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Neuropharmacology

Antiallodynic action of phosphodiesterase inhibitors in a mouse model of neuropathic pain

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Abstract:	<p>Neuropathic pain arises as a consequence of a lesion or disease affecting the somatosensory nervous system. It is accompanied by neuronal and non-neuronal alterations, including alterations in intracellular second messenger pathways. Cellular levels of 3',5'-cyclic adenosine monophosphate (cAMP) and 3',5'-cyclic guanosine monophosphate (cGMP) are regulated by phosphodiesterase (PDE) enzymes. Here, we studied the impact of PDE inhibitors (PDEi) in an animal model of neuropathic pain. In mice, we induced neuropathic pain by placing a cuff around the main branch of the sciatic nerve, and tested mechanical thresholds using von Frey filaments. Mechanical hypersensitivity was relieved by sustained treatment with the non-selective PDEi theophylline and ibudilast (AV-411), with PDE4i rolipram, etazolate and YM-976, and with PDE5i sildenafil, zaprinast and MY-5445, but not by treatments with PDE1i vinpocetine, PDE2i EHNA or PDE3i milrinone. Using pharmacological and knock-out approaches, we show a preferential implication of delta opioid receptors in the action of the PDE4i rolipram and of both mu and delta opioid receptors in the action of the PDE5i sildenafil. Calcium imaging highlighted a preferential action of rolipram on dorsal root ganglia non-neuronal cells, through PDE4B and PDE4D inhibition. Rolipram had anti-neuroimmune action, as shown by its impact on levels of the pro-inflammatory cytokine tumor necrosis factor-α (TNFα) in the dorsal root ganglia of mice with neuropathic pain, as well as in human peripheral blood mononuclear cells (PBMCs) stimulated with lipopolysaccharides. This study suggests that PDEs, especially PDE4 and 5, may be targets of interest in the treatment of neuropathic pain.</p>
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05 July 2021

Dr. Michel Barrot, PhD, DR CNRS

Dr. Ipek Yalcin, PhD, DR CNRS

To the Editors of *Neuropharmacology*

Dear Guest Editors Michelle Roche and David Finn,

Please find attached our manuscript entitled “**Antiallodynic action of phosphodiesterase inhibitors in a mouse model of neuropathic pain**” which we would like to submit to *Neuropharmacology* for the special Issue on “*Advances in mechanisms and therapeutic targets relevant to pain*”.

This work aimed at studying the impact of phosphodiesterase inhibitors (PDEi) on neuropathic pain. The rationale for studying PDE as targets was in part based on previous studies demonstrating that PDE4i can display antidepressant-like effects (antidepressant drugs being one of 1st line treatment of neuropathic pain), that PDE4 mediates the hydrolysis of cAMP following activation of β_2 adrenoceptors (which are receptors contributing to antidepressant drug action against neuropathic pain), that PDE4i can suppress the activation of glial cells and act on cytokine production, and that inhibitors of another class of PDE (PDE5) were also shown to potentially act on neuropathic pain in animal models.

Accordingly, we did a large screening of various PDEi, which showed that only PDE4i and PDE5i significantly relieved neuropathic allodynia in a dose-dependent manner. Using pharmacological and knock-out approaches, we then showed an implication of different opioid receptors in the antiallodynic effect of the PDE4i rolipram and of the PDE5i sildenafil. Calcium imaging highlighted a preferential action of rolipram on dorsal root ganglia non-neuronal cells, through PDE4B and PDE4D inhibition. In addition, long-term treatment with rolipram decreased levels of the pro-inflammatory cytokine TNF α in the dorsal root ganglia. This was completed by a study on human blood cells, further confirming the inhibitory action that rolipram can exert on TNF α production.

We think that this work is putting new light on the effect of PDEi in chronic pain and might be worth your consideration for publication in *Neuropharmacology*. We hope that you will find it of interest for the broad readership of your journal.

Sincerely,

Ipek Yalcin & Michel Barrot



Antiallodynic action of phosphodiesterase inhibitors in a mouse model of neuropathic pain

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Keywords: antidepressant, phosphodiesterase inhibitors, allodynia, TNF α , neuronal, non-neuronal, rolipram, sildenafil

Abstract

Neuropathic pain arises as a consequence of a lesion or disease affecting the somatosensory nervous system. It is accompanied by neuronal and non-neuronal alterations, including alterations in intracellular second messenger pathways. Cellular levels of 3',5'-cyclic adenosine monophosphate (cAMP) and 3',5'-cyclic guanosine monophosphate (cGMP) are regulated by phosphodiesterase (PDE) enzymes. Here, we studied the impact of PDE inhibitors (PDEi) in an animal model of neuropathic pain. In mice, we induced neuropathic pain by placing a cuff around the main branch of the sciatic nerve, and tested mechanical thresholds using von Frey filaments. Mechanical hypersensitivity was relieved by sustained treatment with the non-selective PDEi theophylline and ibudilast (AV-411), with PDE4i rolipram, etazolate and YM-976, and with PDE5i sildenafil, zaprinast and MY-5445, but not by treatments with PDE1i vinpocetine, PDE2i EHNA or PDE3i milrinone. Using pharmacological and knock-out approaches, we show a preferential implication of delta opioid receptors in the action of the PDE4i rolipram and of both mu and delta opioid receptors in the action of the PDE5i sildenafil. Calcium imaging highlighted a preferential action of rolipram on dorsal root ganglia non-neuronal cells, through PDE4B and PDE4D inhibition. Rolipram had anti-neuroimmune action, as shown by its impact on levels of the pro-inflammatory cytokine tumor necrosis factor- α (TNF α) in the dorsal root ganglia of mice with neuropathic pain, as well as in human peripheral blood mononuclear cells (PBMCs) stimulated with lipopolysaccharides. This study suggests that PDEs, especially PDE4 and 5, may be targets of interest in the treatment of neuropathic pain.

Antiallodynic action of phosphodiesterase inhibitors in a mouse model of neuropathic pain

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Neuropathic pain arises as a consequence of a lesion or disease affecting the somatosensory nervous system. It is accompanied by neuronal and non-neuronal alterations, including alterations in intracellular second messenger pathways. Cellular levels of 3',5'-cyclic adenosine monophosphate (cAMP) and 3',5'-cyclic guanosine monophosphate (cGMP) are regulated by phosphodiesterase (PDE) enzymes. Here, we studied the impact of PDE inhibitors (PDEi) in an animal model of neuropathic pain. In mice, we induced neuropathic pain by placing a cuff around the main branch of the sciatic nerve, and tested mechanical thresholds using von Frey filaments. Mechanical hypersensitivity was relieved by sustained treatment with the non-selective PDEi theophylline and ibudilast (AV-411), with PDE4i rolipram, etazolate and YM-976, and with PDE5i sildenafil, zaprinast and MY-5445, but not by treatments with PDE1i vinpocetine, PDE2i EHNA or PDE3i milrinone. Using pharmacological and knock-out approaches, we show a preferential implication of delta opioid receptors in the action of the PDE4i rolipram and of both mu and delta opioid receptors in the action of the PDE5i sildenafil. Calcium imaging highlighted a preferential action of rolipram on dorsal root ganglia non-neuronal cells, through PDE4B and PDE4D inhibition. Rolipram had anti-neuroimmune action, as shown by its impact on levels of the pro-inflammatory cytokine tumor necrosis factor- α (TNF α) in the dorsal root ganglia of mice with neuropathic pain, as well as in human peripheral blood mononuclear cells (PBMCs) stimulated with lipopolysaccharides. This study suggests that PDEs, especially PDE4 and 5, may be targets of interest in the treatment of neuropathic pain.

1. Introduction

3',5'-cyclic adenosine monophosphate (cAMP) and 3',5'-cyclic guanosine monophosphate (cGMP) are important second messengers implicated in many biological processes (Friebe et al., 2020; Gancedo, 2013; Houslay et al., 2010). Their cellular levels are regulated by phosphodiesterase (PDE) enzymes belonging to an eleven-family group (PDE1-PDE11) that can be further divided into multiple isoforms, encoded by different genes and/or resulting from alternative splicing (Ahmad et al., 2015; Azevedo et al., 2014; Francis et al., 2001; Soderling and Beavo, 2000). The PDE families differ in their ability to hydrolyze cAMP and/or cGMP, in their tissue and subcellular distribution, as well as in their sensitivity to pharmacological inhibitors (Ahmad et al., 2015; Azevedo et al., 2014; Francis et al., 2011). Either clinically or in preclinical models, these PDE inhibitors (PDEi) have been shown to be effective in a variety of pathological conditions, including major depressive disorder, sexual dysfunction, chronic obstructive pulmonary dysfunction, psoriasis and chronic pain (Ahmad et al., 2015; Azevedo et al., 2014; Bollenbach et al., 2019; Keravis and Lugnier, 2012; Mayer et al., 2005; Page and Spina, 2011; Renau, 2004; Wittmann and Helliwell, 2013).

Neuropathic pain is a chronic pain arising as a consequence of a lesion or a disease affecting the somatosensory nervous system (Scholz et al., 2019; Treede et al., 2019). Interestingly, antidepressant drugs acting on monoamine uptake are among first line treatments for neuropathic pain (Attal et al., 2010; Attal, 2019; Barrot et al., 2009; Wright and Rizzolo, 2017). This action has been shown to be mediated by the secondary recruitment of β_2 adrenoceptors (Choucair-Jaafar et al., 2009; Barrot et al., 2010; Bohren et al., 2013; Kremer et al., 2018; Yalcin et al., 2009a; Yalcin et al., 2009b) and a reduction of the cytokine tumor necrosis factor (TNF)- α in the dorsal root ganglia (Bohren et al., 2013; Kremer et al., 2018). Since: 1) PDE4 inhibitors have been preclinically investigated for their antidepressant effects (Bobon et al., 1988); 2) PDE4

has been shown to be the main PDE to mediate the hydrolysis of cAMP formed by the activation of β_2 adrenoceptors (Ye et al., 1997); 3) increasing cAMP levels suppresses the activation of glial cells and the production of proinflammatory cytokines such as TNF α , and PDEi can enhance the anti-TNF α activity of a β_2 adrenoceptor agonist (Christiansen et al., 2011), we sought to investigate whether a long-term treatment with PDEi could alleviate neuropathic pain.

In this study, our pharmacological screening showed that only PDE4i and PDE5i significantly relieved neuropathic allodynia in a dose-dependent manner. Using pharmacological and knock-out approaches, we further showed a differential implication of opioid receptors in the antiallodynic effect of the PDE4i rolipram and the PDE5i sildenafil. Calcium imaging highlighted a preferential action of rolipram on dorsal root ganglia non-neuronal cells, through PDE4B and PDE4D inhibition. In addition, long-term treatment with rolipram decreased levels of the pro-inflammatory cytokine TNF α in the dorsal root ganglia.

2. Material and methods

2.1. Animals

Experiments were performed in adult male mice, housed three to five per cage under a 12 h light/dark cycle with *ad libitum* access to water and food, and weighing 25-30 g at surgery time. C57BL/6J mice (Chronobiotron UMS3415, Strasbourg, France), as well as wild-type (WT) and MOR^{-/-}, DOR^{-/-}, KOR^{-/-} littermate mice were used. The generation of mice lacking the μ (MOR), δ (DOR) or κ (KOR) opioid receptor has been previously described (Filliol et al., 2000; Simonin et al., 1998). These mice were backcrossed on a C57BL/6J background for at least 10 generations, and heterozygote mice (MOR^{+/-}, DOR^{+/-}, KOR^{+/-}) were bred in our facilities and genotyped upon weaning. The animal facilities are legally registered for animal experimentation under Animal House Agreement C67-482-1. Experiments were approved by the regional ethical committee (CREMEAS).

2.2. Model and test

Neuropathic pain was induced by inserting a cuff around the main branch of the right sciatic nerve (Benbouzid et al., 2008b; Yalcin et al., 2014). Surgeries were done under ketamine (17 mg/mL) / xylazine (2.5 mg/mL) anesthesia (intraperitoneal, 4 mL/kg) (Centravet, Taden, France). The common branch of the right sciatic nerve was exposed and a 2 mm section of split PE-20 polyethylene tubing (Harvard Apparatus, Les Ulis, France) was placed around it (Cuff group). The shaved skin was closed using suture. Sham-operated mice underwent the same procedure, without implantation of the cuff (Sham group). Mechanical allodynia being a symptom of neuropathic pain, we measured mechanical thresholds of hindpaw withdrawal using von Frey hairs and the results were expressed in grams (Barrot, 2012; Bohren et al., 2010; Yalcin et al., 2014). Mice were placed in clear Plexiglas® boxes (7 cm × 9 cm × 7 cm) on an elevated

mesh screen and calibrated von Frey filaments (Bioseb, Chaville, France) were applied to the plantar surface of each hindpaw in a series of ascending forces until they bent. Gram value of the lower filament that induced at least 3 paw responses out of 5 trials was considered as the paw withdrawal threshold for this animal.

2.3. Drug Treatments

Ibudilast (AV-411) and MY-5445 were purchased from Tocris Biosciences (Bristol, United Kingdom). Rolipram, theophylline, naltrindole and naloxonazine were purchased from Sigma-Aldrich (St Quentin Fallavier, France). Erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA), etazolate, milrinone, sildenafil, vinpocetine, YM-976 and zaprinast were purchased from Biotrend Chemikalien GmbH (Köln, Germany). For studies in mice, drugs were suspended in 1% methylcellulose in 0.9% NaCl. PDEi and vehicle solutions were injected intraperitoneally, twice per day (morning and evening) in a volume of 5 mL/kg body weight. Chronic treatments began after at least a two-week delay post-surgery and lasted at least 12 days, except for the non-selective PDEi ibudilast that was given for 7 days. In order to measure the effect of chronic treatments, behavioral tests were done in mornings before the first daily injection and at least twice *per* week during treatments. For the study in human peripheral blood mononuclear cells (PBMCs), rolipram was re-suspended in dimethyl sulfoxide (DMSO) and tested at different concentrations (final DMSO concentration was adjusted to 1% maximum for each testing well).

2.4. Immunoblot

After 21 days of rolipram or control treatment, dorsal root ganglia corresponding to lumbar levels L4, L5 and L6 were dissected. Total protein was extracted in 150 μ L of lysis buffer (20 mM Tris pH 7.5; 150 mM NaCl; 10% glycerol (v/v); 1% NP40 (v/v); Protease Inhibitor

EDTA-free (Roche)) and quantified with DC protein assay kits (Bio-Rad). Fifteen μg of proteins were resolved on 12% polyacrylamide-SDS electrophoresis gel under reducing conditions, and transferred to PVDF membranes (Immobilion, Millipore, #IPVH00010). Blots were incubated 1 h in blocking agent (ECL Blocking Agent, Advance Western Blotting Detection System Kit) and overnight with anti-TNF α (1:500, RD systems, #AF-410-NA) and anti- β -tubulin (1:10000, Abcam, #Ab108342) antibodies. Blots were then incubated with anti-goat and anti-rabbit HRP-conjugated secondary antibodies (1:5000, Chemicon, #AF106P and #AP187P) at room temperature, and revealed by chemiluminescence (Advance Detection System Kit, Amersham, #RPN2135) using Hyperfilm substrates (Hyperfilm, Amersham Biosciences, #RPN1674K). Protein expression was quantified using the densitometry tool of Adobe PhotoShop software CS5.

2.5. Dorsal root ganglia culture

Dorsal root ganglia cell cultures were prepared from adult mice. Immediately after dissection, lumbar dorsal root ganglia were collected in a phosphate-buffered saline solution, enzymatically dissociated for 20 min at 37°C with trypsin-EDTA (0.5 g/L, Seromed) and 2 mg/mL of collagenase IA/dispase (Invitrogen). Following enzymatic dissociation, culture medium consisting of MEM α (minimum essential medium α , Gibco, France) with 50 IU/mL penicillin-streptomycin (Gibco) and 10% v/v heat inactivated horse serum (Gibco) was added. Cells were then further dissociated by trituration with polished Pasteur pipettes of decreasing tip-diameter. Dorsal root ganglia cells were then plated on 15 mm glass coverslips coated with poly-D-lysine (0.02 mg/mL), and cultures were placed in a controlled atmosphere (95% O₂, 5% CO₂). Calcium imaging experiments were performed 24 to 36 h after seeding.

2.6. Calcium imaging

Calcium imaging was performed on dorsal root ganglia cell cultures from Sham and Cuff mice. Cells were incubated in a solution containing 2 μ M Fura-2 acetoxymethyl ester (F1201, Molecular Probes, USA) and 0.01% pluronic acid (Molecular Probes) for 1 h at room temperature. During calcium measurements, cells were continuously perfused with an extracellular solution (130 mM NaCl; 5 mM KCl; 1 mM CaCl₂; 1 mM MgCl₂; 10 mM glucose; 10 mM HEPES pH 7.3). Fluorescence measurements were performed on an inverted microscope (Axiovert 35, Zeiss) with a 40X oil-immersion objective (Fluo 40, NA 1.30, Nikon) using a real-time imaging system with a cooled CCD camera (CoolSnap HQ, Photometrics) and an imaging analysis software (Imaging Workbench software 4.0, Axon Instruments). Fluorescence was alternatively excited at 340 nm and 380 nm with a Lambda-10 filter wheel (Sutter Instruments) and the emitted light was collected above 520 nm. Images were acquired every 2 s and intracellular calcium concentration was expressed using the fluorescence ratio F340/F380 after background subtraction. Responses to UTP (50 μ M), KCl (50 mM) and rolipram (1, 10 and 100 μ M) were tested. Neurons and non-neuronal cells were identified using successive application of UTP, a P2Y receptor agonist, and high KCl which triggers calcium responses selectively in neurons. Cells that clearly showed calcium responses to UTP but not to KCl were considered as non-neuronal cells, while cells that responded to KCl with or without responses to UTP were considered as neurons.

2.7. siRNA experiments

In order to determine the PDE4 subtype involved in the neuronal and non-neuronal effects of rolipram, we performed siRNA-mediated knockdown of *Pde4b* and *Pde4d* in dorsal root

ganglia cell cultures. Knockdown of *Pde4b* and *Pde4d* mRNA were performed using ACCELL siRNA (#E-049576-00 and #E-043744-00, respectively) designed and validated by ThermoFisher/Dharmacon (Lafayette, CO, USA). A non-targeting siRNA (#D-001910-10-05) was used as a negative control. Stock solutions for the targeted and scrambled siRNA (#D-001910-10-05) were prepared at 100 μ M in 1x siRNA Buffer (diluted from Dharmacon 5x siRNA Buffer, #B-002000-UB-100). Dorsal root ganglia cells were plated and allowed to attach to culture dishes for 30 min in standard culture medium. Then, medium was replaced by 1 mL of serum-free medium (Thermo scientific, #B-005000-500) containing 1 μ M siRNA or the scrambled negative control. The cultures were tested 24–36 h after transfection.

2.8. Real time quantitative PCR (RT-qPCR)

Quantification of gene expression was done by RT-qPCR. Total mRNA was extracted using RNAeasy kit (Qiagen) and the cDNA was generated using *iScript cDNA synthesis kit* (Biorad). RT-qPCR was performed on a Thermocycler Biorad MyIQ, using Biorad IQ Syber Green Supermix assay, with initialization step at 95°C for 3 minutes followed by amplification for 40 cycles of 20 s 95°C denaturation, 20 s 95°C annealing and 20 s 60°C extension. Experiments were performed with triplicate sample deposits on the amplification plate. Relative abundance of each RNA target gene transcript was normalized to the endogenous control gene *Gapdh* RNA. Data were analyzed according to the standard curve method. Primers were designed from *Mus Musculus* using gene data bank and Primer Blast as follows: *Pde4b*: forward 5'-AAATTCTGAACTTGCTTTGATGT-3' and reverse 3'-GAAAGATGTCGCAGTGTTCCCT-5'; *Pde4d*: forward 5'-AGAGCCTGTCTTGTAAGCATTTC-3' and reverse 5'-GGTGCGGCTCTCAGTAGTTT-3'; and *Gapdh*: forward 5'-GGCCTTCCGTGTTCCCTAC-3' and reverse 3' TGTCATCATACTTGGCAGGTT-5'.

2.9. Anti-TNF α activity in human peripheral blood mononuclear cells (PBMCs)

PBMCs were prepared from the peripheral blood of healthy donors (Etablissement Français du Sang) as described by [Kümmerle et al. \(2012\)](#). PBMCs were isolated using Histopaque gradient (Sigma-Aldrich), washed in HanksamgiS noitulos tlas decnalab '-Aldrich) and were then cultured in RPMI-1640 medium (Sigma-Aldrich) supplemented with 10% (v/v) fetal bovine serum (PAA Laboratories), 100 U/mL penicillin and 100 μ g/mL streptomycin (PAA Laboratories). PBMCs were seeded in 24-well plates (5×10^5 cells/well) and stimulated with 5 μ g/mL lipopolysaccharide (LPS) (Sigma-Aldrich, L2630), in a total volume of 1 mL per well. Cells without LPS stimulation were considered as bio-inactive control (basal level of cytokine). Incubation with rolipram was performed at 37°C (5% CO₂) for 24 hours and TNF α was measured on culture supernatant by ELISA as previously described ([Kümmerle et al., 2012](#)). Cell viability was also controlled using the WST-1 (Ozyme) assay described previously ([Houël et al., 2015](#)). Briefly, after the supernatant was transferred for cytokine determination, WST-1-containing medium was added to cells and cell viability was measured using the Victor³ reader (PerkinElmer) after 2-hour incubation at 37°C. Each measurement was performed in triplicate with three independent experiments, and results expressed as means of the three independent experiments.

2.10. Statistical analysis

Data are expressed as mean \pm SEM. Statistical analyses were performed using multifactorial analysis of variance (ANOVA). Surgical procedures (Sham or Cuff) and treatments (saline vs. drug injections) were taken as between-group factors. When needed, the time of test (either time course or pre-injection vs. post-injection data) was taken as within-subject factor.

Duncan test was used for *post-hoc* multiple comparisons. For calcium imaging experiments, the fraction of cells responding or not to rolipram in sham and neuropathic conditions were compared by a contingency table with a Fischer's exact test. For quantitative PCR and immunoblotting experiments, statistical analyses were done using Kruskal-Wallis non-parametric test and a comparison between groups was performed with Mann-Whitney U test. Significance level was set at $p < 0.05$.

3. RESULTS

3.1. PDEi relieves neuropathic mechanical hypersensitivity

We first tested the non-selective PDEi theophylline which, given intraperitoneally twice a day, relieved mechanical hypersensitivity over time ($F_{27,216}=3.98$, $p<0.001$) (Fig. 1A). Similarly, seven days of twice daily treatment with another non-selective PDEi, ibudilast (AV-411), also suppressed mechanical hypersensitivity ($F_{4,80}=8.14$, $p<0.001$) (Fig. 1B). To assess the respective role of different PDE subtypes, we then tested more selective compounds. Neither the PDE1i vinpocetine ($F_{27,264}=0.96$, $p=0.52$) nor the PDE2i EHNA ($F_{27,242}=0.50$, $p=0.95$) or the PDE3i milrinone ($F_{33,242}=1.42$, $p=0.1$) suppressed mechanical hypersensitivity of the paw with sciatic nerve cuffing (Fig. 1C). However, chronic treatment with PDE4i, such as rolipram ($F_{33,330}=4.47$, $p<0.001$), etazolate ($F_{27,180}=9.90$, $p<0.001$) and YM-976 ($F_{30,264}=6.99$, $p<0.001$) relieved hypersensitivity (Fig. 1D). Besides, we also observed that treatments with PDE5i, including sildenafil ($F_{27,198}=6.51$, $p<0.001$), zaprinast ($F_{33,197}=3.91$, $p<0.001$) and MY-5445 ($F_{18,54}=12.19$, $p<0.001$), reversed mechanical hypersensitivity (Fig. 1E).

3.2. PDE4i and PDE5i reverse mechanical hypersensitivity in a dose-dependent manner

To identify the threshold doses leading to allodynia relief, we then assessed the dose-response effect of the PDE4i rolipram and PDE5i sildenafil. After around two weeks of treatment, rolipram exerted an antiallodynic action at doses 0.25 mg/kg ($F_{27,242}=3.77$, $p<0.001$) and 0.125 mg/kg ($F_{33,208}=2.83$, $p<0.001$), but not at lower doses (0.06 mg/kg: $F_{24,144}=0.32$, $p=0.99$; 0.03 mg/kg: $F_{24,144}=0.42$, $p=0.99$) (Fig. 2A). Chronic sildenafil was effective against mechanical hypersensitivity at doses 0.02 mg/kg ($F_{15,100}=4.03$, $p<0.01$), 0.01 mg/kg ($F_{21,198}=6.51$, $p<0.001$) and down to 0.0075 mg/kg ($F_{15,110}=3.77$, $p<0.01$), but not at 0.005 mg/kg ($F_{24,110}=0.53$, $p=0.72$) (Fig. 2B).

3.3. The opioid system is necessary for PDE4i and PDE5i antiallodynic action

Since the presence of opioid receptors has been shown to be necessary to the antiallodynic action of antidepressant drugs (Benbouzid et al., 2008a; Kremer et al., 2016; Kremer et al., 2018) and of agonists of β 2 adrenoceptors (Choucair-Jaafar et al., 2014; Yalcin et al., 2010), we tested whether opioid receptors also contributed to PDE4i and PDE5i antiallodynic action. For this, we used mice deficient for MOR, DOR and KOR, and opioid receptor antagonists (Fig. 3A). The antiallodynic action (area under the curve over 9 days) of repeated treatment with rolipram (0.5 mg/kg, s.c. twice a day) ($F_{3,23}=19.13$, $p<0.01$) was similar between WT, MOR^{-/-} and KOR^{-/-} mice, while rolipram lost its action in DOR^{-/-} mice (Fig. 3B). Likewise, pharmacological antagonism of DOR by naltrindole 5 mg/kg acutely reversed the therapeutic effect of chronic rolipram ($F_{2,14}=97.9$, $p<0.01$), while there was no relapse following the acute administration of a MOR antagonist, naloxonazine 30 mg/kg ($F_{2,14}=2.16$, $p=0.7$) (Fig. 3C). While only DOR appeared critical to rolipram antiallodynic action, the situation differed for sildenafil. Indeed, sildenafil (0.01 mg/kg, s.c. twice a day) (area under the curve over 6 days; $F_{3,16}=27.26$, $p<0.001$) failed to impact mechanical hypersensitivity in MOR^{-/-} mice as well as in DOR^{-/-} mice, whereas allodynia relief was observed in WT and KOR^{-/-} (Fig. 3D). In agreement, relief of mechanical hypersensitivity by chronic sildenafil was acutely reversed by an injection of either naloxonazine ($F_{6,39}=21.71$, $p<0.001$) or naltrindole ($F_{6,39}=16.74$, $p<0.001$) (Fig. 3E).

3.4. Neuronal PDE4B and non-neuronal PDE4D/4B are implicated in rolipram action

We then further explored the mechanism of the PDE4i rolipram, with the hypothesis that it may share mechanistic features with antidepressant treatment of neuropathic pain (Bohren et al., 2013; Kremer et al., 2018). Using calcium imaging on lumbar dorsal root ganglia, we

observed an increased proportion of neurons (19% vs. 7.2%; $p=0.017$) and of non-neuronal cells (32% vs. 5.2%; $p=0.0389$) responding to rolipram at very high dose (100 μM) in the dorsal root ganglia of mice with neuropathic pain (Fig. 4A,B). A dose-response study, however, supports the idea that non-neuronal cells were preferential targets. Indeed, the neuronal response disappeared at lower doses (1.4% at 10 μM ; 4.7% at 1 μM ; $p<0.01$ vs. 100 μM in both cases); whereas the proportion of responding non-neuronal cells remained similar at 1 μM (32% at 10 μM ; 25% at 1 μM ; $p>0.05$ vs. 100 μM in both cases) (Fig. 4C). To test whether responses to rolipram depended on extracellular calcium, we used a calcium-free medium. This led to a dramatic decrease in the proportion of responding cells (rolipram 100 μM : neurons: 2.6% vs. 16.5%, $p=0.0055$; non-neuronal cells: 5.4% vs. 21.9%, $p=0.0051$) (Fig. 4D).

PDE4 is a family of proteins encoded by 4 different genes. Antidepressant's action in neuropathic pain depends on β_2 -adrenoceptors (Bohren et al., 2013; Kremer et al., 2018; Yalcin et al., 2009a; Yalcin et al., 2009b), and cAMP regulation downstream of β_2 -adrenoceptors has been preferentially associated with *Pde4b* and *Pde4d*. We thus used siRNA to selectively knockdown these genes in dorsal root ganglia cell cultures from neuropathic animals. The selectivity of siRNA approach was controlled by RT-qPCR (*Pde4b*: $F_{3,16}=40.09$, $p<0.0001$. *Pde4d*: $F_{3,15}=99.54$, $p<0.0001$) (Fig. 4E). The neuronal response present at a very high concentration of rolipram (100 μM) was prevented by *Pde4b* (5.8% vs. 14.2%; $p=0.036$) but not *Pde4d* siRNA (Fig. 4F); while both *Pde4b* ($p=0.0136$) and *Pde4d* ($p=0.048$) contributed to the response of non-neuronal cells at low rolipram concentration (1 μM) (Fig. 4F).

3.5. The PDE4i rolipram decreases TNF α expression in mouse dorsal root ganglia and human peripheral blood mononuclear cells (PBMCs)

The antiallodynic action of chronic antidepressant treatment relies on an inhibition of neuropathy-induced neuroimmune mechanisms, including the inhibition of dorsal root ganglia TNF α production (Bohren *et al.*, 2013; Kremer *et al.*, 2018). This indirect anti-TNF α action depends upon β_2 adrenoceptors (Bohren *et al.*, 2013). We thus tested whether chronic rolipram action would impact this cytokine levels. Our results confirmed that neuropathic pain increased levels of membrane-bound TNF α (mTNF α) in dorsal root ganglia and chronic rolipram (0.5 mg/kg, s.c. twice a day) suppressed this overexpression ($H_{2,18}=9.36$, $p=0.0039$) (Fig. 5A,B). We then tested rolipram action on TNF α production in human PBMCs stimulated with LPS. Rolipram blocked TNF α release from PBMCs in a dose-dependent manner with an IC $_{50}$ of 133 nM (Fig. 5C).

4. Discussion

The present study demonstrates that a long-term treatment with non-selective PDEis, as well as PDE4i and PDE5i, alleviates mechanical hypersensitivity in a dose-dependent manner in a model of neuropathic pain. This action requires the endogenous opioid system. For PDE4i, we show that rolipram preferentially recruits dorsal root ganglia non-neuronal cells, acting through both PDE4B and PDE4D, and reverses TNF α overexpression.

Among all the PDEis tested in this study, only PDE4i and PDE5i reversed mechanical hypersensitivity in the model of neuropathic pain induced by sciatic nerve cuffing in mice. The selective PDE4i rolipram has been shown to also be acutely effective in a model of chemotherapy-induced peripheral neuropathy (Kim et al., 2017). However, the dose necessary to obtain such acute and transient action was high (3 mg/kg), which would question the therapeutic potential. In the present study, we showed that a ten-time lower dose was able to completely suppress mechanical hypersensitivity after a twice daily treatment. It was a lasting effect as the mice were tested after an overnight delay following the previous injection. These findings suggest that a long-term treatment may be necessary to achieve disease-modifying properties. Sildenafil, a PDE5i, has also been shown to attenuate mechanical hypersensitivity in a model of peripheral nerve injury (Huang et al., 2010), as well as in a model of painful diabetic neuropathy (Wang et al., 2011) and of alcohol-induced neuropathy (Kaur et al., 2017). Similarly, another PDE5i, zaprinast, attenuated neuropathic pain-related behavior in a model of chronic constriction injury (Rojewska et al., 2019). In agreement with those findings, we showed here that sildenafil, zaprinast, as well as MY-5445, all being selective PDE5i, suppressed mechanical hypersensitivity in mice with sciatic nerve cuffing. Interestingly, we also showed that a sustained treatment with sildenafil produced its effect at doses as low as 7.5 μ g/kg, which is twenty to a hundred times

lower than the doses reported in previous studies to have an acute (Vale et al., 2007) or chronic (Wang et al., 2011) action.

Experimental evidence suggests that the activation of the nitric oxide-cGMP (NO-cGMP) pathway can have both pro- and anti-nociceptive actions (Schmidtko, 2015). PDE5 is the enzyme responsible for cGMP hydrolysis and previous findings reported that spinal injection of a low dose of 8-bromo cGMP, a non-hydrolysable cGMP analog, alleviated inflammatory pain while at higher doses it aggravated hyperalgesia (Tegeeder et al., 2002). High doses of NO donors activate protein kinase G, which has been proposed to underlie the pro-nociceptive effect, while the antinociceptive effect seems to be independent from this activation (Cury et al., 2011). We show here that the antiallodynic action of the PDE5i sildenafil requires both MORs and DORs. Similarly, tadalafil, another PDE5i, has been shown to display an antinociceptive action relying on both NO/cGMP pathway and opioid receptors (Mehanna et al., 2018). This is also in line with previous data showing that NO favored opioid release (Armstead, 1998). The co-requirement of MORs and DORs might reflect a sequential or parallel recruitment of separate pathways, but it could also suggest the implication of cells co-expressing both opioid receptors (Erbs et al., 2015) that have even been proposed to potentially form heterodimers (Derouiche and Massotte, 2019; Zhang et al., 2020).

DORs are necessary to the antiallodynic action of chronic treatments with antidepressant drugs (Benbouzid et al., 2008a; Ceredig et al., 2018; Choucair Jaafar et al., 2014; Kremer et al., 2016; Kremer et al., 2018) and with β_2 adrenoceptor agonists (Ceredig et al., 2020; Choucair Jaafar et al., 2014; Kremer et al., 2020). In agreement with these findings, we found here that the action of a chronic treatment with the PDE4i rolipram on mechanical hypersensitivity also required DORs. Previous reports evidenced a prominent role of the peripheral nervous system (Bohren et al., 2013; Kremer et al., 2018), and more particularly of peripheral DORs present in

dorsal root ganglia neurons expressing Nav1.8 sodium channels (Ceredig et al., 2018; Ceredig et al., 2020), in the antiallodynic effect of chronic treatments with antidepressant drugs or β_2 adrenoceptor agonists. Although the mechanistic link between β_2 adrenoceptor, PDE4i and DORs is not completely understood, data suggest that treatment with a β_2 adrenoceptor can activate the cAMP/PKA pathway and favor the release of opioid peptides from immune cells (Binder et al., 2004).

Using calcium imaging, it has been shown that the efficacy of β_2 adrenoceptor coupling to its transduction pathway was enhanced in lumbar dorsal root ganglia under neuropathic conditions (Bohren et al., 2013). Here, we show that PDE4i dose-dependent action on intracellular free calcium concentration is also amplified in dorsal root ganglia non-neuronal cells from mice with neuropathic pain. Together, these findings suggest that nerve injury can sensitize the cAMP/PKA pathway (Mironov et al., 2009). Interestingly, *in vitro* low concentrations of rolipram (that are likely more relevant to the *in vivo* treatments) only activated the dorsal root ganglia non-neuronal cells, whereas higher concentrations also recruited neurons. Previous findings support a pro-nociceptive role of cAMP in neurons (Huang et al., 2012; Song et al., 2005). Indeed, increasing cAMP in neurons was shown to contribute to sensory neuron hyperexcitability and to behavioral hyperalgesia in a neuropathic pain model. Conversely, elevation of cAMP levels in glial cells was shown to decrease the release of pro-inflammatory cytokines such as TNF- α (Zhu et al., 2001). Interestingly, rolipram was also shown to decrease the production of TNF- α in homogenates of spinal cord, dorsal root ganglia and in activated human mononuclear cells (Guo et al., 2016; Kim et al., 2017; Pearce et al., 2004; Semmler et al., 1993). After peripheral nerve injury, a production of TNF- α has been observed in satellite glial cells of the dorsal root ganglia, which was reversed by stimulation of β_2 adrenoceptors (Bohren et al., 2013). A potential mechanism for such action could rely on the stimulation of the

cAMP/PKA pathway which can inhibit $\text{I}\kappa\text{B}\alpha$ degradation, thus preventing the nuclear translocation of the nuclear factor κB ($\text{NF}\kappa\text{B}$) and the transcription of pro-inflammatory cytokines such as $\text{TNF}\alpha$ (Farmer and Pugin, 2000). Here we observed that a long term treatment with rolipram indeed decreased $\text{TNF}\alpha$ levels in the dorsal root ganglia of mice with neuropathic pain, as well as in lipopolysaccharide-activated human PBMCs.

Rolipram provides a non-selective inhibition of the different PDE4 subtypes, which differ in their cellular and subcellular distribution (Ahmad et al., 2015; Azevedo et al., 2014; Francis et al., 2011; Johansson et al., 2012; Pérez-Torres et al., 2000; Soderling and Beavo, 2000). The PDE4B subtype has been identified in activated microglia under neuropathic pain condition and in bone cancer pain (Guo et al., 2016; Ji et al., 2016; Whitaker et al., 2008), and a selective knock-down of PDE4B attenuated mechanical allodynia (Ji et al., 2016). Here we showed that PDE4B seems necessary for rolipram-induced calcium release in neurons at high dose, while PDE4D and PDE4B are both important in non-neuronal cells. PDE4D is known to be associated with the β_2 adrenoceptor signaling pathway (Bruss et al., 2008), suggesting that in glial cells PDE4D could be the main enzyme responsible for the hydrolysis of cAMP upon activation of β_2 adrenoceptor.

In this study, we showed that a sustained treatment with PDE inhibitors, particularly PDE4i and PDE5i, alleviated neuropathic pain-induced mechanical hypersensitivity in a dose-dependent manner. These actions require components of the endogenous opioid system. Based on present findings and previous data, we propose that antidepressant drugs, β_2 adrenoceptor agonists and PDE4i could all be sharing a common mechanism contributing to the relief of neuropathic pain. This mechanism would involve cAMP signaling in non-neuronal cells of the dorsal root ganglia, inhibiting the production of $\text{TNF}\alpha$. While emetic properties have limited the clinical potential of PDE4i (Girod, 2010), the preclinical action of sustained treatment at low

doses is interesting. Some compounds with PDE4i properties are clinically used against asthma (Ntontsi et al., 2019; Phillips, 2020), and the development of PDE4 inhibitors with improved subtype specificity (Kagayama et al., 2009), dual inhibition properties (Blöcher et al., 2018; Chłoń-Rzepa et al., 2018) and/or with peripheral action may be of interest in the context of peripheral neuropathic pain.

Author contribution

MB and IY designed the study. IY performed surgeries; SM, YB and ES conducted and analyzed behavioral experiments; SH and SM performed and analyzed the imaging studies; SM and AL and VL performed molecular analyses; PV and JJB designed, conducted and analyzed the PBMC study; SD did genotyping and animal care. All authors contributed to the writing of the manuscript.

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Figure legends

Fig. 1. Non-selective PDEi and selective PDE4i and PDE5i relieve mechanical hypersensitivity. Neuropathic pain was induced by inserting a cuff around the main branch of the sciatic nerve. Treatments started after at least 2 weeks postsurgery. Mechanical sensitivity was tested using von Frey filaments. (a,b) Chronic treatment with the non-selective PDEi theophylline (n=5-7/group; post-hoc Duncan: $*p<0.05$ and $***p<0.001$ vs. Cuff Veh Ipsi) or ibudilast (n=5-6/group; post-hoc: $***p<0.001$) relieved mechanical hypersensitivity. (c) Prolonged treatments with vinpocetine (PDEi1), EHNA (PDEi2) or milrinone (PDEi3) (n=5-6/group) did not reverse mechanical hypersensitivity. (d) The PDE4i rolipram suppressed mechanical allodynia after chronic treatment (n=5-6/group; post-hoc: $***p<0.001$ vs. Cuff Veh Ipsi), and this effect was also observed with 2 other PDE4i: etazolate (n=4-5/group; post-hoc: $*p<0.05$ and $***p<0.001$ vs. Cuff Veh Ipsi) and YM-976 (n=5-6/group; post-hoc: $***p<0.001$ vs. Cuff Veh Ipsi). (e) Treatment with the selective PDE5i sildenafil (n=5/group; post-hoc: $*p<0.05$ and $***p<0.001$ vs. Cuff Veh Ipsi), zaprinast (n=5/group; post-hoc: $***p<0.001$ vs. Cuff Veh Ipsi) and MY-5445 (n=5-7/group; post-hoc: $***p<0.001$ vs. Cuff Veh Ipsi) also relieved mechanical hypersensitivity. Data are presented as mean \pm SEM. Contra, contralateral; Ipsi, ipsilateral; PWT, paw withdrawal thresholds; Sal, saline; Veh, vehicle.

Fig. 2. The PDE4i rolipram and PDE5i sildenafil reverse mechanical hypersensitivity in a dose-dependent manner. Neuropathic pain was induced by inserting a cuff around the main branch of the sciatic nerve. Treatments started (time 0) at least 2 weeks postsurgery. Mechanical sensitivity was tested using von Frey filaments. (a) Chronic treatment with rolipram at 0.03 mg/kg and 0.06 mg/kg had no effect, while doses 0.125 mg/kg (post-hoc Duncan: $**p<0.01$

and $***p < 0.001$ vs. Cuff Sal Ipsi) and 0.25 mg/kg (post-hoc: $**p < 0.01$ and $***p < 0.001$ vs. Cuff Sal Ipsi) fully reversed mechanical hypersensitivity. (b) Chronic sildenafil at 0.005 mg/kg had no effect, while it relieved hypersensitivity at doses 0.0075 mg/kg (post-hoc: $*p < 0.05$ and $***p < 0.001$ vs. Cuff Sal Ipsi), 0.01 mg/kg (post-hoc: $**p < 0.01$ and $***p < 0.001$ vs. Cuff Sal Ipsi) and 0.02 mg/kg (post-hoc: $**p < 0.01$ vs. Cuff Sal Ipsi). Data are presented as mean \pm SEM. B, baseline; Contra, contralateral; Ipsi, ipsilateral; PWT, paw withdrawal thresholds; Sal, saline; Veh, vehicle.

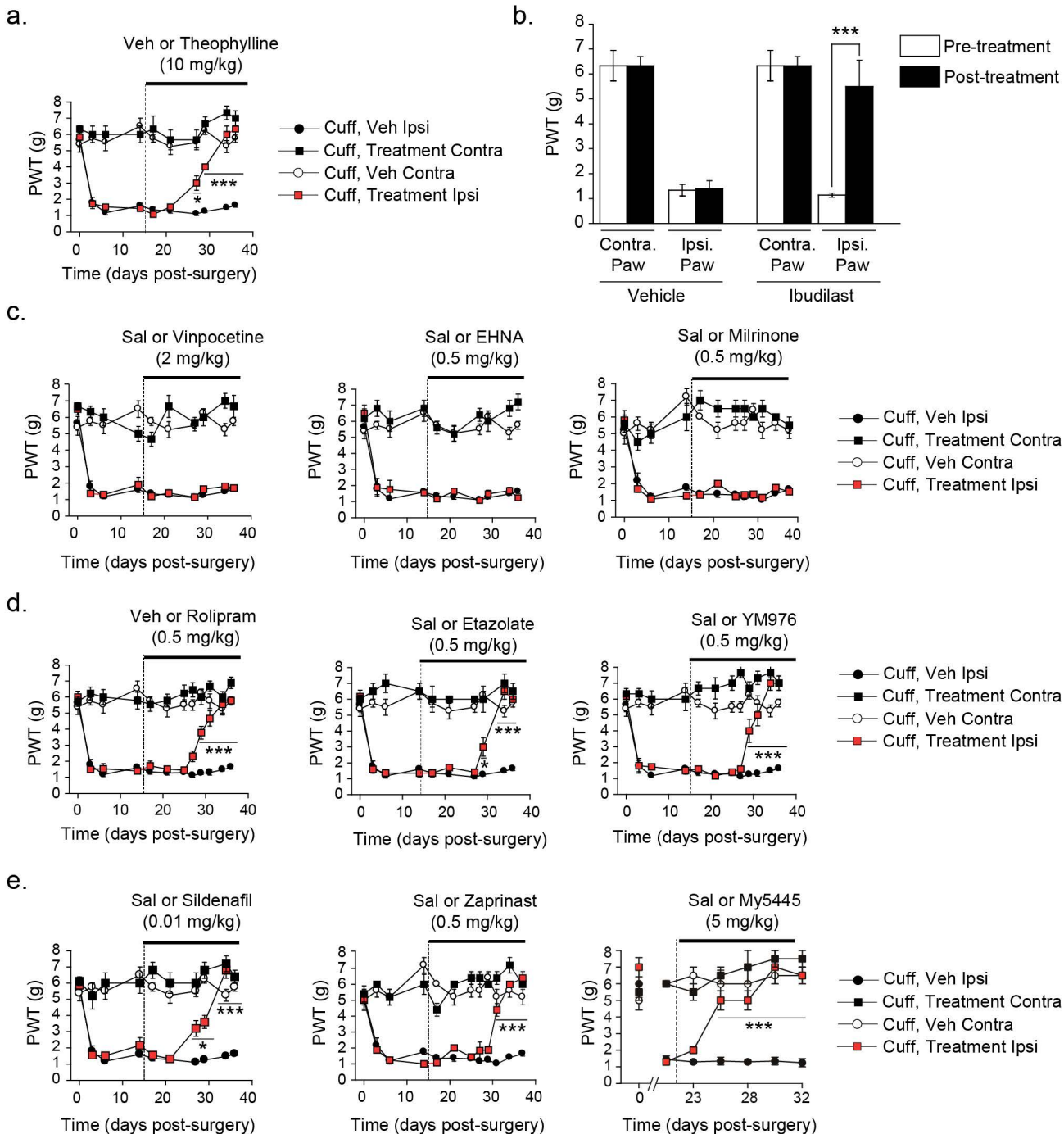
Fig. 3. Opioid receptors are necessary to PDE4i and PDE5i antiallodynic action. (a) Time course of experiments. Neuropathic pain was induced by cuffing the sciatic nerve and treatments started at least 2 weeks postsurgery. Mechanical sensitivity was tested using von Frey filaments. Opioid receptor implication was assessed using WT, MOR^{-/-}, KOR^{-/-} and DOR^{-/-} mice, and, in a separate set of experiments, by testing the MOR antagonist naloxonazine (30 mg/kg, s.c) and the DOR antagonist naltrindole (5 mg/kg, s.c) compared to vehicle injection (methylcellulose 1%, s.c) in mice that had fully recovered from mechanical hypersensitivity with chronic rolipram or sildenafil treatment. All data are presented for the right paw, ipsilateral to the surgery. (b) Data are expressed as area under the curve (AUC, g.day) over the period of treatment. Rolipram had an antiallodynic action in WT, MOR^{-/-} and KOR^{-/-} mice, but was ineffective in DOR^{-/-} mice (n=5-6/group; post-hoc Duncan: $***p < 0.001$; ns, non-significant). (c) Acute injection of naloxonazine failed to block chronic rolipram-induced alleviation of mechanical hypersensitivity (n=5), while naltrindole suppressed rolipram effect (n=5-6; post-hoc Duncan: $***p < 0.001$ vs. Sham Vehicle and vs. Cuff Rolipram at time 0). (d) Long-term sildenafil treatment had an antiallodynic action in WT and KOR^{-/-} mice, while no effect was present in MOR^{-/-} and DOR^{-/-} mice (n=6-7; post-hoc: $***p < 0.001$). (e) Acute injection of either naloxonazine (n=5-7; post-hoc: $***p < 0.001$) or

naltrindole (n=5; post-hoc: *** $p<0.001$) induced a relapse of allodynia in sildenafil treated mice. Data are presented as mean \pm SEM.

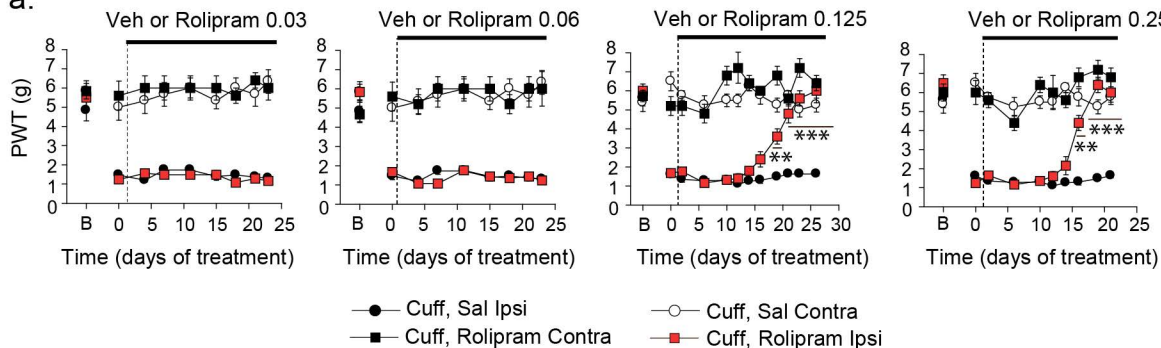
Fig. 4. PDE4B and PDE4D are main targets of rolipram in dorsal root ganglia. For all calcium imaging data, the numbers of responding cells and of analyzed cells are indicated over bars; the number of animals is detailed below. A) Rolipram at 100 μ M increased intracellular calcium concentration in dorsal root ganglia neurons and non-neuronal cells from mice with neuropathic pain (n=10-12 mice/group; Fisher's exact test: * $p<0.05$). B) Representative trace showing a non-neuronal cell responding to an application of UTP while no change in intracellular calcium was observed after application of 50 mM KCl. C) Rolipram induced a calcium response at 100 μ M, but not at 10 or 1 μ M in neurons (n=3 mice/group; Fisher's exact test: ** $p<0.01$), while a similar proportion of non-neuronal cells responded at the 3 doses. D) Rolipram-induced calcium response was dependent on extracellular calcium. (n=4 mice/group; Fisher's exact test, ** $p<0.01$). E) RT-qPCR analysis confirmed siRNA selectivity for *Pde4b* and *Pde4d* (post-hoc: **** $p<0.0001$). F) *In vitro* treatment with siRNA targeting *Pde4b* and *Pde4d* abolished calcium responses in non-neuronal cells (Fisher's exact test, * $p<0.05$), while only *Pde4b* influenced neuronal response at a high dose of rolipram (Fisher's exact test, * $p<0.05$). Rol, rolipram; Scrambld, scrambled control; UTP, uridine-5'-triphosphate.

Fig. 5. Rolipram decreases TNF α production in mouse dorsal root ganglia and in human peripheral blood mononuclear cells (PBMCs). A,B) Membrane-bound TNF α (mTNF α) expression increased in the lumbar dorsal root ganglia of mice with neuropathic pain, which was reversed by chronic rolipram (0.5 mg/kg, s.c. twice a day) treatment (n=6/group; post-hoc:

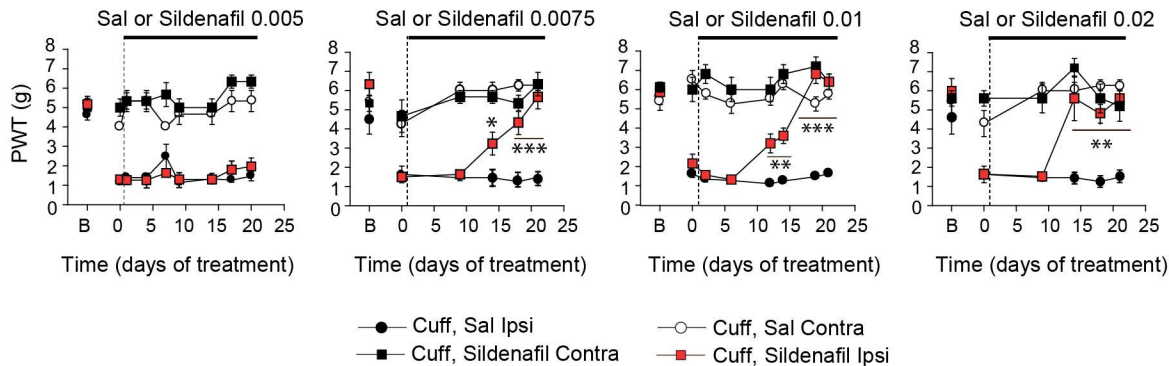
* $p < 0.05$). Rol, rolipram; Sal, saline. C) In human PBMC, rolipram inhibited TNF- α secretion induced by LPS in a dose-dependent manner ($IC_{50} = 133$ nM).



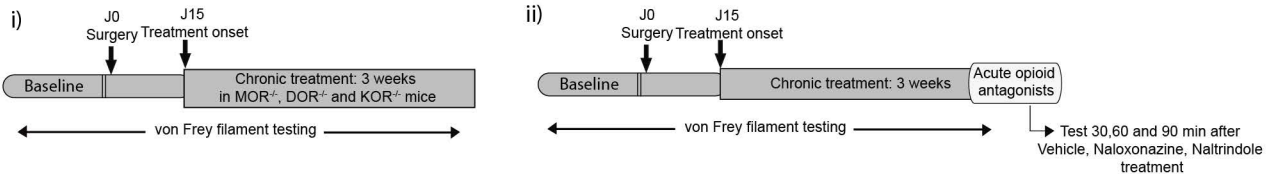
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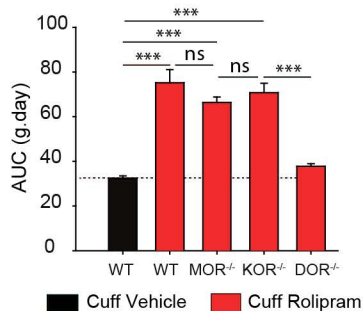
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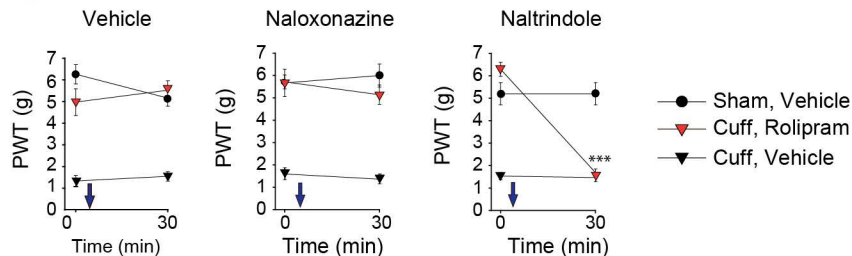
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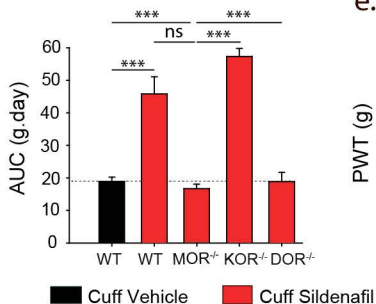
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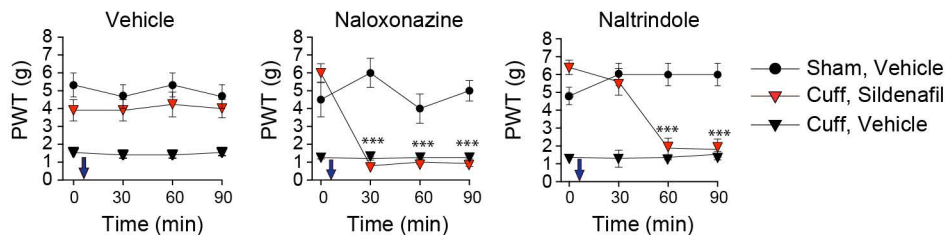
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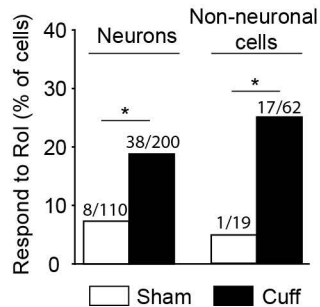
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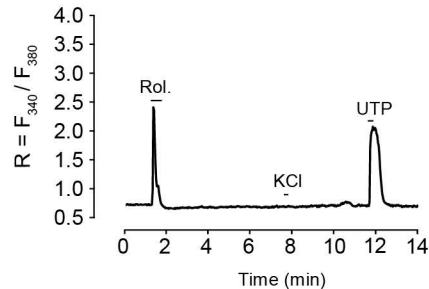
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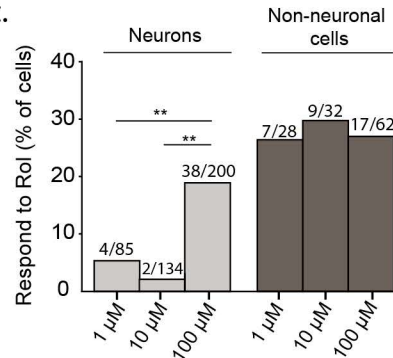
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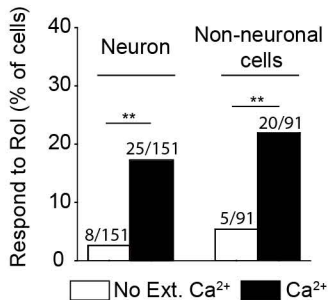
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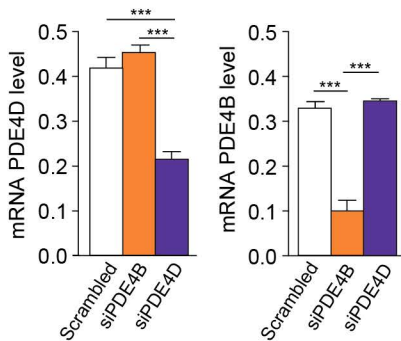
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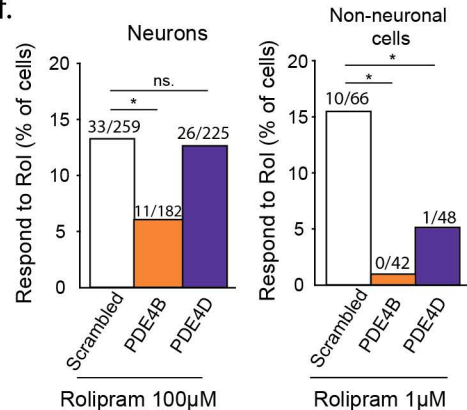
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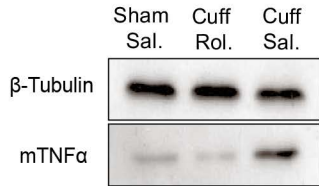
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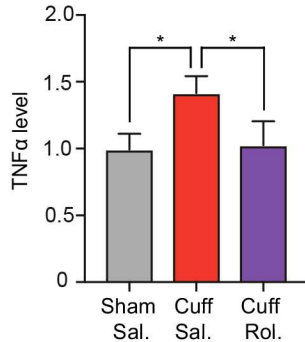
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