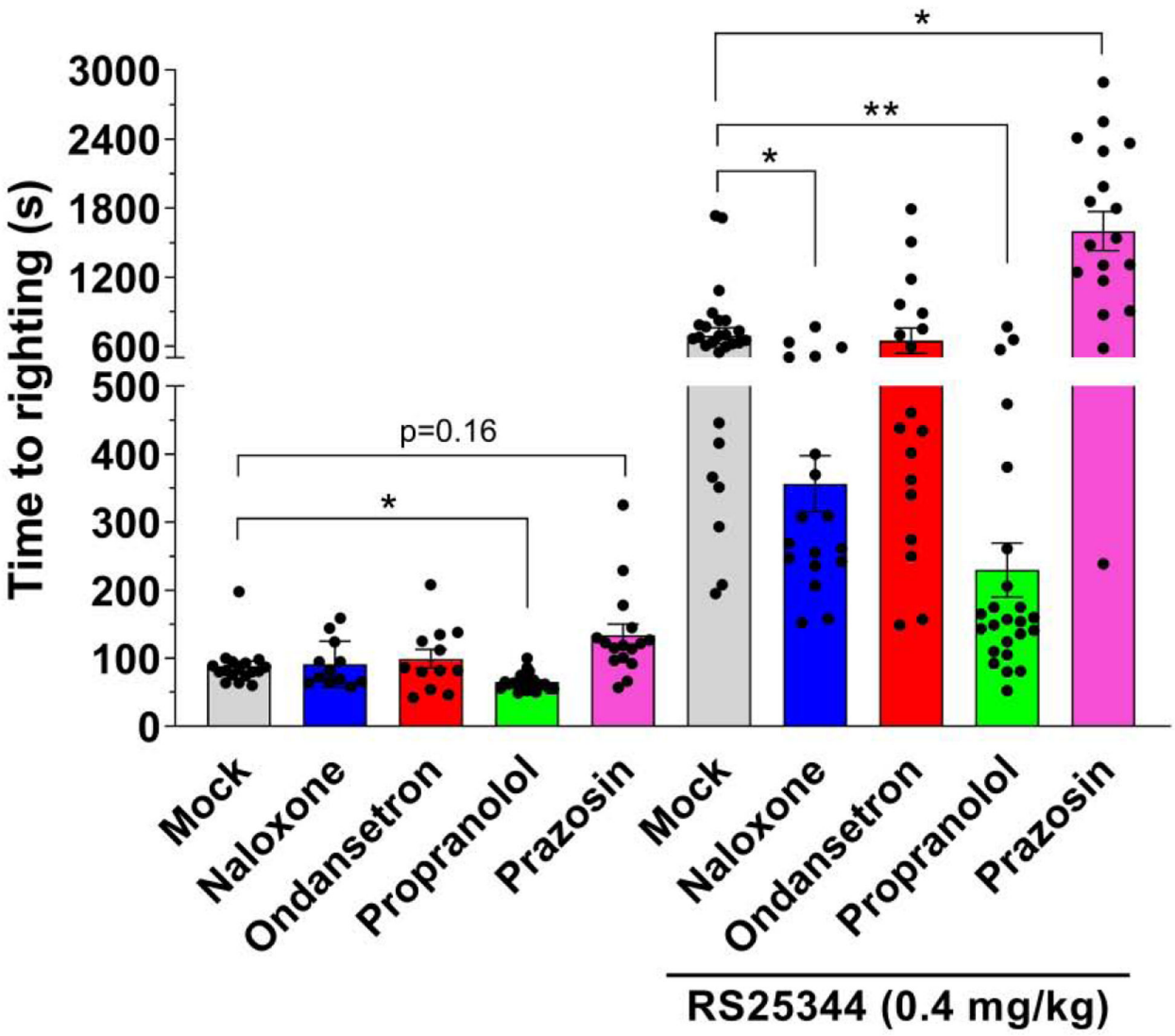

Fig. 6. Blockade of opioid- and β-adrenergic receptors modulates the potentiating effect of PDE4 inhibition on Isoflurane anesthesia.

Mice were injected with the indicated drugs including the opioid-receptor antagonist Naloxone (5 mg/kg, i.p.), the 5-HT_3 serotonin receptor blocker Ondansetron (5 mg/kg), the β-adrenoceptor blocker Propranolol (5 mg/kg, i.p.), the α_1-adrenoceptor blocker Prazosin (1 mg/kg, i.p.), the PDE4 inhibitor RS25344 (0.4 mg/kg, i.p.) and/or solvent control (Mock). Thirty min later, mice were placed in an anesthesia induction chamber ventilated with 3% Isoflurane in 100% oxygen at 1 L/min for 10 min. Mice were then removed from the anesthesia chamber, placed on their backs into new cages and the time to first righting was recorded. In the absence of RS25344, treatment with Propranolol significantly reduced the duration of Isoflurane anesthesia, whereas Naloxone or Ondansetron had no effect, and Prazosin trends to extend Isoflurane anesthesia. In the presence of the PDE4 inhibitor RS25344, Naloxone and Propranolol significantly shortened the duration of anesthesia, whereas Ondansetron had no effect, and Prazosin significantly delayed the recovery from Isoflurane anesthesia also in the presence of RS25344. Data represent the mean ± SEM.

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Statistical significance was determined using Kruskal-Wallis followed by Dunn's post hoc test and is indicated as * ($p<0.05$); and ** ($p<0.01$).

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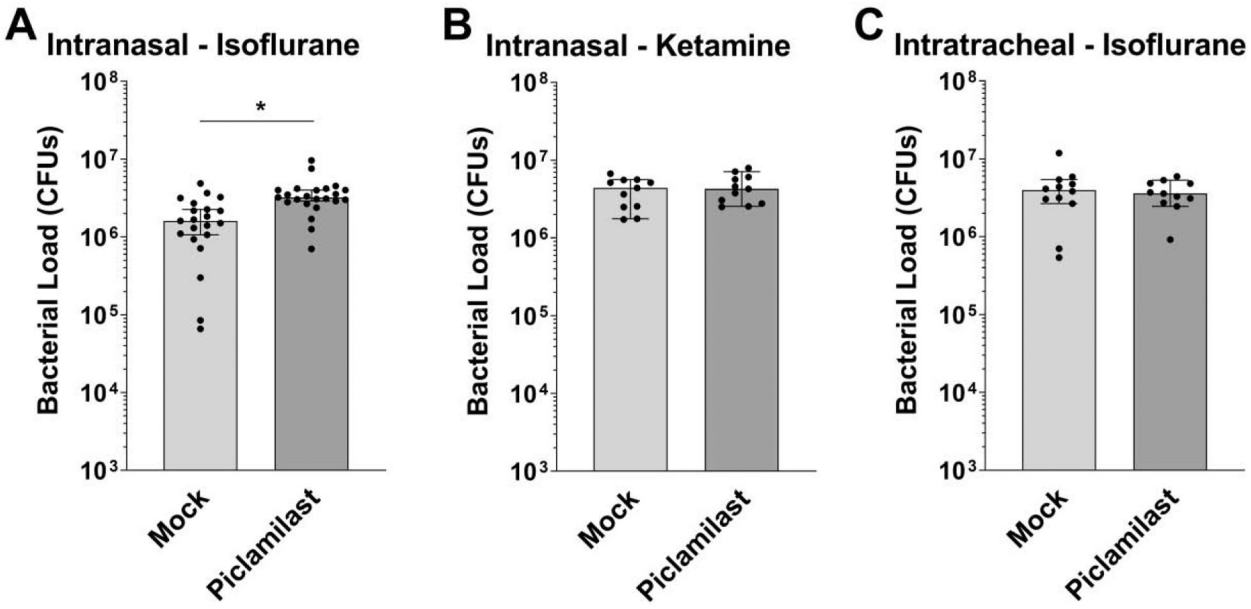

Fig. 7. The potentiation of Isoflurane anesthesia correlates with an increase in bacterial load in the lungs of PDE4 inhibitor-treated mice infected intranasally.

One hour after injection with the PAN-PDE4 inhibitor Piclamilast (5 mg/kg, i.p.) or solvent control (Mock), mice were inoculated with 5×10^6 *cfu* of *Pseudomonas aeruginosa* strain *PA01* suspended in 50 µl of PBS *via* intranasal (**A/B**) or intratracheal (**C**) routes and using Isoflurane (**A/C**) or Ketamine/Xylazine (**B**) for anesthesia. Mice were euthanized 45 min after infection, the lungs extracted, and serial dilutions of tissue extracts plated on agar plates to determine bacterial titers. (**A**) Bacterial load in lungs of mice infected intranasally under Isoflurane anesthesia. (**B**) Bacterial load in lungs of mice infected intranasally under Ketamine/Xylazine anesthesia. (**C**) Bacterial load in lungs of mice infected intratracheally under Isoflurane anesthesia. Data represent the mean ± SEM. Statistical significance was determined using Mann-Whitney test with 95% confidence interval and is indicated as * ($p<0.05$).