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Abstract

The dual enkephalinase inhibitor PL37, a small molecule that protects enkephalins from their rapid degradation, has demonstrated analgesic properties in animal pain models and in early human clinical trials. This study tested the antimigraine potential of PL37 on cutaneous mechanical hypersensitivity affecting cephalic regions in migraineurs. Using behavioral testing and c-Fos immunoreactivity in male rats, we investigated the effects of single (oral or intravenous) and repeated oral administration of PL37 on changes in cutaneous mechanical sensitivity and sensitization of the trigeminocervical complex induced by repeated administration of the nitric oxide donor, isosorbide dinitrate. In naive rats, single or repeated administration of PL37 or vehicle had no effect on cephalic mechanical sensitivity. However, single oral PL37 treatment effectively inhibited isosorbide dinitrate-induced acute cephalic mechanical hypersensitivity. Single intravenous but not oral PL37 administration inhibited chronic cephalic mechanical hypersensitivity. Daily oral administration of PL37 prevented cephalic mechanical hypersensitivity and decreased touch-induced c-Fos expression in trigeminocervical complex following repeated isosorbide dinitrate administration. These data reveal the therapeutic potential of the dual enkephalinase inhibitor PL37 as an acute and prophylactic treatment for migraine. Protecting enkephalins from their degrading enzymes therefore appears as an innovative approach to treat migraine.

Keywords: headache; encephalin; opioid; mechanical hypersensitivity; nitric oxide

Abbreviations: ANP = aminopeptidase N, CGRP = calcitonin gene related peptide**,** DENKI = Dual ENKephalinase Inhibitor, DOR = delta opioid receptors, ISDN = isosorbide dinitrate, MH = mechanical hypersensitivity, MOR = mu opioid receptors, NEP = neutral endopeptidase, and TCC = trigeminocervical complex.

Introduction

Acute and chronic migraine are incapacitating diseases, and improving their management is a high health priority[1]. Although a range of migraine treatments, including newly developed ones, is currently available, considerable dissatisfaction remains with acute and prophylactic therapies[2]. Moreover, most current acute headache treatments have been linked to medication overuse headache[1], further highlighting the need for new medications.

One promising avenue in the field is to improve the potency of enkephalins, the endogenous opioid peptides that contribute to the physiological system of pain control[3,4]. Enkephalins interact with both the μ- and δ-opioid receptors (MOR and DOR) that are strategically located at various levels of nociceptive pathways[3,4,5]. However, their analgesic effects are transient, as they are rapidly degraded by two membrane-bound exo-metalloproteases, termed neprilysin (neutral endopeptidase; NEP) and aminopeptidase N (APN). It was hypothesized that blocking enkephalin metabolism could increase their extracellular concentrations near their physiological secretion sites only, thus restricting their pharmacological effects to the surrounding opioid receptors. This contrasts with exogenous opiates that bind to all available opioid receptors, thus sustaining side effects such as tolerance, constipation, sedation and respiratory depression[3,4].

Concomitant inhibition of NEP and APN by Dual ENKephalinase Inhibitors (DENKIs) like PL37 was shown to increase enkephalin half-life and induce analgesia in a wide range of rodent preclinical pain models. In humans, it did not result in tolerance or dependence[3,4]. This study explores the antimigraine potential of PL37 [((2S)-3-{[(2S)-2-amino-4-(methylthio)butyl]dithio}-2-(phenylmethyl)propanoyl)amino]acetic acid 1-[(ethoxy-carbonyl) oxy]ethyl ester, using a novel animal model of migraine[6] in which cutaneous hypersensitivity is used as a surrogate for headache[7,8].

Materials and methods

Animals

Male Sprague-Dawley rats weighing 250–275 g (*n* = 174; one rat excluded from the propranolol group because it died after the 5th injection, Charles River, L'Arbresle, France) were housed at 22 ± 1°C in plastic cages (size: 425 x 266 x 185 mm; 3-4 rats per cage) on soft bedding with *ad*

libitum water and food under a 12h/12h light/dark cycle for at least one week before the start of experiments. Every effort was made towards minimizing the number of animals used. The number of animals used in each experiment was selected according to previous experiments[6,7]. All experimenters were blind to treatment conditions. Randomization was performed within individual home cages to avoid any cage effects, and balanced to have approximately equal numbers of each treatment group represented in each home cage[9].

Animal protocols were approved by the Committee of Animal Research at the University of Clermont-Ferrand, France and in accordance with the International Association for the Study of Pain (https://www.iasp-pain.org/resources/guidelines/iasp-guidelines-for-the-use-of-animals-in-research/), the European Directive 2010/63/EU and ARRIVE guidelines.

Behavioral sensory testing

Behavioral sensory testing was performed as previously described[6]. Cephalic cutaneous mechanical sensitivity was assessed before and on test day (Day 1, 2, 3, 4 and 5: before injection and at 30-min intervals for 3-4 h after injection) in groups of animals receiving intraperitoneal isosorbide dinitrate (ISDN, 10 mg/kg, Sanofi-Aventis, France) or saline. Cephalic withdrawal thresholds were determined using von Frey (VF) filaments (0.16, 0.4, 0.6, 1, 1.4, 2, 4, 6, 8, 10 g; Bioseb, France) applied to the forehead midline (between the eyes) *via* the descending-ascending method starting from the 6-g filament[10]. Each filament was applied to the skin, for three seconds (s) 5 times in a row (at 10 s intervals). VF filament threshold (VFT, in grams) was equal to the filament evoking a head withdrawal response in 3 out of 5 trials.

Immunocytochemistry

Immunohistochemistry was performed as previously described[7]. Induction of c-Fos was performed under urethane anesthesia with a 6-g VF filament application during 3 min at 1 Hz on the right periorbital region. Rats were perfused with 4% paraformaldehyde 2 hours after the treatment (see Supplementary material).

Drugs

Treatments were either administered *per os* [PL37 (50 mg/kg, 100 mg/kg, 1 mL/rat volume), rizatriptan benzoate (0.01 mg/kg, 1 mL/rat volume), propranolol hydrochloride (10 mg/kg, 1 mL/rat volume), vehicle for PL37 (10% ethanol in saline)]; or intravenously [sumatriptan succinate (0.3 mg/kg, 0.2 mL/rat volume); PL37 (20 mg/kg, 0.2 mL/rat volume); vehicle for

PL37]. All drugs were dissolved in saline except for PL37 prepared in 90% saline and 10% ethanol. PL37, propranolol (Sigma Aldrich, St Quentin Fallavier, France), rizatriptan (Merck Sharp Dohme-Chibret, Paris, France) and sumatriptan (Sigma Aldrich, St Quentin Fallavier, France), were administered 5 min before ISDN or saline injection. Subcutaneous and intravenous administrations were performed under brief anesthesia (≤ 3 min) using a mask with 2% isofluorane.

Statistical analysis

All data generated throughout the study were analyzed using Matlab (R2014a, The MathWorks Inc) and Statistical Toolbox. Normality and homogeneity of variance were determined using Shapiro–Wilk test and Brown–Forsythe's test, respectively. The comparison between groups were made using two-tailed parametric Student's unpaired t-test, the nonparametric Mann-Whitney test or the two-way repeated-measures ANOVA followed by the Tukey's post-hoc test for multiple-comparisons. For data that did not pass normality testing, comparisons were performed using the aligned rank transformed ANOVA, with repeated measurements and the Tukey's post-hoc test. Data are presented as mean ± standard error of the mean or median and interquartile range (IQR), and n represents the number of animals. The level of significance was set at $P < 0.05$. Statistical details for each quantitative experiment are illustrated in Supplementary Table 1.

Data availability

All data are available upon reasonable request

Results

Effect of PL37 on acute ictal cephalic cutaneous mechanical hypersensitivity

In naive rats, single oral (100 mg/kg, $n = 10$) or intravenous (20 mg/kg, $n = 10$) administration of PL37 or vehicle for PL37 ($n = 10$/group) had no effect on cephalic mechanical sensitivity (Supplementary Fig. 1A, B).

A single ISDN injection induced a reversible cephalic mechanical hypersensitivity (MH) peaking at 1 h and lasting 2 h in control animals but not in PL37- or rizatriptan-treated animals

(*n* = 10-12/group, Fig. 1). One-hour post-administration, VFTs were lower in control [median (IQR): 4.0 (2.0-4.0 g)] than in PL37-50 mg/kg [median (IQR): 6.0 (6.0-6.5 g)], PL37-100 mg/kg [median (IQR): 8.0 (6.5-8.0 g)] and rizatriptan-treated animals [median (IQR): 8.0 (5.5-8.0 g)].

Effect of PL37 on chronic ictal cephalic cutaneous mechanical hypersensitivity

Oral administration of PL37 (50 mg/kg, *n* = 8 or 100 mg/kg, *n* = 8) before the last ISDN injection (*i.e*. on the fifth day) did not inhibit cephalic MH induced by the 5th ISDN injection (Fig. 2A). By contrast, intravenous administration of either PL37 (20 mg/kg) or sumatriptan (0.3 mg/kg) decreased the cephalic MH (Fig. 2B). One-hour post-administration, VFTs were lower in control [median (IQR): 0.6 (0.2-0.7 g)] than in PL37- [median (IQR): 4.0 (1.9-5.0 g)] and sumatriptan-treated animals [median (IQR): 3.0 (1.8-4.5 g)].

Preventive effect of PL37 on ictal and interictal cephalic mechanical hypersensitivity

Rats treated with PL37 (100 mg/kg, po, *n* = 10) or vehicle (*n* = 10) once a day for 5 days did not show changes in cephalic mechanical sensitivity (Supplementary Fig. 1C, D). Interestingly, preventive daily treatment with PL37 or propranolol reduced the cephalic MH observed after the 5th ISDN injection (Fig. 3A). One-hour post-administration, VFTs were lower in control [median (IQR): 0.6 (0.5-0.6 g)] than in PL37-50 mg/kg [median (IQR): 3.0 (1.4-6.0 g)], PL37-100 mg/kg [median (IQR): 4.0 (1.4-8.0 g)] and propranolol-treated animals [median (IQR): 8.0 (2.0-8.0 g)].

In control animals, daily ISDN injections over 4 consecutive days resulted in the progression of persistent interictal (basal) cephalic MH which became persistent after 3 injections and worsened with the following injection (Fig. 3B). Preventive treatment with either oral PL37 (50 mg/kg/day), PL37 (100 mg/kg/day) or propranolol (10 mg/kg/day) suppressed the repeated-ISDN-induced persistent interictal cephalic MH (*n* = 9-12/group, Fig. 3B).

Preventive effect of PL37 on touch-induced c-Fos expression in trigeminocervical complex

Cephalic MH is a manifestation of trigeminovascular neuronal sensitization within the TCC[7]. The fact that repeated oral PL37 prevented the worsening of cephalic MH as well as the chronic

state of cutaneous hypersensitivity following repeated ISDN administration suggests that oral PL37 may block the development of TCC central sensitization induced by repeated ISDN injections. We tested this hypothesis by monitoring neuronal activation evoked by cephalic mechanical stimulation after daily ISDN injections for 5 consecutive days, using c-Fos protein expression in vehicle and PL37- (100 mg/kg, po) treated animals.

In control ISDN animals, innocuous mechanical stimulation of the periorbital region with a 6-g von Frey filament (3 min, 1 Hz) resulted in strong ipsilateral c-Fos expression in the superficial laminae of the TCC (29.1 ± 5.1) (Fig. 4A, B). c-Fos expression was predominant in lamina I and the outer part of lamina II (laminae I-IIo) (22.5 ± 5.1, $n = 6$), revealing which TCC neurons may be responsible for MH. PL37-treatment decreased touch-evoked c-Fos expression in the ipsilateral TCC (13.3 ± 3.5, $n = 6$) as compared to control animals (Fig. 4A, B). The effect was only significant in laminae I-IIo where c-Fos expression was reduced by 54.1 ± 12.1%.

Discussion

This study reveals the therapeutic potential of the DENKI PL37 in migraine treatment. Oral PL37 effectively inhibited acute ictal cephalic MH evoked by a single ISDN injection. Single intravenous, but not oral PL37, inhibited the chronic ictal cephalic MH induced by repeated ISDN injections, which, together with the related touch-induced c-Fos expression in TCC, was fully prevented by daily oral PL37 treatment.

Previous studies reported that unlike exogenous opiates, very high doses of DENKIs in rodents or PL37 in humans do not result in tolerance or dependence[3,4]. Herein daily treatment with PL37 had no effect on cephalic mechanical sensitivity. These findings suggest a low risk of medication overuse headache (MOH) for DENKIs. Accordingly, chronic DOR activation with a daily administration of the DOR agonist SNC80 produces a less severe MOH than sumatriptan[11].

In this report, single oral PL37 was shown to completely inhibit ISDN-induced acute ictal cephalic MH. The effect was rapid, strong and similar to that observed with the active comparator rizatriptan, and to those previously reported using sumatriptan[6,9,12] and olcegepant[6,9]. Compared to its reported short-lasting analgesic effects on MH and other types of pain[4,13], PL37 displayed a prolonged action span on ISDN-induced cephalic MH. As DENKI analgesic effect depends on extracellular concentrations of enkephalins, these data suggest that headache may be associated with high levels of extracellular enkephalins. In accordance, several studies have shown that acute migraine episodes are accompanied by significant

increases in plasma Met-enkephalin levels, which return to normal levels after a pain-free period of several days up to 3 weeks[14,15]. Changes in circulating enkephalin levels could be viewed as an endogenous pain modulation mechanism alleviating the severity of migraine attacks[14]. In this context, the effect of DENKI, by enhancing the endogenous mechanism of pain modulation, may provide physiological pain alleviation. Interestingly, increased levels of enkephalins induce complete analgesia in patients suffering from congenital insensitivity to pain, without any opioid-like side effects, thereby supporting the hypothesis that leveraging the physiological effects of endogenous enkephalins may provide effective and safe activation of the endogenous opioid system[16].

We also found that single oral PL37 did not inhibit chronic cephalic MH when administered after the fourth ISDN injection, a situation similar to that of sumatriptan[6,12]. Accordingly, animal and human studies suggest that once MH is established, triptans show decreased efficacy[17]. Another hypothesis could be that headache severity at treatment intake, more than the establishment of MH, critically influences the efficacy of triptans[18].

When administered intravenously, however, both PL37 and sumatriptan completely inhibited chronic cephalic MH. Similarly, clinical studies revealed differing efficacies of sumatriptan according to the administration route, injectable sumatriptan demonstrating higher efficacy than oral sumatriptan in the treatment of migraine attacks[19]. Analgesic efficacy of intravenous versus oral PL37 could be explained by differing pharmacokinetic characteristics of the prodrug moieties following the different administration routes and a first pass metabolism effect following oral administration. The higher peripheral concentration reached after intravenous administration could result in an increased central maximum concentration, which could be efficient enough to alleviate cephalic MH. Similarly, Poras et *al*.[13] showed that, after rapid hydrolysis of the prodrug, oral PL37 (25 mg/kg) was active on peripheral mechanisms in neuropathic pain models, but had no effect on central mechanisms. However, when administered intravenously, PL37 induced a significant analgesic response starting at 5 mg/kg using the tail flick test, which suggests that the administration route of the prodrug and its hydrolysis affected its ability to provide peripheral and/or central analgesia.

Interestingly, daily oral PL37 treatment completely prevented the cephalic MH induced by recurrent ISDN administration. This effect was comparable to those observed with preventive drugs such as the beta-blocker propranolol (present study;[6,20]) or the anticonvulsant topiramate[21]. PL37 may thus also exert a prophylactic effect on the development of chronic cephalic MH. Similarly, CGRP receptor antagonist (gepants) have shown efficacy in acute migraine treatment, while CGRP monoclonal antibodies have demonstrated their preventive

potential[1]. Furthermore, preventive effect of oral PL37 on touch-induced c-Fos expression in TCC shows that PL37 blocked the development of TCC central sensitization induced by repeated ISDN injections.

The exact mechanism of action of PL37 is currently unknown. PL37 could act by increasing enkephalin levels at the periphery, thus activating peripheral opioid receptor in the dura. Indeed, previous studies using other pain models reported that pretreatment with the peripherally acting opioid receptor antagonist naloxone methiodide reduced, albeit partially, the antinociceptive effect of PL37[4]. Recently, Rice et *al*.[22] found that MOR and DOR are differentially expressed in rat dura, with MOR expression being scarce and observed in a small proportion of CGRP labeled fibers, while the majority of CGRP fibers co-expressed DOR. This is consistent with the findings that DOR agonists may efficiently treat migraine[11,23,24]. In accordance, the increase in CGRP expression in the trigeminal ganglia and TCC induced by chronic nitroglycerin was blocked by the DOR agonist SNC80[24].

PL37 could also exert its effects through the activation of central opioid receptors directly at the TCC level. Immunohistochemical studies demonstrated the presence of enkephalinergic neurons and terminals[25] and a high density of MOR and DOR in the TCC[26]. Direct measurements of endogenous opioid release from the TCC have further shown that trigeminal nociception is associated with changes in TCC Met-enkephalin outflow[27].

Finally, PL37 may also act indirectly through brainstem structures and reinforce descending inhibitory controls that act on TCC. Several brainstem nuclei, including the periaqueductal gray and the nucleus raphe magnus, known to contain enkephalinergic neurons projecting to the TCC[25], have been implicated in the modulation of trigeminal pain[28].

Preliminary clinical findings suggest that inhibiting the breakdown of enkephalins is sufficient to alleviate pain in patients. For instance, intravenous infusion of thiorphan, a selective NEP inhibitor, significantly inhibited both the headache and nausea typically associated with myelography[29]. In a non-controlled open-label study, intrathecal administration of the combination of a selective NEP inhibitor and a nonspecific APN inhibitor elicited marked and lasting pain relief in terminally ill cancer patients unresponsive to morphine[30]. Since the safety and tolerability of oral PL37 have been demonstrated[3], its efficacy in relieving migraine headache could be studied in a proof of concept clinical trial.

In summary, although the experiments described here were exclusively performed in male animals and will require to be extended in females, this study shows that enkephalins are involved in migraine pathophysiology and highlights the therapeutic potential of PL37 as both an acute and prophylactic treatment for migraine.

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Competing interests

H. Poras, M. Wurm and T. Ouimet are employees of Pharmaleads SA. All other authors report no competing interests.

Supplementary material

Supplementary material is available at Brain online.

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Figure legends

Figure 1. **Effect of PL37 or rizatriptan on ISDN-induced acute cephalic mechanical hypersensitivity.** Time-course of von Frey withdrawal thresholds (VFT) of the face in vehicle-treated (n = 10), PL37-treated (50 mg/kg, *n* = 10; 100 mg/kg, *n* = 12) and rizatriptan-treated (0.01 mg/kg, *n* = 10) rats after a single intraperitoneal injection of isosorbide dinitrate (ISDN). Drugs were administered *per os* 5 min before ISDN injection. ISDN produced cephalic mechanical hypersensitivity (vehicle for PL37), which was significantly attenuated by PL37 and rizatriptan (two-way repeated measures ANOVA followed by Tukey's post-hoc test, $P < 0.001$ on time, treatment and time×treatment).*$P < 0.05$ compared to vehicle with two-way repeated measures ANOVA followed by Tukey's post-hoc test. Data are presented as box-and-whisker plots depicting median, interquartile interval, minimum and maximum. The empty square represent the medians and the boxes present the quartiles.

Figure 2. Effect of PL37 or sumatriptan on ISDN-induced chronic ictal cephalic mechanical hypersensitivity. (**A**) Time-course of von Frey withdrawal thresholds (VFT) of the face in vehicle-treated (*n* = 8), PL37-treated (50 mg/kg, *n* = 8), and PL37-treated (100 mg/kg, *n* = 8) rats after the 5th intraperitoneal injection of isosorbide dinitrate (ISDN). Drugs or vehicle for PL37 were administered *per os* 5 min before the 5th ISDN injection. Single oral administration of PL37 (50 mg/kg or 100 mg/kg) did not inhibit the chronic ictal cephalic mechanical hypersensitivity induced by the 5th ISDN injection. (**B**) Time-course of VFT of the face in vehicle-treated (*n* = 8), PL37-treated (20 mg/kg, *n* = 8), and sumatriptan-treated (0.3 mg/kg, *n* = 8) rats after the 5th ISDN injection. Drugs or vehicle for PL37 were administered intravenously 5 min before the 5th ISDN injection. The chronic ictal cephalic mechanical hypersensitivity induced by the 5th ISDN injection was significantly attenuated by PL37 and sumatriptan (two-way RM ANOVA followed by Tukey's post-hoc test, $P = 0.028$ on treatment, $P < 0.001$ on time, and $P < 0.001$ on time×treatment, *n* = 8/group).* $P < 0.05$ compared to vehicle for PL37 with two-way repeated measures ANOVA followed by Tukey's post-hoc test. Data are presented as box-and-whisker plots depicting median, interquartile interval, minimum and maximum. The empty square represent the medians and the boxes present the quartiles**.**

Figure 3. Preventive effects of PL37 or propranolol on chronic ictal and interictal cephalic mechanical hypersensitivity. (**A**) Time–course of VF withdrawal thresholds (VFT) of the face following the 5th intraperitoneal (IP) injection of isosorbide dinitrate (ISDN) in rats treated with either vehicle for PL37 (n = 10), PL37 (50 mg/kg, n = 10 or 100 mg/kg, n = 12) or propranolol (n = 9) once a day for 5 days. Drugs or vehicle for PL37 were administered daily, *per os*, 5 min before ISDN injections. Daily treatment with PL37 and propranolol significantly reduced (two-way repeated measures ANOVA followed by Tukey's post-hoc test, $P < 0.001$ on treatment, $P < 0.001$ on time, $P = 0.434$ on time×treatment) the cephalic mechanical hypersensitivity induced by 5 ISDN injections. *$P < 0.05$ compared to vehicle for PL37 with two-way repeated measures ANOVA followed by Tukey's post-hoc test. (**B**). Time-course of VFT of the face after each daily ISDN injection in rats treated with vehicle for PL37 (n = 10), PL37 (50 mg/kg, n = 10 or 100 mg/kg, n = 12) or propranolol (10 mg/kg, n = 9). Drugs or vehicle for PL37 were administered daily, *per os*, 5 min before ISDN injection. Daily treatment with PL37 or propranolol completely inhibited the interictal cephalic mechanical hypersensitivity (two-way repeated measures ANOVA followed by Tukey's post-hoc test, $P < 0.001$ on day, treatment and treatment×day) induced by 4 ISDN, daily injections. * $P < 0.05$ compared to vehicle for PL37 with two-way repeated measures ANOVA followed by Tukey's post-hoc test. Data are presented as box-and-whisker plots depicting median, interquartile interval, minimum and maximum. The empty square represent the medians and the boxes present the quartiles.

Figure 4. Effect of PL37 on touch-induced c-Fos expression in the trigeminocervical complex. (**A**) Examples of c-Fos immunoreactivity in the trigeminocervical complex (TCC) following innocuous mechanical stimulation of the ipsilateral periorbital region with a 6-g von Frey filament (3 min, 1 Hz) after daily intraperitoneal injections of isosorbide dinitrate (ISDN) 5 consecutive days, in vehicle for PL37 and PL37-treated (100 mg/kg, *per os* for 5 days) rats. (**B**) Distribution of c-Fos-immunoreactive cells within the different laminae of the ipsilateral and contralateral TCC in vehicle for PL37 and PL37-treated rats (n = 6/group) after daily ISDN injections for 5 consecutive days. Comparisons between the two groups were performed using a two-way repeated measures ANOVA followed by Tukey's post-hoc test, $P < 0.049$ on treatment, $P < 0.001$ lamina, $P = 0.050$ on lamina×treatment. In vehicle-treated rats, innocuous mechanical stimulation resulted in strong ipsilateral c-Fos expression in superficial laminae of the TCC (left). c-Fos expression was predominant in laminae I-IIo. In contrast, only minor expression was found in the contralateral side of the TCC (right). Oral administration of PL37 resulted in a significant reduction in the touch-evoked c-Fos expression in the superficial

laminae of the TCC. I-IIo: laminae I and outer II; IIi-IIIo: laminae inner II and outer III. Values are means ± s.e.m; *$P < 0.05$ compared to vehicle for PL37 with Mann-Whitney non-parametric test. Bar is 100 μm.

Supplementary Material

Supplementary methods

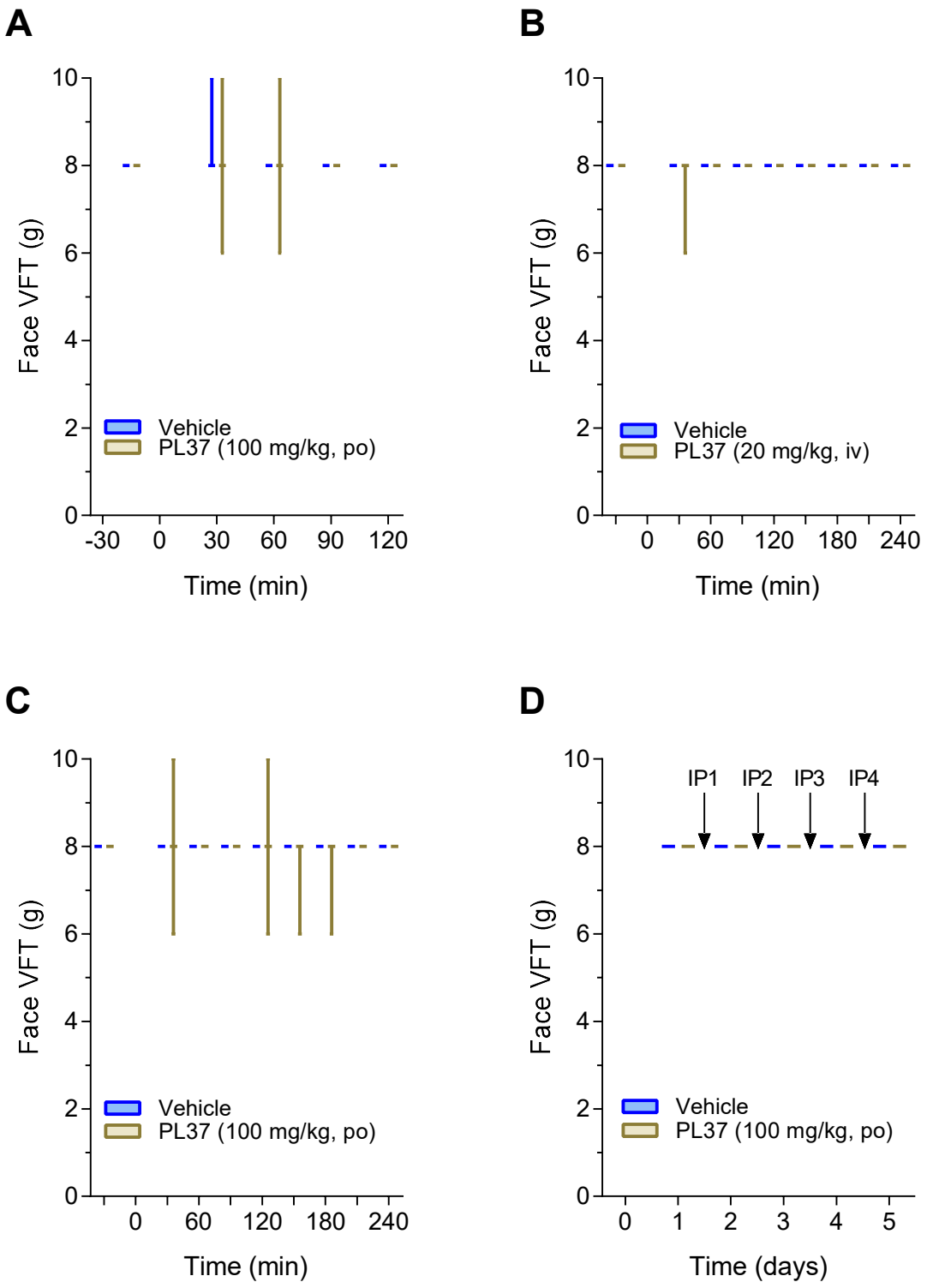
Immunocytochemistry

Neuronal activation within the trigeminocervical complex (TCC) was assessed using the anatomical marker of activation, c-Fos protein. Animals were randomly assigned to 2 groups (6 rats/group) treated with a daily administration of either vehicle for PL37 or PL37 (100 mg/kg, po) for 4 days. Five min after each treatment administration, animals were injected intraperitoneally with ISDN. On the day of the fifth injection, rats were anaesthetized with urethane (1.5 g/kg i.p.). Twenty minutes after injection, the depth of the anesthesia was tested, and animals received either vehicle for PL37 or PL37. Five min after, rats were intraperitoneally injected with ISDN. One hour after ISDN injection, innocuous mechanical stimulation was applied using a 6-g von Frey filament during 3 min at a frequency of 1 Hz on the right periorbital region. Two hours later, animals were transcardially perfused with warm (37°C) heparinized saline (100 mL, 25 IU heparin/ml) followed by cold (10°C) phosphate-buffered solution (0.1 M, pH 7.6) containing 4% paraformaldehyde (350 mL) for 15 minutes. Brainstem were removed and transferred in the same paraformaldehyde solution. After 2 hours, the brainstem were transferred into 30% sucrose-azide solution and left overnight. Coronal sections (30 µm) were cut on a freezing microtome and collected in a 0.05 M Tris-buffered saline (TBS). Free-floating sections were placed in normal horse serum diluted in Tris-buffered saline containing 0.25% bovine serum albumin and 0.3% Triton X-100 for 30 minutes before incubation with a polyclonal rabbit primary anti c-Fos antibody (1:3000, Oncogene Science, San Diego, CA) diluted in Tris-buffered saline containing 0.25% bovine serum albumin and 0.3% Triton X-100

overnight at room temperature. Sections were thereafter incubated for 30 min with the secondary antibody horse anti-rabbit conjugated with peroxidase (1:400, Vector Laboratories, Les Ulis, France). Immunoreactivity was revealed using nickel-diaminobenzidine (Vector Laboratories). The sections were rinsed in Tris-buffered saline several times, between and after each incubation.

The number of c-Fos immunoreactive nuclei within the different TCC laminae of each treated animal were computed from 7 different rostrocaudal planes, between levels 0 and 2400 µm caudal to the obex. Given the 400-µm interval between planes, cells could not be counted twice. Data are expressed as the sum of the total number of labeled cells counted from every one of the 7 sections analyzed from each animal.

Supplementary Figure 1

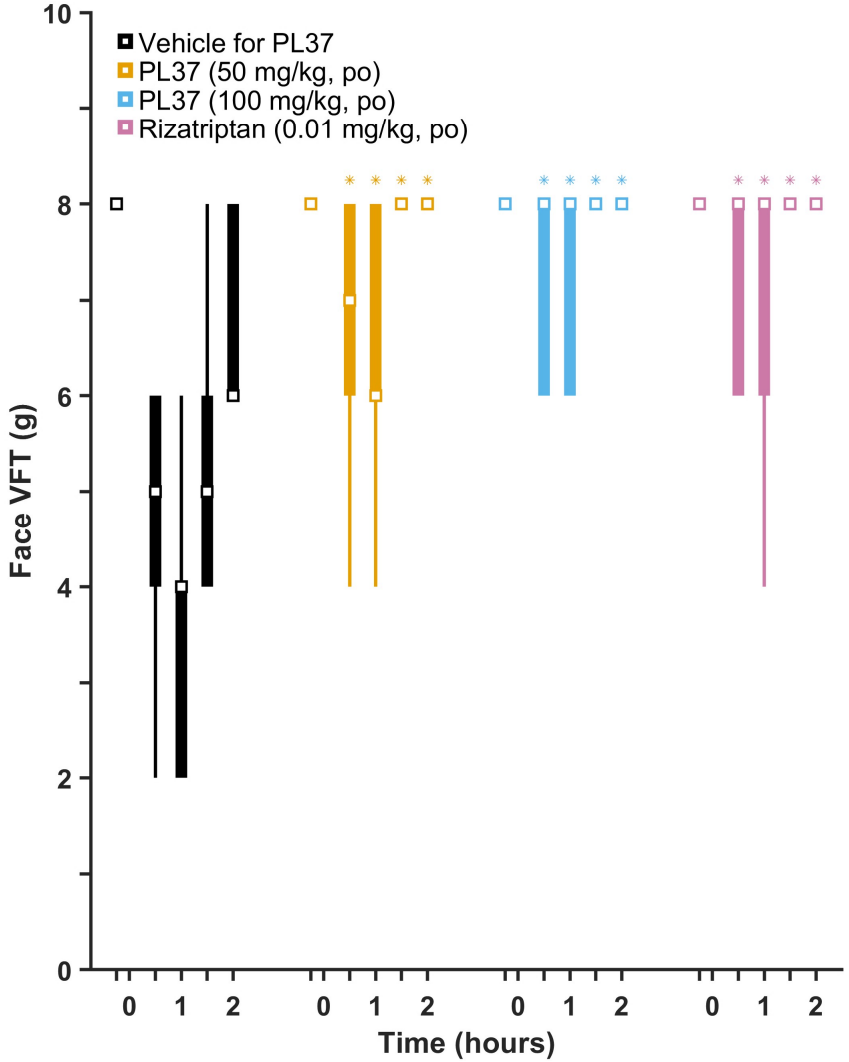


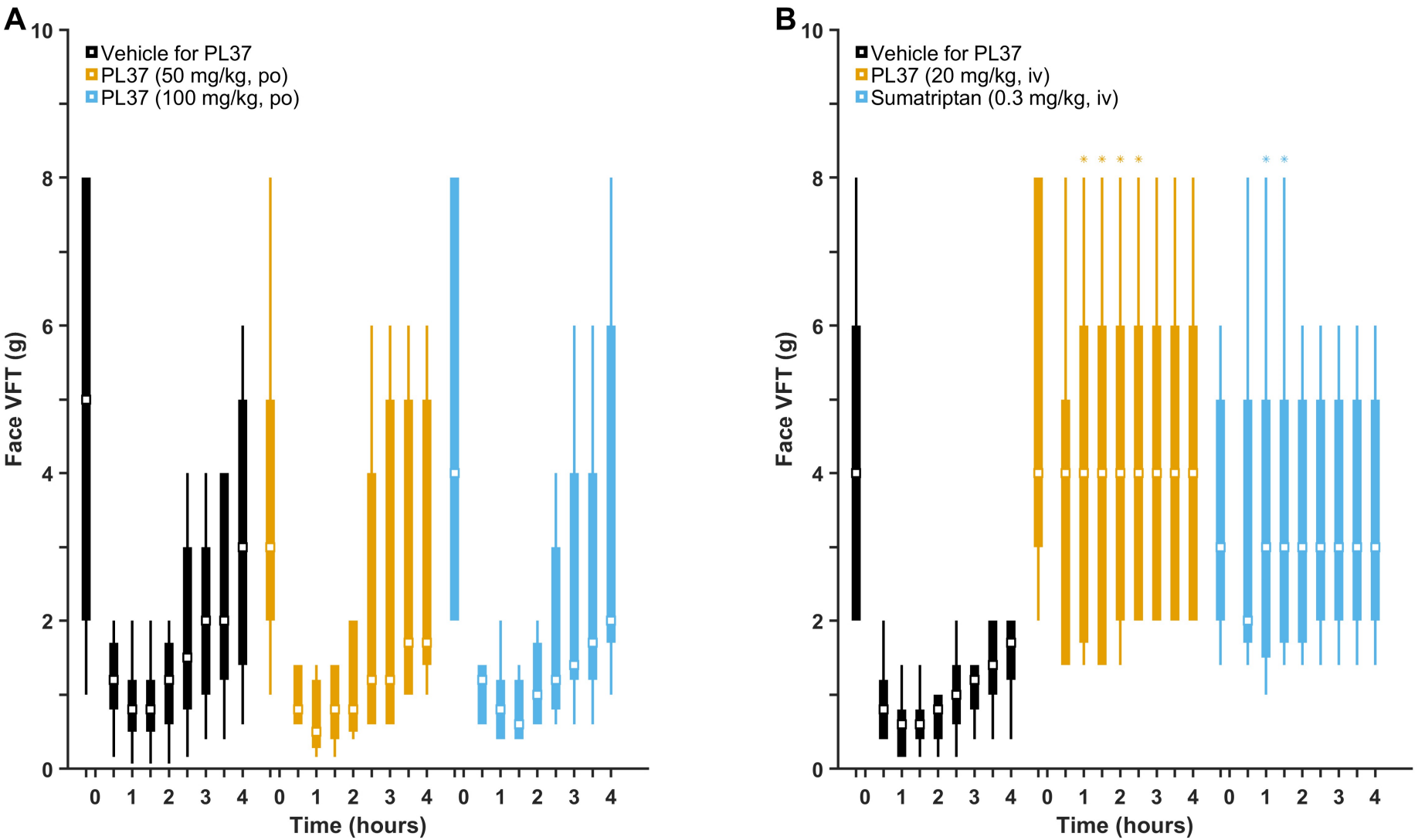
Supplementary Figure 1. Effect of PL37 on basal cephalic mechanical sensitivity in naive rats. (**A**) Time-course of von Frey withdrawal thresholds (VFT) of the face in vehicle-treated (n = 10) or PL37-treated (100 mg/kg, n = 10) rats after a single intraperitoneal (IP) injection of saline. PL37 or vehicle for PL37 were administered *per os* (po) 5 min before saline injection. Single oral administration of PL37 had no effect on cephalic mechanical withdrawal responses to mechanical stimulation. (**B**) Time-course of VFT of the face in vehicle-treated (n = 10) or PL37-treated (20 mg/kg, n = 10) rats after the 5th saline injection. PL37 or vehicle for PL37 were administered intravenously (iv) 5 min before the 5th saline injection. Single intravenous administration of PL37 had no effect on cephalic mechanical withdrawal responses to mechanical stimulation. (**C**) Time-course of VFT of the face following the 5th saline injection on the 5th day of treatment following repeated oral administration of vehicle for PL37 (n = 10) or PL37 (100 mg/kg, n = 5). PL37 or vehicle for PL37 were administered *per os*, 5 min before saline injection, once a day for five consecutive days. Daily treatment with PL37 had no effect on cephalic mechanical withdrawal responses to mechanical stimulation. **(D**) Time-course of VFT of the face over 4 days after each daily ISDN injection, following vehicle for PL37 (n = 10) or PL37 (100 mg/kg, n = 10) administration. PL37 or vehicle were administered daily, *per os*, 5 min before each ISDN injection. Daily oral treatment with PL37 had no effect on cephalic mechanical withdrawal responses to mechanical stimulation. Data are presented as box-and-whisker plots depicting median, interquartile interval, minimum and maximum. The red or green dashes represent the medians and the whiskers present the minimum and maximum values.

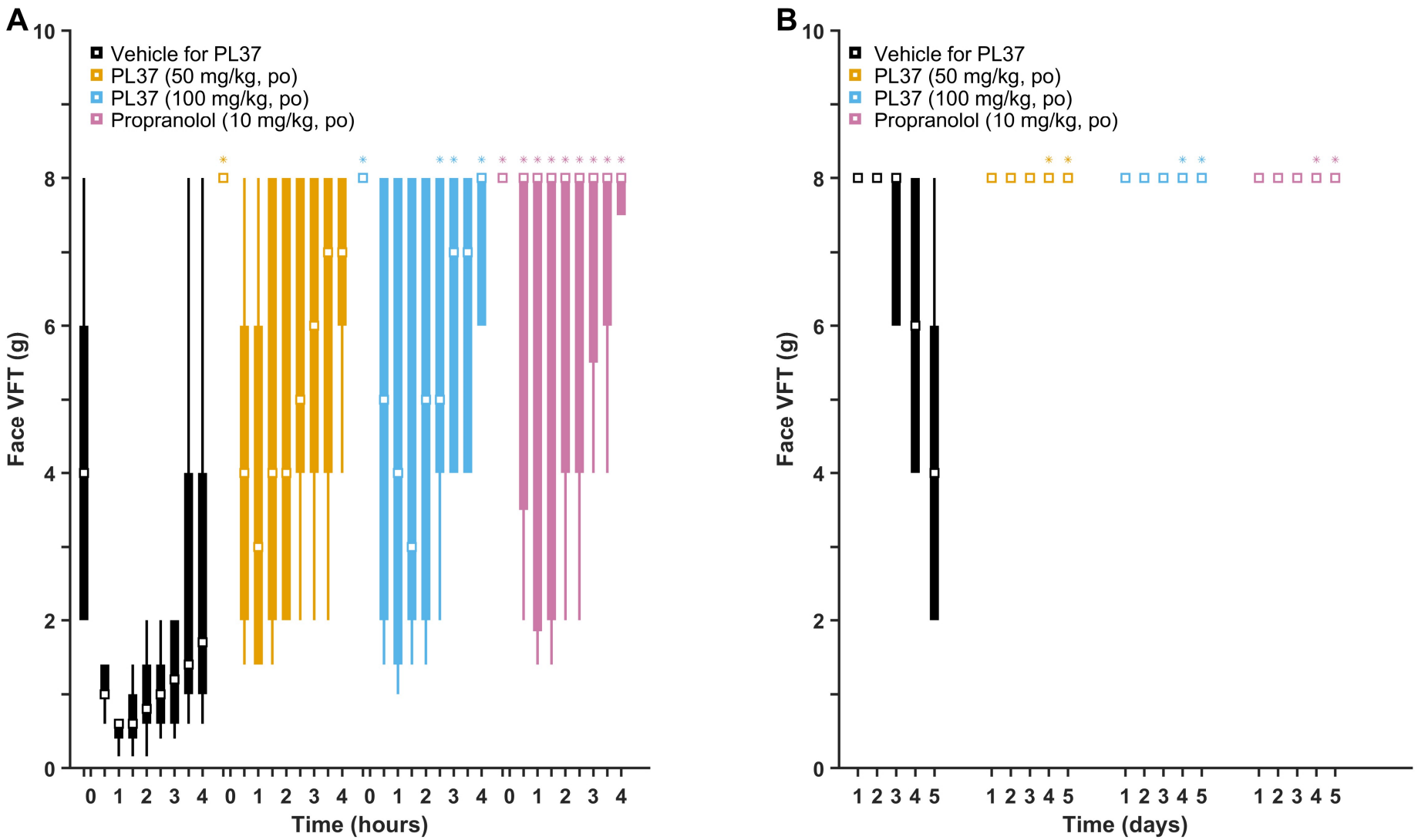
Supplementary Table 1. Summary of statistical analysis

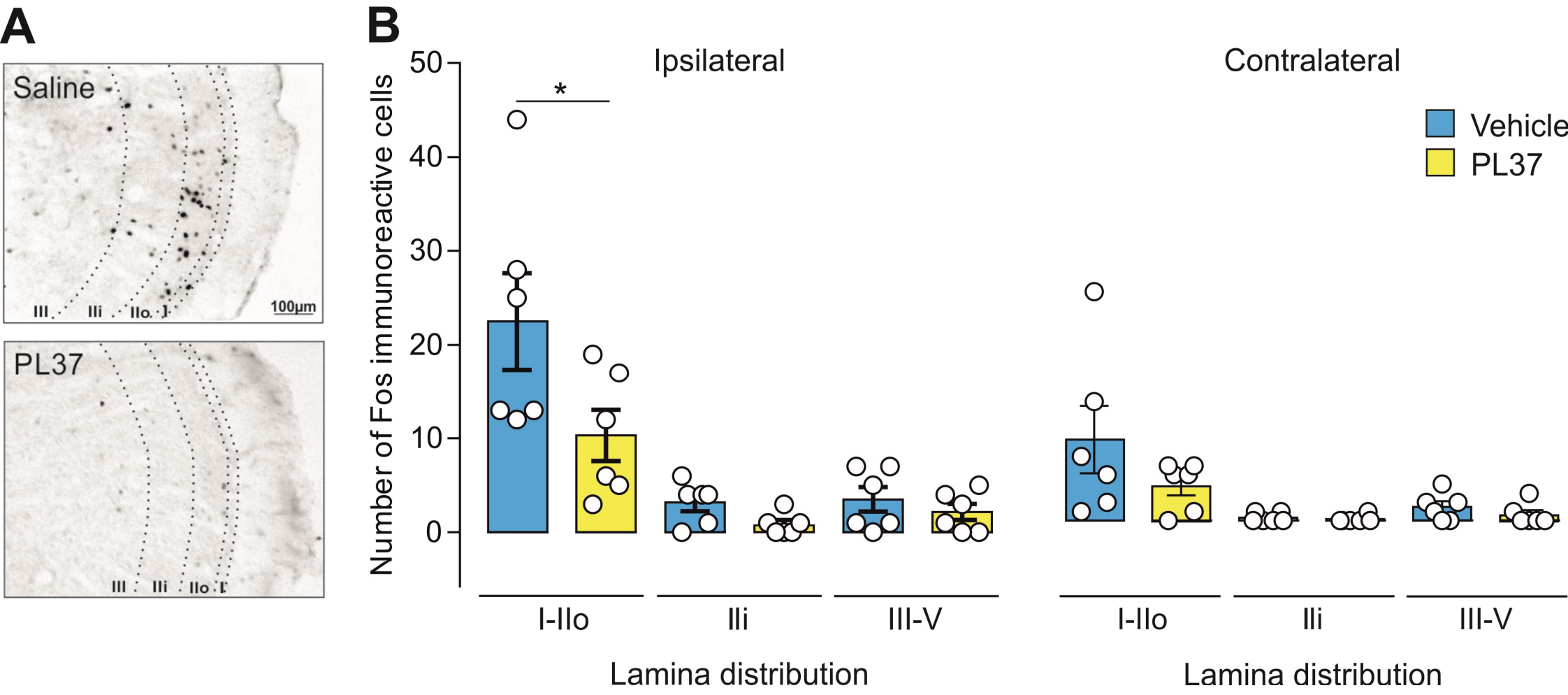
Figures	N/group	Analysis (post-hoc test)	Factors analyzed	Normality	F-ratios	*P* values
1	10-12	2-way ANOVA (Tukey)	Treatment x Time	< 0.05	Treatment F(3,38) =17.960	**<0.001**
					Time F(4,152) = 78.138	**<0.001**
					Interaction F(12,152) = 10.332	**<0.001**
2A	8	2-way ANOVA (Tukey)	Treatment x Time	< 0.05	Treatment F(2,21) = 0.061	0.940
					Time F(8,168) = 70.489	**<0.001**
					Interaction F(16,168) = 0.417	0.976
2B	8	2-way ANOVA (Tukey)	Treatment x Time	< 0.05	Treatment F(2,21) =7.131	**0.004**
					Time F(8,168) = 14.101	**<0.001**
					Interaction F(16,168) = 5.254	**<0.001**
3A	9-12	2-way ANOVA (Tukey)	Treatment x Time	< 0.05	Treatment F(3,37) = 10.394	**<0.001**
					Time F(8,296) =35.916	**<0.001**
					Interaction F(24,296) =1.354	0.128
3B	9-12	2-way ANOVA (Tukey)	Treatment x Day	< 0.05	Treatment F(3,36) = 46.713	**<0.001**
					Day F(4,144) = 117.313	**<0.001**
					Interaction F(12,144) = 25.732	**<0.001**
4 ipsilateral	6	2-way ANOVA (Tukey)	Treatment x Lamina	0.074	Treatment F(1,10) = 5.000	**0.049**
					lamina F(2,20) = 25.462	**<0.001**
					Interaction F(2,20) = 3.483	0.050
4 contralateral	6	2-way ANOVA (Tukey)	Treatment x Lamina	< 0.05	Treatment F(1,10) = 2.617	**0.137**
					lamina F(2,20) = 8.079	**0.003**
					Interaction F(2,20) = 1.274	0.301
Supplementary Fig 1A	10	2-way ANOVA (Tukey)	Treatment x Time	< 0.05	Treatment F(1,18) =0.223	0.643
					Time F(4,72) = 0.176	0.950
					Interaction F(4,72) = 0.176	0.950
Supplementary Fig 1B	10	2-way ANOVA (Tukey)	Treatment x Time	< 0.05	Treatment F(1,18) =1.000	0.331
					Time F(8,144) = 1.000	0.439

					Interaction F(8,144) = 1.000	0.439
Supplementary Fig 1C	10	2-way ANOVA (Tukey)	Treatment x Time	< 0.05	Treatment F(1,18) =0.474	0;500
					Time F(8,144) = 0.283	0.971
					Interaction F(8,144) = 0.283	0.971
Supplementary Fig 1D	10	2-way ANOVA (Tukey)	Treatment x Day	< 0.05	Treatment F(1,18) =0.000	1.000
					Time F(4,72) = 0.000	1.000
					Interaction F(4,72) = 0.000	1.000


Vehicle for PL37
PL37 (50 mg/kg, po)
PL37 (100 mg/kg, po)
Rizatriptan (0.01 mg/kg, po)
Face VFT (g)
10
8
6
4
2
0
0 1 2 0 1 2 0 1 2 0 1 2
Time (hours)


A
Vehicle for PL37
PL37 (50 mg/kg, po)
PL37 (100 mg/kg, po)
Face VFT (g)
Time (hours)
B
Vehicle for PL37
PL37 (20 mg/kg, iv)
Sumatriptan (0.3 mg/kg, iv)
Face VFT (g)
Time (hours)


A
Vehicle for PL37
PL37 (50 mg/kg, po)
PL37 (100 mg/kg, po)
Propranolol (10 mg/kg, po)
Face VFT (g)
0
2
4
6
8
10
0 1 2 3 4 0 1 2 3 4 0 1 2 3 4 0 1 2 3 4
Time (hours)
B
Vehicle for PL37
PL37 (50 mg/kg, po)
PL37 (100 mg/kg, po)
Propranolol (10 mg/kg, po)
Face VFT (g)
0
2
4
6
8
10
1 2 3 4 5 1 2 3 4 5 1 2 3 4 5 1 2 3 4 5
Time (days)


A
Saline
III
IIi
IIo
I
100µm
PL37
III
IIi
IIo
I
B
Ipsilateral
Contralateral
Vehicle
PL37
Number of Fos immunoreactive cells
50
40
30
20
10
0
*
I-IIo
IIi
III-V
Lamina distribution
I-IIo
IIi
III-V
Lamina distribution