Research Paper

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Dual enkephalinase inhibitor PL265: a novel topical treatment to alleviate corneal pain and inflammation

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Abstract

Ocular pain is a core symptom of inflammatory or traumatic disorders affecting the anterior segment. To date, the management of chronic ocular pain remains a therapeutic challenge in ophthalmology. The main endogenous opioids (enkephalins) play a key role in pain control but exhibit only transient analgesic effects due to their rapid degradation. The aim of this study was to explore the antinociceptive and anti-inflammatory effects of topical administration of PL265 (a dual enkephalinase inhibitor) on murine models of corneal pain. On healthy corneas, chronic PL265 topical administration did not alter corneal integrity nor modify corneal mechanical and chemical sensitivity. Then, on murine models of corneal pain, we showed that repeated instillations of PL265 (10 mM) significantly reduced corneal mechanical and chemical hypersensitivity. PL265-induced corneal analgesia was completely antagonized by naloxone methiodide, demonstrating that PL265 antinociceptive effects were mediated by peripheral corneal opioid receptors. Moreover, flow cytometry (quantification of CD11b+ cells) and in vivo confocal microscopy analysis revealed that instillations of PL265 significantly decreased corneal inflammation in a corneal inflammatory pain model. Chronic PL265 topical administration also decreased Iba1 and neuronal injury marker (ATF3) staining in the nucleus of primary sensory neurons of ipsilateral trigeminal ganglion. These results open a new avenue for ocular pain treatment based on the enhancement of endogenous opioid peptides' analgesic effects in tissues of the anterior segment of the eye. Dual enkephalinase inhibitor PL265 seems to be a promising topical treatment for safe and effective alleviation of ocular pain and inflammation.

Keywords: Enkephalins, DENKI, Corneal pain, Ocular inflammation

1. Introduction

To date, management of chronic ocular pain remains a therapeutic challenge in ophthalmology and there are, for instance, no specific ophthalmic pain treatments. However, although topical anesthetics reduce acute pain, their long-term use is precluded due to their shortcomings (corneal ulceration).[18,46]

The cornea is the most powerful pain generator in the body.[3,17] Pain sensation is conveyed to the brain by primary sensory neurons that express chemotransducers, thermotransducers, and mechanotransducers at their distal-end nerve ending. They have the potential to be sensitized by repeated stimulation or by chronic inflammation.[3] Cell bodies of sensory neurons are located in the trigeminal ganglion (TG).[29,36] Their central afferents project to the sensory trigeminal brainstem complex.

Pain sensations are modulated by the opioid peptides (enkephalins, endorphin, and dynorphins), which in turn bind to mu, delta, and kappa opioid receptors widely distributed in the nervous[35] and immune systems.[39]

Opioid drugs such as morphine act through mu and delta opioid receptors to induce analgesia. Although opioids remain the gold standard for treating pain, they can induce debilitating or even life-threatening side effects.[4] In addition, chronic treatments with opioids induce tolerance (lowering of analgesic effect), which in turn require increasing the dose to maintain the therapeutic effect.[19]

In spite of these caveats, systemic opioids continue to be used to provide pain relief after refractive surgery, despite the contradictory evidence of their efficacy across randomized clinical studies (for review, see Ref. 42). Topical morphine administration has been assessed as a safer alternative to systemic treatment to alleviate corneal pain but has shown conflicting results. For instance, although antihyperalgesic effects of topical 5-μM morphine on ocular inflammatory pain in rat were reported,[56,62] a recent clinical study in rats and dogs showed no measurable analgesic effects of topical 1% morphine.[58]

Enkephalins exhibit an analgesic efficacy comparable with that of morphine without its adverse effects.[47] All the elements of the endogenous opioid system (opioid receptors and enkephalins) are expressed in ocular surface tissues.[52,62] The endogenous Met- and Leu-enkephalin peptides are released by corneal nerve

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fibers as well as by immune cells (lymphocytes, dendritic cells, and monocytes)[6–8] recruited to the inflamed site.[10,25,43] Once released, enkephalins bind to both mu and delta opioid receptors expressed in the cornea.[61,62] However, enkephalins evoke local and transient analgesic effects due to their rapid degradation[27] by the concomitant action of neutral endopeptidase neprilysin (NEP, EC 3.4.24.11) and aminopeptidase N (APN, EC 3.4.11.2).[47,48] The antinociceptive effects elicited by the local protection of enkephalins with the prodrug dual enkephalinase inhibitor (DENKI), PL265, have been reported in somatic pain.[16,63] The active drug released by PL265, PL254, inhibits NEP, APN, as well as LTA4 hydrolase (LTA4H) enzymatic activities with nanomolar affinity in vitro.[4]

This study explored the antinociceptive and anti-inflammatory effects of topical PL265 administration on corneal nociception in mice. Involvement of ocular opioid receptors was investigated through pharmacological blockade using a specific antagonist. The effects of topical administration PL265 on corneal and TG inflammation as well as neuronal injury were also determined.

2. Material and methods

2.1. Animals

Adult male C57BL/6 mice (30 g; Janvier Labs, Le Genest Saint Isle, France) were maintained under controlled conditions (22 ± 1°C, 60 ± 10% relative humidity, 12/12-hour light/dark cycle, food and water ad libitum). All animal procedures were performed in strict accordance with institutional guidelines for the care and use of experimental animals approved by the European Communities Council Directive 2010/63/UE.

2.2. Drugs

Benzalkonium chloride (BAC) 50% solution was purchased from Sigma-Aldrich (Saint Quentin Fallavier, France). This solution was diluted in sterile 0.1 M phosphate-buffered saline (PBS), pH 7.4 (PBS), to obtain a final 0.2% solution of BAC.

Lipopolysaccharide (LPS) (*Escherichia coli* O111:B4) was purchased from Sigma-Aldrich. A solution of 50 μg/10 μL was prepared in sterile PBS.

PL265, 2-(2-Biphenyl-4-ylmethyl-3-(hydroxyl-(1-(1-isobutyryloxy-ethoxycarbonylamino)-ethyl)-phosphinoyl)-propionylamino))-propionic acid disodium salt, synthesized and developed by Pharmaleads, was dissolved in sterile PBS and administered topically in the eye in a final volume of 10 μL at 10 mM, 1 mM, or 100 μM.

Capsaicin (Sigma-Aldrich) was dissolved in 100% ethanol (1-M solution) and then diluted in isotonic saline to obtain a 100-μM solution.

Naloxone methiodide was purchased from Sigma-Aldrich. Naloxone methiodide (100 μM) was dissolved in sterile PBS and administered topically in the eye in a final volume of 10 μL. This nonselective opioid receptor antagonist is unable to cross the blood–brain barrier.

2.3. Biomicroscopic evaluation of the cornea

Fluorescein staining of the corneal epithelium was used to evaluate corneal integrity, as previously described.[30] On the last experimental day (1 hour after the last instillation), ocular surfaces were evaluated using the fluorescein test. A drop of fluorescein sodium (Fluorescein Faure 0.5%; Novartis France, Rueil-Malmaison, France) was placed in the treated eye of anesthetized mice. The ocular surface was photographed using a SteREO Lumar microscope (Carl Zeiss Meditec, Marly Le Roi, France). The biomicroscope was calibrated for control (PBS) mice before acquisitions in PL265-treated mice. The microscopic analysis of corneal integrity (and inflammation) was performed in a blinded fashion.

2.4. In vivo confocal microscopy

A laser-scanning in vivo confocal microscope (IVCM, Heidelberg Retina Tomography II/Rostock Cornea Module; Heidelberg Engineering GmbH, Heidelberg, Germany) was used to examine the corneal integrity of anesthetized mice. The first layer of superficial epithelium, the stroma, and the endothelium were considered. A minimum of 200 serial TIFF images (400 × 400 μm) were acquired per animal. The increased laser reflectivity of the activated resident dendritic and invading immune cells (ie, monocytes/macrophages/lymphocytes) was visualized on IVCM images. The microscopic analysis of corneal integrity (and inflammation) was performed in a blinded fashion.

2.5. Behavioral tests

2.5.1. Eye-wiping test

The eye-wiping test was used to evaluate corneal chemical sensitivity.[11] The same experimenter performed all experiments in a test room close to the colony room, to minimize stress. Behavioral experiments were conducted in single-blind conditions (the experimenter was blinded to the treatment group). During the adaptation period, animals were placed in an individual Plexiglas chamber (where the tests took place) for 10 minutes every day for 4 days before the experiments. Eye-wiping tests were systematically performed 15 minutes after the last instillation of either PBS or PL265. For the experiment, a 10-μL drop of 2M NaCl was applied to the treated (right) eye, the animal placed in an individual cage, and the number of wipes counted for a period of 30 seconds by an experimenter blinded to the treatment group. Normal facial grooming episodes were not considered as wipes.

2.5.2. von Frey filament test

Fifteen minutes (except for the time course study) after the last instillation, mechanical corneal sensitivity was measured using von Frey filaments. For this purpose, a series of von Frey hairs corresponding to applications of either of 0.008, 0.02, and 0.04 g were used. The mechanical threshold response was determined by assessing the first treated-eye blinking response evoked by calibrated von Frey filaments of increasing force (0.008-0.04 g) in the center of the cornea of immobilized mice. This behavioral test resembles the Cochet-Bonnet esthesiometer used in the clinic to measure corneal mechanical sensitivity.[9] The experimenter was blinded to the treatment arm.

2.5.3. Corneal sensitivity to capsaicin

To assess chemical corneal sensitivity, one drop (10 μL) of 100-μM capsaicin (a TRPV1 receptor agonist) was applied to the treated (right) eye 15 minutes after the last instillation of either PBS or PL265. Animals were then placed in individual Plexiglas chambers, and the palpebral fissure closure time was measured for 5 minutes by a blinded experimenter.

2.6. Preclinical models of corneal injury

To minimize animal suffering, only one eye was injured and/or treated per animal. Thus, although the right eye was injured and/or treated, the left remained undisturbed throughout the experiments of this study.

2.6.1. Corneal scraping model

Under isoflurane anesthesia, a corneal scraping was performed on the right eye using a 1.5-mm trephine (Beaver-Visitec International, Waltham, MA) and an interdental brush to remove superficial corneal epithelium.[32] The right eyes of the animals with scraped cornea were instilled twice daily with PBS or PL265 (10 mM). On Day (D) 5, corneal sensitivity was evaluated using von Frey filament tests 15 minutes after the last topical administration of either PBS or PL265. To analyze capsaicin corneal sensitivity, 10 μL of 100-μM capsaicin solution were instilled and the closure time of the palpebral fissure then measured as described below.

2.6.2. Corneal injury induced by chronic topical administration of benzalkonium chloride

In the first treatment phase, mice were gently restrained and both solutions (10-μL 0.2% BAC solution or PBS) were instilled twice a day at 10 AM and 2 PM, for 7 days. In the second phase, mice received twice daily 0.2% BAC at 10 AM and 2 PM, and 2 hours after BAC instillation, mice were instilled with PBS or PL265 for 5 days. Chemical and mechanical corneal sensitivities were evaluated at D5 of the second phase using the wiping test and von Frey filaments, 15 minutes after the last instillation of either PBS or PL265. The experimenter was blinded to the treatment group.

2.6.3. Corneal inflammatory model induced by corneal injury and lipopolysaccharide instillation

A corneal epithelial lesion was created (as described above) on the right eye using a 1.5-mm trephine combined with an interdental brush on isoflurane-anesthetized animals. A drop (10 μL) of LPS (50 μg/10 μL) was then placed on the injured area. The first topical treatment with PBS or with PL265 (10 mM, 1 mM, and 100 μM) was given 2 hours after the first LPS instillation. Nonoperated and operated mice were treated twice a day with either a drop of PL265 (10 mM) or with PBS (control animals) in the right eye for 5 days. On D3 (AM), a second drop of LPS solution (50 μg/10 μL) was placed on the cornea 2 hours before treatment with either PBS or PL265. At D5 (15 minutes after the last instillation), corneal mechanical sensitivity was evaluated using the von Frey filament test. To analyze capsaicin corneal sensitivity, a 10-μL drop of capsaicin (100 μM) was then placed on the treated eye, and the closure time of the palpebral fissure was measured. Corneal integrity and inflammation were evaluated using in vivo confocal microscopy performed at D5, 15 minutes after the last instillation of either PBS or PL265 and using separate groups of animals. The experimenter was always blinded to the treatment group.

2.7. Pharmacological studies

2.7.1. Effects of naloxone methiodide

In LPS-induced inflammatory corneal pain experiments, animals received twice-daily naloxone methiodide (100 μM), a nonselective opioid receptor antagonist, which does not cross the blood–brain barrier, 15 minutes before each topical instillation of PBS or PL265 (10 mM). At D5, mechanical corneal sensitivity was evaluated using von Frey filaments 15 minutes after last topical instillation of either PBS or PL265. The experimenter was blinded to the treatment arm.

2.7.2. PL265 dose response on lipopolysaccharide-induced inflammatory corneal pain

After corneal scraping and LPS topical administration, operated mice were treated twice daily with either a drop of PL265 at 10 mM, 1 mM, or 100 μM, or PBS in the right eye for 5 days. At D5, mechanical corneal sensitivity was evaluated by a blinded experimenter using von Frey filaments 15 minutes after the last topical instillation.

2.7.3. Time course of PL265 effects on lipopolysaccharide-induced inflammatory pain model

Using the previously described inflammatory pain model, operated mice were treated twice daily with either one drop of PL265 at 10 mM or PBS in the right eye for 3 or 5 days. At D3 or D5 (10, 20, 40, and 120 minutes after the last ocular instillation), mechanical corneal sensitivity was evaluated using von Frey filaments. Time 0 values at D3 and D5 correspond to mechanical threshold measured just before the second daily PBS or PL265 instillation.

2.7.4. Corneal dissection and immunoconjugation for flow cytometry analysis

At D5, corneas were dissected and rinsed in PBS. Enzymatic dissociation was performed by transferring corneas into DMEM with Liberase (Roche, Indianapolis, IN) at 100 μg/mL at +37°C, 5% CO_2 for 4 hours. Cell suspension was centrifuged and washed in PBS before being used extemporaneously. The monoclonal antibodies used were phycoerythrin (PE)-conjugated CD11b (MCA711PE, Serotec) and PE-conjugated CD4 (1767PE, Serotec). For each condition, 10,000 cells were suspended in 100-μL PBS containing 1 μL of fluorescent antibody and incubated for 30 minutes at +4°C. The cells were then washed in PBS and suspended in 250-μL PBS before FCM analysis (Gallios; Beckman Coulter). Graphs and data were analyzed using FlowJo 10 software.

2.8. Immunohistological studies

2.8.1. Tissue preparation

Two hours after the last topical instillation, mice were anesthetized using ketamine (80 mg/kg body weight) and xylazine (8 mg/kg body weight, Virbac, Carros, France) and transcardially perfused with 10 mL of 0.9% NaCl solution followed by 40 mL of 4% (wt/vol) paraformaldehyde in PBS. The treated eye and ipsilateral TGs were carefully removed. Eyes were frozen in liquid nitrogen and stored in −80°C. Trigeminal ganglions were immersed in the same fixative solution for 48 hours and then placed in 15% (wt/vol) sucrose solution in PBS (overnight), and then in 30% (wt/vol) sucrose solution before freezing at −20°C in 7.5% gelatin and 10% sucrose. Transverse frozen corneal and TG sections (14 μm) were cut in a cryostat (Leica Microsystems, Wetzlar, Germany) and mounted on Superfrost slides and kept at −20°C until use. To avoid sampling the same cells, one TG section every 5 was used for immunolabelling.

2.8.2. Immunofluorescence labeling in the cornea and trigeminal ganglion

After being rinsed in PBS 3 times and blocked in PBS containing 3% normal donkey serum and 0.1% triton X-100 for 1 hour at room temperature, the sections were incubated with primary antibody at 4°C for 24 hours. The primary antibodies used in this study were rabbit anti-ATF3 (Santa Cruz Biotechnology, Heidelberg, Germany: Lot #K1912, 1:250), rabbit anti-ionized calcium-binding adaptor molecule-1 (Iba1) (Wako, Richmond, VA: Cat. #019-19741, 1:250), and rat anti-mouse CD4 (Biorad, Hercules, CA, #MCA 1767, 1:250). Corneal sections immunolabelled with the CD4 antibody were rinsed 3 times and then incubated with Alexa Fluor 488–conjugated goat anti-rat antibody (1:500) and DAPI (1:1000) for 1 hour 30 minutes and washed 3 times. For ATF3 and Iba1 staining, TG sections were washed 3 times, and ATF3 and Iba1 labeling were amplified using biotin-conjugated horse anti-rabbit antibody (1:500; Vector Laboratories, Burlingame, CA) for 1 hour and finally revealed by incubation with streptavidin–Alexa Fluor 488 (1:500; Invitrogen Life Technologies SAS, Courtaboeuf, France).

2.8.3. Information about primary antibodies used

Rat anti-mouse CD4 monoclonal antibody (clone YTS 191.1) is raised against CD4 (L3/T4; Ly4) antigen, and is highly recommended for detecting CD4 of mouse origin by immunofluorescence and flow cytometry.

Anti-ATF3 polyclonal antibody is raised against a peptide mapping the C-terminus of ATF-3 of human origin. Negative controls confirm that this anti-ATF3 antibody detected the antigen of appropriate sequence since incubation (before immunostaining and Western blot) with the specific antigenic peptide abolished staining[38] and led to a disappearance of the single band of 22 kDa found to be expressed in nuclear protein extracts of neonatal rat spinal cord[38] or mouse dorsal root ganglia.[49]

Anti-Iba1 polyclonal antibody is raised in rabbit using a synthetic peptide corresponding to the C terminus of Iba1 as immunogen (N′-PTGPPAKKAISELP-C′). This antiserum stains a single band of 17 kDa molecular weight on Western blot performed on rat microglia (manufacturer's technical information) and on mouse spinal cord.[49]

Specificity of the CD4 (monoclonal rat antibody), ATF3 (polyclonal rabbit IgG antibodies), and Iba1 (polyclonal rabbit IgG antibodies) primary antibodies was demonstrated by incubating TG or corneal sections with purified rabbit IgG (Biorad) or purified rat IgG2b (Life Technologies) using the same concentrations as those used with the various primary antibodies. This was followed by incubation with the appropriate secondary antibodies and detection reagents (as described above). The sections were then analyzed in a blinded manner.

2.8.4. Microscopic analysis

Tissue sections were examined either with a Zeiss M1 epifluorescence microscope or an inverted Olympus FV1000 laser-scanning confocal microscope. The epifluorescence microscope (Axio ImagerM1; Carl Zeiss, Iéna, Germany) is equipped with a digital camera (Axio Cam HRC; Carl Zeiss) and image acquisition software (Zen; Carl Zeiss). The inverted Olympus FV1000 confocal microscope is equipped with an argon (488 nm) ion laser and laser diodes (405 and 559 nm). Images were acquired sequentially, line-by-line, to reduce excitation and emission crosstalk. Step size was defined according to the Nyquist–Shannon sampling theorem (1024 × 1024 pixels). Objective used was a PlanApoN (20×/0.85 NA, oil immersion) objective lens (Olympus). The microscope settings were established using a control section and kept unchanged for all subsequent acquisitions. TIFF images were recorded. For the characterization of injured sensory neurons, slides containing 4 to 6 sections of TG for each animal were immunostained with ATF3 antibody and visualized at ×10 to 20 magnification. The number of neurons with cell body nuclei positively stained for ATF3 was counted in the image field (4 mice in each group were used). To quantify Iba1 labeling density, images were processed using the ImageJ Program as previously described.[44] Using the thresholding function to discriminate objects of interest from the surrounding background, the total surface occupied by immunoreactive structures (ie, total stained pixels) above this set threshold was estimated within a standard area. For each mouse, 5 different areas within the TG were sampled, and 4 mice were analyzed in the PBS- and PL265-treated groups. The microscopic analysis was performed by an experimenter blinded to the treatment group.

2.9. Statistical analysis

Differences between 2 groups were analyzed using nonparametric test (Mann and Whitney) when normality or equal variance failed. Multiple group means of parametric data sets were compared using either a 1- or 2-way analysis of variance (ANOVA) after it was determined that the data conformed to a normal distribution with equal variances. If an overall significance was found, a multiple-comparisons test was used: The Tukey post hoc test after 1-way ANOVA or 2-stage step-up methods (Benjamini–Krieger–Yekutieli) by controlling the false discovery rate ($Q = 0.05$) after 2-way ANOVA, respectively. One-way ANOVA on ranks (the Kruskal-Wallis test) with post hoc comparison (Dunn method) was applied to data when tests for normality or equal variance failed. All statistical analyses were performed using the commercial statistical software GraphPad Prism 7.0. The levels of significance were set at $P < 0.05$ (*), $P < 0.01$ (**), or $P < 0.001$ (***).

3. Results

3.1. Effect of chronic PL265 topical administration on healthy cornea

The effect of a 7-day twice-daily topical instillation of PL265 (10 mM) or PBS on corneal integrity was investigated in healthy mice (**Fig. 1A**). Slit-lamp examination with fluorescein showed that chronic topical administration of PL265 did not alter the corneal epithelium (**Fig. 1B**, bottom panel). Indeed, PL265-treated corneas were similar to those of control animals receiving topical PBS. The analysis of the in vivo confocal microscopy images showed no difference between control (PBS) and PL265-treated mice in the different layers of the cornea (ie, superior and basal epithelium and stroma) (**Fig. 1C**).

3.2. Effect of PL265 on mechanical and chemical sensitivity of healthy cornea

Twice-daily topical instillations of PL265 at 10 mM for 7 consecutive days in cornea of healthy mice did not modify mechanical sensitivity assessed using von Frey filaments compared with mice receiving PBS (0.050 ± 0.006 g vs 0.056 ± 0.004 g) (**Fig. 1D**). In addition, chronic instillations of PL265 (10 mM) did not alter corneal chemical sensitivity measured with the eye-wiping test. After the instillation of a drop of NaCl solution

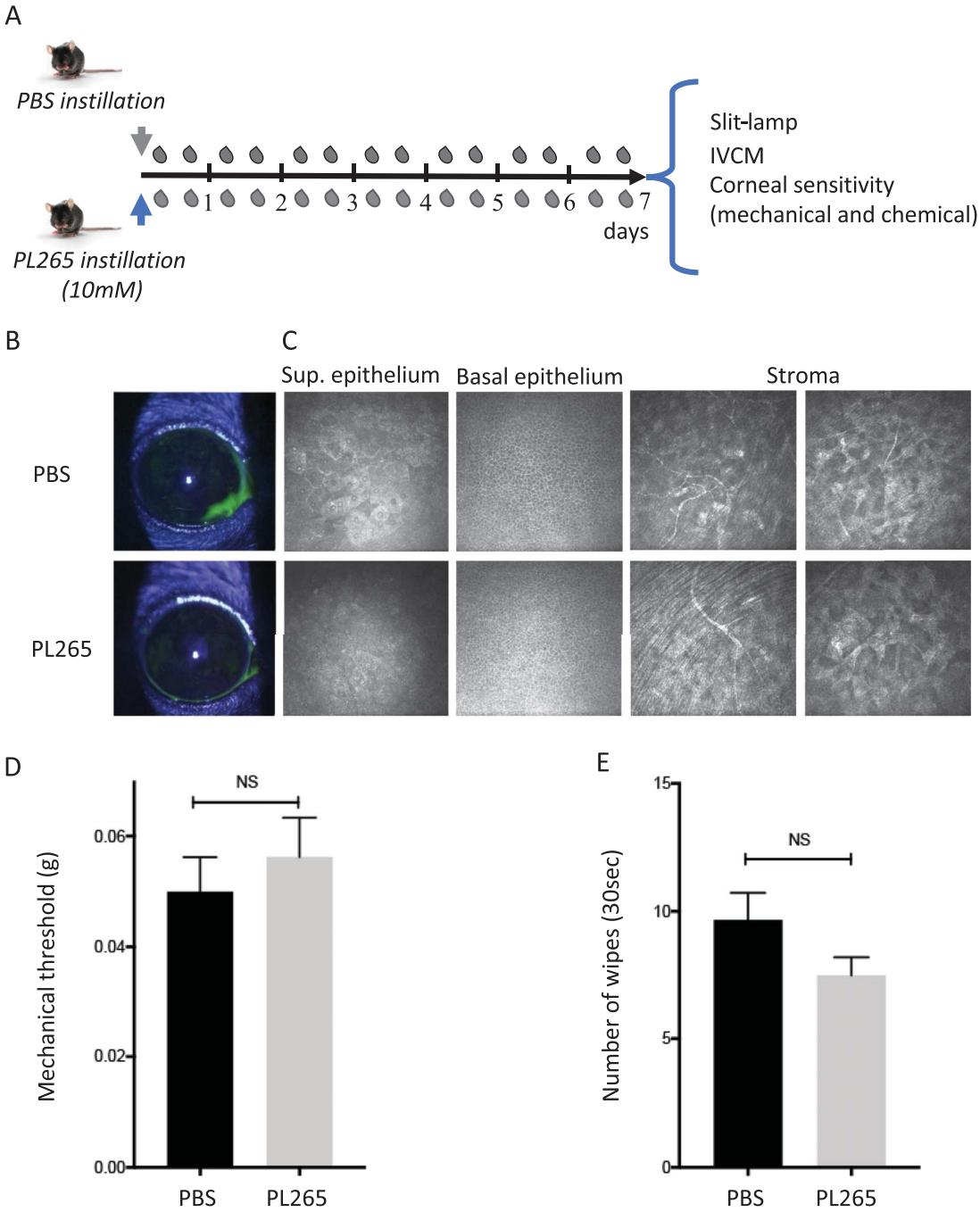

Figure 1. Ocular safety of topical PL265 administration in mice. Schematic diagram of the experimental protocol (A). Corneal surface integrity was investigated using slit-lamp biomicroscopy examination with fluorescein (B), in vivo confocal microscopy (C), mechanical (D), and chemical (E) corneal sensitivity. Slit-lamp and IVCM images were acquired after 7 days of twice-daily instillations of PBS or PL265 (10 mM). Note the similarity of slit-lamp and IVCM images between the 2 groups. Chronic instillations of PL265 on healthy cornea did not modify the mechanical sensitivity measured with von Frey filaments (D) or the chemical sensitivity measured with the eye-wiping test (E) compared to the PBS group. Data are presented as mean ± SEM. (n = 6-11 mice per group); differences between groups (PBS vs PL265) were analyzed using the Mann and Whitney test. IVCM, in vivo confocal microscope; NS, nonsignificant.

(2M), the number of wipes counted for 30 seconds was not significantly different between the groups (9.66 ± 1.05 for PBS-treated vs 7.50 ± 0.70 for PL265-treated mice) (**Fig. 1E**).

3.3. Effect of PL265 on corneal integrity in animals treated with 0.2% benzalkonium chloride

Twice-daily topical instillations of PL265 (10 mM) or PBS was also performed on 0.2% BAC-treated corneas (**Fig. 2A**). Animals receiving 0.2% BAC and PBS exhibited greater corneal alterations than those instilled with 0.2% BAC in the presence of PL265 (10 mM). Indeed, slit-lamp examination with fluorescein showed that the keratitis accompanied by central ulceration (white arrow) was detected in mice cornea instilled with 0.2% BAC + PBS, whereas only slight superficial diffuse keratitis with no corneal ulceration was observed in animals treated with PL265 (10 mM) (**Fig. 2B**).

In vivo confocal microscopy exploration revealed numerous hyperreflective inflammatory cells (eg, dendritic cells, macrophages, T lymphocytes) in the stroma layer (**Fig. 2C**, orange

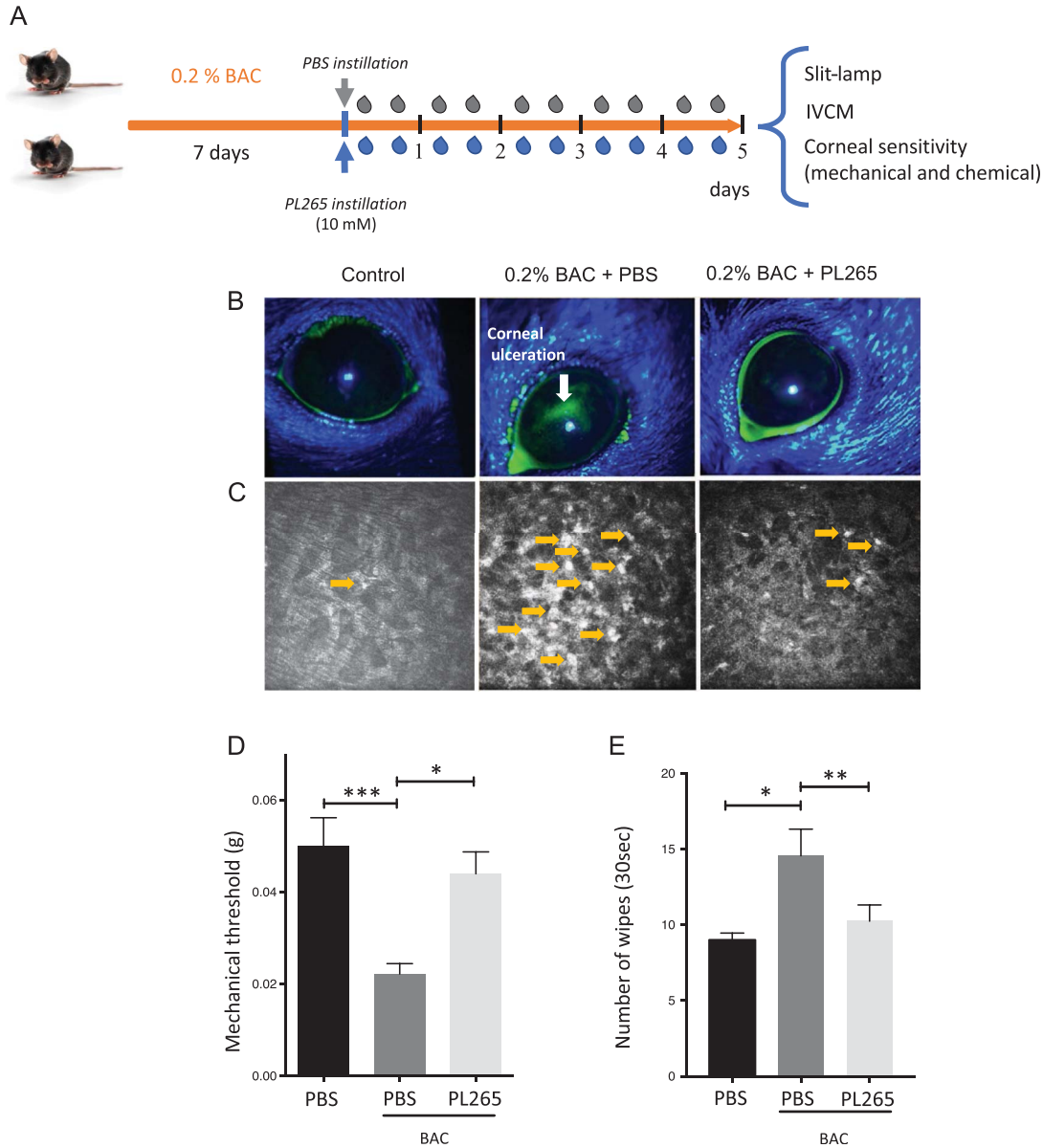

Figure 2. Topical administration of PL265 reduces corneal injury, inflammation, and corneal hypersensitivity after exposure to benzalkonium chloride (BAC). Schematic diagram of the experimental protocol (A). Fluorescein staining (B) confirmed BAC-induced corneal ulceration (white arrow), which was reduced after topical PL265 (10 mM) administration. In vivo confocal microscope images showed (C) immune cell infiltration (orange arrows) in the stroma of mice receiving BAC + PBS. Note the subsequent anti-inflammatory effect in PL265-treated mice. Mechanical (D) and chemical (E) sensitivity were measured with von Frey filaments and the eye-wiping test 15 minutes after the last topical administration. A significant antinociceptive effect (an increase of mechanical threshold response and a reduced number of wipes) was observed after topical PL265 compared to PBS. Data were presented as mean ± SEM (n = 10-11 mice per group); differences between groups were analyzed using nonparametric 1-way ANOVA test on ranks (Kruskal and Wallis) followed by Dunn multiple-comparison test. Levels of significance were $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$, respectively. ANOVA, analysis of variance.

arrows) of corneas of mice instilled with 0.2% BAC + PBS, whereas the corneas of PL265-treated mice contained only sparse immune cells, suggesting higher corneal inflammation in 0.2% BAC + PBS compared with PL265-treated mice (**Fig. 2C**).

3.4. Effect of PL265 on corneal mechanical allodynia and chemical sensitivity in animals treated with 0.2% benzalkonium chloride

Mechanical allodynia was tested using von Frey filaments on D5 of the second experimental phase, 15 minutes after the last instillation of PBS or PL265 (10 mM) in both 0.2% BAC-instilled mice and in control mice (instillation of PBS alone) (**Fig. 2D**). Corneal instillation of 0.2% BAC significantly reduced the mechanical threshold by 46% (0.027 ± 0.005 g) ($^{***}P < 0.001$) compared to control mice (0.050 ± 0.006 g) instilled with PBS alone. The topical administration of PL265 at 10 mM significantly reduced the BAC-induced mechanical allodynia by 62% ($^{*}P < 0.05$) (0.027 ± 0.005 g for BAC + PBS vs 0.044 ± 0.004 g for BAC + PL265). Interestingly, the mechanical threshold of PL265-treated mice returned to control values.

At D5 of the second experimental phase, a wiping test was performed to measure chemical corneal sensitivity in the 3 animal groups. As shown in **Fig. 2E**, 0.2% BAC instillation significantly increased the number of wipes (by 60%, $^{*}P < 0.05$) compared to mice instilled with only PBS (9.00 ± 0.46 vs 14.57 ± 1.76, for

BAC + PBS). Interestingly, PL265 twice-daily instillation, 2 hours after each 0.2% BAC instillation, significantly reduced the number of wipes by 30% (**$P < 0.05$) as compared to 0.2% BAC + PBS (14.57 ± 1.76 vs 10.25 ± 1.06 for BAC + PL265). The chemical sensitivity in PL265-treated mice was again found similar to that measured in control mice receiving PBS alone.

3.5. Effect of PL265 on mechanical sensitivity after mechanical corneal de-epithelization

Corneal scraping was performed as described in the **Figure 3A** and the injured eye was subsequently treated with twice-daily instillations of either PBS or 10 mM PL265 (**Fig. 3A**). Mechanical corneal allodynia developed 5 days after corneal de-epithelization (corneal scraping) (**Fig. 3B**). A pronounced 40% decrease (*$P < 0.05$) of the mechanical threshold was measured compared to uninjured cornea instilled with PBS (0.050 ± 0.006 g vs 0.030 ± 0.004 g for corneal scraping + PBS). Twice-daily topical instillations of PL265 at 10 mM for 5 consecutive days after mechanical de-epithelization of the cornea significantly reduced the mechanical sensitivity increase of the threshold response (by 65%, **$P < 0.01$) compared to corneal scraping in PBS-treated mice (0.030 ± 0.004 g vs 0.049 ± 0.004 g for corneal scraping + PL265; **Fig. 3B**).

At D5, the palpebral closure time after topical capsaicin administration was measured as an index of chemical corneal sensitivity (**Fig. 3C**). A highly significant 115% increase (***$P < 0.001$) of the palpebral closure time after corneal scraping in PBS-treated mice (91 ± 9 seconds for control vs 196 ± 18 seconds for corneal scraping + PBS) was observed. Topical administration of PL265 (10 mM) significantly decreased, by 48% (***$P < 0.001$), the palpebral closure time after corneal scraping (103 ± 12 seconds as compared to the value of corneal scraping + PBS-treated mice of 196 ± 18 seconds). Moreover, the palpebral closure time measured in the corneal scraping + PL265-instilled mice was not statistically different from that obtained in control mice (PBS alone).

3.6. Effects of topical PL265 on lipopolysaccharide-induced inflammatory pain model

3.6.1. Recruitment of $CD4^+$ cells in the cornea after a corneal scraping and lipopolysaccharide instillation

At D5, corneal $CD4^+$ cells were quantified by flow cytometry in control (healthy) and in mice subjected to corneal scraping and LPS treatment. Representative flow cytometry dot plot for $CD4^+$ cells (T cells and dendritic cells) extracted from corneas of the different groups of mice (**Fig. 4A**) demonstrate that corneal scraping + LPS induced infiltration of $CD4^+$ cells in the cornea. Indeed, the results show that corneal scraping + LPS caused a significant, 85-fold increase in the number of $CD4^+$ cells as compared to control cornea (**Fig. 4B**). The corneal scraping and LPS-induced increase of $CD4^+$ cells was further confirmed by immunohistochemical analysis. Confocal images reveal that

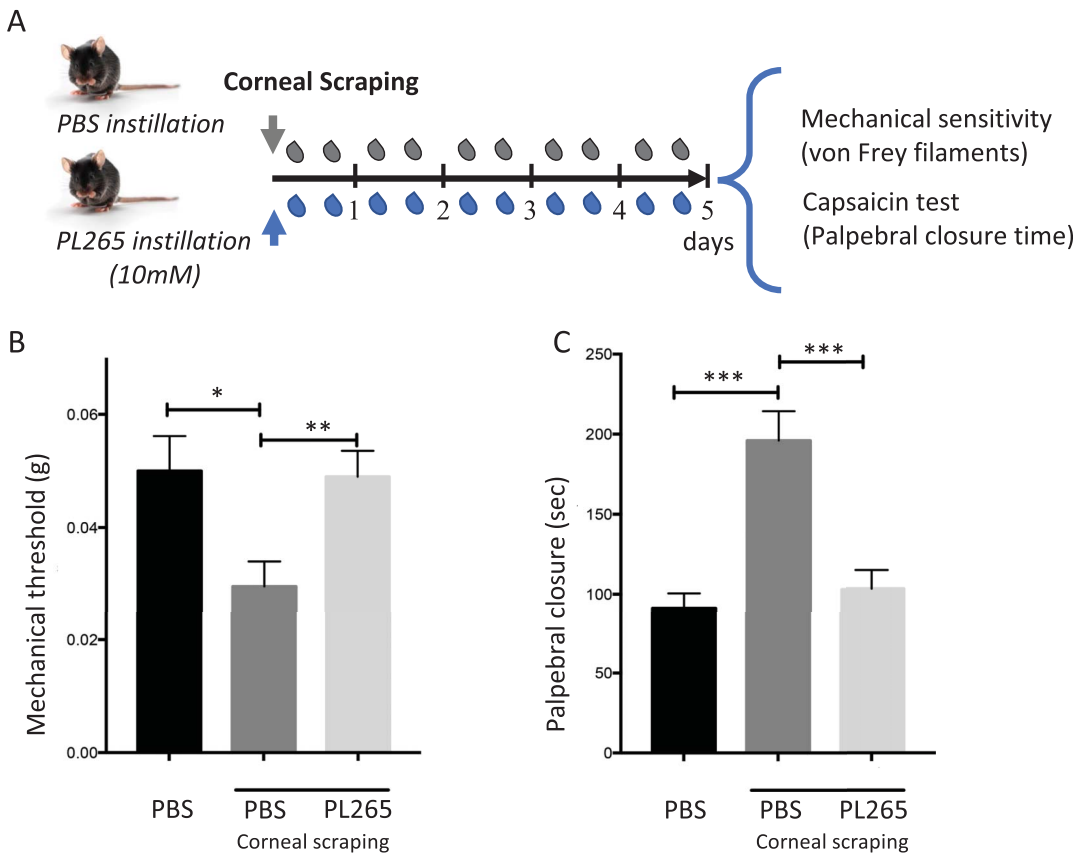

Figure 3. Antinociceptive effects of topical administration of PL265 after corneal scraping. Schematic diagram of the experimental protocol (A). Mechanical (B) and chemical (C) corneal sensitivity after PBS and PL265 topical administration after corneal scraping. Mechanical sensitivity was measured 15 minutes after the last instillation with von Frey filaments. Animals receiving PBS after corneal scraping showed reduced mechanical threshold response and increased palpebral closure time after capsaicin compared to control PBS animals. Topical PL265 administration had a clear and significant antinociceptive effect using both behavioral tests. Data are expressed as mean ± SEM (n = 10-11 mice per group). Differences between groups were analyzed using parametric 1-way ANOVA test followed by the Tukey post hoc test. Levels of significance were *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$, respectively. ANOVA, analysis of variance.

although DAPI staining of control sections shows the presence of multiple cells, very few are stained with the anti-CD4 antibody (**Fig. 4C**, top panels). However, when the cornea was subjected to scraping and LPS treatment, there was a clear infiltration of $CD4^+$ cells (T cells and dendritic cells [**Fig. 4C**, bottom panels, white arrows]). Thus, immunostaining and FCM results provide clear evidence of an infiltration of $CD4^+$ cells in the cornea after corneal scraping + LPS. Primary antibody specificity control experiment (Fig. S1, available at http://links.lww.com/PAIN/A677) demonstrated that immunostaining obtained with CD4 primary antibody was not caused by nonspecific interactions of the antibody in the corneal tissue. Indeed, no staining was noted in corneal sections (from control [panel A] and LPS-treated [panel B] mice) incubated with purified rat IgG2b and revealed by Alexa Fluor 488–conjugated goat anti-rat antibody.

3.6.2. PL265 dose response in lipopolysaccharide-induced inflammatory pain model

In this set of experiments, corneal scraping was performed followed by 2 instillations of LPS (50 μg) at D3 and D5 (**Fig. 5A**). The effects of 3 concentrations (10 mM, 1 mM, and 100 μM) of PL265 were evaluated using mechanical sensitivity in LPS-induced corneal inflammatory pain. Thus, the von Frey filament test was performed at D5, 15 minutes after the last instillation of PBS or PL265. As shown in **Figure 5B**, corneal scraping and LPS treatment induced a highly significant, 72% decrease of mechanical threshold (**$P < 0.01$) compared with PBS-instilled uninjured cornea (0.047 ± 0.004 g vs 0.013 ± 0.003 g for the corneal scraping + LPS + PBS instillation group). After corneal scraping and LPS treatment, mice treated with 10-mM PL265 had a significantly higher response threshold (up to 300%, **$P <$ 0.01), corresponding to a decrease of mechanical sensitivity, than animals treated by PBS alone (0.013 ± 0.003 g vs 0.052 ± 0.007 g for corneal scraping + LPS + PL265 group). In addition, instillations with PL265 at the concentrations of 1 mM and 100 μM also induced a slight but nonsignificant increase in the threshold response compared to that observed in animals instilled with PBS alone (**Fig. 5B**). Thus, PL265 at 10 mM seemed to be the most effective concentration to efficiently decrease mechanical sensitivity in the LPS-induced inflammatory corneal pain model.

3.6.3. Time course effects of PL265 on the lipopolysaccharide-induced inflammatory pain model

The most efficacious, 10-mM PL265 concentration given topically twice daily was chosen for the remaining behavioral experiments. Mechanical thresholds were then evaluated at different time points: before instillation (basal), and 10, 20, 40, 60, and 120 minutes after instillation. On D3, the basal value (corresponding to the mechanical threshold before the first,

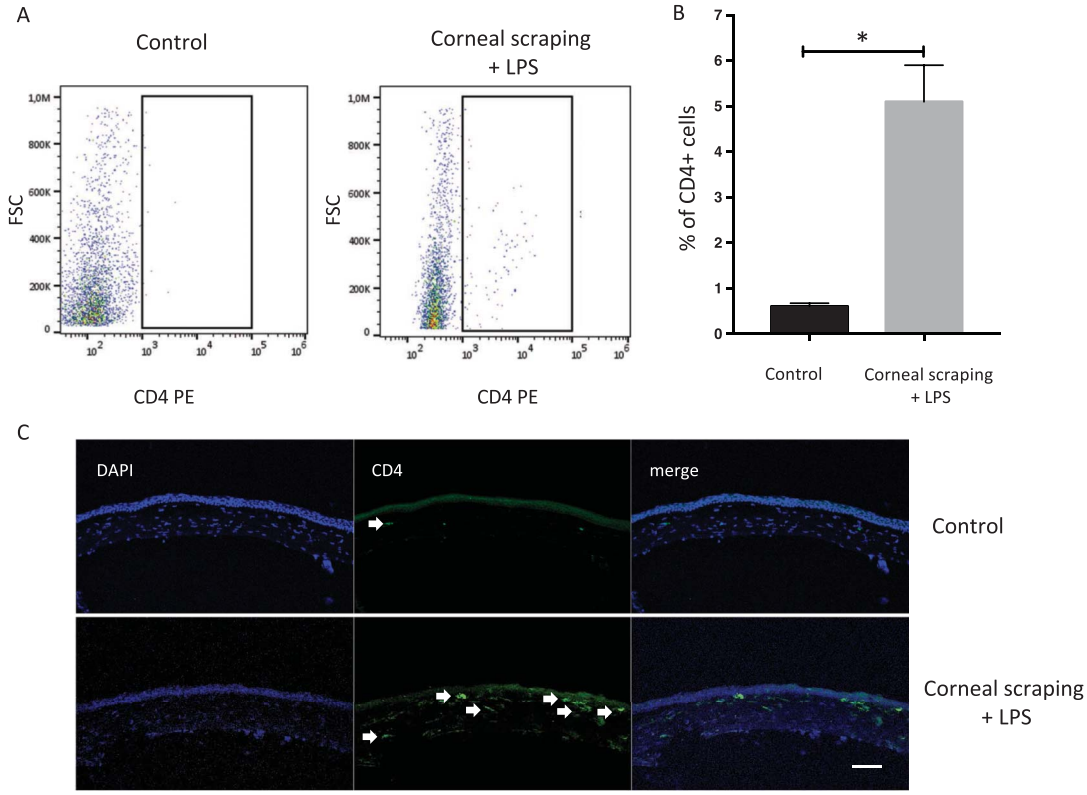

Figure 4. Recruitment of $CD4^+$ cells in the cornea after corneal scraping and LPS instillation. Representative flow cytometry dot plot for $CD4^+$ cells (T cells and dendritic cells) in corneas from different groups of mice (A and B) and immunohistochemistry (C) in control cornea and in cornea from mice with corneal scraping and LPS. Corneas from mice were dissected at day 5. (A) Detection of $CD4^+$ cells in corneas from control mice and mice subjected to scraping + LPS treatment by flow cytometry by forward-scattered light (FSC) method. Cell suspensions prepared from corneas from individual mice were assessed using anti-CD4-PE mAb. (B) Graph illustrating the % of $CD4^+$ cells (T cells and dendritic cells) in the cornea in both groups of mice. Corneal scraping + LPS triggers infiltration of $CD4^+$ cells in the cornea. Data are presented as mean ± SEM (n = 4 mice per group); difference between groups was analyzed using the Mann and Whitney test. Level of significance was *$P < 0.05$. (C) Confocal images illustrating $CD4^+$ cells (T cells and dendritic cells) and DAPI staining on corneal sections performed as described in Materials and Methods (n = 3 mice per group; scale bar = 100 μm). $CD4^+$ cells appeared in green (white arrows). Note the increase of $CD4^+$ cells in cornea of mice that underwent a corneal scraping and LPS instillation. LPS, lipopolysaccharide.

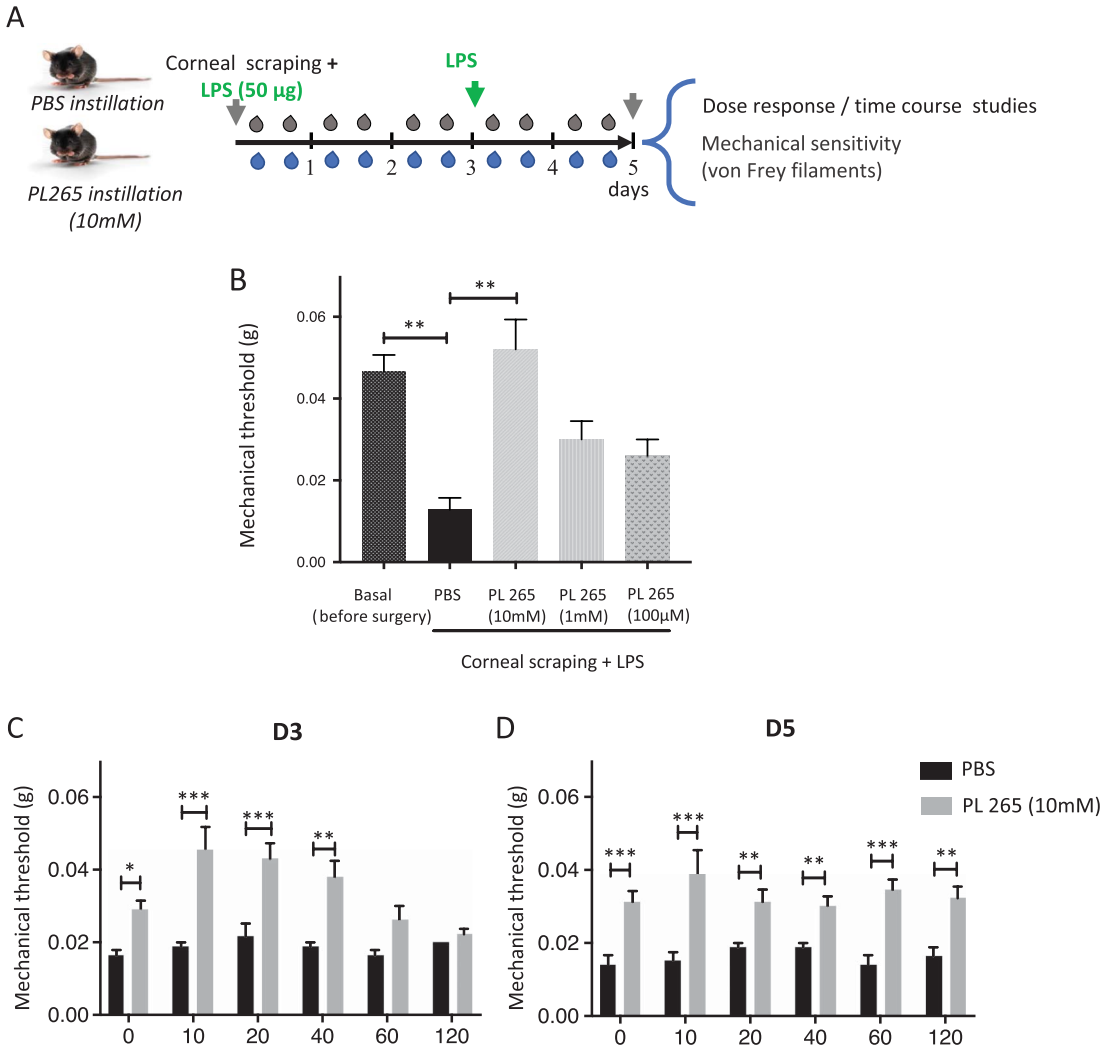

Figure 5. Dose response and time course of PL265 in a corneal inflammatory pain model. Schematic diagram of the experimental protocol (A). Corneal scraping and LPS-induced mechanical corneal hypersensitivity, which was prevented by topical administration PL265 at the concentration of 10 mM (B). Note that the concentrations of 1 mM and 100 µM tend to reduce corneal hypersensitivity. Basal value corresponded to the mechanical threshold measured before the surgery. Data presented as mean ± SEM (n = 5 mice per group); difference between groups was analyzed using nonparametric 1-way ANOVA test on ranks followed by Dunn post hoc test for multiple-comparison test. To analyze differences between groups after corneal scraping/LPS, we used 1-way ANOVA test followed by multiple-comparison test (Kruskal and Wallis). Level of significance was $^{**}P < 0.01$. Time course of the antinociceptive effects of PL265 at D3 (C) and D5 (D). Time 0 value corresponded to the mechanical threshold measured before the last daily PBS or PL265 instillation. Corneal mechanical thresholds were measured with von Frey filaments at different time points: 10, 20, 40, 60, and 120 minutes after the topical administration of PBS and PL265. Note the significant antinociceptive effect of PL265 at all the time points analyzed at D5 compared to D3. Data were presented as mean ± SEM (n = 5 mice per group); to analyze difference between groups, a parametric 2-way ANOVA test followed by multiple-comparison (Benjamini–Krieger–Yekutieli) test by controlling the false discovery rate (Q = 0.05) was used. Levels of significance were $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$, respectively. ANOVA, analysis of variance; LPS, lipopolysaccharide.

morning instillation) was already significantly higher ($^{*}P < 0.05$) in mice treated with PL265 (0.029 ± 0.003 g) in comparison to animals treated with PBS (0.015 ± 0.001 g) (**Fig. 5C**). On D3, mechanical allodynia measured 10 minutes post-instillation was significantly reduced (increased threshold) in PL265 (10 mM)-treated mice, whereas instillation with PBS had no effect on the threshold across all the time points studied. However, instillation with PL265 significantly increased the thresholds measured at 10, 20, and 40 minutes. The analgesic effect of PL265 on mechanical allodynia was no longer significant 60 minutes post-instillation.

On D5, the basal mechanical threshold was still significantly higher in mice treated with PL265 (0.031 ± 0.003 g) than in mice receiving PBS (0.014 ± 0.003 g) (**Fig. 5D**). This significant difference between the 2 groups was observed at all time points analyzed until 120 minutes. These results demonstrate that 5 days of twice-daily instillations of PL265 (10 mM) significantly reduce the mechanical hypersensitivity in an LPS-induced inflammatory pain model.

3.6.4. Reversion of the antinociceptive effect of PL265 by naloxone methiodide on lipopolysaccharide-induced inflammatory pain model

In these behavioral experiments, operated mice were divided into 4 groups: mice receiving PBS or PL265 in presence of PBS or naloxone methiodide, a nonselective opioid receptor antagonist that does not cross the blood–brain barrier (**Fig. 6A**). Again, topical administration of PBS/PL265 (10 mM) significantly decreased mechanical hypersensitivity ($^{*}P < 0.05$) compared to mice treated with PBS/PBS (0.036 ± 0.009 g vs 0.016 ± 0.002 g). The PL265-induced corneal analgesia was totally prevented by

naloxone methiodide administration (100 μM; *$P < 0.05$) (0.016 ± 0.002 g vs 0.036 ± 0.009 g), demonstrating that the analgesic effect of PL265 is mediated by peripheral opioid receptors on the ocular surface (**Fig. 6B**). Finally, the topical administration of naloxone methiodide in PBS-treated mice (0.016 ± 0.002 g) had no effect on mechanical sensitivity, which was similar to that of the PBS/PBS (0.016 ± 0.002 g) and naloxone methiodide/PL265 animals (0.016 ± 0.002 g).

3.6.5. Topical PL265 administration decreases inflammation and neuronal injury in the cornea and trigeminal ganglion

To evaluate the anti-inflammatory action of PL265, flow cytometry experiments were performed to quantify mononuclear phagocyte (defined as $CD11b^+$ cells) population in corneas from different groups of mice. Representative flow cytometry dot plot for $CD11b^+$ cells in corneas of mice (**Fig. 7A**) after corneal scraping + LPS clearly demonstrated an abundant infiltration of myeloid cells in the cornea. The graph illustrating the percentage of corneal inflammatory monocytes/macrophages $CD11b^+$ in the corneas from the different groups of mice (**Fig. 7B**) shows that topical PL265 administration significantly decreases, by 2-fold (*$P < 0.05$) the $CD11b^+$ cells in the cornea. The corneal inflammation was also evaluated by in vivo confocal microscopy in the LPS-induced corneal pain model. Images taken at D5 from PBS-treated mice showed cellular alterations in the superficial corneal epithelium (orange arrowheads), and numerous hyperreflective immune cells (orange arrows) were observed (**Fig. 7C**). In contrast to PBS mice, the superficial epithelium from PL265-treated mice was less reflective, reinforcing the corneal injury reduction effect of PL265. In addition, the stroma of PL265-instilled mice contained fewer hyperreflective immune cells, suggesting an anti-inflammatory effect of topical administration of PL265. The decreased corneal inflammation correlated with the decreased hypersensitivity after PL265 treatment (as previously showed in **Fig. 5**). Primary sensory neuronal injury and inflammation were also assessed in the ipsilateral TG through ATF3 and Iba1 by the semiquantitative analysis of immunoreactive levels on D5. For ATF3 experiments, the number of ATF3-positive neurons in the ophthalmic branch of the ipsilateral TG was quantified. Trigeminal ganglion sections from PL265-treated mice exhibited

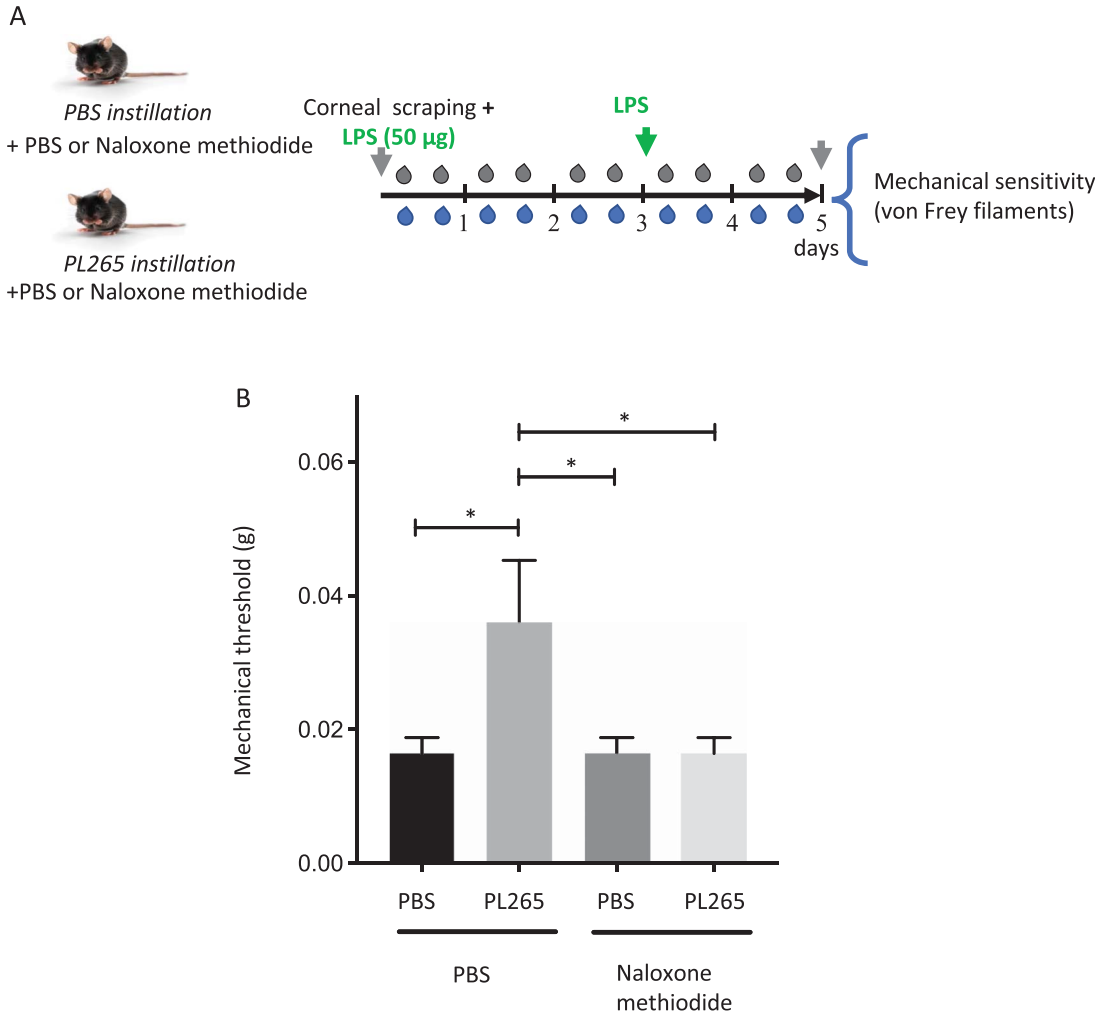

Figure 6. The antinociceptive effects of PL265 are antagonized by naloxone methiodide. Schematic diagram of the experimental protocol (A). Animals with corneal scraping + LPS were topically instilled with either PBS or PL265 in the presence or the absence of naloxone methiodide (100 μM), a nonselective antagonist of opioid receptors, unable to cross the blood–brain barrier. (B) Mechanical sensitivity was measured with von Frey filaments, 15 minutes after the last instillation. Note that PL265 significantly reduced corneal hypersensitivity compared to PBS. The antinociceptive effect of PL265 was completely reversed by topical administration of naloxone methiodide. Results were expressed as means ± SEM. Differences between groups were analyzed using parametric 1-way ANOVA test followed by the Tukey post hoc test. Level of significance was *$P < 0.05$; n = 5 animal per group. ANOVA, analysis of variance; LPS, lipopolysaccharide.

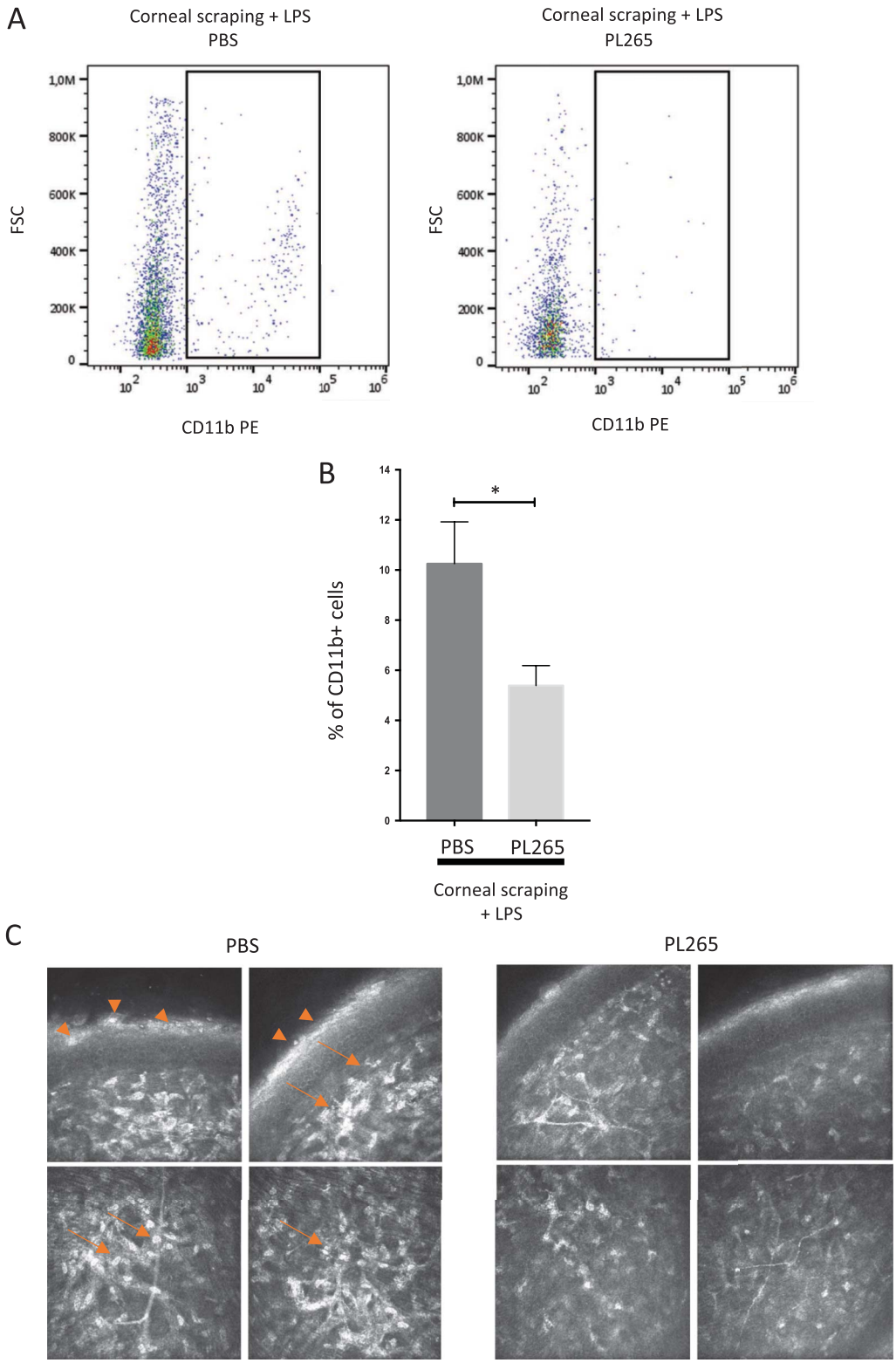

Figure 7. Topical administration of PL265 decreases corneal inflammation. Representative flow cytometry dot plot for myeloid populations in corneas from different groups of mice with mononuclear phagocytes defined as $CD11b^+$ cells (A). Corneas were extracted at day 5 from mice with a corneal scraping + LPS receiving PBS (left panel) and from mice with a corneal scraping + LPS receiving PL265 (right panel). Corneas were dissected at day 5 and digested for flow cytometry analysis by forward-scattered light (FSC) method. Cell suspensions of cornea from individual mice were assessed using anti-CD11b-PE mAb. Graph illustrating the % of $CD11b^+$ cells in the different groups of mice (B). After corneal scraping + LPS, topical PL265 treatment significantly decreased the number of $CD11b^+$ cells in the cornea as compared to the PBS group. Corneal IVCM images from PBS and PL265 animals at D5 after corneal scraping + LPS (C). Superficial epithelium alteration (orange arrowheads) and inflammatory cells (orange arrows) were noted in PBS-treated animals. Corneal inflammation was clearly reduced after topical administration of PL265. Data are presented as mean ± SEM (n = 4 mice per group); difference between groups were analyzed using the nonparametric Mann and Whitney test. Level of significance was $^*P < 0.05$. IVCM, in vivo confocal microscope; LPS, lipopolysaccharide.

a significantly lower number of ATF3-IR neurons as compared to mice receiving PBS (8 vs 1, $^{***}P < 0.001$; **Fig. 8A**). For Iba1 immunofluorescent staining analysis, the staining surface percentages of Iba1 were calculated in both groups. As the TG is located outside the blood–brain barrier, the infiltrating inflammatory cells labelled with Iba1 only derive from circulating monocytes, ie, macrophages. The results clearly showed a significant decrease of Iba1 immunostaining in the ipsilateral TG in mice treated with PL265 compared to the PBS group percentage (0.058 ± 0.006 AU vs 0.020 ± 0.001 AU, $^{**}P < 0.01$) (**Fig. 8B**), indicating a reduced number of infiltrating cells (monocytes/macrophages) under topical PL265 treatment. Furthermore, Iba1-IR cells in the ipsilateral TG of PBS-treated mice had enlarged and amoeboid morphological features of activated macrophages (**Fig. 8B**, arrows). By contrast, a typical resting macrophage morphology was noted in the ipsilateral TG of PL265-treated mice (**Fig. 8B**, arrowheads).

Primary antibody specificity control experiment (Fig. S2, available at http://links.lww.com/PAIN/A677) demonstrated that immunostaining observed with ATF3 and Iba1 primary antibodies was not caused by nonspecific interactions of the antibodies in the TG tissue. Indeed, no staining was noted in TG sections (from control and LPS-treated animals) incubated with purified rabbit IgG and revealed with biotin-conjugated horse anti-rabbit antibody and streptavidin–Alexa Fluor 488.

4. Discussion

In this study, we evidenced the antinociceptive and anti-inflammatory properties of PL265, a dual inhibitor of the enkephalin-degrading enzymes NEP and APN, in several animal models of corneal pain. The data suggest that the activation of corneal opioidergic receptors by endogenous enkephalins protected from their physiological degradation could be an effective approach for reducing the hypernociceptive responses observed after corneal injuries (toxic, traumatic, and inflammatory). Indeed, predicting the usefulness of DENKIs as a new class of topical analgesics devoid of the side effects of exogenous opiates is based on the reasonable assumption that they are to increase extracellular concentrations of enkephalins, whether released tonically or after stimulus-evoked depolarization (phasic release).[47] The originality of this pharmacological concept is based on the fact that, unlike exogenous opiates that directly stimulate all available opioid receptors, DENKIs, which do not bind to opioid receptors,[47] act specifically where endogenous enkephalins are released in response to a noxious stimulus. A

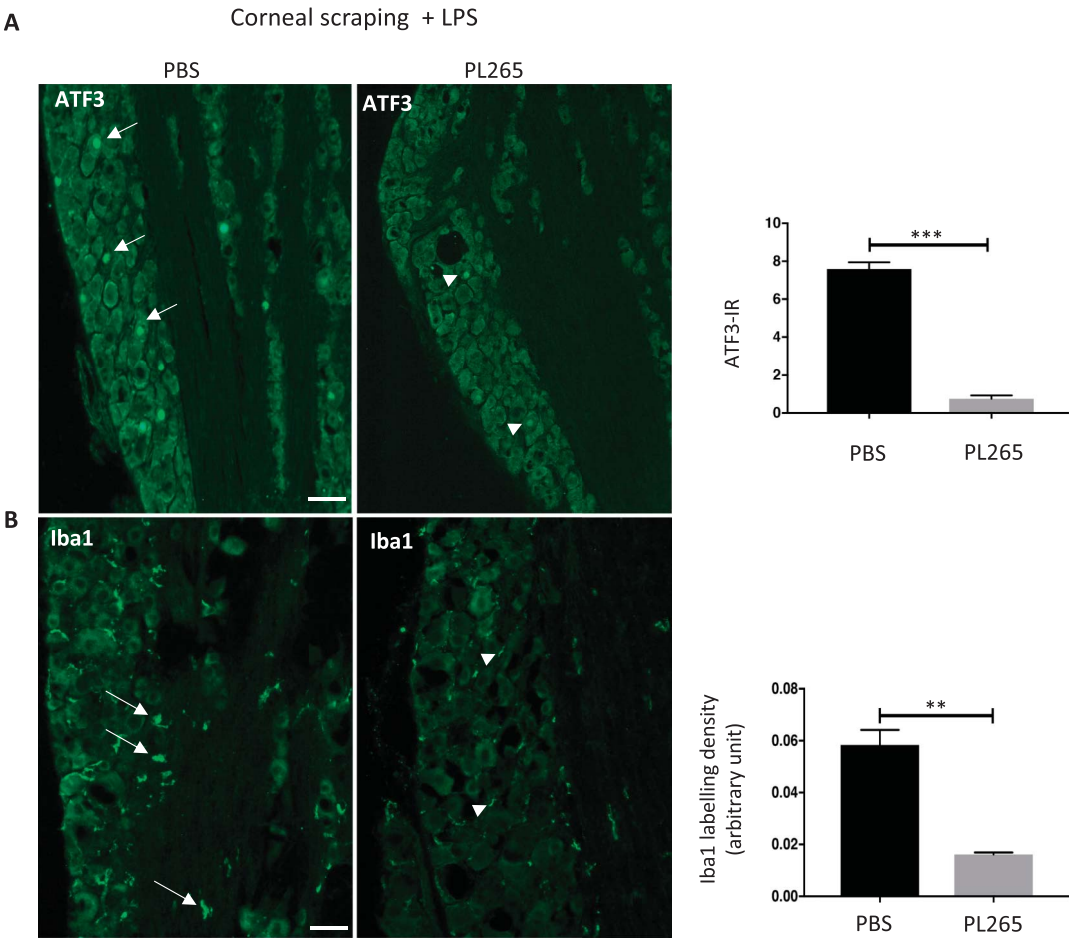

Figure 8. Topical administration of PL265 reduces ATF3 and Iba1 immunostaining in the ipsilateral trigeminal ganglion (TG) in LPS-induced inflammatory corneal pain. Microscopic images showing ATF3-positive nuclei of primary sensory neurons (A) and Iba1-positive cells (monocytes/macrophages) (B) in the ipsilateral TG in mice receiving topical administration of PL265 (white arrowheads) or PBS (white arrows). Graphs illustrate quantifications of ATF3-positive cells and Iba1 immunoreactivity in PBS- and PL265-treated animals. Note that topical PL265 significantly downregulated both markers in the ipsilateral TG. Results are expressed as means ± SEM. Data are presented as means ± SEM; 1 section every 5 sections was used to quantify both staining, (n = 3 mice per group); difference between groups was analyzed using the nonparametric Mann and Whitney test. Levels of significance were $^{**}P < 0.01$ and $^{***}P < 0.001$, respectively. LPS, lipopolysaccharide.

number of studies have shown that nociceptive nerve injury induced a local increase in enkephalin secretion[55,57] from injured nerve fibers[45] or from lymphocytes attracted to the inflamed site.[1,15] The analgesic effects of PL265 mediated by the prevention of enkephalin degradation can be related to studies showing the potent analgesic effects of enkephalin-encoding viral vector infection in pain models, which produce an artificial increase of enkephalin synthesis.[34] In this study, the use of 2 different approaches (flow cytometry analysis and immunohistochemistry experiments) concurred in demonstrating that corneal scraping and LPS treatment induced a significant recruitment of $CD4^+$ cells in the cornea, which in turn could be responsible for the local release of endogenous opioids.[1]

Alleviating corneal pain by a DENKI-mediated sustained increase in local enkephalin concentrations should provide better efficacy and safety than exogenous opiates. Therefore, the preliminary step of our studies aimed at confirming that PL265 had no harmful effects on corneal integrity after repeated instillations in a healthy eye. Slit-lamp examination (fluorescein test) and IVCM analysis of PL265-treated cornea confirmed that repeated instillations of PL265 did not induce any corneal alterations. This lack of corneal toxicity confirms the results of previous studies, which explored the safety of PL265 administered through other oral or intravenous routes.[5] Moreover, nociceptive defense behaviors were not observed when PL265 was administered repeatedly on cornea.

Although topical instillation of morphine derivatives has no favorable effects on corneal wound healing in dogs,[56] we show here, using slit-lamp examination, that 5 days of repeated instillations of PL265 on injured cornea (BAC model, corneal scraping, and LPS treatment) improved corneal wound healing. These results contrast with those of Zagon's group, which established that opioid growth factor (OGF) also called Met-enkephalin, had a negative influence on the growth and repair of corneal epithelial cells through the activation of its specific receptor (OGF receptor).[65] Indeed, these authors demonstrated that blocking the OGF-receptor axis by topical administration of the nonselective opioid antagonist naltrexone accelerates corneal wound healing after corneal scraping in rats. In addition, naltrexone stimulated in vitro epithelial outgrowth and accelerated re-epithelization after corneal scraping,[49] in contrast to OGF, which depressed corneal cell division and wound healing.[37] However, a recent study suggests that Leu-enkephalin (derived from the same proenkephalin precursor as Met-enkephalin and from prodynorphin) promotes wound repair through regulation of matrix metalloproteases (MMP-2 and MMP-9).[64] Binding studies demonstrated that Met-enkephalin binds to both μ and OGF receptors, whereas Leu-enkephalin has a higher binding affinity toward δ receptors than toward μ receptors. Further studies using specific μ and δ receptor antagonists will clarify the opposite action reported for Met- and Leu-enkephalins in the wound healing process after corneal injury. Finally, in relation with studies reporting the role of aminopeptidases APN/LTA4 hydrolase (LTA_4H) in extracellular matrix degradation,[13] the inhibition of APN/LTA4H activity by PL254[5] could prevent the actions of extracellular matrix proteases (such as heparanase and MMP-9), hence slowing down the extracellular matrix degradation process.[21,28]

Under inflammatory condition (corneal scraping + LPS), FCM experiments revealed a noticeable increase of inflammatory monocytes/macrophages $CD11b^+$ population in the cornea in PBS-treated mice. Flow cytometry analysis also clearly demonstrated that topical PL265 treatment significantly reduced the inflammatory monocytes/macrophages $CD11b^+$ population in the cornea, demonstrating an anti-inflammatory effect of the DENKI. In addition to these flow cytometry analyses, IVCM images obtained after corneal scraping and LPS treatment show that repeated, 5-day instillations of PL265 drastically decreased corneal inflammation, suggesting that the active drug PL254 is endowed with anti-inflammatory properties, most likely due to increased concentrations of enkephalins. These anti-inflammatory effects of high concentrations of enkephalins, induced either by inhibiting their degrading enzymes or by enkephalin-encoding viral vectors, have been observed in numerous pain models such as complete Freund's adjuvant monoarthritis,[34] neuropathic pain,[5,22] and experimental colitis.[26] Furthermore, immunoreactivity for Iba1, which is the most widely used marker for microglia,[23] is significantly attenuated in the ipsilateral TG of PL265-treated mice compared to mice receiving topical PBS. This anatomical result confirms the anti-inflammatory action of the topical DENKI.

In addition to the anti-inflammatory effect of topical PL265, we carefully characterized the activating transcription factor 3 (ATF3), a sensitive cellular marker of nerve injury,[59] to reveal the engagement of the primary afferent fibers after corneal nerve fiber injury. An increase in ATF3 staining in the ipsilateral TG has recently been reported in a corneal injury model using BAC administration.[30] Here, we show that corneal scraping with LPS administration led to an increase of ATF3 expression in the nucleus of the primary sensory neurons of the TG. There was thus a correlation between ATF3 expression and pain behavior.

To demonstrate the specificity of primary antibodies used in this study, we realized negative control by a substitution of serum- or isotype-specific immunoglobulins at the same protein concentration as the primary antibody[20] (on corneal and TG sections). No immunoreactivity was detected on all tissues of interest in all conditions. Furthermore, as correctly stated in an open letter on the use of antibodies written by Saper,[50] we also checked that the anti-CD4, anti-ATF3, and anti-Iba1 antibodies used in this study produced patterns of staining that are identical to other antiserum directed to each of these proteins.[2,33,49] It is also important to note that anti-CD4, anti-ATF3, and anti-Iba1 antibodies used in this work stained cells exhibiting the classic morphology and distribution of $CD4^+$ T cells, primary sensory neurons, and monocytes/macrophages, respectively.

A large number of in vitro investigations have found evidence that endogenous opioids can reduce the release of proinflammatory molecules such as cytokines or of molecules related to plasma extravasation, vasodilation, immune mediators, expression of adhesion molecules, and tissue destruction.[54]

Moreover, in tissues where painful stimuli occurred, enhanced APN and NEP activities are generally correlated with an increase in the inflammatory process.[14,24] Thus, use of DENKIs in peripheral inflamed tissues could also have a direct potential to reduce inflammation.[51]

Another explanation is based on the anti-inflammatory effects of PL265 through the inhibitory potential of the PL254 on LTA_4H activity.[5] LTA_4H is a ubiquitously expressed enzyme that catalyzes the synthesis of leukotriene B4 (LTB4), a potent proinflammatory lipid mediator derived from arachidonic acid. LTB4 is assumed to be a potent chemoattractant and activator on leukocytes mediating innate inflammatory responses. With LTB4 receptors present on multiple cell types, including T lymphocytes and antigen-presenting dendritic cells, the inhibition of LTB4 synthesis through inhibition of LTA_4H activity by the PL265-derived drug PL254[5] could have the potential to directly target the inflammatory process.[12]

Finally, repeated topical administration of PL265, in all murine models used in this study, did not induce any mechanical hypersensitivity, a side effect commonly observed after repeated peripheral administration of morphine.[53,60] In addition, the antinociceptive effects of PL265 on corneal pain models were not altered over time, suggesting that no tolerance develops with DENKI-protected enkephalins. The absence of tolerance may be explained by the fact that a local DENKI-induced increase of enkephalin did not downregulate opioid receptors.[31,40,41] By contrast, we observed an increase in efficacy, the analgesic effect of PL265 being sustained and present 120 minutes after instillation on D5, although the PL265 effect lasted only 40 minutes after the instillation on D3.

The array of results on corneal pain models clearly suggests that PL265 increases extracellular concentrations of enkephalins, which in turn counteract hyperalgesia by specifically stimulating peripheral opioid receptors present on the cornea. Indeed, PL265 does not cross the blood–brain barrier and its activity remains strictly peripheral.[5] A complete reversion of the antinociceptive effects of PL265 by naloxone methiodide (a nonselective antagonist of opioid receptors unable to cross the blood−brain barrier) was observed in an LPS-induced inflammatory corneal pain model. However, in the experiment showing that naloxone methiodide antagonized the PL265-induced antinociceptive effects in LPS-induced inflammatory pain model, no difference was observed in mechanical threshold between mice receiving topical administration of naloxone methiodide in PBS-treated mice vs mice receiving only PBS (PBS/PBS-treated mice). In accordance with the relatively short half-life of enkephalins,[27] this result suggests that the level of endogenous enkephalins in the injured cornea (which depends on the balance between the release and the degradation of the endogenous enkephalins) is not sufficient to induce a significant analgesia. By contrast, we observed that in PL265-treated animals, the mechanical threshold increased significantly, suggesting that PL265 efficiently protected enkephalins from degradation, thereby increasing their endogenous concentrations in injured cornea. Taken together, these results could explain why, in PBS-treated mice, naloxone methiodide did not induce a more sustained corneal mechanical hypersensitivity.

In conclusion, the present results open a totally new avenue for ocular pain treatment based on the enhancement of endogenous opioid peptide concentration in tissues of the anterior segment of the eye. Dual enkephalinase inhibitor–protected enkephalins circumvent the risk of overstimulation of opioid receptors by excessive amounts of exogenous agonists. Thus, PL265 seems to be a promising topical medication for safe and effective alleviation of ocular pain and inflammation.

Conflict of interest statement

The authors have no conflict of interest to declare.

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Author contributions: A. Reaux-Le Goazigo, S. Melik Parsadaniantz, H. Poras, and M. Wurm participated in the conception of the study. The experiments were managed at the Therapeutic Department of the Vision Institute by C. Ben-Dhaou, A. Reaux-Le Goazigo, and S. Melik Parsadaniantz. All authors contributed to the interpretation of the data. A. Reaux-Le Goazigo, S. Melik Parsadaniantz, H. Poras, M. Wurm, T. Ouimet, and C. Baudouin drafted the manuscript and all authors approved the final version of the manuscript.

Appendix A. Supplemental digital content

Supplemental digital content associated with this article can be found online at http://links.lww.com/PAIN/A677.

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