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Role of Type 4 Phosphodiesterases in Thermoregulation and Salivation in Mice

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ROLE OF TYPE 4 PHOSPHODIESTERASES IN THERMOREGULATION AND SALIVATION IN MICE

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LIST OF ABBREVIATIONS

AC	=	Adenylyl Cyclase
Atr	=	Atropine
cAMP	=	Cyclic Adenosine Monophosphate
CF	=	Cystic Fibrosis
CFTR	=	Cystic Fibrosis Transmembrane Conductance Regulator
CNS	=	Central Nervous System
cGMP	=	Cyclic Guanosine Monophosphate
CNG	=	Cyclic Nucleotide-Gated Ion Channel
DMSO	=	Dimethyl Sulfoxide
DTT	=	Dithiothreitol
EDTA	=	Ethylenediaminetetraacetic acid
EIA	=	Enzyme Immunoassay
EPAC	=	Exchange Protein Activated by cAMP
ERK	=	Extracellular Signal-Related Kinases
FBS	=	Fetal Bovine Serum
FSK	=	Forskolin

GAF	=	Guanine Nucleotide Exchange Factor
GEF	=	Guanine Nucleotide Exchange Factor
G_i	=	G Protein Inhibitory for Adenylyl Cyclase
GPCR	=	G Protein Coupled Receptor
G_s	=	G Protein Stimulatory for Adenylyl Cyclase
HARBS	=	High-Affinity Rolipram Binding State
HEPES	=	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IP	=	Immunoprecipitation; Intraperitoneal
Iso	=	Isoprenaline
KO	=	Knockout
O.G.	=	Oral Gavage
PA	=	Phosphatidic Acid
PA	=	*Pseudomonas aeruginosa*
PANS	=	Parasympathetic Autonomic Nervous System
PAS	=	Per (Period Circadian Protein) Arnt (Aryl Hydrocarbon Receptor Nuclear Translocator Protein), Sim (Single-Minded Protein)
PBS	=	Phosphate-Buffered Saline
PDE	=	Phosphodiesterase
Pilo	=	Pilocarpine
PONV	=	Post-Operative Nausea and Vomiting
Prop	=	Propranolol
PKA	=	Protein Kinase A
Roli	=	Rolipram

RPMI	=	Roswell Park Memorial Institute
SANS	=	Sympathetic Autonomic Nervous System
SDS-PAGE	=	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
TBST	=	Tris-Buffered Saline-Tween
TNFα	=	Tumor Necrosis Factor α
UCR	=	Upstream Conserved Region
WT	=	Wildtype

ABSTRACT

Boyd, Abigail Grace, M.S., University of South Alabama, August 2022. Role of Type 4 Phosphodiesterases in Thermoregulation and Salivation in Mice. Chair of Committee: Wito Richter, Ph.D.

Type 4 cAMP phosphodiesterases (PDE4s) comprise a family of four isoenzymes, PDE4A to D, that hydrolyze and inactivate the second messenger cAMP. Non/PAN-selective PDE4 inhibitors, which inhibit all four subtypes simultaneously, produce many promising therapeutic benefits, such as anti-inflammatory or cognition- and memory-enhancing effects. However, unwanted side effects, principally, nausea, diarrhea, and emesis, have long hampered their clinical and commercial success. Targeting individual PDE4 subtypes has been proposed for developing drugs with an improved safety profile, but which PDE4 subtype(s) is/are actually responsible for nausea and emesis remains ill-defined. In mice treated with PAN-selective inhibitors, there is substantial impairment of autonomic nervous system functions, including gastroparesis (retention of food in the stomach), and hypothermia, both of which are associated with nausea and emesis in humans. Selective inactivation of any of the four PDE4 subtypes does not induce gastroparesis in the mice, nor does it change their body temperature, suggesting that these adverse effects are not mediated by a single subtype, but require concurrent inhibition of multiple (at least two) PDE4 subtypes. As mice are anatomically incapable of vomiting, we have identified these effects as novel correlates of nausea in these animals.

Importantly, these studies suggest that subtype-selective inactivation of individual subtypes may be free of certain adverse effects and thus have an improved safety profile relative to PAN-selective inhibition.

CHAPTER I

INTRODUCTION TO CAMP SIGNALING AND PHOSPHODIESTERASES

1.1 Introduction

3',5'-Cyclic adenosine monophosphate (cAMP) is a ubiquitous second messenger that regulates a plethora of physiologic paradigms ranging from the fight-or-flight response to metabolism and mammalian reproduction, and from immune responses to cognition and memory. cAMP is produced in response to a large number of extracellular signals, including hormones, neurotransmitters and drugs, that act as agonists or antagonists on G protein-coupled receptors (GPCRs), which in turn transduce their activation via stimulatory ($G\alpha_s$) or inhibitory ($G\alpha_i$) heterotrimeric G-proteins to modulate the rate of cAMP synthesis by adenylyl cyclases (Fig. 1). cAMP then initiates a cellular response to the external stimuli via activation of several effector proteins including protein kinase A (PKA), cyclic nucleotide-gated ion channels (CNG), the exchange protein activated by cAMP (EPAC), and, more recently, Popeye domain-containing proteins (POPDCs).[1]

The concentration of cAMP in the cell is not solely determined by the rate of its production, but by the equilibrium between the rate of its production by adenylyl cyclases and the rate of its degradation. The latter is catalyzed by cyclic nucleotide phosphodiesterases (PDEs) (Fig. 1), a superfamily of isoenzymes that hydrolyze the

second messengers cAMP and cGMP. The mammalian PDEs are encoded by 21 genes, which in turn are grouped into 11 PDE families by sequence homology[2-4]. Three PDE families – PDE4, PDE7, and PDE8 – are comprised of enzymes that exclusively hydrolyze cAMP. Three PDE families – PDE5, PDE6, and PDE9 – comprise enzymes that selectively hydrolyze cGMP; the remaining PDE families can hydrolyze both cyclic nucleotides. All PDEs share a conserved domain structure with a relatively conserved C-terminal catalytic domain that determines substrate specificity and inhibitor sensitivity, and highly divergent N-terminal regulatory domains that mediate the regulation of PDE activity and subcellular localization via a multitude of post-translational and allosteric regulations[5-11].

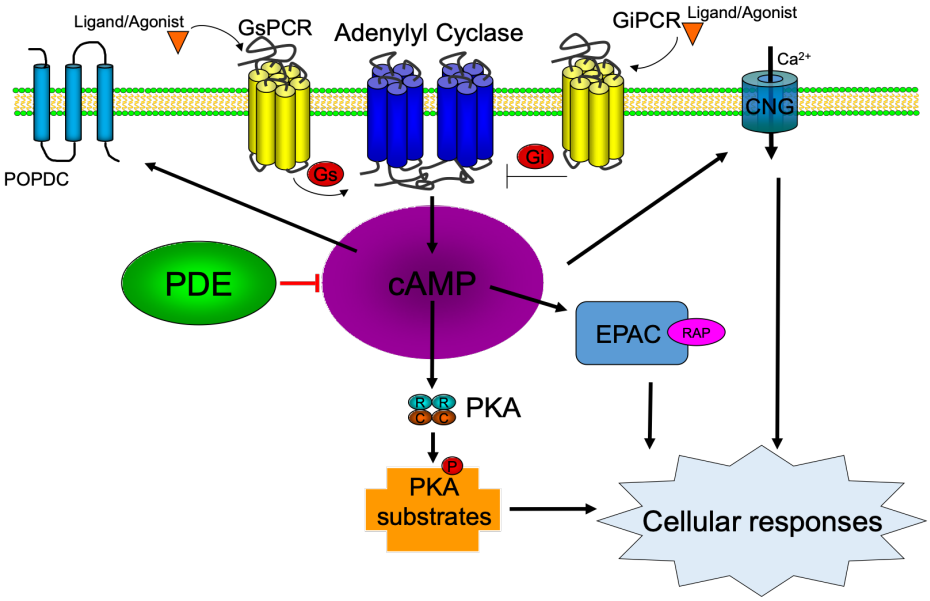

Figure 1. The cAMP Signaling Pathway.

Binding of a first messenger, such as a hormone or drug, to a G_s-protein coupled receptor eventually activates cAMP synthesis catalyzed by adenylyl cyclases. cAMP then exerts its downstream effects via activation of protein kinase A (PKA), exchange protein activated by cAMP (EPAC) or cyclic nucleotide-gated channels (CNG). Cyclic nucleotide phosphodiesterases (PDEs) hydrolyze and inactivate cAMP, thereby playing a critical role in the spatial and temporal control of cAMP signals.

The PDE4 family is the largest and arguably most widely expressed of all PDE families. It comprises four genes that are each expressed as a number of distinct protein variants by use of alternative promotors and transcription start sites, so that in total likely >25 distinct PDE4 proteins are expressed in a cell- and tissue-specific manner[2, 11-14]. Given the ubiquitous expression of PDE4s, it is not surprising that non-/PAN-selective inhibition of PDE4s produces an array of potentially therapeutic effects including memory- and cognition-enhancing, anti-inflammatory or anti-neoplastic effects[11, 14-17]. However, the clinical and commercial success of PAN-PDE4 inhibitors has been somewhat muted given a number of adverse effects, principally nausea, emesis, and diarrhea, that generate a narrow therapeutic window[18-19]. The genetic ablation of individual PDE4 subtypes in mice, or their knockdown in cell culture, has clearly demonstrated that each PDE4 subtype has unique and non-overlapping roles in the body. Thus, targeting individual PDE4 subtypes or protein variants is a promising approach to separate the therapeutically beneficial from the adverse effects of currently available PAN-PDE4 inhibitors [9,12,17,20-22]

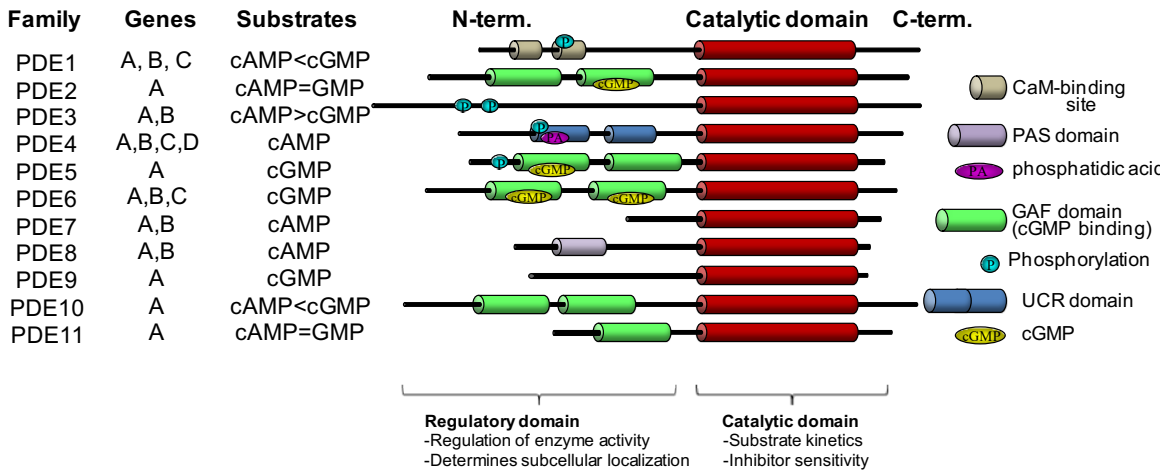

Figure 2. The Superfamily of the Mammalian Phosphodiesterases.

Indicated are the domain structures, number of genes, and substrate specificity of the eleven mammalian PDE families. Domains are represented by 'barrels' connected by 'wires' indicating linker regions. Phosphorylation sites are indicated by circles. The catalytic domain is highly conserved among PDE subtypes, while the N-terminal regulatory domains are subtype-specific. CaM, (Ca^{2+}-calmodulin); GAF (cGMP-activated PDEs, adenylyl cyclase, and Fh1A), UCR (upstream conserved regions); PAS (period, aryl-hydrocarbon receptor nuclear translocator (ARNT), and single minded).

Towards this goal, I have spearheaded or contributed to multiple projects that utilized mouse models to delineate the roles of PDE4s in multiple physiologic paradigms ranging from the protective effect of PDE4 inactivation in settings of *Pseudomonas aeruginosa* (*PA*) lung infection[21], to the role of PDE4s in gastric motility[19], body temperature regulation[23-24], salivation[25], or the response to anesthesia[26]. During experiments to infect mice with *Pseudomonas aeruginosa,* we noticed that mice pre-treated with PAN-selective PDE4 inhibitors were cold to the touch, suggesting an effect of PDE4 inhibition on body temperature regulation, and also exhibited excessive salivation. We expanded these observations, each physiologically significant in its own right, into separate projects that I am reporting here.

Targeting individual PDE4 subtypes has been proposed for developing drugs with an improved safety profile, but which PDE4 subtype(s) is/are actually responsible for nausea and emesis has remained ill-defined. Highly subtype-selective PDE4 inhibitors are not currently available to test this question in humans. Genetic tools, such as the genetic deletion of individual PDE4s, have been restricted to mice and rats, two species that are anatomically unable to vomit. A further complication is the fact that nausea is the more prevalent, and hence the more critical adverse effect of PDE4 inhibitor treatment in humans, compared to emesis, and it is now well-established that nausea is driven by neuronal and molecular pathways that are partly distinct and extend beyond the mechanisms that drive the emetic reflex. Based on the observation that nausea is often accompanied by hypothermia in humans and other mammals, I have led efforts to establish PAN-PDE4 inhibitor-induced hypothermia as a novel correlate of drug/PDE4 inhibitor-induced nausea[23]. As detailed in Chapter II, we found that selective inactivation of any of the four PDE4 subtypes did not change the body temperature of mice, suggesting that PAN-PDE4 inhibitor-induced hypothermia (and hence nausea in humans) requires the concurrent inhibition of multiple PDE4 subtypes. This suggests that the selective inhibition of any individual PDE4 subtype may be free of nausea and emesis. In Chapter III, I report on efforts to further explore the initial observation that treatment with PAN-PDE4 inhibitors induces salivation in mice[25]. Of particular note is the observation that PDE4 inhibitor-induced salivation is dependent upon cystic fibrosis transmembrane conductance regulator (CFTR) activity, as the effect of PDE4 inhibition of saliva secretion is completely ablated in mice carrying the inactivating ΔF508-CFTR mutation. This observation confirms in an *in vivo* model that PDE4 exerts a critical role

in the regulation of CFTR activity, which has been reported previously in cell culture systems[27-28], and underlines the potential of inhibiting PDE4 as a therapeutic approach in Cystic Fibrosis.

CHAPTER II

ASSESSMENT OF PDE4 INHIBITOR-INDUCED HYPOTHERMIA AS A CORRELATE OF NAUSEA IN MICE

2.1 Introduction

Type 4 cyclic nucleotide phosphodiesterases (PDE4s) comprise a group of four isoenzymes, PDE4A to D, that hydrolyze and inactivate the second messenger cAMP. PDE4s are widely expressed throughout mammalian cells and tissues[29-33], and diverse therapeutic benefits result from their non/PAN-selective inhibition. Preclinical studies of PAN-PDE4 inhibitors have established their potent anti-inflammatory, memory- and cognition-enhancing, anti-depressant and anti-psychotic, metabolic, and cardiovascular properties [30, 34-40]. However, the clinical application and commercial success of PDE4 inhibitors have been limited due to adverse effects, particularly nausea, diarrhea, and emesis. These adverse effects are characteristic for PAN-PDE4 inhibitors, leaving only two PDE4 inhibitors currently approved for systemic administration: Roflumilast, for moderate to severe chronic obstructive pulmonary disease, and Apremilast, for the treatment of psoriasis [30]. However, nausea remains one of the most common side effects of both Roflumilast and Apremilast (frequency of 28.7% and 8.9%, respectively; from [41-42]).

A multitude of studies have now shown that the genetic knockdown or deletion of individual PDE4 subtypes in cells and/or animals produces unique phenotypes, indicating

that each PDE4 exerts unique and non-overlapping roles in the body[11, 20- 21, 25, 30, 43-50]. Thus, the development of subtype-selective PDE4 inhibitors has been proposed to avert the adverse effects of currently available PAN-PDE4 inhibitors [29, 51]. While individual PDE4 subtypes have already been identified as therapeutic targets for a variety of inflammatory, cardiovascular, neuronal or metabolic conditions [14, 18, 30, 34-36, 50, 52-58], the PDE4 subtype(s) that mediate the adverse effects of PAN-PDE4 inhibitors remain ill-defined. The main obstacle to their identification is that highly subtype-selective PDE4 inhibitors are not yet available to test and address this question in humans. In addition, the tools that are available, such as the genetic deletion or knockdown of PDE4 subtypes, have been restricted to mice and rats; two species that are anatomically unable to vomit, which is the prevailing preclinical correlate of nausea. Further complicating this analysis is the fact the nausea is by far the more prevalent, and hence the more critical, adverse effect of PDE4 inhibitor treatment in humans, compared to emesis[41-42]. It is now well-established that nausea is driven by neuronal and molecular pathways that are partly distinct and extend beyond the mechanisms that drive the emetic reflex [59-63]. In addition, since nausea is at its core a feeling or sensation (e.g., stomach awareness), it is difficult to assess in any animal, including in species that are able to vomit. Thus, while dogs or ferrets are obvious choices to study emesis (compared to mice and rats), rodents may yet prove equally useful in assessing nausea, given a proper correlate.

We have shown recently that PDE4s play a critical role in the autonomic regulation of body temperature in mice and that treatment with PAN-PDE4 inhibitors induces a fast-onset (within 10 min), substantial (up to −5 °C) and long-lasting (up to 5 h) hypothermia in the animals[24]. Intriguingly, an increasing body of research closely

correlates nausea with a significant disturbance in thermoregulation in mammals[64-68]. In humans, nausea is frequently associated with signs of hypothermia, including cold sweats, clammy hands, sometimes shivering and may include a modified perception of ambient temperature and cold-seeking behavior (e.g., cold/fresh air), as well as a reduction in core body temperature [59,65]. Impaired thermoregulation parallels nausea induced by a variety of triggers, including motion, poison, surgery/anesthesia (post-operative nausea and vomiting (PONV)), as well as drug/chemotherapy- or radiation-treatment in humans. Despite the variety of triggers for nausea, all are consistently associated with hypothermia in various animal species, whether they possess the emetic reflex (e.g., ferrets and shrews) or not (e.g., mice and rats), and can be similarly detected by a reduction in the animals' core body temperature, cutaneous vasodilation (e.g., tail skin vasodilation), reduced thermogenesis and/or cold-seeking behaviors [64-66,68-70]. Thus, diverse mammalian species share neuronal mechanisms that link nausea to hypothermia, thus providing a valuable correlate to study nausea. The relationship between nausea and hypothermia appears reciprocal, in that nausea, such as upon induction of motion sickness, predisposes to hypothermia[71-72], while vice versa, forced (externally-induced) hypothermia can trigger or predispose to nausea[67].

Utilizing the close association between nausea and hypothermia, the present study was designed to assess core body temperature in mice as a potential correlate of nausea and emesis induced by PDE4 inhibitors in humans. We aimed to elucidate the role of individual PDE4 subtypes in mediating these adverse effects and to begin exploring their molecular mechanism(s). Finally, we contrast our findings on PDE4 inhibitor-induced hypothermia with the effects of similar treatments and PDE4 subtype ablation on the

duration of Ketamine/Xylazine-induced anesthesia[73], a paradigm previously proposed as a correlate of the adverse effects of PDE4 inhibitors in animals.

2.2 Materials and Methods

2.2.1 Drugs

RS25344 [74] (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), Ondansetron ((RS)-1,2,3,9-Tetrahydro-9-methyl-3-(2-methylimidazol-1-ylmethyl)carbazol-4-one) from Sigma-Aldrich (St. Louis, MO, USA) and YM976[75] (4-(3-chlorophenyl)-1,7-diethylpyrido[2,3-d]pyrimidin-2-one) from Tocris/Bio-Techne (Minneapolis, MN, USA). Rolipram [76] (4-(3-cyclopentyloxy-4-methoxyphenyl)pyrrolidin-2-one), Piclamilast[77,78] (RP73401; 3-(Cyclopentyloxy)-N-(3,5-dichloropyridin-4-yl)-4-methoxybenzamide), Roflumilast [79-81] (3-(cyclopropylmethoxy)-N-(3,5-dichloropyridin-4-yl)-4-(difluoromethoxy)benzamide), Clonidine (N-(2,6-dichlorophenyl)-4,5-dihydro-1H-imidazol-2-amine), Yohimbine (methyl (1S,15R,18S,19R,20S)-18-hydroxy-1,3,11,12,14,15,16,17,18,19,20,21-dodecahydroyohimban-19-carboxylate) and Metoclopramide (4-amino-5-chloro-N-[2-(diethylamino)ethyl]-2-methoxybenzamide) were from Cayman Chemical (Ann Arbor, MI). Metoclopramide was dissolved in 1% methylcellulose in water and administered via oral gavage. Clonidine, Yohimbine, Ondansetron and all PDE inhibitors were applied by intraperitoneal (i.p.) injection (100 µL per 20 g body weight). The drugs were initially dissolved in DMSO, and 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).

2.2.2 Animals

All mice were maintained on a C57BL/6 background and in a temperature-controlled (22–23 °C) vivarium with a 12 h light/dark cycle. Animals were group-housed up to four mice per cage and had ad libitum access to food and water. Adult mice ≥18 g of body weight and ≥10 weeks of age were used for experimentation by evenly and randomly dividing cage littermates into experimental groups. Wildtype C57BL/6 mice were generated in-house using breeders obtained from Charles River Laboratories (Wilmington, MA). Mice carrying genetic deletions of PDE4A[45], PDE4B [9] or PDE4D[6] were generated by Drs. S.-L. Catherine Jin and Marco Conti (Stanford University, CA; [11]), whereas PDE4C knockout mice (Pde4c$^{tm1.1(KOMP)Wtsi/J}$) were generated by the National Institutes of Health (NIH) Knockout Mouse Program. The animals were distributed via the Mutant Mouse Resource and Research Centers (MMRRC, http://www.mmrrc.org) and the KOMP repository (KOMP; www.komp.org) of the University of California at Davis, respectively. Experimenters were blinded to the genotypes of the mice and the identity of injected drugs until data acquisition and analyses were completed. All experiments and procedures were approved by the University of South Alabama Institutional Animal Care and Use Committee and were conducted in accordance with the guidelines described in the Guide for the Care and Use of Laboratory Animals (National Institutes of Health, Bethesda, MD, USA). For euthanasia, EUTHASOL® Euthanasia Solution (Patterson Veterinary, Greeley, CO, USA) was injected i.p., followed by cervical dislocation.

2.2.3 Duration of Ketamine/Xylazine Anesthesia

The duration of Ketamine/Xylazine-induced anesthesia was measured as described previously [73] with minor modifications. In short, mice were anesthetized with a combination of Ketamine (80 mg/kg) and Xylazine (10 mg/kg) administered by intraperitoneal (i.p.) injection. Upon loss of righting (2–3 min), the mice were then placed in dorsal recumbency, and the time to first righting was measured (see Figure 3A,B). Experiments were ended at 120 min after Ketamine/Xylazine injection and sleep duration for animals that did not right themselves by that time was recorded as 120 min. To probe the effect of drug treatment, the α_2-adrenoceptor antagonist Yohimbine, the PAN-PDE4 inhibitors Rolipram or Piclamilast/RP73401 or Mock/solvent control were injected (i.p.) into anesthetized mice at 10 min after Ketamine/Xylazine administration.

2.2.4 Measurement of Core Body Temperature

Core body temperature was measured as described[24] using a thermocouple thermometer (MicroTherma 2T) with mouse rectal probe (RET-3), both from Braintree Scientific (Braintree, MA, USA), following the manufacturer's instructions.

2.2.5 Data and Statistical Analysis

Statistical analyses were performed using the GraphPad Prism 9 software (GraphPad Software Inc, San Diego, CA, USA). To compare two treatment groups, the Mann–Whitney test with 95% confidence interval was applied. To determine differences between more than two treatment groups, the Kruskal–Wallis test followed by Dunn's post hoc test was applied. Statistical differences are shown as # (not significant; $p > 0.05$), * ($p < 0.05$), ** ($p < 0.01$) and *** ($p < 0.001$). All data are expressed as the mean

± SEM and n numbers indicate the number of individual animals assessed and are represented by individual dots in the scatter plots.

2.3 Results

2.3.1 Selective Ablation of PDE4D, but Not Ablation of PDE4A, PDE4B, or PDE4C Mimics the Effect of PAN-PDE4 Inhibitors to Shorten the Duration of Ketamine/Xylazine-Induced Anesthesia in Mice

Xylazine is thought to promote anesthesia via its agonism of α_2-adrenoceptors present at presynaptic sites. Activation of these G_i-coupled receptors leads to inhibition of adenylyl cyclase activity and a decrease of intracellular cAMP levels, which in turn impairs the release of various neurotransmitters and the resulting suppression of neuronal signaling facilitates anesthesia [82]. As shown previously, treatment with PAN-PDE4 inhibitors counteracts the hypnotic effects of Xylazine, reflected in a shorter duration of Ketamine/Xylazine-induced anesthesia, in various animal species[73, 82-86], given that PDE4 inhibition increases cAMP signaling and thus acts as a physiologic antagonist of α_2-adrenoceptor signaling. As shown in Figure 3C, the archetypal PDE4 inhibitor Rolipram, as well as the second-generation PDE4 inhibitor Piclamilast/RP73401, both shorten the duration of Ketamine/Xylazine anesthesia in mice, although with distinct potencies. As little as 0.1 mg/kg Rolipram significantly shortens Ketamine/Xylazine-induced anesthesia, whereas doses higher than 1 mg/kg Piclamilast are required to produce a significant effect.

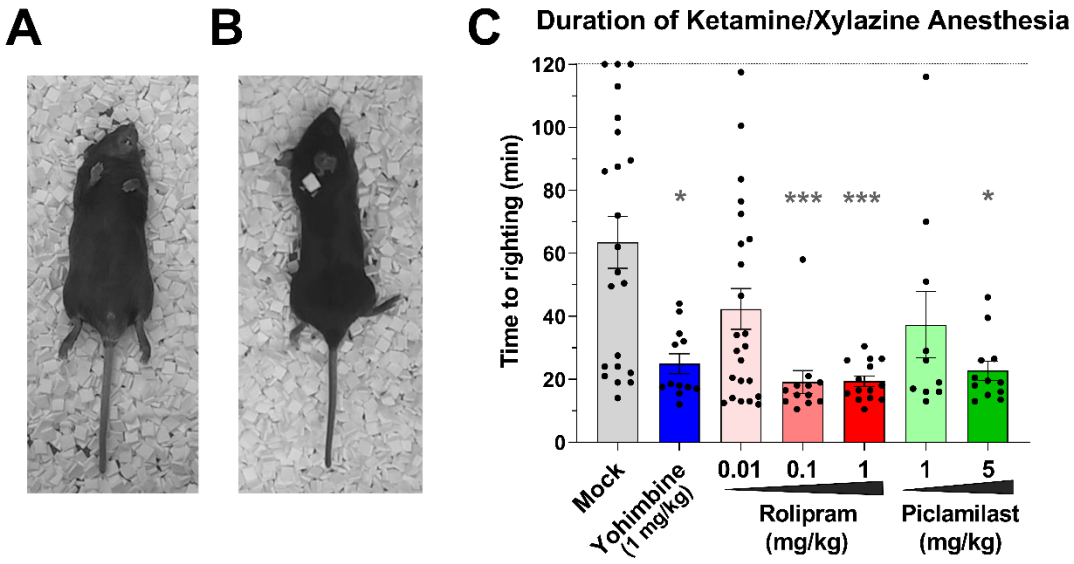

Figure 3. PAN-Selective PDE4 inhibition shortens the duration of Ketamine/Xylazine-induced anesthesia via α_2-adrenoceptor antagonism.

(**A**,**B**) Representative images illustrating the measurement of "Time to righting". Upon induction of anesthesia and the resulting loss of righting (~3 min after Ketamine/Xylazine injection), the unconscious mice are placed on their backs (**A**). As the anesthetic effect of Ketamine/Xylazine wears off and the animals awaken, the righting reflex, an automatic reaction to move the body to its normal/prone position, kicks in and the mice turn onto their abdomen (**B**). The time from loss of righting to the time of first righting is recorded. (**C**) Mice were anesthetized with a combination of Ketamine (80 mg/kg) and Xylazine (10 mg/kg) administered by intraperitoneal injection. Ten minutes later, the animals were injected intraperitoneally with the α_2-adrenoceptor antagonist Yohimbine (1 mg/kg), the PAN-PDE4 inhibitors Rolipram (0.01, 0.1, or 1 mg/kg) or Piclamilast/RP73401 (1 or 5 mg/kg) or solvent control (Mock). The mice were then placed in dorsal recumbency and the time to first righting was measured. Data represent the mean ± SEM. Statistical analysis was determined using the Kruskal–Wallis test followed by Dunn's post hoc test and is indicated as * ($p < 0.05$) or *** ($p < 0.001$).

Shortening the duration of Ketamine/Xylazine-induced anesthesia has previously been suggested as a physiological correlate of the emetic potential of PDE4 inhibitors, given that both PAN-PDE4 inhibitors as well as α_2-adrenoceptor antagonists, such as Yohimbine, induce emesis in ferrets and that treatment of ferrets with α_2-adrenoceptor

agonists, such as Clonidine, alleviates PDE4 inhibitor-induced vomiting [83-84]. The duration of Ketamine/Xylazine-induced anesthesia has subsequently been used as a correlate to assess the emetic potential of PDE4 inhibitors in species that are unable to vomit, including mice and rats [73,82]. In a landmark study probing the critical question of which PDE4 subtypes may be involved in the emetic effects of PDE4 inhibitors, it was shown that in mice the genetic ablation of PDE4D, but not the ablation of PDE4B, shortens the duration of Ketamine/Xylazine anesthesia, mimicking the effect of PDE4 inhibitors[73]. This finding has led to the conclusion that PDE4D inactivation mediates the emetic effects of PDE4 inhibitors and is irrevocably tied to inducing emesis. This, in turn, has led to efforts to develop PDE4 inhibitors with selectivity for PDE4B over PDE4D [54,87-92], at the same time forgoing numerous therapeutic benefits that may be derived from PDE4D inhibition. Here, we confirm and extend the finding that PDE4D is the sole and principal PDE4 subtype involved in shortening the duration of Ketamine/Xylazine-induced anesthesia in mice. As shown in Figure 4, selective ablation of either PDE4A, PDE4B or PDE4C in mice has no effect on the duration of Ketamine/Xylazine-induced anesthesia (Figure 4A–C), whereas selective ablation of PDE4D (Figure 4D) shortens anesthesia to levels similar to the administration of PAN-selective PDE4 inhibitors (see Figure 3C). While recent studies have confirmed the value of the Ketamine/Xylazine anesthesia test as a model of α_2-adrenoceptor antagonism, its value as a physiological correlate of nausea and/or emesis in humans is less clear [85]. We thus explored alternative correlates of emetic potential in mice.

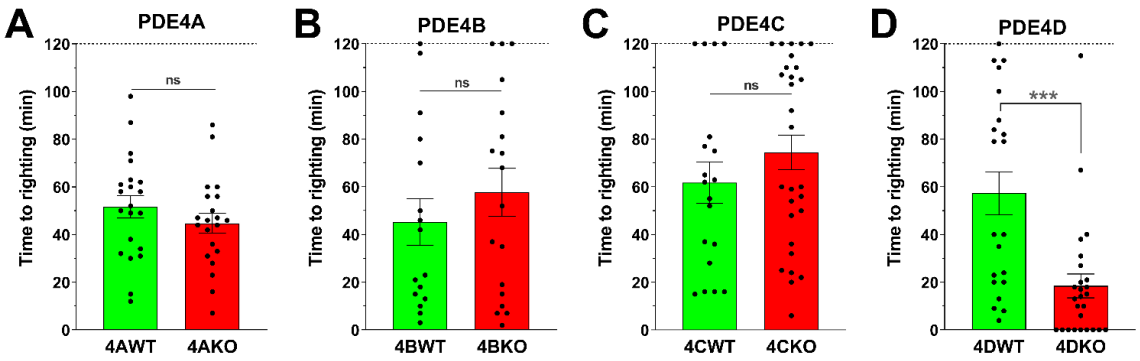

Figure 4. Genetic ablation of PDE4D, but not ablation of PDE4A, PDE4B or PDE4C, shortens the duration of Ketamine/Xylazine-induced anesthesia in mice.

Mice were anesthetized with a combination of Ketamine (80 mg/kg) and Xylazine (10 mg/kg) administered by intraperitoneal injection. Upon loss of righting (~3 min), the mice were placed in dorsal recumbency and the time to first righting was measured. Mice that never lost the righting reflex were counted as 0 min. Data represent the mean ± SEM. Statistical significance was determined using the Mann–Whitney test with 95% confidence interval and is indicated as *** ($p < 0.001$) or ns (not significant; $p > 0.05$).

2.3.2 Treatment with PAN-PDE4 Inhibitors Induces Hypothermia in Mice

Prior studies have shown that treatment with PAN-PDE4 inhibitors induces a fast-onset, substantial, and long-lasting hypothermia in various animal species, including mice, rats and rabbits [24,93-94]. As shown previously[24], this is a class effect of PDE4 inhibitors as it is induced by various, structurally distinct PAN-PDE4 inhibitors (see Figures 5A and 7. Intriguingly, an increasing body of evidence causally links nausea and emesis with hypothermia[65-66, 68]. Hence, we explored the measurement of core body temperatures to re-evaluate the association of individual PDE4 subtypes with emetic potential.

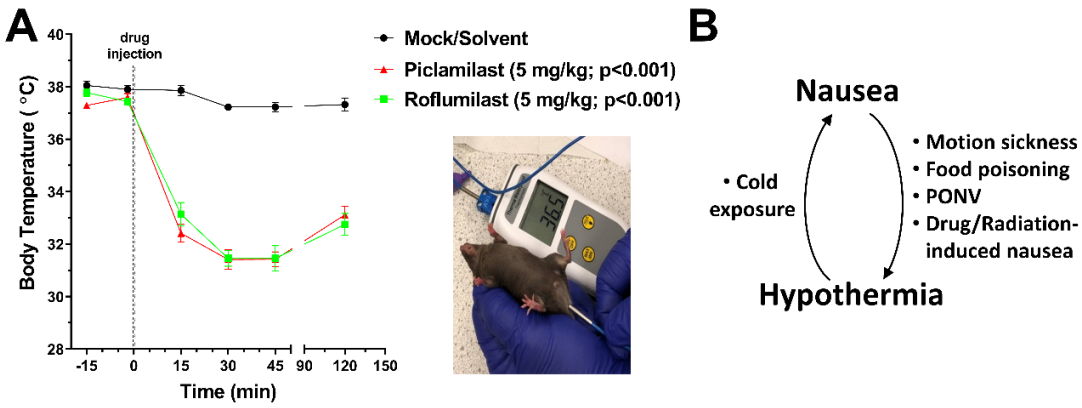

Figure 5. Treatment with PDE4 inhibitors induces hypothermia.

(**A**) The core body temperature of mice was measured at the indicated time points prior to and after injection of the PAN-PDE4 inhibitors Piclamilast/RP73401 or Roflumilast (both 5 mg/kg; i.p.) or solvent control. Data represent the mean ± SEM. Statistical significance was determined using two-way ANOVA with Sidak's post hoc test. The picture inset illustrates the measurement of core body temperature using a rectal thermometer. (**B**) Scheme illustrating the reciprocal relationship between nausea and hypothermia frequently observed in humans and animals. Nausea induced by motion, poison, surgery/anesthesia (post-operative nausea and vomiting (PONV)), drug- or radiation treatment is frequently accompanied by hypothermia. Conversely, aberrant body temperature regulation (including cold exposure) can predispose to nausea[25, 41-43].

2.3.3 PDE4 Inhibitor-Induced Hypothermia Results from the Concurrent Inhibition of Multiple PDE4 Subtypes

To determine if one of the four PDE4 subtypes (PDE4A to D) is predominantly responsible for the PDE4 inhibitor-induced hypothermia phenotype, we compared the body temperature of mice deficient in PDE4A, PDE4B, PDE4C or PDE4D to the body temperature of their respective wildtype littermates. As shown in the solid columns of Figure 8A–D, genetic ablation of any of the four PDE4 subtypes did not significantly change the baseline body temperature of mice. To exclude the possibility that

compensatory changes may have obscured the loss of one PDE4 in the control of body temperature, the effect of treatment with the PDE4 inhibitor Piclamilast (5 mg/kg, i.p.) was then tested in each knockout strain and their respective wildtype littermate controls. As shown in Figure 6A–D, treatment with a high dose of Piclamilast produced substantial hypothermia in each knockout strain that was similar in extent to that of the matching wildtype controls. Together, these data suggest that ablation of a single PDE4 subtype does not cause hypothermia, which instead likely results from the simultaneous inhibition of multiple (at least two) PDE4 subtypes. How many and which PDE4 subtypes are involved in mediating the effect of PAN-PDE4 inhibitors on body temperature regulation, and thus associate with emetic potential, remains to be determined.

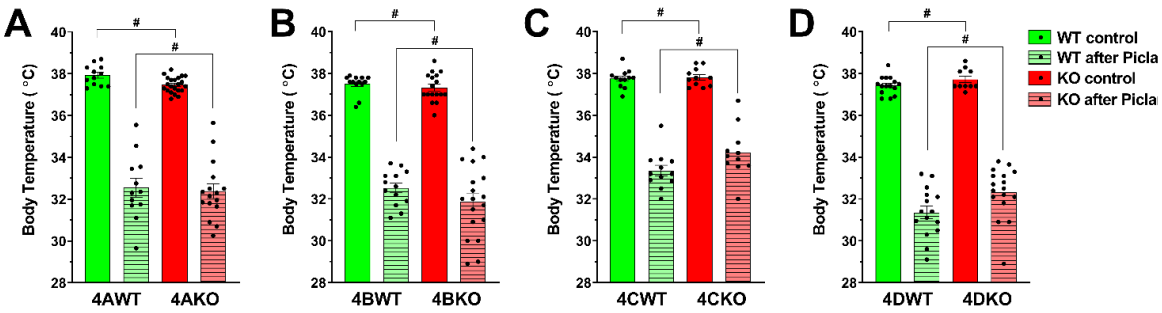

Figure 6. PDE4 inhibitor-induced hypothermia results from the concurrent inactivation of multiple (at least two) PDE4 subtypes.

The core body temperature of PDE4 knockout (KO, red bars) mice and their respective wildtype (WT, green bars) littermates was measured just prior to (control, solid colors) and again 30 min after i.p. injection of the PAN-PDE4 inhibitor Piclamilast/RP73401 (after Piclamilast; striated bars; 5 mg/kg). Injection of Piclamilast produced a statistically significant drop in body temperature in all genotypes ($p < 0.01$ for 4CKO; $p < 0.001$ for all others). Conversely, the body temperature of mice deficient in (**A**) PDE4A (4AKO), (**B**) PDE4B (4BKO), (**C**) PDE4C (4CKO) or (**D**) PDE4D (4DKO) was not different from that of their respective wildtype (WT) littermates either prior to or after injection of the PDE4 inhibitor Piclamilast. Data represent the mean ± SEM. Statistical significance was determined using the Mann–Whitney test with 95% confidence interval and is indicated as # ($p > 0.05$).

As far as the role of individual PDE4 subtypes, and in particular PDE4D, is concerned, the two correlates of emetic potential studied here, hypothermia (Figure 6) and the duration of α_2-adrenoceptor-dependent anesthesia (Figure 4), produce contrary results. α_2-adrenoceptor-dependent anesthesia is exclusively mediated by inactivation of PDE4D, whereas hypothermia is not. Given the lack of any effect of PDE4D ablation on hypothermia, we wished to exclude the possibility that the substantial hypothermia induced by a high dose of Piclamilast (5 mg/kg; Figure 6) may have overshadowed a potential, more subtle effect of genetic PDE4D ablation on body temperature regulation. Thus, we initially repeated body temperature measurements in PDE4D-KO mice and their respective wildtype controls using submaximal doses of the PAN-PDE4 inhibitors

Piclamilast and Rolipram as follow-up experiments. Intriguingly, the dose of the PDE4 inhibitors used and/or the amplitude of hypothermia induced by PDE4 inhibitor treatment did not alter the pattern of responses in PDE4D-WT versus PDE4D-KO mice. For example, upon treatment with Piclamilast, there was no difference in the levels of hypothermia between PDE4D-WT and PDE4D-KO mice whether a high dose of the drug was used and hypothermia was more substantial (5 mg/kg Piclamilast; −5°C; Figure 6D) or if a lower drug dose was used, producing a more subtle hypothermia (1 mg/kg Piclamilast; −2 °C; Figure 7C). However, we observed that the type of PDE4 inhibitor used affected responses in PDE4D-WT and PDE4D-KO mice. Upon treatment with Piclamilast, Roflumilast or YM976 (Figure 7C–E), the level of hypothermia induced by the inhibitors was the same in PDE4D-WT and PDE4D-KO mice. Conversely, PDE4D-KO mice were partially protected from hypothermia induced by treatment with Rolipram or RS25344 (Figure 7A,B).

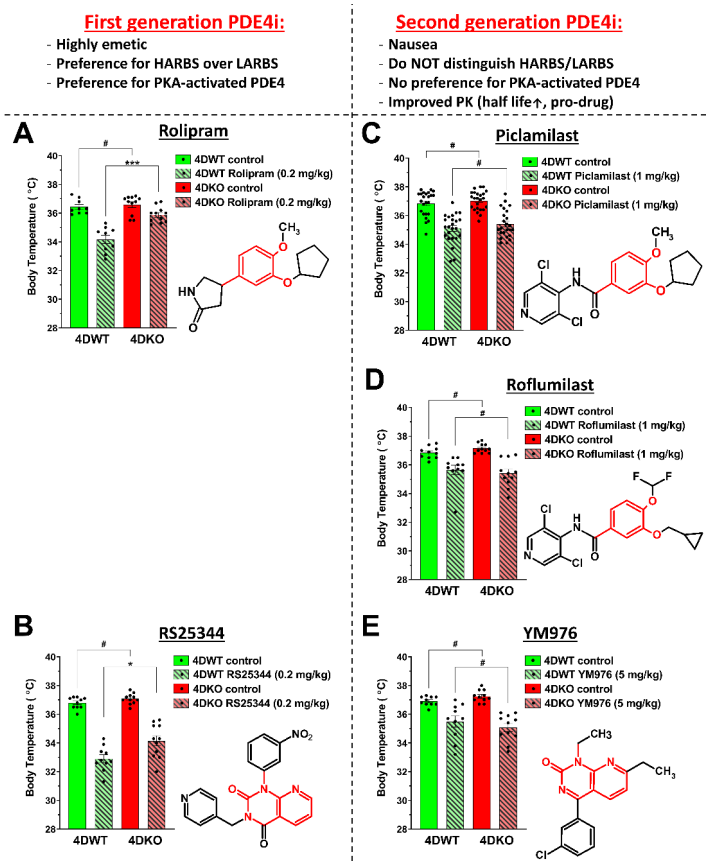

Figure 7. Genetic deletion of PDE4D in mice alleviates the hypothermia induced by first-generation PDE4 inhibitors Rolipram and RS25344.

(**A**–**E**) Core body temperatures of PDE4D knockout (4DKO) mice and their respective wildtype (4DWT) littermates were measured just prior to (control) and again 30 min after i.p. administration of the PAN-PDE4 inhibitors (**A**) Rolipram (0.2 mg/kg), (**B**) RS25344 (0.2 mg/kg), (**C**) Piclamilast/RP73401 (1 mg/kg), (**D**) Roflumilast (1 mg/kg) or (**E**) YM976 (5 mg/kg). Data represent the mean ± SEM. Statistical significance was determined using the Mann–Whitney test with 95% confidence interval and is indicated as # ($p > 0.05$; not significant), * ($p < 0.05$) or *** ($p < 0.001$). The chemical structures of the PAN-PDE4 inhibitors tested are shown for comparison. Compounds are grouped into the first-generation PDE4 inhibitors, Rolipram and RS25344 (**A**,**B**), on the left, and the second-generation PDE4 inhibitors, Piclamilast, Roflumilast and YM976 in (**C**–**E**), on the right. First-generation inhibitors are distinguished from second-generation drugs by high emetic potential, selectivity for HARBS (high-affinity Rolipram-binding state—a unique conformation of PDE4 proteins) and their preference for inhibition of PKA-phosphorylated/activated PDE4[26,42,74,80]. While all listed PDE4 inhibitors do engage in paradigms that require brain-penetrance (e.g., anesthesia, dyskinesia, hypothermia [42,78]), the effects of Rolipram and RS25344 appear more rapid in onset and more potent compared to second-generation PAN-PDE4 inhibitors, suggesting that Rolipram and RS25344 exhibit a more rapid and profound brain-penetrance.

The separation of PDE4 inhibitors into one group that produces distinct effects in PDE4D-WT/KO mice (Rolipram and RS25344) and another group that does not (Piclamilast, Roflumilast, YM976) is not dependent upon the chemical core structure of the drugs (highlighted in red in Figure 7A–E), given that Roflumilast and Piclamilast are derivatives of Rolipram and that YM976 is a derivative of RS25344 (Figure 7). However, the compounds cluster well by some of their established pharmacodynamic and pharmacokinetic differences, in that Rolipram and RS25344 are more potently emetic and somewhat more brain-penetrant than the second-generation PDE4 inhibitors Roflumilast, Piclamilast and YM976. In addition, Rolipram and RS25344 exhibit a preference for binding to the HARBS (high-affinity Rolipram-binding state [95]) conformation of PDE4s, as well as an increased potency to inhibit the PKA-phosphorylated/activated PDE4 long forms, whereas Roflumilast, Piclamilast and YM976 do not[96-97] (Figure 7).

2.3.4 Role of α_2-Adrenoceptor Signaling in Body Temperature Regulation

Given that distinct PDE4 subtypes appear to mediate the effect of PAN-PDE4 inhibitors on the duration of Ketamine/Xylazine-induced anesthesia (Figure 4) or hypothermia (Figures 6 and 7), we wished to assess the level of similarity or divergence of the molecular mechanisms underlying these two phenotypes. To this end, we tested whether the PDE4 inhibitors' ability to mimic α_2-adrenoceptor antagonism, which drives their effect on the duration of Ketamine/Xylazine-induced anesthesia, may also contribute to their induction of hypothermia. As shown in Figure 3C, treatment with the α_2-adrenoceptor blocker Yohimbine (1 mg/kg; i.p.) mirrors the effect of PAN-PDE4 inhibitors and potently shortens the duration of Ketamine/Xylazine anesthesia. Conversely, the same dose of Yohimbine does not have any effects on baseline body

temperature (Figure 8A) and thus does not replicate the effect of PAN-PDE4 inhibitors in this paradigm. Intriguingly, however, treatment with the α_2-agonist Clonidine induced dose-dependent hypothermia in mice (Figure 8A). Clonidine-induced hypothermia was effectively alleviated by pre-treatment with the α_2-blocker Yohimbine (Figure 8B). Conversely, neither pre-treatment with the α_2-blocker Yohimbine nor pre-treatment with the α_2-agonist Clonidine alleviated PAN-PDE4 inhibitor-induced hypothermia (Figure 8C), suggesting that α_2-adrenoceptor signaling is not involved in the body temperature perturbance produced by PAN-PDE4 inhibitors. Taken together, these data suggest that distinct molecular pathways mediate the effect of PDE4 inhibitors on the two correlates of emetic potential studied here. While the effect of PAN-PDE4 inhibitors on the duration of Ketamine/Xylazine-induced anesthesia is mediated via α_2-adrenoceptor antagonism, the effect of PDE4 inhibitors on hypothermia is independent of α_2-adrenoceptor signaling, and it is thus understandable that distinct patterns of PDE4 subtypes are involved in these two paradigms.

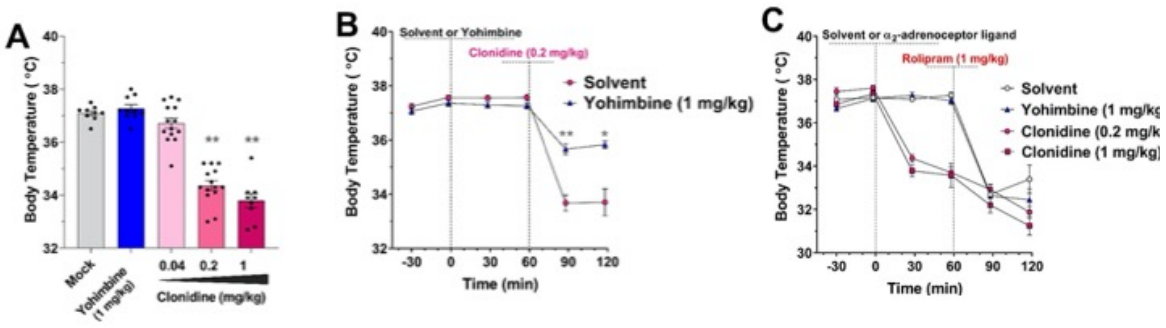

Figure 8. Agonism at α_2-adrenoceptors induces hypothermia, but neither agonism nor antagonism of α_2-adrenoceptors protects from PDE4 inhibitor-induced hypothermia in mice.

(**A**) The effect of treatment with the α_2-adrenoceptor antagonist Yohimbine (1 mg/kg; i.p.) or the α_2-adrenoceptor agonist Clonidine (0.04, 0.2 and 1 mg/kg; i.p.) on core body temperature measured 30 min after drug injection. Each dot represents a different animal. (**B**) Pre-treatment with the α_2-adrenoceptor antagonist Yohimbine (1 mg/kg; i.p., at 0 min) protects from hypothermia induced by the α_2-adrenoceptor agonist Clonidine (administered i.p. 60 min after pretreatment) (n = 8). (**C**) Neither pre-treatment with the α_2-adrenoceptor antagonist Yohimbine (1 mg/kg; i.p.) nor the α_2-adrenoceptor agonist Clonidine (0.2 or 1 mg/kg; i.p.) protects from or alters PDE4 inhibitor-induced hypothermia (Rolipram, 1 mg/kg, administered i.p. at 60 min after pretreatment) (n = 8). All data represent the mean ± SEM. Statistical significance was determined using the Kruskal–Wallis test and Dunn's post hoc test for the bar graph in (**A**) and using two-way ANOVA with Sidak's post hoc test for the time courses in (**B**,**C**) and is indicated as * ($p < 0.05$), ** ($p < 0.01$), or *** ($p < 0.001$).

2.3.5 Treatment with the Antiemetic Metoclopramide Alleviates PAN-PDE4 Inhibitor-Induced Hypothermia in Mice

If hypothermia represents a close correlate of the emetic potential of PAN-PDE4 inhibition in mammals, then anti-emetic medications may also be effective in reducing PDE4 inhibitor-induced hypothermia. To test this idea, we explored the role of two anti-emetics used to treat nausea due to chemo- or radiation-therapy: the 5-hydroxytryptamine 3 (5-HT_3)-serotonin receptor blocker Ondansetron and the prokinetic Metoclopramide, which acts via D_2-dopamine receptor antagonism and 5-HT_4-serotonin receptor agonism.

As shown in Figure 7, treatment with Metoclopramide partially alleviated hypothermia induced by the PAN-PDE4 inhibitor Rolipram (Figure 9A), whereas Ondansetron had no effect (Figure 9B).

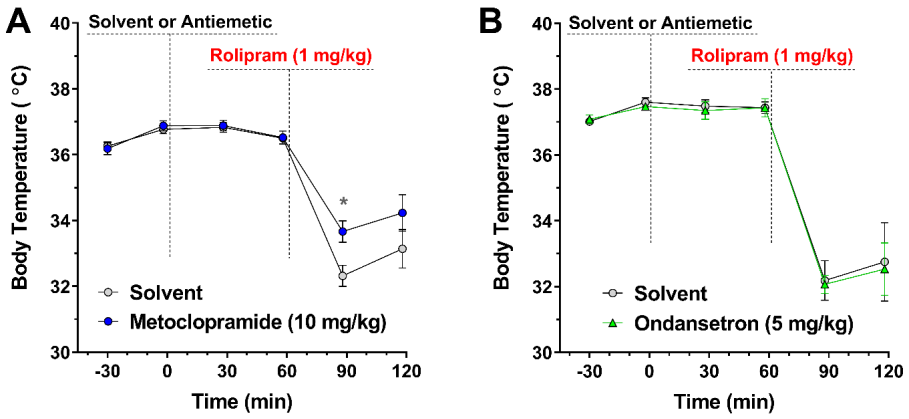

Figure 9. Effect of the antiemetics metoclopramide and Ondansetron on PDE4 inhibitor-induced hypothermia.

After measurement of baseline body temperature (at −30 and −1 min), mice were treated with the antiemetics Metoclopramide (10 mg/kg; o.g.; $n = 12$) or Ondansetron (5 mg/kg; i.p.; $n = 6$) or their respective solvent controls (Solvent), followed 60 min later by injection of the PDE4 inhibitor Rolipram (1 mg/kg; i.p.). Body temperature was measured at the indicated time points using a rectal probe thermometer. Pre-treatment with the prokinetic Metoclopramide (**A**), but not pre-treatment with Ondansetron (**B**), alleviated PDE4 inhibitor-induced hypothermia. All data represent the mean ± SEM. Statistical significance was determined using two-way ANOVA with Sidak's post hoc test and is indicated as * ($p < 0.05$).

2.4 Discussion

2.4.1 Assessing Hypothermia as a Correlate of PDE4 Inhibitor-Induced Nausea and Emesis in Mice: Comparison of First- and Second-Generation PDE4 Inhibitors

Nausea and/or emesis induced by a variety of triggers, from motion-sickness to radiation-treatment, anesthesia and/or surgery, or treatment with distinct classes of drugs, can all produce features of impaired thermoregulation[64-68]. This begs the question of whether PDE4 inhibitor-induced hypothermia may also represent an accurate and thus valuable correlate of the emetic potential of this particular class of drugs? Intriguingly, the potency of PAN-PDE4 inhibitors to induce nausea and/or emesis in humans [80,95] the potency of the same drugs to induce vomiting in animals[83, 98-99], as well as the potency of these drugs to induce correlates of emesis in animals, such as shortening the duration of α_2-adrenoceptor-dependent anesthesia [73, 82-83] and gastric retention [19,75], as well as hypothermia [24], all align to indicate that first-generation PDE4 inhibitors, such as Rolipram or RS25344, are significantly more emetic than second-generation PDE4 inhibitors. This pattern is confirmed in the present study. As shown in Figure 3C, the first-generation PDE4 inhibitor Rolipram is more potent than Piclamilast in the Ketamine/Xylazine anesthesia model. For hypothermia, the higher potency of first-generation PDE4 inhibitors was reported previously [24] but is also apparent in Figure 5, as doses of 0.2 mg/kg Rolipram or RS25344 (Figure 7A,B) produce more substantial hypothermia compared to higher doses of various second-generation PDE4 inhibitors (Figure 7C–E).

Importantly, the order of potencies by which they induce vomiting or correlates of emetic potential does not match the order of potency by which these drugs inhibit PDE4 activity in vivo or in vitro. This is illustrated by the fact that Rolipram is the least potent in inhibiting PDE4 of the drugs tested here but is one of the most emetic [74,78]. Nevertheless, there are several well-established properties that clearly distinguish first- and second-generation PDE4 inhibitors. The former, exemplified by Rolipram and RS25344, exhibit a preference for binding to HARBS (high-affinity Rolipram-binding state [78]), a particular conformation of PDE4 proteins, and both drugs also exhibit increased potency to inhibit PKA-phosphorylated/activated PDE4 compared to the non-phosphorylated enzymes [74,78,96]. Neither of these properties are shared by the second-generation PDE4 inhibitors, such as Roflumilast or Piclamilast[74, 80, 96-97] (Figure 7). Rolipram and RS25344 are arguably also more brain-penetrant [26, 75]. Thus, it seems plausible that a preference for HARBS and/or PKA-phosphorylated/activated PDE4 and/or high brain-penetrance may increase the emetic potential of PDE4 inhibitors such that they should be avoided in drug development. As these three properties strongly pattern together, it is currently difficult to clearly identify the main driver of the high emetic potential of first-generation PDE4 inhibitors among them. Perhaps the latter is not necessary, as these three properties may define the same pool of PDE4. Given that PDE4 is expressed at high levels in the brain, that cAMP/PKA-signaling is critical for neuronal signaling and that HARBS is highly enriched in the brain [78,100-103], the high emetic potential of first-generation PDE4 inhibitors may be due to engagement of a single pool of PKA-activated, neuronal PDE4 that preferentially exists in HARBS conformation. Taken together, PDE4 inhibitor-induced hypothermia in mice replicates differences in the

emetic potential of first- and second-generation PDE4 inhibitors that are well-established in humans and animals, suggesting that hypothermia in mice may represent a useful correlate of the emetic potential of PDE4 inhibitors. This conclusion is further supported by the finding that treatment with a clinically used anti-emetic, Metoclopramide (Figure 9A), alleviates PDE4 inhibitor-induced hypothermia. That both anti-emetics tested here did not alleviate PDE4 inhibitor-induced hypothermia (e.g., Ondansetron; Figure 9B) does not refute this argument, as it is well-known that most anti-emetics do not exhibit a broad-spectrum clinical efficacy. Instead, the efficacy of a particular class of anti-emetics is generally closely tied to the particular cause and hence the molecular mechanism that induced nausea/emesis. Prior reports have shown that this concept extends to the efficacy of anti-emetics to alleviate hypothermia induced by distinct triggers, such as provocative motion, radiation or drugs [59,69-70,104].

2.4.2 Role of Individual PDE4 Subtypes in Mediating the Side Effects of PAN-PDE4 Inhibitors: Comparison of Distinct Correlates of Emetic Potential in Mice

Given that development of subtype-selective PDE4 inhibitors is a promising approach to mitigate the adverse effects of PAN-PDE4 inhibitors, while retaining their therapeutic benefits, we wished to employ hypothermia in mice as a correlate to identify the specific PDE4 subtype(s) that predominantly mediate(s) the emetic potential of PDE4 inhibitors. To this end, we assessed body temperatures in mice deficient in each of the four PDE4 subtypes, PDE4A to D (Figure 6). These experiments revealed that selective inactivation of each PDE4 subtype per se does not produce hypothermia, which instead likely results from the concurrent inhibition of multiple (at least two) PDE4 subtypes. Alternatively, one could also postulate that a critical role of a particular PDE4 subtype on

body temperature may have been obscured in the response of the respective PDE4 knockout mouse by compensatory changes in the expression or activity of the three other PDE4 isoforms. We consider this unlikely, however, given the large body of data suggesting that PDEs are not functionally interchangeable [31,105-106] and because such compensatory changes in PDE4 expression were not observed in various primary cells or tissues of PDE4-KO mice reported previously [9,17,29,44,46-47 ,73 107-108].

The finding that ablation of any individual PDE4 subtype does not replicate PAN-PDE4 inhibitor-induced hypothermia mirrors the effect of subtype-selective PDE4 ablation on gastric retention reported previously [19]. Moreover, pre-treatment with the pro-kinetic Metoclopramide has been shown to alleviate acute gastric retention induced by PAN-PDE4 inhibition [19] and also alleviates PAN-PDE4 inhibitor-induced hypothermia (Figure 9). Given prior reports that blockade of $D_{2/3}$-dopamine receptors alleviates PDE4 inhibitor-induced hypothermia [24], this may suggest that Metoclopramide, which has some affinity for multiple receptor classes, including D_2-dopamine, muscarinic and serotonergic receptors [109-111], alleviates both gastric retention and hypothermia via its blockade of $D_{2/3}$-dopamine receptors.

In contrast to the effect of subtype-selective PDE4 ablation on body temperature and gastric motility, the effect of PAN-PDE4 inhibition on the duration of α_2-adrenoceptor-dependent anesthesia [82-84] is exclusively mediated and replicated by inactivation of PDE4D [73] (Figure 4). This difference in the role of the PDE4D subtype is paralleled by distinct molecular mechanisms involved in these correlates of emetic potential. The Ketamine/Xylazine model tests whether PDE4/PDE4D inactivation can act via physiological α_2-adrenoceptor antagonism to shorten/counteract α_2-adrenoceptor-

dependent anesthesia (see Figure 3C). Conversely, as shown in Figure 8, PDE4 inhibitor-induced hypothermia is not mediated by or dependent upon α_2-adrenoceptor antagonism. Intriguingly, both hypothermia as well as gastric retention [19] induced by PDE4 inhibitors are unaffected by α_2-adrenoceptor antagonism (Figure 8A), whereas α_2-adrenoceptor agonists, such as Clonidine, induce hypothermia (Figure 8A) as well as gastric retention [19]. Thus, gastric retention and hypothermia share multiple molecular mechanisms, including the absence of a predominant role of PDE4D and the effect of $D_{2/3}$-dopamine receptor antagonists, as well as the effects of α_2-adrenoceptor agonists and antagonists in these paradigms.

The role of PDE4D in curtailing α_2-adrenoceptor-dependent anesthesia may lead to the assumption that inhibition of PDE4D is irrevocably tied to adverse effects and that any therapeutic benefits that may be derived from targeting PDE4D must be forfeited. In contrast, the observation that the concurrent inhibition of multiple PDE4 subtypes is required to induce hypothermia (Figure 6) or gastric retention suggests that the selective inhibition of any individual PDE4 subtype, including the selective inhibition of PDE4D, may be free of nausea and emesis. This may hold true even if PDE4D should be one of the PDE4 subtypes that must be inhibited simultaneously to produce these adverse effects, and thus suggests that PDE4D may be targeted for therapeutic benefits. This idea is validated to some extent by the reduced emetogenic potential of allosteric PDE4D-selective inhibitors in animal models reported previously [112-113].

Considering the distinct effect of PDE4D ablation on Ketamine/Xylazine-induced anesthesia versus hypothermia, it is tempting to speculate which of the two is a more accurate correlate of emetic potential. However, as a short historic perspective on the

emetic effects of other drug classes (e.g., chemotherapeutics [59]) would quickly reveal, such speculations are likely futile and error-prone at present. Only after candidate PDE4 inhibitors (whether they are designed to be subtype/PDE4D-selective, conformation-selective, or perhaps exhibit unique pharmacokinetic properties) demonstrate the absence of adverse effects compared to PAN-PDE4 inhibitor in humans may these compounds also serve to identify the more appropriate animal model that reflects the emetic potential of PDE4 inhibition, which may subsequently serve as a standard in drug development. Moreover, just as individual patients taking PDE4 inhibitors may experience variable levels of nausea and emesis, it is also possible that distinct molecular mechanisms contribute to nausea and emesis in individual patients experiencing these adverse effects, which opens the possibility that different animal models capture the emetic potential of PDE4 inhibitors reflective of distinct patient populations.

2.4.3 Variable Responses of PDE4D-KO Mice to Hypothermia Induced by First- and Second-Generation PAN-PDE4 Inhibitors

The observation that two first-generation PDE4 inhibitors, Rolipram and RS25344 (Figure 7A,B), produce less severe hypothermia in PDE4D-KO mice compared to wildtype controls is noteworthy, given that this involves PDE4D somehow in the hypothermia paradigm. There are various possible explanations for this observation, all of which remain to be tested experimentally, however. For example, given the necessity of maintaining normal body temperature for the survival of the organism, one may speculate that the long-term deletion of PDE4D in the KO animals, even if acute PDE4D inactivation were to affect body temperature, would trigger an adaptation and compensatory mechanisms to restore body temperature back to 37 °C. Even if so, PDE4D

must not play a predominant role, however, given that all PDE4 inhibitors, including the first-generation PDE4 inhibitors Rolipram and RS25344, produce significant hypothermia in PDE4D-KO mice. This clearly shows that these inhibitors act in PDE4D-KO mice via inhibition of PDE4s other than PDE4D to induced hypothermia. But why are there differences between first-generation PDE4 inhibitors, which distinguish between WT and PDE4D-KO mice, and second-generation PDE4 inhibitors, which do not? These differences suggest that while PDE4D is mechanistically linked to the highly emetic effects exhibited by Rolipram and RS25344, there is no such mechanistic link associating PDE4D with the emetic potential that remains in current second-generation PDE4 inhibitors. In other words, first- and second-generation PDE4 inhibitors induce emesis via inhibition of distinct, though likely overlapping, pools of PDE4 in the body. By avoiding a particular pool of PDE4, which is likely predominated by PDE4D (e.g., a pool of PDE4D in HARBS conformation of the central nervous system), second-generation PDE4 inhibitors present with an improved safety profile. However, PDE4D does not seem to play a predominant role in mediating the remaining adverse effects of second-generation PDE4 inhibitors. Thus, for further improvement in the safety profile of this class of drugs (e.g., development of third-generation PDE4 inhibitors) there is little concrete evidence to suggest that their action on PDE4D must be avoided.

2.5 Conclusions

Given the growing body of evidence that links nausea and emesis to disturbances in thermoregulation in mammals, we here explored PDE4 inhibitor-induced hypothermia as a novel correlate of nausea in mice. Using knockout mice for each of the four PDE4 subtypes, we show that selective inactivation of individual PDE4 subtypes does not produce hypothermia, which must instead require the concurrent inactivation of multiple (at least two) PDE4 subtypes. This finding contrasts with prior reports that proposed PDE4D as the subtype mediating these adverse effects of PAN-PDE4 inhibitors and suggests that inhibitors that selectively target any individual PDE4 subtype, including those targeting PDE4D, may be free of nausea and emesis.

Intriguingly, the potency of distinct PDE4 inhibitors to induce hypothermia correlates well with the reported potency of these drugs to induce nausea and/or emesis in humans, as well as their potency to engage correlates of emetic potential in animals. In addition, hypothermia mirrors some of the facets of PDE4 inhibitor-induced gastric retention reported previously [19], in that both require the inactivation of multiple PDE4 subtypes and both are alleviated by $D_{2/3}$-dopamine receptor blockers. Finally, treatment with a clinically used anti-emetic, Metoclopramide, alleviates PDE4 inhibitor-induced hypothermia in mice, providing a mechanistic link between the two physiological paradigms. Together, these observations suggest that hypothermia may represent a useful correlate for PDE4 inhibitor-induced nausea and thus represents a rapid and cost-effective experimental approach to evaluate novel lead compounds or to explore novel mechanistic insights to facilitate drug development.

CHAPTER III

THE cAMP-PHOSPHODIESTERASE 4 (PDE4) CONTROLS β-ADRENOCEPTOR- AND CFTR-DEPENDENT SALIVA SECRETION IN MICE

3.1 Introduction

It is now well appreciated that in humans and animals alike, saliva is indispensable for oral health, and in extension for the overall health of the organisms[114-116]. While largely comprised of water, saliva contains critical minerals and electrolytes (such as sodium, potassium, calcium, chloride), buffers (bicarbonate) as well as a plethora of proteins including digestive enzymes (e.g. amylase), various antimicrobials (including cystatins, lysozyme, agglutinins, and secretory immunoglobulins/IgA), as well as a large number of glycoproteins, of which mucins (e.g. Muc5B) are the most eminent. Together, these facilitate several critical tasks including the lubrication and moistening of oral mucosal surfaces, the maintenance of microbial homeostasis and innate immune defense, they assist in the digestion of food, bolus formation, swallowing, and taste, and play critical roles in the mineralization of teeth and wound healing[115-117]. Humans produce between 0.5 and 1 L of saliva each day and its importance is plainly demonstrated by the significant discomfort and impairments suffered by patients with salivary gland hypofunction, which include xerostomia, the feeling of dry mouth, a loss

of taste sensation, difficulty chewing, digesting and swallowing food leading to malnutrition/weight loss, an increased incidence of oral infections, sialosis, enamel hypomineralization and dental caries[114-116,118]. Salivary gland hypo/dys-function is caused by various medical conditions or interventions including ageing, radiation therapy (e.g. for head and neck cancers), autoimmune diseases (such as Sjögren's syndrome), infection/inflammation affecting the salivary glands, diabetes, or be caused by a wide range of xerogenic medications (including anticholinergics, anti-histamines, some antidepressants/antipsychotics, or antihypertensives)[118]. Current treatment options are limited and include parasympathomimetics, particularly the $M_{1/3}$-muscarinic-acetylcholine receptor agonists Pilocarpine and Cevimeline, as well as symptomatic (mouth washes or gels) or homeopathic treatments.

Saliva is produced by three main glands, the submandibular gland (SMG), the sublingual gland (SLG), and the parotid gland (PG), as well as a multitude of smaller glands[114], which are regulated by both the sympathetic and the parasympathetic autonomic nervous system and produce secretions with distinct compositions[119]. An intracellular increase in calcium (e.g. in response to M_3AChR or α_1-adrenoceptor activation) induces production of large volumes of a watery saliva that is low in protein content. Conversely, an increase in the second messenger cAMP (e.g. in response to β-adrenergic stimulation) induces secretion of saliva that is high in protein content[116,118].

The cellular concentration of cAMP is principally defined by the interplay between G_s- and G_i-coupled receptor signaling that determines the rate of cAMP synthesis by adenylyl cyclases, but in equal measure by the rate of cAMP degradation that is catalyzed by cyclic nucleotide phosphodiesterases (PDEs). In humans and most

mammalian model species, including mouse and rat, PDEs are encoded by 21 genes, which in turn are grouped into 11 PDE families by sequence homology[30, 32]. The PDE4 family is the largest

of the PDE families, comprising four PDE4 genes or subtypes, PDE4A, PDE4B, PDE4C, and PDE4D[32]. Each PDE4 gene in turn is expressed as a large number of protein variants, that are generated *via* use of alternate promoters and transcription start sites, or by alternative splicing. These variants are distinguished by their cell- and tissue-specific expression patterns, unique post-translational regulation, and their recruitment into distinct macromolecular signaling complexes and subcellular compartments[53]. As a result, individual PDE4 subtypes and protein variants exert unique cellular and physiologic functions. PDE4s are also widely expressed, so that one or more PDE4 variants are found in almost every cell of the body, and combined, PDE4s often contribute a significant amount of total cellular cAMP hydrolytic capacity. Given their wide distribution, it is not surprising that PAN-selective PDE4 inhibition produces an array of potentially therapeutic benefits, including broad-spectrum anti-inflammatory properties, improvement in cognition and memory, metabolic- or cardiovascular effects[29-30, 35,52]. While exploring potential anti-inflammatory benefits of PDE4 inhibition in a model of bacterial lung infection, we noticed an obvious, abnormal salivation in mice pretreated with PDE4 inhibitors when we subsequently handled the animals during intratracheal infection, and have further explored this observation.

3.2 Materials and Methods

3.2.1 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, and Atropine were from Cayman Chemical (Ann Arbor, MI), Isoprenaline, Pilocarpine, and Propranolol from Millipore Sigma (St. Louis, MO, 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). 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) and were applied by intraperitoneal (i.p.) injection (100 µl per 20 g body weight).

3.2.2 Animals

Wild-type C57BL/6 mice for experimentation were generated in-house using breeders obtained from Charles River Laboratories (Wilmington, MA). Mice deficient in PDE4A[45], PDE4B[9] and PDE4D[6] were generated by Drs. S.-L. Catherine Jin and Marco Conti (Stanford University, CA; also see[11]) 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. 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. Please find a description of the PDE4C knockout mouse here[19]; additional details are available on the website of the Mutant Mouse Regional Resource Centers (MMRRC; http://www.mmrrc.org; Stock number 049025-UCD). Mice carrying the ΔF508-CFTR ($Cftr^{tm1Kth}$) mutation, that is common among cystic fibrosis patients, were generated by Dr. Kirk R. Thomas (University of Utah) and kindly distributed *via* The Jackson Laboratory (stock 002515 | ΔF; Bar Harbor, ME, USA). To alleviate their established phenotype of lethality resulting from bowel obstructions, the ΔF508-CFTR colony was maintained on laxative (50% Golytely; Braintree Laboratories, Braintree, MA, USA) in the drinking water. All mice were maintained on a C57BL/6 background and group housed four mice per cage with *ad libitum* access to food and water in a temperature-controlled (22-23°C) vivarium with a 12-h light/dark cycle. Adult mice ≥10 weeks of age and ≥18 g of body weight were used for experimentation by equally and randomly dividing cage littermates into experimental groups. Unless indicated otherwise, experiments were performed using male mice. 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 Institutes of Health, Bethesda, MD, USA) and were approved by the University of South Alabama Institutional Animal Care and Use Committee. For euthanasia, animals were injected i.p. with EUTHASOL® Euthanasia Solution (Patterson Veterinary, Greeley, CO, USA) followed by cervical dislocation.

3.2.3 Scoring salivation in awake mice

An experimenter, blinded to drug treatments or genotypes, scored the mice three times at 10, 20, and 30 min after drug injection as either "normal/not salivating = 0" or "abnormal/increased salivation=1". For each animal, the number/sum of positive "1" scores out of the three performed is reported.

3.2.4 Measurement of saliva secretion rate

Saliva secretion was measured as reported previously with minor modifications[120]. In short, after induction of anesthesia using Ketamine/Xylazine (80 and 10 mg/kg in PBS; i.p.), mice were placed on their sides, their mouths were wiped out with tissue, and narrow, pre-weighed strips of filter paper (4 mm x 20 mm) were placed 7 mm deep into their downward-facing cheek to absorb saliva (see Fig. 12A). The filter papers are replaced every 10 min for a total of 60 min and saliva production is calculated as the increase in weight of the paper strips before and after placement in the mice's mouth. Two different timelines were applied to evaluate the effect of PDE4 inhibitor treatment on saliva production in anesthetized mice. In the short protocol (see scheme in Fig. 12B), PDE4 inhibitors or solvent control are injected (i.p.), and measurement of saliva production is initiated as soon as the mice lose consciousness (3-5 min) after Ketamine/Xylazine administration. In the longer protocol (see Fig. 12C), test drugs (e.g. PDE4 inhibitor, Isoprenaline, Pilocarpine) are injected 15 min after administration of Ketamine/Xylazine. For several data sets, both time courses of cumulative saliva production as well as the total amount of saliva produced in 60 min. is reported. Saliva production after treatment with high-dose Pilocarpine (1 mg/kg) is so substantial, that filter papers are soaked past the 7 mm depth within 2-5 min. Thus, for Pilocarpine only,

filter papers were replaced once or twice within a given 10 min time period as needed. If animals started to recover from anesthesia during the 60 min time course of the experiment (as detected by independent limb or head movements), additional doses of 25% of the original volume of Ketamine/Xylazine (80 and 10 mg/kg in PBS; i.p.) were administered to maintain anesthesia. PDE inhibitors were generally administered at a dose of 1 mg/kg (i.p.), which has been shown to produce ≥50% of maximal efficacy on a variety of acute phenotypes of PDE4 inhibition in mice[19,26,24], and thus likely reflects ≥50% target engagement.

3.2.5 Measurement of cAMP-PDE activity in salivary glands

Salivary glands were extracted from mice, flash-frozen in liquid nitrogen and stored at -80°C until processing. Tissues were then homogenized in buffer containing 20 mM HEPES (pH 7.4), 1 mM EDTA, 0.2 mM EGTA, 150 mM NaCl, 20% sucrose, HaltTM Protease & Phosphatase Inhibitor Cocktail (Thermo Scientific, Rockford, IL) and 1% Triton X-100 using a Dounce glass homogenizer. After a 30-min rotation at 4°C, cell debris was pelleted with a 10-min centrifugation at 20,000 g at 4°C, and soluble extracts were then subjected to cAMP-PDE activity assays following a protocol described previously[121] with minor modifications. In brief, samples were assayed in a reaction mixture of 200 µl containing 40 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 1.34 mM β-mercaptoethanol, 1 µM cAMP, and 0.1 µCi [^{3}H]cAMP (Perkin Elmer, Waltham, MA) for 10 min at 37°C followed by heat inactivation in a boiling water bath for 1 min. The PDE reaction product 5′-AMP was then hydrolyzed by incubation of the assay mixture with 50 µg *Crotalus atrox* snake venom (Sigma-Aldrich, St. Louis, MO) for 20 min at 37°C and the resulting adenosine was afterwards separated by anion exchange chromatography on

1 ml of AG1-X8 resin (Bio-Rad Laboratories, Hercules, CA) and quantitated by scintillation counting. PDE4 activity was defined as the fraction of total cAMP-PDE activity inhibited by 10 μM of the archetypal PDE4 inhibitor Rolipram compared to solvent control (final concentration of 1% DMSO in the assay reaction).

3.2.6 Data and Statistical Analysis

All data are expressed as the mean ± SEM and n numbers indicate the number of individual animals assessed and are represented by individual dots in the scatter plots. 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 Bonferroni's post hoc test. Statistical differences are indicated as # (not significant; $p>0.05$), * ($p<0.05$), ** ($p<0.01$), and *** ($p<0.001$).

3.3 Results

3.3.1 Treatment with PAN-PDE4 inhibitors induces salivation in awake/conscious mice

While performing intratracheal infections, we noted that mice pretreated with PDE4 inhibitors exhibited an unusual, elevated salivation (see representative images in Fig. 10A/B). The effect was replicated by several, structurally-distinct PAN-PDE4

inhibitors including Rolipram, Roflumilast, Piclamilast/RP73401, and RS25344 (all at 1 mg/kg; i.p.), as scored by an experimenter blinded to drug treatments at 10, 20, and 30 min after drug injection (Fig. 10C). Conversely, treatment with the PDE3-inhibitor Cilostamide had no effect (Fig. 10C). Together, these data suggest that increased salivation is a class-effect of PAN-selective PDE4 inhibition in mice. It is well established that activation of M_3-muscarinic acetylcholine receptors (M_3-AChR) or β-adrenoceptors may induce salivation *via* central effects and/or by acting directly on salivary gland cells. Indeed, treatment with the M_3-mAChR agonist Pilocarpine induced visible salivation in the mice that was blunted by treatment with the muscarinic acetylcholine receptor (mAChR) antagonist Atropine, whereas the β-adrenoceptor agonist Isoprenaline induced salivation that was sensitive to treatment with the β-blocker Propranolol (Fig. 10D). Intriguingly, salivation induced by treatment with two structurally-distinct PDE4 inhibitors, Rolipram and RS25344 (see formulas in Fig. 10C), was ablated by blockade of either β-adrenoceptor signaling or mAChR signaling (Fig. 10E), suggesting that PDE4 inhibitor-induced salivation requires both receptor pathways.

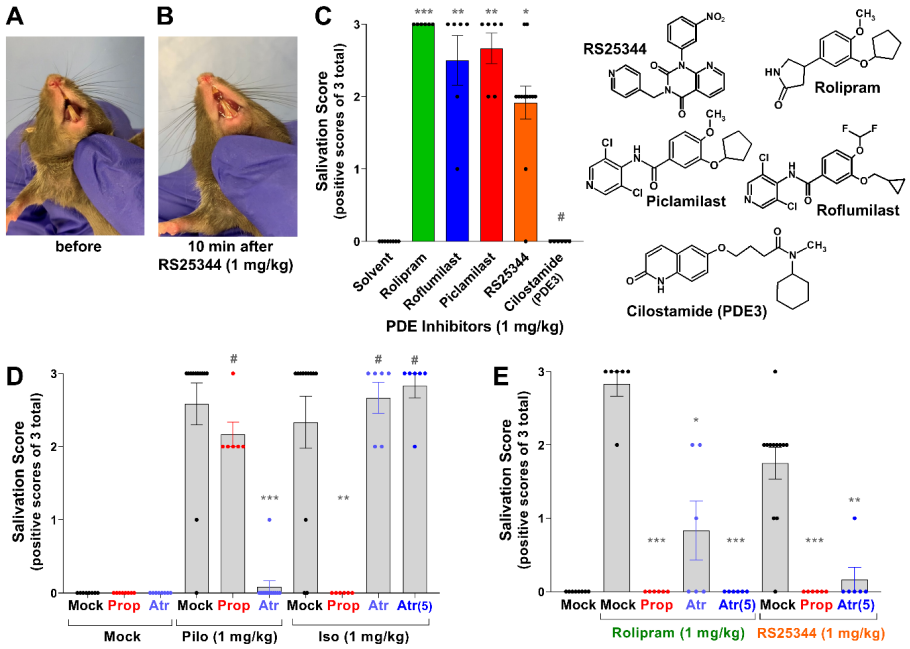

Fig. 10. Salivation is a class effect of PAN-selective PDE4 inhibitors in mice.

Male C57Bl6 mice were injected intraperitoneally with distinct PDE inhibitors, the M_3 muscarinic receptor agonist Pilocarpine (Pilo, 1 mg/kg), the β-adrenoceptor agonist Isoprenaline (Iso, 1 mg/kg) or solvent controls (Mock). Mice were scored at 10, 20 and 30 min after drug injection as either "1" elevated/abnormal salivation, or "0" no elevated/abnormal salivation and the sum of the three scores is reported for each mouse. (**A**/**B**) Representative images of the same mouse shortly before drug injection showing no abnormal salivation (**A**) and at 10 min after treatment with the PDE4 inhibitor RS25344 (1 mg/kg) (**B**) exhibiting abnormal drug-induced salivation. (**C**) Mice were injected with the PAN-PDE4 inhibitors Rolipram, Roflumilast, Piclamilast/RP73401, or RS25344 (all 1 mg/kg, i.p.), the PDE3 inhibitor Cilostamide (1 mg/kg) or solvent and salivation was scored three times at 10, 20 and 30 min after drug injection. Treatment with any PDE4 inhibitor produced salivation, whereas inhibition of PDE3 did not. (**D**/**E**) 30 min after pre-treatment with the mAchR blocker Atropine at a dose of 1 mg/kg (Atr) or 5 mg/kg (Atr(5)), the β-adrenoceptor blocker Propranolol (Prop, 5 mg/kg, i.p.) or solvent control (Mock), mice were injected with the M_3 muscarinic receptor (M_3R) agonist Pilocarpine (Pilo, 1 mg/kg) or the β-adrenoceptor (βAR) agonist Isoprenaline (Iso, 1 mg/kg) (**D**), or the PDE4 inhibitors Rolipram (1 mg/kg) or RS25344 (1 mg/kg) (**E**), and salivation was scored at 10, 20 and 30 min after drug injection. Salivation induced by M_3R agonism was ablated by Atropine, but not Propranolol. Salivation induced by Isoprenaline was selectively ablated by Propranolol, but unaffected by Atropine. Salivation induced by PDE4 inhibitors Rolipram or RS25344 was ablated by treatment with either Propranolol or Atropine, suggesting that both β-adrenergic and muscarinic signaling mediate PDE4 inhibitor-induced salivation. Data represent the mean ± SEM. Statistical significance was determined using Kruskal-Wallis and Dunn's post hoc tests and is indicated as # (not significant; $p>0.05$), * ($p<0.05$), ** ($p<0.01$) and *** ($p<0.001$).

In mice and other mammals, saliva is produced by three main glands, the submandibular gland (SMG), the sublingual gland (SLG), and the parotid gland (PG), as well as a multitude of smaller glands[114]. To assess the relative expression of PDE4 in these tissues, we measured cAMP-PDE activity in detergent extracts prepared from these glands in the presence or absence of the PDE4 inhibitor Rolipram (10 μM) *in vitro*. As shown in Fig. 11, PDE4 activity, defined as a fraction of cAMP-PDE activity inhibited by the archetypal PDE4 inhibitor Rolipram, contributes a major portion of total cAMP-PDE activity in each of these glands. This indicates that PDE4 inhibitor-induced salivation may result, at least in part, from direct action of PDE4 inhibitors in the gland cells, rather than exclusively from indirect effects, such as from PDE4 inhibition in sensory- or central nervous system components of salivary regulation.

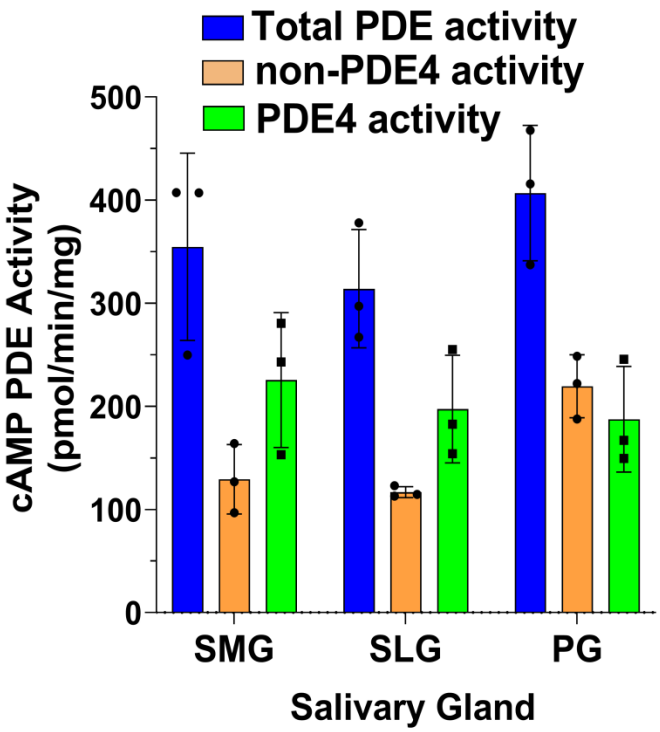

Figure 11. PDE4 contributes the majority of cAMP-PDE activity in mouse salivary glands.

Detergent extracts prepared from submandibular (SMG), sublingual (SLG), and parotid (PG) salivary glands were subjected to *in vitro* cAMP-PDE activity assays in the presence or absence of the PDE4 inhibitor Rolipram (10 μM). Total cAMP-PDE activity is defined as the rate of cAMP hydrolysis measured in the absence of Rolipram, whereas PDE4 and non-PDE4 activity are defined as the fraction of total activity that is either inhibited or that is insensitive to inhibition by Rolipram, respectively. All data represent the mean ± SEM. In the scatter plot, each dot represents a gland isolated from a different animal (n=3)

3.3.2 In Anesthetized Mice, PDE4 Inhibition Potentiates Saliva Production Induced by β-adrenoceptor-, but Not Muscarinic Receptor Stimulation

We next aimed to quantify glandular saliva production in response to PDE4 inhibitor treatment. To this end, mice were anesthetized using Ketamine/Xylazine and saliva production was assessed by the weight increase of thin filter paper strips that were placed in the mouths of mice for 10 min at a time (see representative image in Fig. 12A). If PDE4 inhibitors were injected as soon as the animal was unconscious (3-5 min after Ketamine/Xylazine; see timeline in Fig. 12B), highly variable rates of saliva production were observed (Fig. 12D and two left bars of Fig. 12E). In some mice, PDE4 inhibition hardly induced any saliva production over solvent controls, whereas in others, a substantial saliva production was induced. Conversely, if PDE4 inhibitor administration was delayed until 15 min after induction of anesthesia (see timeline in Fig. 12C), responses were more consistent, but PDE4 inhibition produced only a miniscule increase in saliva production over solvent controls (two right bars in Fig. 12E). Our interpretation of this finding is that inhibition of neuronal activity during deep anesthesia ablates a neurotransmitter signal that is required for, and that is amplified by PDE4 inhibition in awake mice. Given that blockade of β-adrenoceptor- or mAChR-signaling ablated PDE4 inhibitor-induced salivation in awake mice, we thus further explored these two pathways. Treatment with the β-agonist Isoprenaline (Iso) dose-dependently induced salivation in deeply anesthetized mice that plateaued at ~4 mg/g/h (Fig. 13A and B).

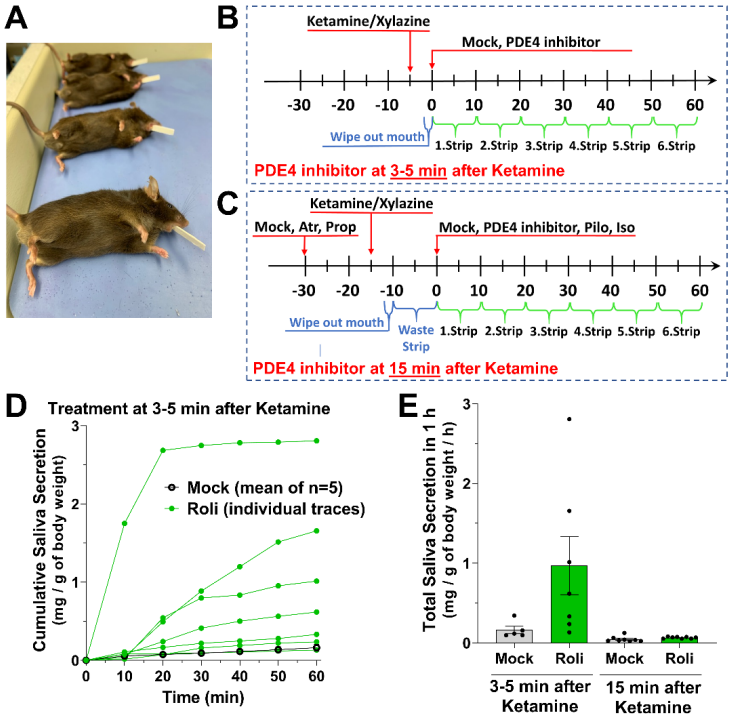

Figure 12. Measurement of saliva production in anesthetized mice.

(**A**) A representative image of the approach to measure saliva production. (**B**/**C**) Approach of measuring saliva production with PDE4 inhibitor injection following either 3-5 min (**B**) or 15 min (**C**) after Ketamine/Xylazine anesthesia. In the shortened timeline (**B**), mice were put on their sides as soon as they became unconscious (3-5 min after Ketamine/Xylazine), their mouth was wiped with a tissue to remove baseline saliva, followed by injection of PDE4 inhibitor or solvent and immediate placement of the first of 6 pre-weighed filter paper strips. In the longer timeline (**C**), mice were placed on their side at 5 min after Ketamine/Xylazine, their mouth was wiped and a filter strip was placed in their mouths for 10 min to absorb baseline saliva. Ten min later, this filter strip was discarded, PDE4 inhibitors or other drugs were injected, followed by placement of the first pre-weighed filter. (**D**) Mice treated with the PDE4 inhibitor Rolipram (1 mg/kg, i.p.) immediately after achieving anesthetic plane (3-5 min after Ketamine/Xylazine injection; see (**B**)) exhibit variable rates of saliva production over an hour, with some mice producing substantial saliva, whereas others do not show elevated production. (**E**) PDE4 inhibitor-induced saliva production depends on the timing of drug injection. Shown is total saliva production after i.p. injection of the Rolipram (Roli; 1 mg/kg) or solvent as soon as animals are anesthetized by Ketamine/Xylazine (3-5 min; left two bars), or if the drugs were injected at 15 min after Ketamine/Xylazine. Data represent the mean ± SEM. In scatter plots, each dot represents a different mouse. Statistical significance was determined using Mann-Whitney test with 95% confidence interval and is indicated as * ($p<0.05$).

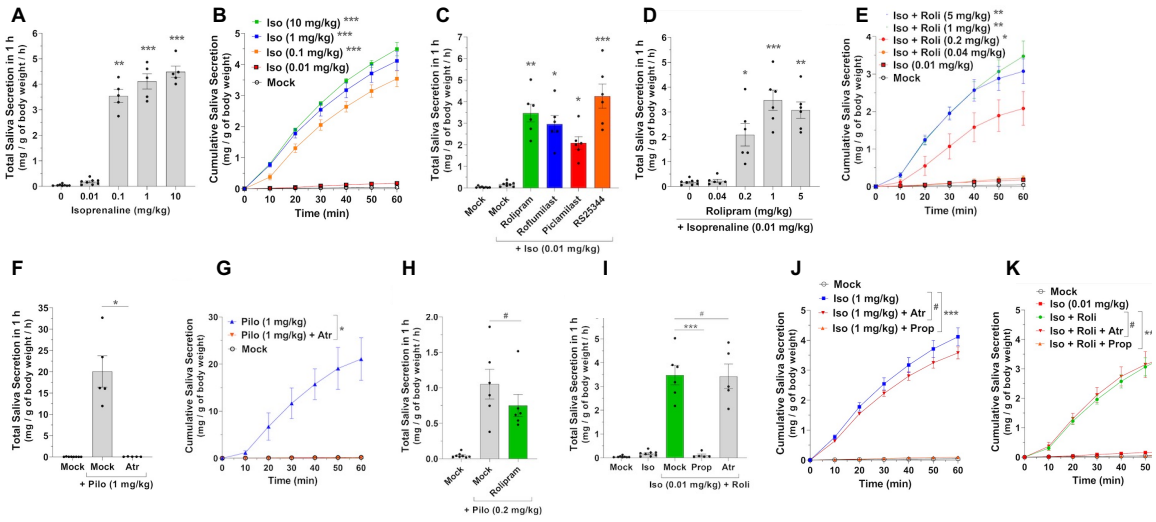

Figure 13. PDE4 inhibition potentiates β-adrenoceptor-dependent salivation in anesthetized mice.

Fifteen minutes after Ketamine/Xylazine-anesthesia, mice were injected i.p. with the indicated drugs and saliva production was measured for 60 min. (**A**) Dose-dependent induction of salivation by the β-adrenoceptor agonist Isoprenaline (Iso). (**B**) Cumulative saliva production induced by distinct doses of Iso (n=5) compared to solvent (Mock) control. (**C**) Potentiation of salivation in the presence of low-dose Iso (0.01 mg/kg) is induced by PDE4 inhibitors Rolipram, Roflumilast, Piclamilast/RP73401, and RS25344 (all 1 mg/kg), suggesting a class-effect these drugs. (**D**) Total saliva produced in 60 min by distinct doses of Rolipram with low-dose Iso (0.01 mg/kg). (**E**) With of low-dose Iso (0.01 mg/kg), Rolipram (Roli; n=6) dose-dependently induces significant saliva production. (**F**) Total saliva production in mice treated with the muscarinic agonist Pilocarpine (Pilo; 1 mg/kg) with or without the mAChR blocker Atropine (Atr; 5 mg/kg). **(G)** Pilo (1 mg/kg; n=5) induces substantial salivation that is ablated by pretreatment with Atropine (Atr; 5 mg/kg; $p<0.001$). **(H)** Co-treatment with Rolipram does not enhance saliva production induced by low-dose Pilo (0.2 mg/kg). **(I)** Pretreatment with the β-blocker Propranolol (Prop; 5 mg/kg), but not Atropine (Atr, 5 mg/kg) prevents salivation induced by Roli (1 mg/kg) in the presence of low-dose Iso (0.01 mg/kg). **(J)** Pre-treatment with the Prop (5 mg/kg), but not Atr (5 mg/kg) prevents salivation induced by high-dose Iso (1 mg/kg; n=5) **(K)** Time course of saliva production in mice treated with Rolipram (Roli; 1 mg/kg) and/or low-dose Iso (0.01 mg/kg). Some mice were pre-treated with the Propranolol (Prop; 5 mg/kg) or Atr (5 mg/kg) at 30 min prior to Iso/Roli injection).All data represent the mean ± SEM. In scatter plots, each dot represents a different mouse. For total saliva production shown in bar graphs, statistical significance was determined using Kruskal-Wallis and Dunn's post hoc tests; for time courses, two-way ANOVA and Bonferroni's post hoc tests were used. Statistical significance is indicated as # (not significant; $p>0.05$), * ($p<0.05$), ** ($p<0.01$), and *** ($p<0.001$).

In the presence of a low dose of Isoprenaline (0.01 mg/kg), which by itself did not induce significant salivation, co-treatment with the PDE4 inhibitor Rolipram produced a substantial potentiation of saliva production in a dose-dependent manner (Fig. 13D/E). Potentiation of β-agonist-dependent salivation in mice was replicated by distinct PDE4 inhibitors, including Roflumilast, Piclamilast, or RS25344, confirming it as a class effect of PAN-PDE4 inhibitors (Fig. 13C). Moreover, β-agonist-dependent salivation in anesthetized mice was ablated by treatment with the β-blocker Propranolol, whereas blockade of mAChR signaling with Atropine had no effect (Fig. 13I-K). Treatment with the M_3-mAChR agonist Pilocarpine produced substantial saliva production in Ketamine/Xylazine-anesthetized mice, that was ablated by Atropine (Fig. 13 F/G). However, contrary to β-adrenergic stimulation, salivation induced by a low/submaximal dose of Pilocarpine was not further enhanced by co-treatment with the PDE4 inhibitor Rolipram (Fig. 13H). Taken together, these data suggest that under conditions of neuronal depression induced by anesthesia, direct inhibition of PDE4 in the salivary glands does not produce significant salivation by itself (Fig. 13E), but potently enhances salivation induced by minimal activation of receptors that lead to increased cAMP production (e.g. β-adrenoceptors) (Fig. 13E). Conversely, salivation induced by mACh receptors that couple to G_q and intracellular calcium release (e.g. M_3-AChR) is not potentiated by PDE4 inhibition in anesthetized animals (Fig. 13K).

Prior reports have suggested some sex-differences in the amount of saliva produced in mice[122]. While male mice were primarily used throughout this study, we have repeated critical findings using female C57BL/6 mice and observed a similar pattern of spontaneous salivation after PDE4 inhibitor treatment in awake mice (Fig. 14A), and a

substantial potentiation of salivation in the presence of low-dose Isoprenaline in anesthetized mice (Fig. 14 B/C).

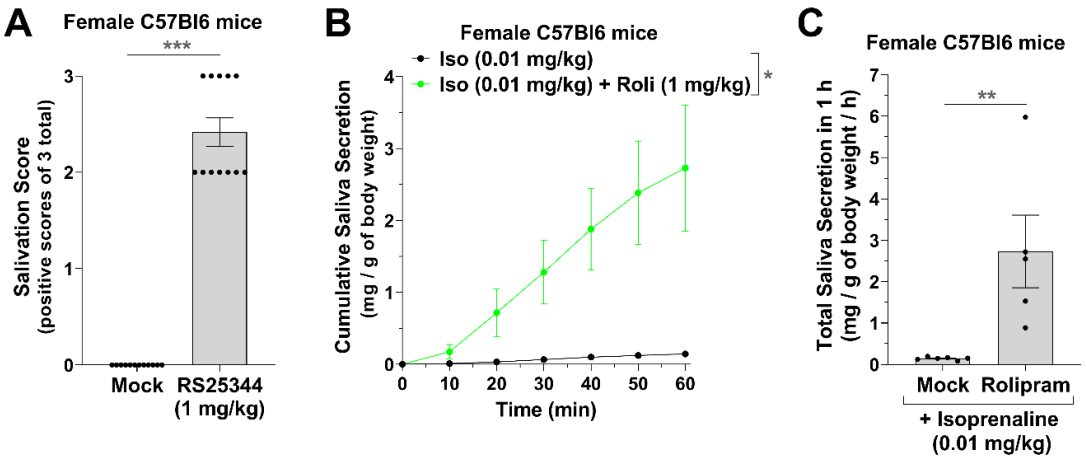

Figure 14. Treatment with PAN-PDE4 inhibitors induces salivation in female mice.

(**A**) Female C57BL/6 mice were treated (i.p.) with the PAN-PDE4 inhibitor RS25344 (1 mg/kg, n=12) or solvent control (Mock, n=12) and salivation was scored at 10, 20, and 30 min later. The number of positive salivation scores out of the three scorings performed is reported for each animal. (**B/C**) Fifteen minutes after induction of Ketamine/Xylazine-anesthesia, female C57BL/6 mice were injected (i.p.) with the β-adrenoceptor agonist Isoprenaline (Iso, 0.01 mg/kg) either by itself (n=6), or in combination with the PAN-PDE4 inhibitor Rolipram (Roli, 1 mg/kg, n=5), and saliva production was measured for the next 60 min. Shown is time course of saliva production (**B**), or the total amount of saliva produced in 1 h (**C**), in response to Iso/Roli. Data represent the mean ± SEM. In scatter plots, each dot represents a different animal. Statistical significance was determined using Mann-Whitney test with 95% confidence interval (**A/C**) or using two-way ANOVA and Bonferroni's post hoc test (**B**) and is indicated as * ($p<0.05$), ** ($p<0.01$), and *** ($p<0.001$).

3.3.3 PDE4 inhibitor-induced salivation is CFTR-dependent

Prior reports indicated that β-adrenoceptor-dependent salivation in mice is dependent upon, and proportional to cystic fibrosis transmembrane conductance regulator

(CFTR) function[120, 122]. Thus, the cAMP/PKA-mediated activation of CFTR current likely gates β-adrenoceptor-induced salivation. To assess whether PDE4 inhibitor-induced salivation is dependent upon functional CFTR, and may reflect a cAMP/PKA-mediated activation of the channel, we explored salivation in mice homozygous for the common cystic fibrosis mutation ΔF508-CFTR, which do not express functional CFTR. As shown in Fig. 15A, PDE4-inhibitor induced salivation observed in awake wild-type mice was significantly reduced in homozygous ΔF508-CFTR mice. In addition, anesthetized homozygous ΔF508-CFTR mice did not produce any measurable saliva levels in response to β-adrenergic stimulation with Isoprenaline, nor to co-treatment with Isoprenaline and the PDE4 inhibitor Rolipram, compared to heterozygous mice (Fig. 15B-D).

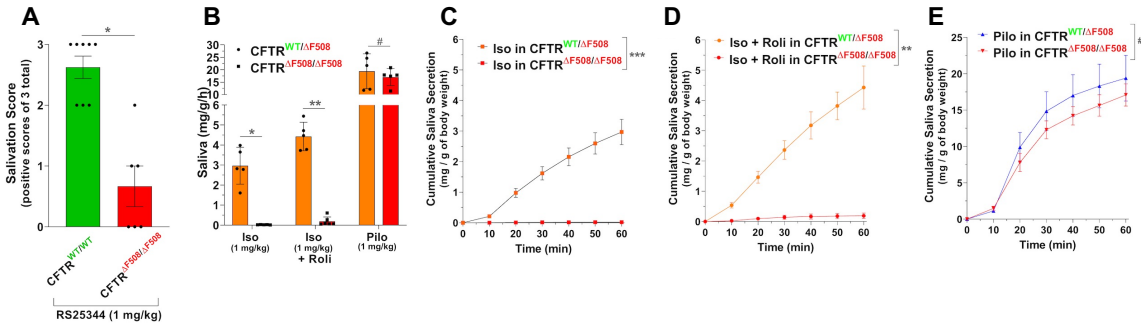

Fig. 15. PDE4 inhibitor-induced salivation is CFTR-dependent.

Fifteen minutes after induction of Ketamine/Xylazine-anesthesia, mice were injected i.p. with the β-adrenoceptor agonist Isoprenaline (Iso; 1 mg/kg), the PDE4 inhibitor Rolipram (Roli; 1 mg/kg), or the muscarinic receptor agonist Pilocarpine (Pilo; 1 mg/kg) and saliva production was measured for the next 60 min. (**A**) Homozygous ΔF508-CFTR mice ($CFTR^{\Delta F508/\Delta F508}$), which lack functional CFTR, or wildtype controls ($CFTR^{WT/WT}$) were injected with the PAN-PDE4 inhibitor RS25344 (1 mg/kg; i.p.) and animals were scored at 10, 20, and 30 min after drug injection for abnormal salivation by an experimenter blinded to the treatments. The sum of positive salivation scores for the three scorings performed is reported for each mouse. (**B**) Fifteen minutes after induction of Ketamine/Xylazine-anesthesia, mice were injected with the β-adrenoceptor agonist Isoprenaline (Iso; 1 mg/kg), the PDE4 inhibitor Rolipram (Roli; 1 mg/kg), or the muscarinic receptor agonist Pilocarpine (Pilo; 1 mg/kg) and saliva production was measured for the next 60 min. Saliva production in response to Iso in mice heterozygous for the ΔF508-CFTR mutation ($CFTR^{WT/\Delta F508}$) was slightly reduced compared to wildtype mice (see Fig. 3A). Conversely, neither Iso by itself, nor combination treatment with Iso and Roli induced significant saliva production in homozygous ΔF508-CFTR mice ($CFTR^{\Delta F508/\Delta F508}$). However, saliva production in response to the muscarinic agonist Pilocarpine (Pilo; 1 mg/kg) is preserved in homozygous ΔF508-CFTR mice. (**C**) Saliva production in response to Iso in mice heterozygous for the ΔF508-CFTR mutation ($CFTR^{WT/\Delta F508}$) was slightly reduced compared to wildtype mice (see Fig. 4A) but was completely ablated in homozygous ΔF508-CFTR mice ($CFTR^{\Delta F508/\Delta F508}$) which lack functional CFTR. (**D**) Saliva production in response to combination treatment with Iso and Roli is also ablated in homozygous ΔF508-CFTR mice. (**E**) Saliva production in response to the muscarinic agonist Pilocarpine is preserved in homozygous ΔF508-CFTR mice compared to heterozygous controls. All data represent the mean ± SEM. Statistical significance was determined using two-way ANOVA and Bonferroni's post hoc tests and is indicated as # (not significant; $p>0.05$), and *** ($p<0.001$). For A/B, statistical significance was determined using Mann-Whitney test with 95% confidence interval (**A**) or Kruskal-Wallis followed by Dunn's post hoc test (**B**) and is indicated as # (not significant; $p>0.05$), * ($p<0.05$), and ** ($p<0.01$).

Conversely, treatment with the M_3-agonist Pilocarpine induced comparable levels of saliva production in both heterozygous and homozygous ΔF508-CFTR mice (Fig. 15E). These data indicate that potentiation of the cAMP/PKA-mediated activation of CFTR is one mechanism whereby PDE4 inhibition induces salivation.

3.3.4 PDE4 inhibitor-induced salivation results from the concurrent inactivation of multiple PDE4 subtypes.

The PDE4 family comprises four subtypes, PDE4A, PDE4B, PDE4C, and PDE4D, and each have been shown to play unique roles in the body. To discern whether one of the four PDE4 subtypes exerts a predominant role in the regulation of salivation, we assessed salivation in systemic/global PDE4-KO mice for each of the four PDE4 subtypes. Visual scoring of the mice revealed that none of the four PDE4-KO mouse lines replicated the abnormal salivation induced by treatment with PAN-PDE4 inhibitors (no positive salivation scores in n=8 for each line). Measurement of saliva production in anesthetized mice produced a similar pattern. In the presence of low-dose Isoprenaline, none of the four PDE4KO mouse lines replicated the substantial amount of saliva produced by injection with a PDE4 inhibitor (Rolipram; 1 mg/kg), nor did ablation of any PDE4 subtype protect the animals from salivation induced by treatment with the PDE4 inhibitor Rolipram (Fig. 16A). That none of the PDE4KO mouse lines replicates the amount of saliva produced by injection of a PAN-PDE4 inhibitor, nor is protected from the effect of PDE4 inhibitor, suggests that PDE4 inhibitor-induced salivation results from the concurrent inhibition of multiple (at least two) PDE4 subtypes. Saliva production in PDE4D-KO mice, while clearly minor compared to the effect of PAN-PDE4 inhibitor treatment, trended to be higher compared to wild-type controls in mice treated with 0.01

mg/kg Iso, and was significantly increased over wild-type controls in mice treated with the higher dose of 0.04 mg/kg Iso (Fig. 16B). These data suggest that PDE4D, while not the sole PDE4 subtype involved, is likely the primary, or at least one of the PDE4 subtypes involved in the direct regulation of saliva secretion in the salivary glands of mice.

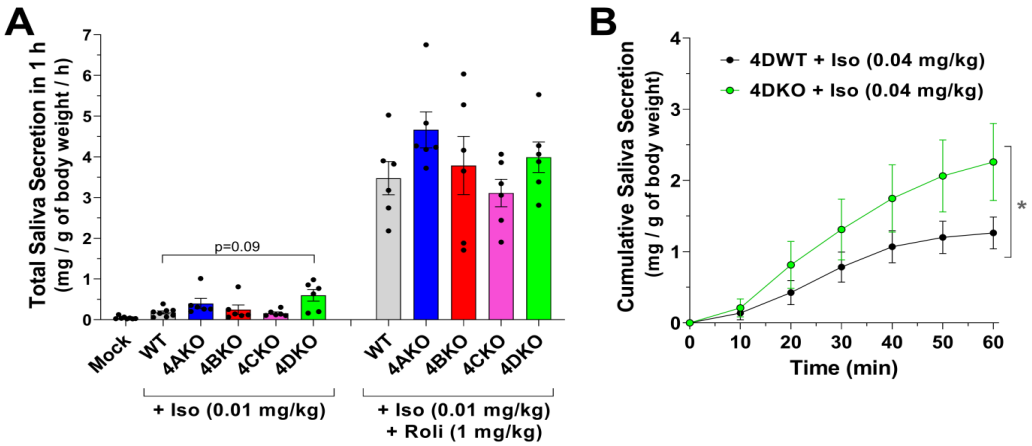

Figure 16. PAN- but not subtype-selective PDE4 inactivation induces salivation. Fifteen minutes after induction of Ketamine/Xylazine-anesthesia, mice were injected with the PDE4 inhibitor Rolipram (1 mg/kg) and/or the β-adrenoceptor agonist Isoprenaline (Iso; 0.01 mg/kg in (**A**), 0.04 mg/kg in (**B**)) and saliva production was measured for the next 60 min. (**A**) Shown is the total amount of saliva produced within 60 min of drug injection in mice deficient in PDE4A (4AKO), PDE4B (4BKO), PDE4C (4CKO) or PDE4D (4DKO), or in wildtype (WT) controls. There were no significant differences ($p>0.05$) in saliva production between any PDE4-KO mice and wild type controls after treatment with either Iso or after treatment with Iso+Roli. Conversely, treatment with Iso+Roli produced significantly more saliva than treatment with Iso alone ($p<0.001$) in any of the PDE4-KO lines or in wild type controls. (**B**) Cumulative saliva secretion in PDE4D-KO mice and wildtype littermates in response to injection of 0.04 mg/kg Iso. All data represent the mean ± SEM. In scatter plots, each dot represents a different animal. For total saliva production shown in bar graphs, statistical significance was determined using Kruskal-Wallis and Dunn's post hoc tests; for time courses, two-way ANOVA and Bonferroni's post hoc tests were used. Statistical significance is indicated as * ($p<0.05$).

3.4 Discussion

3.4.1 Inducing salivation is a class effect of PAN-PDE4 inhibitors in mice.

We report here that inhibition of PDE4, but not inhibition of PDE3, stimulates elevated saliva production in mice, as detected by observational scoring (Fig. 10A-C) or by measuring saliva secretion rates in anesthetized mice (Fig. 14C). Salivation is dose-dependently induced by the archetypal PDE4 inhibitor Rolipram (Fig. 14B) as well as several other, structurally-distinct PAN-PDE4 inhibitors, including Roflumilast, Piclamilast or RS25344 (all 1 mg/kg i.p.; Figs. 10C and 14C), suggesting that salivation is a class-effect of PDE4 inhibition in mice. While not mechanistically explored, salivation has been noted previously during toxicity studies of PDE4 inhibitors in rat[123] and dog[124], as well as monkeys[125-126], suggesting the effect may be conserved among mammalian species. Thus, if it extends to humans, our findings may suggest a therapeutic potential of PDE4 inhibition for conditions associated with salivary gland hypofunction and xerostomia.

The PDE4 family comprises four subtypes, PDE4A to PDE4D, that each have been shown to exert distinct and non-overlapping physiological and pathophysiological roles[11, 30]. We show here that selective ablation of any of the four individual PDE4 subtypes in mice does not replicate the substantial salivation observed by PAN-PDE4 inhibitor treatment, suggesting that inactivation of several (at least two) distinct PDE4 subtypes is required for maximal stimulation of salivation (Fig. 16A). While significantly less efficacious than PAN-PDE4 inhibition, genetic deletion of PDE4D in mice does

significantly increase saliva production compared to wild-type littermates (Fig. 16A/B), suggesting that PDE4D is one of the PDE4 subtypes that affect salivation; perhaps even the most critical one.

Individual PDE4 subtypes are distinguished by a multitude of unique post-translation regulations as well as the differential recruitment in distinct subcellular compartments and macromolecular signaling complexes. As a result, individual PDE4 subtypes mediate distinct functions even if expressed in the same cell. Thus, PDE4 inhibition may induce salivation by inhibiting several (or the same) PDE4 subtype(s) located in distinct cell types, and inactivation producing salivation *via* physiologic additivity or synergism. But salivation may also be produced by inactivation of several PDE4 subtypes in the same cell, thereby leading to additive/synergistic effects in elevating intracellular cAMP/PKA signaling in salivary gland cells.

Given that the expression of several PDE4 isoforms has been shown to be cAMP responsive[127-129], it is theoretically possible that the actual effect/impact of ablating one of the four PDE4 subtypes on salivary secretion (see Fig. 16) may be partly obscured by compensatory changes in expression of other PDE4 isoforms. We consider this less likely given the large body of data suggesting that PDEs are functionally not interchangeable, and because such compensatory changes in PDE4 expression were not observed in other primary cells or tissues of PDE4-KO mice[11]. Nevertheless, the possibility of compensatory changes in PDE isoform expression or activity in the salivary glands of the different PDE4-KO mouse lines remains to be explored in future studies.
Development of subtype-selective PDE4 inhibitors is a promising approach to retain the many therapeutically beneficial effects of the PAN-PDE4 inhibitors available to date (e.g.

anti-inflammatory, memory/cognition-enhancing) while alleviating their common side effects, mainly nausea and emesis. Thus, PDE4D should be the primary target for future development of subtype-selective PDE4 inhibitors to induce salivation.

Given a lack of highly subtype-selective PDE4 inhibitors, it remains unclear which PDE4 subtypes mediate the adverse effects of PAN-PDE4 inhibitors in humans. A prior report revealed that genetic ablation of PDE4D shortens the duration of xylazine/ketamine-induced anesthesia in mice[73], thus replicating the effects of PAN-PDE4 inhibitors in this paradigm, which has been proposed as a correlate of nausea and emesis in species that are anatomically unable to vomit (e.g. mice/rats). If this model were an accurate predictor of the role of PDE4D in mediating the adverse effect of PAN-PDE4 inhibitors, it would be challenging to derive any therapeutic benefits from the inhibition of PDE4D, including the mitigation of hyposalivation. However, while the xylazine/ketamine-anesthesia test is a reliable measure of α_2-adrenoreceptor antagonism, it does have limitations as a predictor of emetic potential as reviewed recently[85]. Moreover, at least one study has shown that inhibitors with some selectivity for PDE4D exhibited reduced vomiting compared to PAN-PDE4 inhibitors in several species[112]. And finally, using gastric retention as an alternative correlate of nausea and emesis in mice, a recent report suggested that it is the concurrent inhibition of multiple PDE4 subtypes that is responsible for the adverse effects of PAN-PDE4 inhibitors[19]. Thus, in our opinion, the association of individual PDE4 subtypes with emesis and/or nausea remains inconclusive.

3.4.2 Distinguishing central and direct effects of PDE4 inhibition on salivation.

While inhibition of PDE4 promotes salivation in both awake (Fig. 10) and anesthetized mice (Fig. 14), there are some differences in the molecular mechanisms involved. First,

in awake mice, treatment with PDE4 inhibitors induced substantial salivation by itself (Fig. 10A-C), whereas in deeply anesthetized mice, salivation induced by PDE4 inhibition *per se* is minor (see Fig. 12E) and inducing substantial levels of PDE4 inhibitor-induced salivation requires priming with low doses of a β-agonist (Fig. 13D/E). Second, PDE4 inhibitor-induced salivation in awake mice is dependent upon both muscarinic- and β-adrenergic signaling, given it is blocked by either Atropine or Propranolol (Fig. 10E), whereas PDE4 inhibition potentiates β-adrenoceptor-dependent salivation in anesthetized mice, but has no effect on M_3-dependent salivation (Fig. 13I). Given that anesthesia principally involves the inhibition of neuronal activity, it is tempting to speculate that PDE4 inhibitor-induced salivation in anesthetized mice results directly from PDE4 inhibition in salivary glands and not from effects on neuronal regulation of salivation (see scheme in Fig. 17). The fact that PDE4 contributes the major portion of total cAMP-hydrolytic capacity in all three major salivary glands (Fig. 11) supports this notion. And indeed, a prior study has shown that inhibition of PDE4 promoted the release of amylase from parotid acinar cells in culture[130], which is consistent with the notion that β-adrenoceptor- and cAMP-dependent saliva secretions are rich in protein[114-115]. Conversely, we propose that in awake mice, PDE4 inhibition may induce salivation *via* additional, central/neuronal regulations, in line with the high expression of PDE4 in brain[18] and the fact that PDE4 inhibition has been shown to affect other autonomic nervous system regulations including control of body temperature or gastrointestinal motility[19,24]. In an intriguing parallel, although the most widely used drug to induce salivation, Pilocarpine, can induce salivation by acting directly on the salivary glands (see Fig. 13F/G), the drug may yet mediate its pronounced effect on salivation

largely *via* central mechanisms. This is supported by the observation that intracerebroventricular (i.c.v) injection of Pilocarpine induces salivation in rats[131-132], and that *vice versa*, lesions in the medial preoptic area and the lateral hypothalamus, or i.c.v.-injection of muscarinic blocker Atropine, impair salivation is response to peripheral Pilocarpine[133-135]. Curiously, central α_2-adrenoceptor agonism has been shown to inhibit the central actions of Pilocarpine on salivation[136-138]. Given that PAN-PDE4 inhibitors act as potent, physiologic α_2-adrenoceptor blockers in mammals[73,82,84], it is possible that in awake mice, PDE4 inhibition mediates salivation by releasing a central, α_2-adrenoceptor-dependent inhibition of muscarinic signaling, which may explain why Atropine is effective in ablating PDE4 inhibitor-induced salivation in awake mice (Fig. 13G). Furthermore, and in light of the observation that muscarinic stimulation has been shown to increase cGMP signaling in rabbit parotid acinar cells[139], the role of PDE families other than PDE3 and PDE4, as well as the role of cGMP signaling in the regulation of saliva secretion remain to be explored.

3.4.3 PDE4, CFTR, and Cystic Fibrosis

β-agonist-induced saliva production in mice has been shown to be gated by CFTR in the salivary glands and be dependent upon both expression level and the cAMP/PKA-mediated activation of the channel[120,122] (Fig. 17). The rate of β-adrenoceptor-induced saliva secretion has thus been proposed as a suitable *in vivo* correlate of CFTR function in mice, given that mice do not sweat, and the classical sweat-chloride test that is used to assess CFTR function in humans, cannot be applied. On this basis, our study is also the first report that inhibition of PDE4, but not inhibition of PDE3, stimulates CFTR function in an *in vivo* model (Fig. 17). The critical role of PDE4 in regulating CFTR function in

salivary glands aligns with prior reports identifying PDE4 as the predominant PDE family regulating CFTR function in airway, intestinal, and renal epithelial cells[27,28,140-142]. Activation of PDE4 using small-molecule allosteric activators has thus been proposed as a therapeutic approach to alleviate the cAMP/PKA-mediated hyperactivation of CFTR and its associated cyst formation in models of autosomal dominant polycystic kidney disease (ADPKD)[142]. Conversely, inhibition of PDE4, which is widely expressed throughout the cells of the bronchi and lung parenchyma[143], has been shown to activate CFTR in non-CF as well as ΔF508-CFTR human bronchial airway epithelial cells in culture [27], thus confirming PDE4 as a promising target to potentiate the effects of CFTR correctors and potentiators to restore CFTR function in CF patients. In this context, it is worth noting that several prior reports suggested a predominant role for the PDE4 subtype PDE4D in controlling CFTR activity in both airway and intestinal epithelial cells[27,140-141,144]. In line with our finding of a pre-eminent, but not exclusive role of PDE4D in controlling β-adrenoceptor- and CFTR-dependent salivation (Figs. 14 and 17), these data suggest that PDE4D may serve a conserved role in controlling CFTR function across various cells and tissues throughout the body, and that targeting PDE4D may thus serve to alleviate CFTR hypofunction.

3.5 Conclusions

3.5.1 Targeting PDE4 in settings of salivary gland hypofunction.

There remains a critical need for effective treatments for salivary gland hypofunction, particularly for patients presenting with severe symptoms such as in Sjögren's syndrome. While the parasympathomimetics Pilocarpine and Cevimeline are widely prescribed, their use can be limited by significant side effects resulting directly from their muscarinic agonism, and they certainly cannot be prescribed if xerostomia is the result of anti-cholinergic medications to begin with[114-115]. Thus, inhibition of PDE4/PDE4D may represent a novel therapeutic approach for xerostomia, particularly since in awake mice, inhibition of PDE4 in the brain (Fig. 10E) and inhibition of PDE4 in salivary glands (Fig. 11) appear to synergize in the induction of salivation (Fig. 17). In addition, while not measured here, the composition of saliva produced by muscarinic activation or PDE4 inhibition is expected to be distinct, and the effects of both treatments may thus be complementary. Muscarinic activation (e.g. with Pilocarpine) is well-known to produce large volumes of watery saliva with low protein content, thus principally ameliorating hydration of the oral mucosa. Conversely, saliva produced upon β-adrenergic stimulation (and thus likely also upon PDE4/PDE4D inhibition) is high in protein content and may be better suited to ameliorate deficits in food digestion or innate defense that are associated with salivary gland hypofunction. Indeed, a prior report has shown that PDE4 inhibition potentiates the β-adrenoceptor-dependent release of amylase from parotid glands *in vitro*[130]. Moreover, underlining the significance of saliva produced upon β-adrenergic-, cAMP- and CFTR-dependent salivation, an increased incidence of dental caries has been

reported in ΔF508-CFTR cystic fibrosis mice, which lack functional CFTR[145]. Finally, although the causes of salivary gland dysfunction are varied, there is often an inflammatory component. This is obvious in Sjögren's syndrome, an autoimmune disease, but also in salivary gland dysfunction caused by infections, diabetes or ageing. Targeting PDE4, particularly the PDE4 subtypes PDE4B and PDE4D[44, 46-47,108], is well established to exert broad-spectrum anti-inflammatory properties and to dampen both innate and adaptive immune responses. Therefore, in addition to stimulating salivary secretions, thus alleviating the symptoms of salivary gland hypofunction, PDE4 may also exert therapeutic benefits by alleviating the inflammatory responses that cause salivary gland dysfunction.

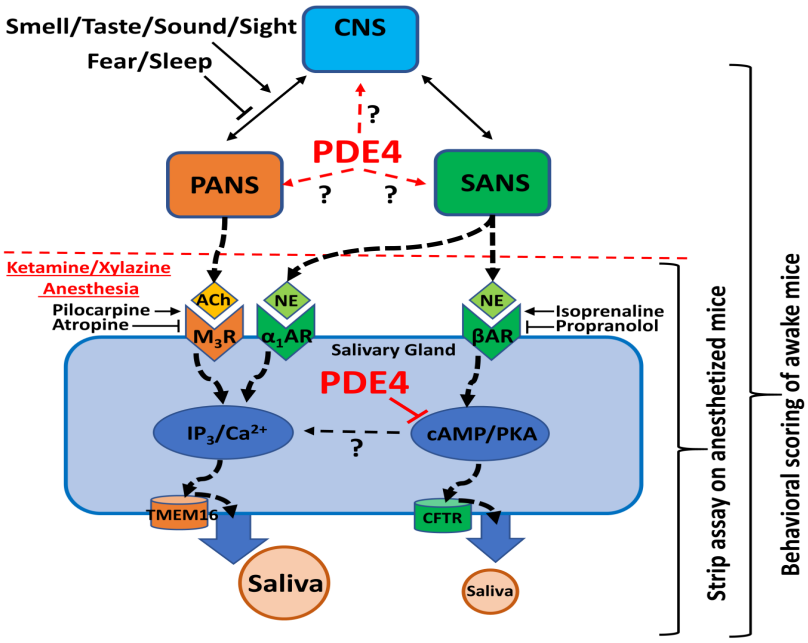

Figure 17. Scheme illustrating the role(s) of PDE4 in the regulation of salivation.

The scheme illustrates the known mechanisms of autonomic nervous system control and the intracellular signaling events that mediate saliva secretion, as well as the potential role(s) of PDE4 in inducing salivation. As shown within the blue rectangle representing the salivary glands, direct stimulation of glandular G_q-coupled receptors, such as M_3-muscarinic acetylcholine receptors (M_3R) or α_1-adrenergic receptors (α_1AR), and the subsequent rise in intracellular Ca^{2+} mediates the release of high volumes of watery saliva that is dependent upon activation of the calcium-activated chloride channel TMEM16A[146-147]. In addition, stimulation of intracellular cAMP signaling, such as upon activation of β-adrenergic receptors (βAR), promotes saliva secretion *via* a pathway dependent upon the cAMP/PKA-activation of the anion channel CFTR[120,122]. Under conditions of inhibited neuronal signaling due to Ketamine/Xylazine-induced anesthesia, only the events shown below the red striated line are detected. Under these conditions, inhibition of PDE4/PDE4D selectively potentiates a β-adrenoceptor-, cAMP-, and CFTR-dependent saliva secretion (Fig. 13B/C), which is completely ablated in homozygous ΔF508-CFTR mice (Fig. 15B), that lack functional CFTR, or by treatment with the β-blocker Propranolol (Fig. 13D). On the other hand, in anesthetized mice, PDE4 inhibition does not affect salivation induced by the direct action of the M_3R agonist Pilocarpine on salivary glands. However, in awake/conscious mice, inhibition of PDE4 induces salivation *via* an additional, central/neuronal mechanism that remains to be defined and may involve the sympathetic autonomic nervous system (SANS), the parasympathetic autonomic nervous system (PANS), or the central nervous system (CNS) , but is ablated by Atropine (Fig. 10E), and only partially dependent upon CFTR (Fig. 15A).

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