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Inhibition of Type 4 cAMP-phosphodiesterases (PDE4s) in mice induces hypothermia *via* effects on behavioral and central autonomous thermoregulation

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Abstract

Inhibitors of Type 4 cAMP-phosphodiesterases (PDE4s) exert a number of promising therapeutic benefits, including potent anti-inflammatory, memory- and cognition-enhancing, metabolic, and antineoplastic effects. We report here that treatment with a number of distinct PDE4 inhibitors, including Rolipram, Piclamilast, Roflumilast and RS25344, but not treatment with the PDE3-selective inhibitor Cilostamide, induces a rapid (10-30 min), substantial (-5°C) and long-lasting (up to 5 h) decrease in core body temperature of C57BL/6 mice; thus, identifying a critical role of PDE4 also in the regulation of body temperature. As little as 0.04 mg/kg of the archetypal PDE4 inhibitor Rolipram induces hypothermia. As similar or higher doses of Rolipram were used in a majority of published animal studies, most of the reported findings are likely paralleled by, or potentially impacted by hypothermia induced by these drugs. We further show that PDE4 inhibition affects central body temperature regulation and acts by lowering the cold-defense balance point of behavioral (including posture and locomotion) and autonomous (including cutaneous tail vasodilation) cold-defense mechanisms. In line with the idea of an effect on central body temperature regulation, hypothermia is induced by moderate doses of various brain-penetrant PDE4 inhibitors, but not by similar doses of YM976, a PDE4 inhibitor that does not efficiently cross the blood-brain barrier. Finally, to begin delineating the mechanism of drug-induced

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WM, JR and WR designed the experiments; WM, JR, IVA, LA and WR performed core body temperature measurements; LA and IVA acquired, and AR analyzed infrared thermography images; IVA, AB, AK and WR generated the animals and maintained the colonies; all authors contributed to data analyses; WR wrote a draft and all authors edited and approved the manuscript.

Credit Author Statement

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hypothermia, we show that blockade of $D_{2/3}$ -type dopaminergic, but not β -adrenergic, H_1 -histaminergic or opiate receptors, can alleviate PDE4 inhibitor-induced hypothermia. We thus propose that increased $D_{2/3}$ -type dopaminergic signaling, triggered by PDE4 inhibitor-induced and cAMP-mediated dopamine release in the thermoregulatory centers of the hypothalamus, is a significant contributor to PDE4 inhibitor-induced hypothermia.

Graphical Abstract

PDE4 inhibition induces substantial hypothermia in mice (A), that involves central autonomous thermoregulatory processes including cutaneous tail vasodilation (B), and is alleviated by centrally-acting $D_{2/3}$ -type dopamine receptor blockers (C).

Keywords

Spiperone; hypothermia; Rolipram; cutaneous tail vasodilation; D_2 -dopamine receptor

1. INTRODUCTION

In mammals and other homeotherms, the maintenance of body temperature is a critical and tightly regulated process^{1, 2}. To safeguard vital cellular and physiological functions, and hence the survival of the organism, their body temperature is kept relatively constant within a narrow range (generally around 37°C) independent of changes in ambient temperatures. A significant elevation of body temperature above the normal range (hyperthermia) may be harmful due to denaturation of proteins at temperatures higher than ~44°C, whereas an abnormal reduction in body temperature (hypothermia) may diminish fitness by lowering the speed of enzymatic reactions and/or ion transport, or by reducing membrane fluidity. The significance of body temperature control is plainly illustrated by the fact that in some small mammals (such as mice) which have a high rate of heat dissipation due to a large body surface-to-volume ratio, up to a third of total energy expenditure, and thus a third of all food calories consumed, may be dedicated to heat production during cold exposure³.

Hypothalamic regions, including the pre-optic area, serve as the main centers for thermoregulation. They receive input from receptors that track temperature at the body's core as well as from receptors in the skin that sense environmental temperatures, and integrate these with other inputs, such as energy homeostasis and behavioral states, to direct appropriate sets of thermoeffectors to produce and conserve, or to dissipate body heat,

respectively⁴. Thermoregulation involves principally two types of effector responses: behavioral and autonomic. Cold exposure triggers heat-seeking or heat-conserving behaviors such as seeking shelter or warmth (e.g. sun exposure), nesting, huddling in groups, assuming a ball-like posture that reduces body surface and thus heat dissipation, as well as curling the tail around, or hiding it under the body (in rodents) or putting your sweater on (in humans)⁵. Conversely, seeking shade is a behavioral response to high environmental- or body temperatures. Autonomic cold defenses include cutaneous vasoconstriction, in particular on surfaces not covered with fur (such as the paws, ears and tail of mice), shivering, and increased non-shivering thermogenesis (e.g. brown fat thermogenesis). Conversely, autonomic heat defense mechanisms include enhanced heat dissipation *via* cutaneous vasodilation as well as sweating or panting. These defense mechanisms are engaged in a certain order¹. Behavioral defenses are recruited very early and may even be proactive (e.g. dressing for the weather before stepping outside). Conversely, autonomic responses are engaged only after a change in body or environmental temperature has occurred. Mechanisms that come at no or at little biological cost, such as vasodilation or constriction, are employed first, while mechanisms with a higher biologic cost, such as thermogenesis (energy consuming) or sweating (water loss), are recruited later¹. Each defense mechanism is engaged in a semi-autonomous manner if body temperatures veer off certain thresholds, or balance points (e.g. cold-defense or heat-defense balance points). These reference points themselves are not fixed, but may vary with the circadian rhythm⁶, injury and trauma, behavioral states, during torpor or hibernation, or in response to drug treatment (e.g. methamphetamines and anesthetics raise and lower cold-defense balance points, respectively)¹.

Although signaling *via* the ubiquitous second messenger cAMP mediates and/or gates many physiological processes that are recruited during body temperature regulation, from thermogenesis (e.g. lipolysis in brown fat⁷) and energy homeostasis⁸, to regulation of behaviors⁹ or vascular dilation/contraction¹⁰, surprisingly little is known on the role of cAMP signaling in central body temperature regulation under normal physiologic conditions. The arguably most well-known role for cAMP in body temperature regulation is in mediating the effects of PGE₂, the central mediator in the fever response, that eventually acts through activation of G_i-coupled prostaglandin EP₃ receptors to lower cAMP signaling in order to raise the cold-defense balance point, thereby elevating body temperature^{11, 12}. In addition to a myriad of G protein-coupled receptors (GPCRs) that act through activation of G_s or G_i proteins to control the rate of cAMP production by adenylyl cyclases, cAMP signaling is tightly controlled by the activity of cyclic nucleotide phosphodiesterases (PDEs), the enzymes that hydrolyze and inactivate the cyclic nucleotide second messengers cAMP and cGMP. In mammals, PDEs comprise a superfamily of isoenzymes that are grouped into 11 PDE families based on sequence homology as well as their kinetic and pharmacologic properties¹³. Of these, the PDE4 family is the largest, comprising four genes, PDE4A to D, that together generate likely over 25 protein variants *via* use of alternate promoters and alternative splicing^{14, 15}. PDE4s are widely expressed throughout mammalian cells and tissues and are kinetically and pharmacologically defined by their selectivity for cAMP over cGMP as substrate, and their sensitivity to inhibition by the archetypal PDE4 inhibitor Rolipram. By way of controlling cAMP signals, PDE4s affect a large number of

physiologic and pathophysiological processes; as a result, regulation of their activity through small molecule inhibitors or activators^{16, 17} produces a significant number of potential therapeutic benefits including anti-inflammatory effects, as well as memory- and cognition-enhancing, anti-depressant and anti-psychotic, anti-neoplastic, cardiovascular, and metabolic effects^{16, 18-28}. However, PDE4 inhibitors are also associated with some adverse effects, in particular nausea and emesis, that have constrained their clinical utility and commercial success until now.

While exploring potential anti-inflammatory benefits of PDE4 inhibition in a model of bacterial lung infection in mice, we noticed that treatment with a PDE4 inhibitor *per se* caused a substantial and long-lasting reduction of body temperature in the animals. As such a significant decrease in body temperature may by itself exert critical effects on immune responses, and because the role of PDE4 in body temperature regulation remains poorly understood, we have further explored this observation.

2. MATERIAL AND METHODS

2.1. Drugs

Piclamilast/RP73401^{29, 30}, Rolipram³¹, Roflumilast³², Cilostamide³³, Chlorcyclizine³⁴, Spiperone³⁵, and Pimozide³⁶ were from Cayman Chemical (Ann Arbor, MI, USA). YM976³⁷ was obtained from Tocris/Bio-Techne (Minneapolis, MN, USA), Naloxone³⁸ from MP Biomedicals (Irvine, CA), Domperidone³⁹ from Alfa Aesar (Haverhill, MA, USA), Ketanserin³⁵ from TCI America (Portland, OR, USA), Propranolol^{40, 41} from Millipore Sigma (St. Louis, MO, USA), and RS25344⁴² from Santa Cruz Biotech (Santa Cruz, CA, USA). All drugs were initially dissolved in DMSO, subsequently diluted into phosphate-buffered saline (PBS), pH 7.4, containing final concentrations of 5% DMSO and 5% Cremophor EL (Millipore Sigma, St. Louis, MO, USA), and were applied by intraperitoneal injection (100 µl per 20 g body weight).

2.2. Animals

C57BL/6 mice for experimentation were generated in-house using breeders obtained from Charles River Laboratories (Wilmington, MA, USA). The animals were housed in groups of four mice per cage with *ad libitum* access to food and water and were maintained in a temperature-controlled (22-23°C) vivarium with a 12-h light/dark cycle (6:30 am–6:30 pm). Adult mice 10 weeks of age and of either sex were used for experimentation by equally dividing cage littermates into experimental groups. Experiments were generally performed in the mornings (9:00 am – 12:00 pm) and 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.

2.3. Measurement of core body temperature

Body temperature was measured at the sternum of scruffed mice using an infrared thermometer (IR-CF, Braintree Scientific, Braintree, MA, USA), or using a thermocouple thermometer (MicroTherma 2T) with mouse rectal probe (RET-3), both from Braintree Scientific (Braintree, MA, USA), following the manufacturers' instructions. The noninvasive measurement of body temperature with an infrared thermometer was chosen for the time course in Fig. 1 to limit any discomfort to the animals given the frequent measurements. Subsequent tests at a single time point confirmed that the body temperature measured at the sternum using an infrared thermometer was similar to the body temperature measured with a rectal probe thermometer, which is generally considered the gold standard for measurement of "core" body temperature.

2.4. Measurement of tail temperature using infrared thermography

Mice were individually placed in new cages for one hour to allow for habituation before experimentation. Images were then acquired at the time points indicated from a height of 30 cm above the cage floor using an infrared camera (FLIR-C3, FLIR Systems, Wilsonville, OR, USA). Images were transferred to a computer and the temperature of the tails at a distance of 3 cm from the base of the tail was then quantified in infrared images using FLIR Tools, the software provided by the camera manufacturer (FLIR Systems, Wilsonville, OR, USA).

2.5. Measurement of locomotion using SmartCage™ technology

After intraperitoneal injection of the indicated drugs, mice were immediately placed in a new cage with ad libitum access to food and water and locomotion was assessed using the SmartCage™ system (AfaSci Research Laboratories, Redwood City, CA, USA) which tracks the animals position and movement in the cage based on the animals disruption of an array of infrared light beams. The travel distance in cm per 5 min time interval was used as readout of locomotor activity and is plotted over time from the moment the mouse is placed in the cage.

2.6. Scoring of tail and body posture

An experimenter blinded to the treatments scored the mice 30 min after injection of PDE4 inhibitor or solvent controls based on their tail posture ("normal/not extended nor erected = 0"; versus "abnormal, extended or erected = 1") and body posture ("normal, not extended nor low to the ground = 0"; versus "abnormal, extended surface or low to the ground = 1"). We find that none of the animals in the solvent group received a positive ("1") score for either tail extension or body surface/posture, suggesting that the postures induced by high doses of PDE4 inhibitors are quite characteristic.

2.7. Statistics

All data are expressed as the mean $\pm$ SEM and n numbers indicate the number of individual animals assessed. The GraphPad Prism 8.3 software (GraphPad Software Inc, San Diego, CA, USA) was used to perform statistical analyses and one-way ANOVA or two-way ANOVA followed by Tukey's multiple comparisons post hoc test was used to determine

differences between groups at a single time point or in time courses, respectively. Statistical significance is indicated as * ($p<0.05$), ** ($p<0.01$), and *** ($p<0.001$).

3. RESULTS

3.1. Treatment with Piclamilast induces hypothermia in mice

Upon noticing that mice injected with the PDE4 inhibitor Piclamilast felt cold to the touch, we used the measurement of body temperature at the sternum of mice using an infrared thermometer as a first approach to gauge the amplitude and duration of hypothermia induced by PDE4 inhibition. As shown in Fig. 1., treatment with Piclamilast (5 mg/kg, i.p.) caused a decrease in body temperature that was rapid in on-set and substantial, declining by $\sim 5^{\circ}\text{C}$ within ~ 30 min compared to solvent- or non-injection controls. Hypothermia induced by PDE4 inhibition is also long-lasting, with body temperature of Piclamilast-treated mice being significantly lower than that of solvent control mice for up to 5 h after drug administration. When measured in the same animals ($n=8$) at 30 min after Piclamilast (5 mg/kg) injection, the body temperature measured at the sternum of mice using an infrared thermometer ($32.2\pm 0.5^{\circ}\text{C}$) was similar to the body temperature measured using a rectal probe thermometer ($31.8\pm 0.5^{\circ}\text{C}$), suggesting that the Piclamilast-induced drop in temperature is not limited to the body surface, but reflects a reduction in core body temperature.

3.2. Hypothermia is a class effect of PDE4 inhibitors in mice

To determine whether the substantial hypothermia is an effect unique to Piclamilast, or a class effect of PDE4 inhibition, we next treated mice with a number of distinct PAN-PDE4 inhibitors, including the archetypal PDE4 inhibitor Rolipram, as well as Piclamilast, Roflumilast, and RS25344 (each 1 mg/kg; i.p.), as well as the PDE3-selective inhibitor Cilostamide (1 mg/kg, i.p.). As shown in Fig. 2, all PDE4 inhibitors induced significant hypothermia, thus mirroring the effect of Piclamilast, whereas the PDE3-selective inhibitor, Cilostamide, had no effect.

Detailed dose-response curves for Rolipram and Piclamilast revealed that as little as 0.04 mg/kg Rolipram (Fig. 3A) or 0.2 mg/kg Piclamilast (Fig. 3B) induced a significant reduction in body temperature in the animals. With few exceptions, studies in mice use the same or higher doses of these PDE4 inhibitors to induce potentially therapeutic effects, such as anti-inflammatory, cardiovascular-, metabolic- or behavioral effects, suggesting that these studies are generally accompanied, and their effects are paralleled by significant hypothermia^{16, 18, 27, 28, 43-45}.

3.3. PDE4 inhibition affects central body temperature regulation

Rolipram and RS25344 are distinguished from Roflumilast and Piclamilast by their high brain penetrance, and their preference for binding to a conformational state of PDE4 termed HARBS (high affinity Rolipram binding state), which is also enriched in the brain over peripheral tissues^{46, 47}. We thus pursued the idea that their more pronounced effect on body temperature at a dose of 1 mg/kg (Fig. 2) and the left-shifted dose response curve for Rolipram compared to Piclamilast (Fig. 3A/B) may indicate that hypothermia is due to

inhibition of PDE4 in the brain, rather than direct effects of PDE4 inhibition in thermoeffector organs in the periphery. To this end, we determined the dose-dependence of hypothermia induced by YM976, a PAN-PDE4 inhibitor that has a similar potency in inhibiting cAMP hydrolysis by PDE4s compared to Roflumilast, Piclamilast and Rolipram, but is distinguished by its apparent poor brain penetrance³⁷.

As shown previously by Aoki et. al., YM976 has similar potency compared to Rolipram in read-outs of anti-inflammatory benefits in the periphery (e.g. such as reducing TNF α production in mice) or elevating cAMP levels in the periphery (e.g. cAMP levels in peritoneal leukocytes), but was unable to increase cAMP levels in brain, suggesting that YM976 does not efficiently cross into the brain. As shown in Fig. 3C, YM976 induces hypothermia in mice, but its dose-response curve is significantly right-shifted compared to Rolipram or Piclamilast (Fig. 3A/B), suggesting that the portion of PDE4 involved in body temperature regulation is protected, at least in significant part, by the blood-brain barrier.

Moreover, as shown in Fig. 4, the rapid onset of PDE4 inhibitor-induced hypothermia by itself (-4°C within the first 10 min) suggests that multiple heat-generating and heat-preserving mechanisms are globally, temporarily deactivated, as could be achieved by central effects such as a lowering the cold-defense balance point, but are unlikely to result from an effect of PDE4 inhibition on an individual thermoeffector. Indeed, during this initial phase, the rate of body temperature loss is similar to that seen after euthanasia of the animal. By about 15 min after PDE4 inhibitor administration, some cold-defense mechanisms are (re)-activated and body temperature begins to stabilize at a new cold defense balance point ($\sim 32^{\circ}\text{C}$), whereas the temperature of euthanized animals continues to decrease.

3.4. Autonomous and behavioral effects associated with PDE4 inhibition

Given its low biologic cost, cutaneous vasoconstriction is generally the first autonomous cold-defense mechanism employed. Indeed, if mice are kept at an ambient temperature (22°C in this study) that is below their thermoneutral range ($\sim 30^{\circ}\text{C}$ for mice¹), then the cutaneous tail vasculature remains continuously constricted, save for short intermittent control. To determine if inactivation of tail vasoconstriction contributes to PDE4 inhibitor-induced hypothermia, we assessed tail temperatures of mice by infrared thermography. As shown in Fig. 5A, the temperature of the mouse's tail is much lower than that of its body, in line with the idea that cutaneous vasoconstriction is continually engaged to minimize heat loss; thus, limiting energy expenditure. However, as shown in Figs. 5B/C, treatment with the PDE4 inhibitor Rolipram (1 mg/kg, i.p.) promotes a rapid cutaneous vasodilation, reflected in increased tail temperatures, that reaches its peak within ~ 5 min. Thus, loss of tail vasoconstriction is one of the thermoeffectors that are temporarily deactivated by central PDE4 inhibition and contributes to hypothermia shown in Figs. 1-4.

In line with the idea that this is an effect of PDE4 inhibition on central body temperature regulation, and not a more direct effect on the tail vasculature itself, the peripherally acting PDE4 inhibitor YM976³⁷, which does not cross the blood-brain barrier, did not induce tail vasodilation. Tail temperatures return to basal levels by about 15 min after Rolipram injection, suggesting that tail vasoconstriction is recruited as cold-defense mechanism at this time point to stabilize body temperature at a new cold defense balance point. Indeed, (re)-

activation of tail vasoconstriction coincides with the timing by which the decrease in core body temperature after PDE4 inhibitor-treatment diverges from that of the euthanized animal (see Fig. 4). Between 20 and 60 min after Rolipram injection, tail temperatures in PDE4 inhibitor-treated mice then trend below those of control (solvent) mice (Fig. 5C), likely because the core temperature of the mice, and thus the temperature of the blood that circulates and warms the tail, is below that of controls (see Figs. 1-4).

In addition to autonomous effects, such as cutaneous vasodilation, treatment with PDE4 inhibitors induces characteristic behavioral effects, such as changes in posture and locomotion, that may contribute to, and/or be a response to hypothermia. The images in Fig. 6A are representative of postures of mice after treatment with doses of 1 mg/kg of any of the brain-penetrant PDE4 inhibitors (including Rolipram, Piclamilast, Roflumilast, and RS25344). Despite the significant, PDE4 inhibitor-induced decrease in body temperature at the time the images were taken (core body temperature $\sim 32^{\circ}\text{C}$), the animals do not assume postures that may serve to limit heat loss. For example, their tails are frequently extended, sometimes erected (Fig. 6A) and this phenotype was observed at 30 min after drug/solvent injection in 29% (10 mice of 34 tested) and 47% (8 mice of 17 tested) of mice treated with 1 mg/kg and 5 mg/kg Rolipram (i.p.), respectively. RS25344 (1 mg/kg, i.p.) induced this phenotype in 24% (4 mice of 17 tested) of the animals, whereas none of mice treated with solvent (0 mice of 34 tested) showed this abnormal tail extension/erection. In addition to the altered tail posture, mice treated with PDE4 inhibitors also assume distinct body postures. Their bodies are often extended, paws are frequently extended away from the body, and some animals appear to lay on their abdomen, rather than standing on their feet. At 30 min after drug/solvent injection, this phenotype was observed in 21% (7 mice of 34 tested) and 71% (12 mice of 17 tested) of animals treated with 1 mg/kg and 5 mg/kg Rolipram, respectively, as well as in 59% (10 mice of 17 tested) of mice treated with 1 mg/kg RS25344, whereas none of the solvent controls (0 mice of 34 tested) exhibited this phenotype. Both the extension/erection of the tail as well as the extended body posture are behaviors that may further increase heat loss *via* radiation or conduction through the cage bottom/bedding. This is in stark contrast to the posture assumed by mice that are hypothermic due to fasting. Mice fasted overnight readily enter torpor in the early morning hours, which is characterized by a significant decrease in body temperature³, presumably to conserve energy that would otherwise be dedicated to heat production. As shown in Fig. 6B, mice in torpor assume clearly distinct postures (compared to PDE4 inhibitor-treated mice (Fig. 6A) or mice kept at room temperature) that serve to minimize heat-loss including attempts at nesting, balled-up posture, hiding the tail or curling it around the body, and huddling together. Thus, postures unsuitable for cold defense (Fig. 6A) likely extend both amplitude and duration of PDE4 inhibitor-induced hypothermia.

Another characteristic behavior induced by PDE4 inhibition that is of relevance to thermoregulation is reduced locomotion. At doses of 1 mg/kg of the brain-penetrant PDE4 inhibitors, the animals stop moving entirely shortly after PDE4 inhibitor treatment, although the animals will walk, though slowly and haltingly, if prompted. To illustrate, we measured travel distance of mice placed in a new cage (Fig. 6C). In solvent-treated mice, locomotion is initially high as the animals explore the new environment. Locomotion gradually decreases, so that within ~ 2 h, the animals reach a steady state of locomotor activity. Conversely,

treatment with PDE4 inhibitors rapidly and dramatically reduces locomotion. The onset of effects is again more profound with Rolipram, than Piclamilast, likely due to rapid distribution of Rolipram into the brain^{47, 48} and effects last for about 120 min. Given that involuntary muscle movement (shivering) as well as voluntary muscle movement (e.g. during exercise) are well-known to contribute to thermogenesis, this reduction in locomotion likely magnifies the amplitude and duration of PDE4 inhibitor-induced hypothermia.

3.5. Blockade of central D_{2/3}-dopamine receptors alleviates PDE4 inhibitor-induced hypothermia

To begin exploring the molecular mechanism(s) of PDE4 inhibitor-induced hypothermia, we tested whether blockade of distinct receptor pathways, that are known to be regulated by PDE4 in other physiologic paradigms and/or are involved in thermoregulation, may alter PDE4 inhibitor-induced hypothermia. The specific receptor antagonists and their doses were chosen based on their reported efficacy in animal studies. As shown in Fig. 7A-C, blockade of β -adrenergic receptors (Propranolol^{40, 41}, 1 mg/kg), opioid receptors (Naloxone³⁸, 1 mg/kg), or H₁-histamine receptors (Chlorcyclizine³⁴, 5 mg/kg) had no significant effect on hypothermia induced by Rolipram (1 mg/kg).

Conversely, pre-treatment with the antipsychotic Spiperone³⁵, at doses up to 0.2 mg/kg (Fig. 7D), while having no effect on baseline body temperature *per se*, did dose-dependently alleviate PDE4 inhibitor-induced hypothermia by up to 40% (at 0.2 mg/kg Spiperone). At higher doses (0.5 and 1 mg/kg Spiperone), treatment with the drug continued to ameliorate Rolipram-induced hypothermia; however, its effect is more limited as Spiperone *per se* induced hypothermia at these doses (Fig. 7E). The partial effect of Spiperone in alleviating PDE4 inhibitor-induced hypothermia is mechanistic, not dependent on the dose of PDE4 inhibitor used or the amplitude of the resulting hypothermia. As shown in Fig. 7F, even on milder hypothermia induced by a lower dose of Rolipram (0.2 mg/kg), Spiperone had also just a partial (49%) effect (Fig. 7F). Spiperone is established to act as antagonist on D_{2/3}-dopamine as well as 5-HT₂ serotonin receptors^{49, 50}. To distinguish between the two receptor subtypes, we tested a selective 5-HT₂ blocker, Ketanserin³⁵, as well as the selective D_{2/3} blocker, Pimozide³⁶, respectively. Ketanserin dose-dependently induced hypothermia in mice *per se*, but did not alleviate PDE4 inhibitor-induced hypothermia, indicating that 5-HT₂ antagonism does not protect from PDE4 inhibitor-induced hypothermia, but may mediate the hypothermic effects observed with high doses of Spiperone *per se* (Fig. 7G). Conversely, Pimozide did not induce hypothermia by itself, but did protect from PDE4 inhibitor induced hypothermia (Fig. 7H), suggesting that blockade of D_{2/3}-dopamine receptors protects from PDE4 inhibitor-induced hypothermia. Finally, the peripheral D_{2/3}-receptor blocker Domperidone³⁹, which does not cross the blood-brain barrier, did not alleviate PDE4 inhibitor-induced hypothermia (Fig. 7I), in line with the idea of a central effect on thermoregulation induced by PDE4 inhibition.

4. DISCUSSION

4.1. Hypothermia is a class effect of PDE4 inhibitors

We report here that treatment with a number of distinct PAN-PDE4 inhibitors, including Piclamilast, Rolipram, Roflumilast and RS25344, but not treatment with the PDE3-selective inhibitor Cilostamide, induces a rapid, substantial, and long-lasting decrease in core body temperature of C57BL/6 mice (Figs. 1, 2, and 3). This suggests that hypothermia is a class-effect of PDE4 family-selective inhibitors in mice. PDE4 inhibitors induce hypothermia at similar or below doses commonly used to test for potentially therapeutic benefits in mice (e.g. 0.04 mg/kg Rolipram; Fig. 3A); thus, most published findings on the effect of PDE4 inhibitors in mice are paralleled by, or perhaps affected by hypothermia induced by these drugs. PDE4 inhibition affects central body temperature regulation, likely lowering the cold-defense balance point of behavioral (including posture and locomotion; Fig. 6) and autonomous (including cutaneous tail vasodilation; Fig. 5) cold-defense mechanisms. Finally, to begin delineating the mechanism of drug-induced hypothermia, we show that blockade of D_{2/3}-type dopaminergic, but not β -adrenergic, H₁-histaminergic, or opiate receptors, can alleviate PDE4 inhibitor-induced hypothermia. We thus propose that increased D₂-type dopaminergic signaling, likely triggered by PDE4 inhibitor-induced and cAMP-mediated dopamine release in the thermoregulatory centers of the hypothalamus, is a significant contributor to PDE4 inhibitor-induced hypothermia.

This is not the first report that treatment with a PDE4 inhibitor can induce hypothermia in animals. On the contrary, in one of the very first reports on the pharmacologic effects of the drug Rolipram in animals, and many years before Rolipram was used to define and classify the PDE4 family, Przegalinski et al. showed in 1981 that Rolipram induced hypothermia in rats and rabbits⁵¹. Nevertheless, the mechanism of Rolipram-induced hypothermia, and the potential relevance of this observation to the therapeutic potential of PDE4 inhibition, has remained largely unexplored. This may be partly due to the fact that the maximal amplitude of hypothermia reported was subtle, including -1.25°C in rat (up to 10 mg/kg Rolipram⁵¹), -1.56°C in rabbit (up to 10 mg/kg Rolipram⁵¹), and -1.1°C in guinea pig (up to 25 mg/kg Rolipram⁵²). Conversely, the amplitude of hypothermia we observed in C57BL/6 mice is more substantial; core temperatures decrease on average by -5°C when using similar doses (5 mg/kg) of PDE4 inhibitors (see Figs. 1 and 3A/B). Moreover, in some animals, body temperature decreases below 30°C upon PDE4 inhibitor treatment (Figs. 3A/B) and these animals clearly feel cold to the touch. This disparity in the amplitude of Rolipram-induced hypothermia may reflect a species difference. Mice are the smallest of the species tested. They thus have a larger body surface-to-volume ratio and lose proportionately more heat by radiation or conduction. As a result, effects on central body temperature regulation may produce a proportionally larger impact in mice at ambient temperatures (22°C), compared to larger species. However, in light of known strain differences in body temperature regulation, such as due to dietary restriction⁵³, it remains to be determined whether there may also be strain differences that affect the impact of PDE4 inhibition on hypothermia in mice.

Most studies exploring cAMP signaling in central body temperature regulation find an inverse correlation: increasing cAMP levels reduces body temperature and *vice versa*. For

example, PGE₂ is thought to act through G_i-coupled prostaglandin EP₃ receptors to lower cAMP signaling in order to increase body temperature during the fever response^{11, 12}. Conversely, increasing cAMP signaling by treatment with the adenylyl cyclase activator Forskolin or treatment with dibutyryl-cAMP, a cell-permeable cAMP analog that stimulates multiple cAMP effectors⁵⁴, has been shown to induce hypothermia, and similarly to PDE4 inhibition (Fig. 6C), also reduces locomotion in mice and rats^{55, 56}. It is thus tempting to speculate that all these mechanisms target the same pool of cAMP, thus utilizing the same cellular and molecular mechanisms to regulate body temperature. For example, the xanthine-derivative Theophylline has been shown to produce hypothermia in mice, an effect that was antagonized by the D₂-type antagonists Pimozide and Sulpiride⁵⁷. Given that among a number of its molecular targets, Theophylline acts as a low-affinity, non-selective PDE inhibitor may suggest that Theophylline induces hypothermia *via* inhibition of hypothalamic PDE4. However, given that cAMP signaling occurs in every cell of the body, and that PDEs can control distinct physiologic functions often by acting in separate subcellular compartments of the same cells, it is likely that there are multiple separate mechanisms whereby cAMP signaling in general, and PDEs/PDE4s in particular, may affect body temperature. For example, Caffeine and related xanthine-derivatives, which also mediate some of their effects *via* non-selective inhibition of PDEs, have also been shown to produce hypothermia in mice⁵⁸. However, hypothermia induced by Caffeine⁵⁹ has been shown to be ablated by the opioid-receptor antagonist Naloxone, while this is not the case for PDE4 inhibitor-induced hypothermia (Fig. 7B). In line with this idea, it seems likely that even the PDE4 inhibitor-induced hypothermia we report here is induced by multiple additive and/or synergistic molecular mechanisms, given that D₂-type receptor antagonism ablates only a fraction of PDE4 inhibitor-induced hypothermia (Fig. 7D/F/H).

4.2. PDE4 inhibition affects central thermoregulation

Several lines of evidence suggest that PDE4 inhibition affects central thermoregulation such as by lowering the cold-defense balance point, rather than affecting peripheral temperature sensors or thermoeffectors. First, the rapid on-set of PDE4 inhibitor-induced hypothermia *per se* (see Figs. 1 and 4) suggests a central effect that results in the simultaneous deactivation of multiple cold defense mechanisms. Indeed, we show here that both behavioral (posture and locomotion; Fig. 6) as well as autonomous cold-defense mechanisms (cutaneous tail vasodilation; Fig. 5) are inactive at the onset of hypothermia. Given its critical role for the survival of the organism, the maintenance of core body temperature is tightly controlled by several layers of behavioral and autonomic thermoeffectors that are employed in sequential order as body temperature deviates from reference/balance points¹. Thus, a drug-induced inactivation of only one thermoeffector generally induces compensatory upregulation of other cold-defense mechanisms, so that the overall effect on core body temperature is minor. Thus, although not measured here, it seems likely that additional cold-defense mechanisms (e.g. shivering and non-shivering thermogenesis) must also be inactive to induce the substantial decrease in core body temperature at the onset PDE4 inhibitor-induced hypothermia.

Second, we show here that the potency of PDE4 inhibitors to induce hypothermia strongly patterns with their brain penetrance. Rolipram, Piclamilast and YM976, all have similar

potency to inhibit cAMP hydrolysis by PDE4s³⁷. Yet, the highly brain-penetrant inhibitor Rolipram^{47, 48} readily induces hypothermia at as little of 0.04 mg/kg (Fig. 3A), followed by Piclamilast (0.2 mg/kg; Fig. 3B), whereas YM976, a PDE4 inhibitor that does not effectively cross the blood-brain barrier³⁷, does so only at much higher doses (3 mg/kg; Fig. 3C). Moreover, Rolipram, but not YM976 (both at 1 mg/kg), induces cutaneous tail vasodilation (Fig. 5), suggesting this effect results from central autonomic regulation of vasodilation, rather than a direct effect *via* PDE4 inhibition on the tail vasculature. In addition, we show that antagonists at D_{2/3}-dopamine receptors that can enter the brain (Spiperone and Pimozide; Fig. 7D/F/H) alleviate PDE4 inhibitor-induced hypothermia, whereas Domperidone, which does not cross the blood-brain barrier and thus acts only on peripheral D_{2/3}-receptors, is ineffective (Fig. 7I).

Finally, we show that several behavioral effects, including changes in posture and locomotion, are nonconform with normal cold-defense, thus linking another layer of central nervous system effects to PDE4 inhibitor-induced hypothermia. Mice treated with doses of PDE4 inhibitors that induce substantial hypothermia (e.g. Rolipram, Piclamilast, Roflumilast, and RS25344, each at 1 mg/kg, Fig. 2) assume characteristic postures (tails are extended or erected, the body is extended, paws are frequently extended away from the body, some animals lay on their abdomen, rather than standing on their feet) which, together with hypokinesia, allow experimenters to visually distinguish PDE4 inhibitor-treated from control animals with high certainty. These characteristic postures likely contribute to PDE4 inhibitor-induced hypothermia given that they further increase the body's surface area and, thus, increase heat loss *via* radiation or conduction through the cage bottom/bedding.

Increased metabolism due to skeletal muscle contractions generates body heat, whether contractions are induced *via* autonomous motor control, such as during shivering, or somatic control, such as during locomotion. Indeed, increased methamphetamine-evoked locomotion has been suggested as a key contributor to hyperthermia associated with the abuse of these drugs^{60, 61}. Thus, reduced locomotion in mice after PDE4 inhibitor treatment (Fig. 6C) likely enhances the amplitude of the resulting hypothermia. However, this does not establish the mode of causality between the two paradigms, which remains to be determined. For example, PDE4 inhibition may induce cold-seeking behaviors in the mouse (similar to animals entering hibernation or torpor) in which case the extended postures and hypokinesia are the intended means to an end. Alternatively, PDE4 inhibitor-induced hypothermia and posture/hypokinesia may be causally independent and induced by separate mechanism. Finally, the characteristic behaviors of PDE4 inhibitor-treated mice may also be interpreted as some form of sedation; in which case, the animal simply loses control of behaviors associated with thermoregulation, so that only autonomic thermoeffectors remain available to control body temperature. Indeed, use of general anesthetics is well established to induce substantial hypothermia in humans and animals for this reason⁵. However, even the latter idea may be turned on its head. Given that hypothermia *per se* has sedative effects in mammals, PDE4 inhibitor-induced hypothermia may be the cause of the reduced locomotion. For example, mice that enter hypothermia due to caloric restriction (e.g. torpor and/or hibernation; see Fig. 6B) also appear somewhat sedated and initially move very slowly, stiffly when prompted, which is not unlike PDE4 inhibitor-treated mice. To provide another example of hypothermia inducing sedative effects, treatment with the monoamine

reuptake inhibitor Reserpine has been shown to reduce locomotor activity and induce hypothermia in mice at an ambient temperature of 22°C. However, when Reserpine-induced hypothermia was alleviated by housing mice at higher environmental temperatures (32-36°C), Reserpine treatment was no longer sedative⁶². Thus, if and how hypothermia and hypokinesia in PDE4 inhibitor-treated mice are causally related remains to be delineated.

4.3. Blockade of central D_{2/3}-dopamine receptors alleviates PDE4 inhibitor-induced hypothermia

To begin delineating the mechanism of PDE4 inhibitor-induced hypothermia, we show here that blockade of D_{2/3}-type dopaminergic, but not β -adrenergic, H₁-histaminergic, or opiate receptors, can alleviate PDE4 inhibitor-induced hypothermia (Fig. 7). A critical role of hypothalamic D₂-type receptor signaling *per se* in thermoregulation has been well established. For example, intrahypothalamic injections of dopamine have been shown to induce hypothermia in rats^{63, 64}, an effect that was antagonized by treatment with D₂-type antagonists⁶³ and was paralleled by deficits in locomotion⁶⁴ similar to the pattern observed after PDE4 inhibitor treatment (Fig. 6). In a similar vein, central administration of the D₂-type agonist Quinpirole also induced hypothermia in mice, an effect that was abolished by treatment with centrally acting D₂-type antagonists Sulpiride and Spiperone, but not the peripherally acting Domperidone⁶⁵. However, while there is a well-established role for dopaminergic signaling in thermoregulation, little is known to date about the role of PDE4 in the regulation of dopaminergic signaling in the hypothalamus. Conversely, the role of PDE4 in dopaminergic signaling in the frontostriatal system has been more widely explored⁶⁶⁻⁶⁸. It is established that cAMP signaling in general⁶⁹, and PDE4 inhibition in particular, can serve to enhance dopamine synthesis^{66, 68, 70}. Moreover, cAMP signaling in general⁷¹, and PDE4 inhibition in particular, has been shown to promote dopamine release from brain slices⁷². The latter is the more relevant mechanism that may contribute to PDE4 inhibitor-induced hypothermia, given its rapid time course. In opposite to D₁-like receptors, which signal *via* G_s, D₂-like receptors primarily signal through G_i to lower cAMP production, an effect that would be opposed by the effect of PDE inhibition. Thus, we propose that PDE4 inhibition acts upstream of D₂-type receptors, such as by promoting dopamine release, rather than downstream of D₂-receptor-dependent G_i protein signaling. However, like other G protein-coupled receptors, D₂-like receptors have been shown to signal *via* G protein-independent pathways, such as *via* β -arrestin^{73, 74}. As PDE4s have been implicated in modulating β -arrestin signaling of other GPCRs⁷⁵, or modulating the affinity of receptors for their ligand by yet unknown means⁷⁶, alternative modes whereby PDE4 inhibition may affect D₂-type receptor signaling are also possible.

4.4. Potential relevance of hypothermia to the therapeutic benefits and adverse effects attributed to PDE4 inhibitors

Given the significant decrease in body temperature, a potential contribution of hypothermia to the pharmacologic effects (both potentially therapeutic and adverse effects) of PDE4 inhibitors in animal studies should not be ignored, in particular in studies utilizing mice. Development of PDE4 inhibitors is pursued for a number of indications, including for their anti-inflammatory properties, as well as metabolic-, cardiovascular-, antineoplastic-, and behavioral effects^{16, 20, 21, 26, 28, 66, 77-79}. From studies in cells and tissues *ex vivo*, as well as

the use of PDE4 inhibitors that do not induce hypothermia (such as the non-brain penetrant YM976³⁷), it is clear the PDE4 inhibition can exert the above effects independent of its role in thermoregulation. However, when studying intact animals, a contribution of hypothermia to the observed effects is very likely, given that drug doses required to produce therapeutic benefits are the same, or frequently exceed the doses that induce significant hypothermia. In addition, by lowering the speed of many biochemical reactions that are critical for cellular and physiologic functions, hypothermia is certain to leave an impact on many paradigms. For instance, we consider it very likely that the anti-inflammatory properties induced by PDE4 inhibitors in mice are enhanced by the associated hypothermia, given that the rate of synthesis of inflammatory cytokines, or the migration of cells to the site of infection/inflammation, are likely slowed. Indeed, artificially-induced hypothermia is pursued as a therapeutic approach in patients with brain trauma, spinal cord injury, or stroke⁸⁰⁻⁸⁴ with the intent purpose to alleviate inflammatory responses by cooling down the organ/body. In a similar vein, it is well established that hypothermia lowers the metabolic demands of the body in settings of hibernation or torpor in animals, or in settings of therapeutic hypothermia in humans. Thus, PDE4 inhibitor-induced hypothermia will leave a mark on the overall metabolic effects of PDE4 inhibitors in these animals. Similarly, given that behavioral responses play a critical role in central thermoregulation, one can easily imagine that direct effects as well as adaptive responses to PDE4 inhibitor-induced hypothermia may leave their mark in various behavioral models. Finally, PDE4 inhibition is well-known to induce gastrointestinal adverse effects, in particular nausea and emesis. There are surprisingly strong correlations between nausea/emesis and hypothermia^{85, 86}, and often present as cold sweats and clammy hands (cutaneous vasoconstriction). Moreover, drug- or motion sickness-induced hypothermia can be reversed by treatment with anti-emetics⁸⁵. Thus, PDE4 inhibitor-induced hypothermia may well contribute to, result from, or represent a correlate of the established adverse effects of these drugs.

There is significant interest in the pharmacologic modulation of body temperature regulation as a therapeutic approach for a number of indications. This includes the artificial/pharmacologic induction of therapeutic hypothermia^{80, 82, 83}, such as in settings of brain injury, as well as the mitigation of fever^{11, 87} or hot flashes⁸⁸, or the induction of hyperthermia to elevate energy expenditure and induce weight loss¹. The extensive knowledge on the biochemistry and pharmacology of PDE4 enzymes, including the availability of small-molecule PDE4 activators and inhibitors, may provide opportunities to therapeutically target PDE4 given its critical role in body temperature regulation. To this end, further research into the cellular and molecular mechanisms of PDE4 inhibitor-induced hypothermia, the role of individual PDE4 subtypes in mediating the effects of the PAN-PDE4 inhibitors used here, and studies exploring to which extend findings in animals translate into PDE4-dependent body temperature regulation in humans, seems warranted.

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Abbreviations:

DMSO	Dimethyl sulfoxide
D_{2/3}	Dopamine D _{2/3} receptors
HARBS	High-affinity Rolipram binding state
5-HT₂	5-Hydroxytryptamine receptor 2
LARBS	Low-affinity Rolipram binding state
PBS	Phosphate-buffered saline
PDE	cyclic nucleotide phosphodiesterase
PDE4	Type 4 cAMP phosphodiesterase

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Fig. 1. Treatment with the PDE4 inhibitor Piclamilast induces acute hypothermia in mice.

The body temperature of mice was measured at the indicated time points prior to and after intraperitoneal (i.p.) injection of the PDE4 inhibitor Piclamilast/RP73401 (red filled circles, 5 mg/kg; n=8), as well as in solvent controls (open circles, n=12) and non-injection controls (black triangles, n=12) at the sternum of the animals using an infrared thermometer. Data represent the mean $\pm$ SEM. The body temperature of Piclamilast-treated mice is statistically different ($p < 0.001$) from either solvent- or non-injection controls as determined by two-way ANOVA and Tukey's multiple comparison post hoc test at any time point between 5 and 240 min after drug injection.

Fig. 2. Acute hypothermia is a class effect of PDE4 inhibitors.

The core body temperature of mice was measured using a rectal probe thermometer at 30 min after i.p. injection of several distinct PDE4-selective inhibitors (all 1 mg/kg) including Piclamilast/RP73401, Rolipram, Roflumilast, and RS25344, as well as the PDE3-selective inhibitor Cilostamide (1 mg/kg) or solvent controls. Data represent the mean $\pm$ SEM. Statistical significance was determined using one-way ANOVA with Tukey's post hoc test and is indicated as *** ($p < 0.001$). For Cilostamide (1 mg/kg) $p = 0.95$. **(B)** The chemical structures of all PDE inhibitors tested in this study are shown for comparison.

Fig. 3. The dose-dependent induction of hypothermia correlates with the brain penetrance of PDE4 inhibitors.

Mice were injected with the indicated doses of PDE4 inhibitors and core body temperature was measured 30 min later using a rectal probe thermometer. Shown are dose-response curves for the PDE4 inhibitors Rolipram (**A**) and Piclamilast/RP73401 (**B**) which readily distribute into the brain, as well as YM976 (**C**) which does not efficiently cross the blood-brain barrier³⁷. Data represent the mean $\pm$ SEM. Statistical significance was determined using one-way ANOVA with Tukey's post hoc test and is indicated as * ($p < 0.05$), and *** ($p < 0.001$). For 0.2 mg/kg Piclamilast $p = 0.028$.

Fig. 4. The onset of PDE4 inhibitor-induced hypothermia is rapid, suggesting a suspension of cold defense.

Comparison of the time course of core body temperature measured using a rectal probe thermometer, after treatment with the PDE4 inhibitor Rolipram (5 mg/kg; i.p.) to that after euthanasia (carbon dioxide followed by cervical dislocation). The striated line indicates the time point of drug injection or euthanasia. Data represent the mean $\pm$ SEM of $n=8$ animals. * indicates the first time point at which body temperature after euthanasia is significantly different ($p<0.05$) from that after Rolipram treatment as determined by two-way ANOVA followed by Tukey's post hoc test ($p=0.16$ at 10 min, and $p=0.02$ at 15 min after treatment, respectively).

Fig. 5. PDE4 inhibitor-induced hypothermia is associated with tail cutaneous vasodilation. Shown on the left are composite light/infrared images of the same mouse acquired (**A**) shortly before, and (**B**) 4 min after injection of the PDE4 inhibitor Rolipram (1 mg/kg; i.p.). Both images are set to the same pseudocolor scale (23.5°C to 32°C). (**C**) Shown are the tail temperatures of mice treated with the PDE4 inhibitors Rolipram (red filled circles; n=7) or YM976 (black triangles; n=7), each administered at a dose of 1 mg/kg (i.p.), compared to solvent controls (open circles; n=8) computed from infrared images. Data represent the mean $\pm$ SEM. Statistical significance was determined by two-way ANOVA with Tukey's post hoc multiple comparison test. YM976 did not produce a significant difference in tail temperature compared to solvent control. Conversely, Rolipram-treated mice have significantly higher tail temperatures between 2 and 10 min after injection ($p=0.012$ at 2 min; $p<0.001$ at 4, 6 and 8 min; $p=0.046$ at 10 min).

Fig. 6. PDE4 inhibition produces behavioral effects potentially associated with body temperature regulation.

(A/B) PDE4 inhibition affects posture in mice. Shown in (A) are representative images illustrating posture of mice 30 min after injection of the PDE4 inhibitor Rolipram (1 mg/kg; i.p.). After PDE4 inhibitor treatment, animals frequently lay flat on their abdomen, rather than walk/stand on their paws, thus, potentially increasing surface area as well as heat conduction through the cage bedding. In addition, the tails of PDE4 inhibitor-treated mice are characteristically extended, sometimes erected, rather than pulled around or under the body. (B) Representative images of hypothermic mice ($\sim 32^{\circ}\text{C}$) triggered by overnight fasting. Characteristic behaviors include attempted nesting, location to the side or the corner of the cage, as well as hunched posture limiting body surface, tail curling, and huddling. (C) PDE4 inhibition reduces locomotion as reflected by reduced travel distance. Mice were injected (i.p.) with the PDE4 inhibitors Rolipram (1 mg/kg; $n=20$), Piclamilast (1 mg/kg; $n=8$) or solvent controls ($n=12$), placed immediately in a new cage and locomotion was assessed using SmartCageTM technology. Traces represent changes in travel distance (cm per 5 min interval) and are expressed as the mean $\pm$ SEM. Traces for Rolipram or Piclamilast were statistically different ($p < 0.001$) from the solvent control as determined by two-way ANOVA, and Tukey's multiple comparison post hoc test indicates that locomotion in Rolipram- or Piclamilast-treated mice was significantly reduced ($p < 0.05$) compared to solvent controls at any time point between 5 and 80 min.

Fig. 7. Blockade of central $D_{2/3}$ -dopamine receptors alleviates PDE4 inhibitor-induced hypothermia.

Pretreatment with selected receptor antagonists was used to probe the role of various receptor pathways in PDE4 inhibitor-induced hypothermia. After measurement of baseline body temperature (at -30 and -1 min), mice were injected (i.p.; 0 min time point) with the indicated doses of various receptor antagonists, followed 60 min later by injection (i.p.) of the PDE4 inhibitor Rolipram (0.2 mg/kg for panel (F); 1 mg/kg Rolipram for all other panels). The body temperature of mice was measured at the indicated time points using a rectal probe thermometer. Data represent the mean $\pm$ SEM. Statistical significance was determined using two-way ANOVA with Tukey's post hoc test and is indicated as * ($p < 0.05$); ** ($p < 0.01$); *** ($p < 0.001$).

(A-C) Pretreatment with the β -adrenoceptor blocker Propranolol (1 mg/kg, $n=16$), the opioid-receptor blocker Naloxone (1 mg/kg, $n=16$), or the H_1 -histamine receptor blocker Chlorcyclizine (5 mg/kg, $n=13$) did not alleviate hypothermia induced by treatment with the PDE4 inhibitor Rolipram (1 mg/kg). (D-F) Effect of pre-treatment with Spiperone, which acts as antagonist at $D_{2/3}$ -type dopaminergic as well as 5-HT $_2$ serotonin receptors, on PDE4 inhibitor-induced hypothermia. (D) At a dose of 0.2 mg/kg ($n=21$, $p_{90\min} < 0.001$), Spiperone significantly alleviates hypothermia induced by the PDE4 inhibitor Rolipram (1 mg/kg). (E) Spiperone is less effective at higher doses of 0.5 mg/kg ($n=8$, $p_{90\min} = 0.0012$) and 1 mg/kg

(n=21, $p_{90\min}=0.341$), given that Spiperone itself induces hypothermia as seen at the 30 min timepoint prior to Rolipram injection at 0.5 mg/kg (n=8, $p_{30\min}=0.043$) and 1 mg/kg (n=21, $p_{30\min}<0.001$). **(F)** Similar to the effect on high doses of Rolipram (1 mg/kg) tested in **(D)**, Spiperone only partially alleviates PDE4 inhibitor-induced hypothermia also when a lower dose of Rolipram (0.2 mg/kg; n=16, $p_{90\min}=0.002$) is applied. **(G)** Pretreatment with the selective 5HT₂-serotonine receptor blocker Ketanserin at doses of 0.2 mg/kg (n=13, $p_{90\min}=0.032$) and 1 mg/kg (n=6, $p_{30\min}=0.017$, $p_{90\min}=0.005$) induces hypothermia *per se*, but does not protect from hypothermia induced by the PDE4 inhibitor Rolipram (1 mg/kg). **(H/I)** Blockage of D_{2/3}-type dopaminergic receptors with the centrally-acting blocker Pimozide (5 mg/kg, n=14, $p_{90\min}=0.003$) **(H)** replicates the effects of Spiperone in alleviating PDE4 inhibitor-induced hypothermia. Conversely, the peripherally-acting D_{2/3}-type dopaminergic receptor blocker Domperidone (5 mg/kg, n=6) had no effect **(I)**.