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Sigma-1 receptor antagonism as a promising strategy for postoperative pain treatment: A study in laparotomized mice

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ABSTRACT

Postoperative pain remains a major clinical challenge, as it often persists despite analgesic treatment even with opioids. We studied the effects of sigma-1 receptor antagonists (BD-1063 and S1RA), administered alone or in combination with the μ-opioid morphine, on three key aspects of postoperative pain in mice with a transverse laparotomy: tactile allodynia, pain at rest, and movement-induced pain. Sigma-1 antagonism and morphine induced antiallodynic effects sensitive to peripheral opioid antagonism by naloxone methiodide, although only sigma-1 antagonism was sensitive to the sigma-1 agonist PRE-084. The antiallodynic effect of sigma-1 antagonism was also reversed by the μ-opioid antagonist cyprodime and by depletion of neutrophils, which express high levels of proopiomelanocortin, the precursor of the μ-opioid agonist ß-endorphin. Morphine, but not sigma-1 antagonism, reversed pain at rest, and none of the drugs tested improved movement-induced pain. Notably, the combination of S1RA and morphine at doses ineffective when administered alone, fully reversed tactile allodynia, pain at rest, and movement-induced pain, and in a manner sensitive to PRE-084 and naloxone methiodide, indicating the simultaneous participation of both sigma-1 and peripheral opioid receptors. Therefore, sigma-1 antagonism boosts the actions of endogenous opioid peptides from neutrophils only to reverse tactile allodynia, but when combined with morphine, it enhances peripheral opioid analgesia to reverse all aspects of postoperative pain. Finally, S1RA did not enhance morphine-induced inhibition of gastrointestinal transit or rewarding effects. Modulation of opioid analgesia by sigma-1 receptors might have potential clinical application to increase the therapeutic range of opioids in the treatment of postoperative pain.

1. Introduction

More than 300 million people undergo surgery each year worldwide [1]. More than half of patients experience moderate or severe pain in the immediate postoperative period, and it often persists after discharge despite analgesic treatment [2]. In fact, even opioids, the gold standard medication for the treatment of moderate to severe acute pain, often lack efficacy in postoperative pain patients [3,4]. In addition, opioid analgesics produce limiting side effects, which can be mediated both peripherally and centrally, including constipation and abuse potential, respectively. Regarding this latter effect, it is worth noting that the Department of Health and Human Services of USA declared in 2017 a

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public health emergency due to the high increase in deaths related to opioid use [5], which currently accounts for more than 200 deaths per day [6]. The postsurgical care is, for many patients, their first contact with opioid medication, and there is a growing literature suggesting that postsurgical patients are at increased risk for chronic opioid use [7]. Therefore, new therapeutic approaches are needed for postoperative pain management with adequate analgesic efficacy while reducing opioid use.

It has been described that sigma-1 receptor antagonism induces an amelioration of inflammatory pain hypersensitivity [8] through the potentiation of the effects of peripheral endogenous opioid peptides from immune cells (neutrophils) recruited to the inflamed site [9]. Surgical injury induces inflammation as part of the tissue repair process, and therefore postsurgical pain has an obvious inflammatory pain component. However, pain after surgery is not exclusively due to inflammation, as tissue injury itself is also an obvious pain inducer [10]. It is also worth noting that postoperative pain is complex and consists of enhanced cutaneous sensory hypersensitivity, but also on pain at rest (or "stimulus-independent" pain) and movement-induced pain (i.e., provoked or aggravated by movement) [3], and there are marked pharmacological differences in these measures. For instance, opioid drugs, which are highly efficacious in pain at rest, are relatively ineffective for movement-induced pain in both humans [3,4] and rodents [11]. It has never been explored whether the known enhancement of peripherally-mediated immune-driven opioid analgesia induced by sigma-1 antagonism can produce effective pain relief in any of these aspects of postoperative pain.

It is known that sigma-1 antagonism is also able to increase the antinociceptive effect of opioid drugs, such as morphine, at least in nociceptive pain conditions (i.e. in the absence of any sensitization of the nociceptive system) [12–15]. Interestingly, although the antinociceptive effect of opioid drugs has been classically attributed to central actions [16,17], peripheral opioid receptors might also contribute to opioid analgesia [18], and we previously reported that sigma-1 receptor antagonism enhanced peripheral opioid antinociception [14,15,19]. It is thought that the potentiation of the antinociceptive effect of opioid drugs by sigma-1 antagonism is not accompanied by an increase in opioid-induced adverse events [13–15]. Therefore, it could be suggested that sigma-1 antagonism might increase the therapeutic range of opioids. However, the effects of the association of opioids and sigma drugs is rarely studied in clinically relevant pain models, and in fact, this has never been explored in the context of postoperative pain, which is surprising considering that this is one of the major indications for opioid drug treatment, as mentioned above. Therefore, it would be interesting to test whether sigma-1 antagonism, at doses active in postoperative pain, could alter some characteristic opioid side effects.

We hypothesized that sigma-1 antagonism, either alone or in combination with morphine, could improve postoperative pain without producing or increasing typical opioid-mediated side effects. We aimed to study the effects of sigma-1 antagonism, alone or in combination with morphine, on tactile allodynia, pain at rest, and movement-induced pain in mice with postoperative pain, and to determine the peripheral contribution of the effects observed. Furthermore, we also tested the influence of sigma-1 antagonism on morphine-induced inhibition of gastrointestinal transit and on the rewarding properties of this opioid, as an index of the influence of sigma-1 inhibition on clinically relevant opioid side effects.

2. Material and methods

2.1. Experimental animals

The experiments were performed on CD-1 mice (Envigo, Horst, Netherlands) weighing 25–32 g (8–11 weeks old). We used an experimental laparotomy to model postoperative pain (as described in *"2.2 Surgical procedure"*). Considering that open abdominal surgeries are much more common in women than in men (~90 % vs 10 %) [20], this study was performed in female animals. Mice were housed in colony cages (10 per cage) in a temperature-controlled room (22 ± 2 ∘C) with an automatic 12-h light/dark cycle (08:00–20:00). A plastic tunnel was placed in each cage for environmental enrichment. The mice were fed a standard laboratory diet and had free access to tap water until the beginning of the experiments. The behavioral tests were performed during the light phase (9:00–15:00). Testing was conducted at random times throughout the estrous cycle. The mice were handled in accordance with international standards (European Communities Council directive 2010/63), and the experimental protocols were approved by regional (Junta de Andalucía) and institutional (Research Ethics Committee of the University of Granada) authorities. The study was conducted in accordance with the Animal Research: Reporting of *In Vivo* Experiments (ARRIVE) guidelines.

2.2. Surgical procedure

We used an experimental laparotomy to model postoperative pain, as previously described [11]. Mice were anesthetized with 3.5 % isoflurane (IsoVet®, B. Braun, Barcelona, Spain) in oxygen using an induction chamber. Then, anesthesia was maintained with 3 % isoflurane delivered via a nose cone during the procedure. The mice were placed in a supine position to shave the abdominal area. Then, the skin was prepared in a sterile manner with 70 % ethanol solution. Experiments were performed in mice with a transverse laparotomy which consisted of a 1.5 cm incision through the skin and muscle of the lower abdominal area, perpendicular to the midline, using surgical scissors. The surgical wound was stretched to expose the viscera and distend the damaged tissue. We perform single knot sutures to close the muscle and horizontal mattress sutures to close the skin. Sutures were done with Supramid® 5/0 non-absorbable polyamide multifilament thread using a TB15-CT 19-mm needle (Laboratorio Aragó, Barcelona, Spain). The sham procedure involved anesthesia, shaving and sterile preparation of the abdomen, with no incision.

2.3. Administration of drugs and antibodies for in vivo use

Two selective antagonists were used: BD-1063 (1-[2-(3,4-dichlorophenyl)ethyl]−4-methylpiperazine dihydrochloride, Tocris Cookson Ltd., Bristol, United Kingdom) and S1RA (4-[2-[[5-methyl-1-(2-naphthalenyl)−1H-pyrazol-3-yl]oxy]ethyl] morpholine, DC Chemicals, Shanghai, China). Both compounds are chemically different. Although both compounds show a basic amine group, BD-1063 is a rather simple phenetylpiperazine derivative, while S1RA has a more complex structure, featuring a pyrazole ring substituted by a naphthyl and a morpholine groups [21,22]. Both compounds have been repeatedly used as prototypical sigma-1 antagonists in previous studies [8,9,14,23,24]. S1RA is a particularly interesting sigma-1 antagonist, as it has even been tested in phase II clinical trials for the treatment of neuropathic pain [25,26]. In addition to these sigma-1 antagonists, we used the selective agonist PRE-084 (2-[4-morpholinethyl]1-phenylcyclohexanecarboxylate hydrochloride, Tocris).

We also used several opioid receptor ligands, which included the prototypic μ-opioid receptor agonist morphine hydrochloride (Biogen, Madrid, Spain), the centrally penetrant opioid antagonist naloxone hydrochloride and its quaternary derivative naloxone methiodide (both from Sigma-Aldrich, Madrid, Spain), which was used as a peripherally restricted opioid antagonist. Finally, the μ antagonist cyprodime, the κ antagonist nor-binaltorphimine and the δ antagonist naltrindole (all from Tocris Cookson Ltd.) were used as selective antagonists for the three major opioid receptor subtypes.

All drugs were dissolved in sterile physiological saline (0.9 % NaCl) and injected subcutaneously (s.c.) into the interscapular region in a volume of 5 mL/kg. We also tested the effect of combining the sigma-1

antagonist S1RA with morphine. In these experiments, morphine was immediately administered before S1RA. When the sigma-1 agonist PRE-084 or the opioid antagonists were used to reverse the analgesic effects of the sigma-1 antagonists or morphine, they were injected 5 min before the other drug solution. When testing combinations between two or more drugs, each drug was injected into a different area of the interscapular region. Injections with the same volume of sterile physiological saline were used as a control.

The dose of PRE-084 used (32 mg/kg), was selected based on our previous studies [8,9,27], as well as the doses of naloxone (1 mg/kg) and naloxone methiodide (2 mg/kg) [8,9,15]. The doses of cyprodime (15 mg/kg), norbinaltorphimine (10 mg/kg) and naltrindole (5 mg/kg) have been repeatedly reported to reverse opioid effects at the same doses used in our study [8,28,29].

All experiments were carried out in the immediate postoperative period, specifically at 3.5 h after surgery, to allow inflammation to develop [11]. All drugs or drug combinations were injected 1 h before the behavioral evaluation (2.5 h after surgery). Timings for drug administration in the experiments aimed to explore drug effects on gastrointestinal transit and the reward properties of drugs are described in *"2.5 Evaluation of gastrointestinal transit"* and *"2.6 Conditioned place preference"*.

To inhibit neutrophil infiltration, we administered an anti-Ly6G antibody (BE0075–1; Bio X Cell, Lebanon, NH, USA). This antibody was dissolved in physiological saline and injected intraperitoneally (i.p.) at a standard dose (8 μg/0.2 mL) [8,11]. Saline injections and the administration of a nonreactive isotype antibody (BE0089; Bio X Cell) were used as controls. The antibodies or saline were injected 24 h before laparotomy or sham procedure, and the behavioral effect determined 3.5 after surgery.

2.4. Postoperative pain measures

Laparatomized mice or sham controls were placed in the experimental room for a 1 h acclimatization period before the behavioral tests. We evaluated the mechanical threshold to test the development of cutaneous hypersensitivity, facial pain expressions as an index of pain at rest, and alterations in exploratory behavior as a measure of movement-induced pain. Each mouse was used only in one of these tests. The evaluators were blinded to the treatments in all cases.

2.4.1. Assessment of the mechanical threshold

Mechanical thresholds were tested following a previously described protocol [11,30], 3.5 h after laparotomy and 1 h after drug injection. Mice were placed in individual opaque plastic boxes (5 ×9 x 13 cm) on an elevated platform with a wire mesh floor. After a 1 h acclimation period, mechanical threshold was determined using a series of calibrated von Frey filaments (Touch-Test Sensory Evaluators; North coast Medical Inc., Gilroy, CA, USA) with bending forces ranging from 0.02 to 2 g (0.19–19.6 mN). Stimulations were made in the abdomen at approximately 2 mm away from the surgical incision. Filaments were applied three times for 1–2 s. Testing was initiated with the 0.4 g (3.92-mN) von Frey filament. The response to the filament was considered positive if immediate licking/scratching of the application site, sharp retraction of the abdomen, or jumping was observed. If there was a positive response, a weaker filament was used; if there was no response, a stronger stimulus was then selected. The 50 % threshold withdrawal was determined using the up and down method and calculated using the Updown reader software [31].

2.4.2. Evaluation of facial pain expressions

We used a convolutional neural network to score facial pain expressions from video recordings of the mice, using the procedure previously described [11,32]. Mice were placed individually in custom-made, black-walled test compartments (50 ×120 x 60 mm), on an elevated (1.1 m drop) mesh-bottomed platform with a 0.5-cm^2 grid. Test compartments were arranged in arrays composed by four of them, and located at the edge of the platform, with the fourth wall opened and facing to high-resolution (1440 ×1024 pixels) infrared video cameras (Kuman RPi camera, USA) connected to two infrared light emitting diodes (IR-LEDs) each. The arrangement of the test compartments encourages mice to look towards the visual cliff and hence face the cameras. Cameras were placed 25 cm away from the test compartments. Each camera can record simultaneously two mice and is controlled by a raspberry pi zero single-board computer (Kubii, France) that stores the video recordings into USB sticks, which can be transferred to a computer after the recording for analysis. This custom-made device is more deeply described in a previous study [32]. No experimenters were present in the testing room during the evaluation period.

Before the analysis of the facial pain expressions, we trained the DeepLabCut network [33] to recognize the ears, the eyes, and the nose of the animal. Only the frames where all those body parts were simultaneously detected (i.e. when the animal was facing at the camera) with high confidence (>0.9), were used for the analysis of the facial pain expressions, as previously described [11,32]. From these selected frames, a convolutional neural network based on Google's InceptionV3 architecture was trained with manually annotated images classified as "pain" or "no pain", following previously described procedures [32]. After the training was completed, the software was able to examine every frame from new video-recordings from mice with laparotomy and to give a probability value ranging from 0 (no pain) to 1 (pain).

All scripts used for Deeplabcut and InceptionV3 were written in Python (v3.5 Network training and scoring of the video recordings were completed remotely using an Ubuntu Linux computer with an NVIDIA 2080Ti graphics processing unit (GPU).

Recordings of the mice were made 3.5 h after surgery or sham procedure (see *"2.3 Administration of drugs and antibodies for in vivo use"*). The duration of the recordings was always set at 15 min.

2.4.3. Assessment of exploratory behavior

We measured vertical activity (time spent rearing), as it is known to be more sensitive in detecting pain-induced alterations than horizontal locomotion [34]. Exploratory activity was determined with an infrared detector (Med associated Inc., St. Albans, VT, USA) equipped with 48 infrared photocell emitters and detectors, according to a previously described method [11]. Animals were placed individually in transparent evaluation chambers (27.5 cm wide x 27.5 cm long x 20 cm high) and the time spent rearing was recorded during 30 min. Evaluations were made 3.5 h after surgery or sham procedure (see *"2.3 Administration of drugs and antibodies for in vivo use"*). No experimenters were present in the testing room during the evaluation period. Mice were tested only once to avoid habituation to the evaluation chambers, which markedly decreases their locomotor activity.

2.5. Evaluation of gastrointestinal transit

Gastrointestinal transit was evaluated according to previously described methods [14], with minor modifications. Briefly, the mice were fasted between 2 and 3 h with water available *ad libitum*. The water was then removed and the mice received intragastrically 0.3 mL of 0.5 % (w/v) activated charcoal (2–12 μm powder, Sigma-Aldrich) suspended in distilled water. 30 min after ingestion of the activated charcoal, mice were killed by cervical dislocation, and the small intestine was then isolated from the pyloric sphincter to the ileocecal junction and straightened to measure the distance traveled by the leading edge of the charcoal meal. Morphine or its vehicle was s.c. injected 1 h before the administration of the charcoal suspension. In the experiments aimed at testing the effect of S1RA in morphine-induced gastrointestinal transit inhibition, S1RA or saline were s.c. injected right after the administration of morphine or its vehicle.

2.6. Conditioned place preference

The conditioned place preference (CPP) was conducted using modifications of the method previously described [35,36]. The place preference conditioning apparatus (Cibertec SA, Madrid, Spain) consisted of two compartments separated by a guillotine door. One compartment was white and had a floor with a textured surface featuring diamond-shaped stripes, whereas the other was black with a rough floor. Each compartment measured 20 × 15 x 18 cm (length x width x height). Infrared beams were used to determine the time spent in each compartment. The apparatus was located in an isolation chamber (70 × 45 × 40 cm) with thick walls and an air exchange system to minimize the influence of environmental variables in the behavior of the mice.

The place preference conditioning procedure consisted of three phases: (1) the habituation period with a preconditioning test, (2) the conditioning period and (3) the postconditioning test. The habituation period took place during the first two days, when each mouse underwent a 15-min daily session, allowing free exploration of both compartments. On the third day, a preconditioning test was performed, with a 20- min baseline measurement to assess whether the mice exhibited a natural preference for either compartment. The conditioning phase lasted 4 consecutive days. Each day the animals experienced two 30-min conditioning sessions 6 h apart. In each session, the animals received morphine or its vehicle (saline) 3 min before being placed in the apparatus. Morphine administration was always paired with the white compartment, and the administration of its vehicle with the black compartment. Animals receiving morphine in the first daily session were injected with its vehicle in the second daily session, and *vice versa*. The order of compartment placement was counterbalanced, with some animals starting in the morning with the vehicle and others with the opioid. Animals treated only with saline were placed alternately in opposite compartments in the first and second daily sessions, also carefully counterbalancing animals starting in the white and black compartments. To study the influence of sigma-1 antagonism on the rewarding properties of morphine, S1RA or its solvent (saline) was administered 1 h before the administration of morphine or its solvent. S1RA injection was always paired with the white compartment.

The postconditioning test was conducted on day five, when the animals were in a drug-free state, to evaluate their preference for a particular compartment. The guillotine door was removed (as in the baseline measurement), and the mouse was placed in the apparatus with access to both chambers for 20 min. The amount of time spent in each compartment during the 20-min test period was automatically recorded. The difference in the time spent in the drug-paired *versus* the vehicle-paired compartment was used as a measure of the degree of conditioned place preference.

2.7. FACS analysis

Samples containing the incision site and surrounding tissue in the abdominal wall were harvested 3.5 h after laparotomy. Control samples were collected from sham mice. The mice were euthanized by cervical dislocation and abdominal tissue dissected and digested with collagenase IV (1 mg/mL, LS004188, Worthington, Lakewood, NJ, USA) and DNase I (0.1 %, LS002007, Worthington) for 1 h at 37 ºC with agitation. The samples were mechanically crushed over a 70-μm filter and refiltered into a tube with a cell strainer cap (pore size, 35 μm). The rat anti-CD16/32 antibody (1:100, 20 min, 553141, BD Biosciences, San Jose, CA, USA) was used for 20 min to block binding of Fc-γRII (CD32) and Fc-γRIII (CD16) to IgG. The cells were incubated for 30 min on ice with antibodies recognizing the hematopoietic cell marker CD45 (1:200, clone 30-F11, 103108, BioLegend, San Diego, CA, USA), the myeloid marker CD11b (1:100, 101227, BioLegend,), and the neutrophil-specific marker Ly6G (1:100, 127617, BioLegend); a viability dye (1:1000, 65–0865–14, Thermo Fisher Scientific, Massachusetts, USA) was included. A gating strategy was used to identify neutrophils (CD45 + CD11b+ Ly6G+) and macrophages/monocytes (CD45 + CD11b+ Ly6G-) and other immune cells (CD45 + CD11b-). The samples were washed twice in 2 % fetal bovine serum (FBS)/ phosphate buffered saline (PBS) (FACS buffer) after antibody incubation. Finally, they were fixed with 2 % paraformaldehyde for 20 min and washed twice in FACS buffer. On the next day, the samples were assayed on a BD FACSCanto II flow cytometer (BD Biosciences). Compensation beads were used as compensation controls. Fluorescence minus one (FMO) controls were included to determine the level of non-specific staining and autofluorescence associated with different cell subsets. All data were analyzed with FlowJo 2.0 software (Treestar, Ashland, OR, USA).

2.8. Neutrophil sorting

For the quantitative PCR analysis, neutrophils were sorted after FACS was performed on tissue samples from both laparotomized and sham animals 3.5 h after surgery. The tissue was digested in a collagenase IV and DNase I solution in FACS buffer for 1 h at 37 °C and 700 rpm. After mechanical dissociation and filtration through a 70 μm mesh, cell suspensions were centrifuged (450 rcf, 8 min), washed, and filtered again. Cells were stained with anti-CD45-PerCP5.5 (clone 30-F11, BioLegend, 103130), anti-CD11b-FITC (clone M1/70, BD Biosciences, 01714D), and anti-Ly6G-BV510 (clone 1A8, BioLegend, 127633). Cell viability was assessed using the Live/Dead stain (Thermo Fisher, L10119). Following 45 min of incubation, samples were analyzed on a BD LSRII flow cytometer (BD Biosciences). Neutrophils were identified as $CD45^{+}CD11b^{+}Ly6G^{+}$ and sorted using a BD FACSAria II cell sorter directly into lysis buffer for RNA extraction.

2.9. RNA isolation and quantitative PCR

Total RNA was extracted from sorted neutrophils using a magnetic bead-based isolation protocol. Cells were lysed in buffer containing dithiothreitol (Sigma-Aldrich) and processed through binding, DNase digestion, and washing steps. RNA was eluted in RNase-free water and quantified using a Nanodrop spectrophotometer. Reverse transcription was performed using the LunaScript RT SuperMix Kit (E3010, New England Biolabs, Ipswich, MA, USA).

Quantitative PCR (qPCR) was conducted using LunaScript qPCR Master Mix (New England Biolabs, M3003S) in 96-well plates. The qPCR program included an initial denaturation at 95 °C for 60 s, followed by 40 cycles of 95 °C for 15 s and 60 °C for 30 s.

We analyzed the transcripts for proopiomelanocortin (POMC), with a forward primer 5′-GCT GGC ATC CGG GCT TGC AAA CT-3′ and reverse primer 5′-AGC AAC GTT GGG GTA CAC CTT-3′, (expected band size 318 bp), prodynorphin (PDYN), forward primer 5′-GTC CAG TGA GGA TTC AGG ATG GG-3′ and reverse primer 5′-GAG CTT GGC TAG TGC ACT GTA GC-3′, (expected band size 209 bp), and preproenkephalin (PENK), with a forward primer 5′-GAC GAA GAC ATG AGC AAG A-3′ and reverse primer 5′-TCG TCA GGA AGA ATG AGG TAA C-3′, (expected band size 516 bp). Expression was normalized to the housekeeping gene actin, with a forward primer 5′-ATC AGC AAG CAG GAG TAC GA-3′ and reverse primer 5′-GCC ATG CCA ATG TTG TCT CT-3′, (153 bp fragment). Product sizes were verified and imaged using Bio-Rad imaging system and Image Lab 6 software. Basic adjustments to contrast and brightness were applied uniformly to enhance band visibility.

2.10. Data analysis

Data were analyzed using GraphPad Prism 8 (GraphPad Software, Boston, USA). Results are shown as the mean ± SEM. Behavioral and *in vitro* determinations were always made in at least three separate days. Statistical analyses were performed using one-way analysis of variance (ANOVA), with the exception of CPP experiments that were analyzed using repeated measures ANOVA when comparing between pre and post conditioning measures. Before performing ANOVA, the Shapiro-Wilk

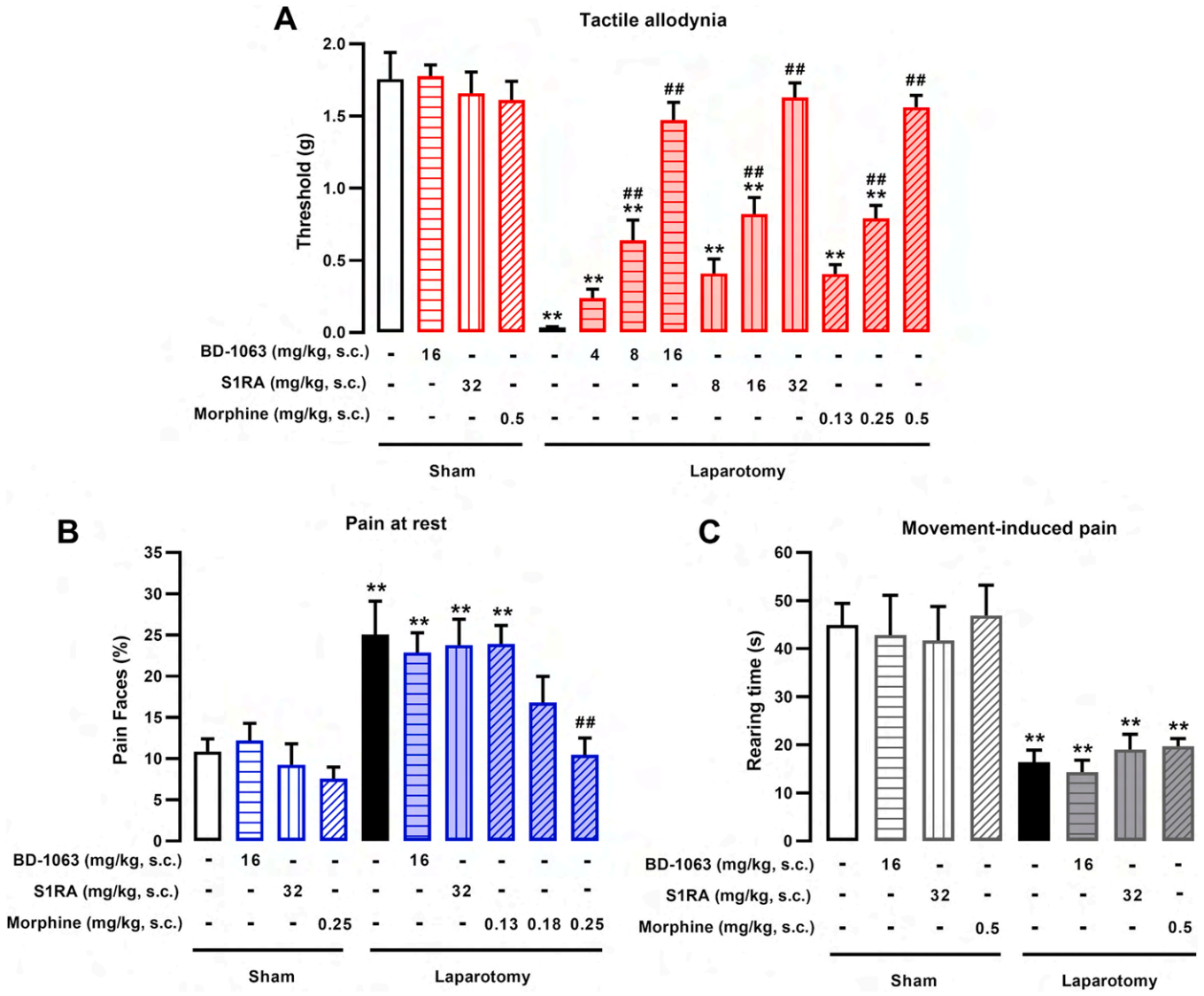

Fig. 1. Effects of sigma-1 antagonists and morphine on different aspects of postoperative pain in laparotomized mice. The results represent the effects of the subcutaneous (s.c.) administration of S1RA, BD1063, morphine, or vehicle (saline) in (A) tactile allodynia (reductions in mechanical withdrawal threshold in abdominal area), (B) pain at rest (presence of facial pain expressions), and (C) movement-induced pain (reductions in time spent rearing). Behavior was evaluated 3.5 h after lapatoromy or sham procedure. Each bar and vertical line represent the mean ± SEM of the results from (A) 7–8, (B) 7–11, and (C) 7–10 mice. One-way ANOVA revealed significant differences in A ($F_{13,91} = 50.27$; $p < 0.001$), B ($F_{10,92} = 8.99$; $p < 0.001$) and C ($F_{7,61} = 10.53$; $p < 0.001$). Student-Newman-Keuls post hoc test found significant differences between the values obtained in sham mice treated with saline (white bars) and the other experimental groups (**$p < 0.01$), and between the values obtained in laparotomized mice treated with saline (black bars) or the drugs tested (##$p < 0.01$). The original experimental data shown in A and C were square-rooted, and data in B were log-transformed to meet the ANOVA assumptions.

test was used to assess whether data sets were normally distributed, and the Brown-Forsythe test was used to assess equality of variances. If data did not meet these assumptions of the ANOVA, they were mathematically transformed. Mathematical transformations were made depending on the type of data. Data from facial pain expressions and FACs analyses were log-transformed, and data from von Frey testing or movement-induced pain were square-rooted. In some CPP experiments, data were transformed using the function $(600 + x)^2$. In all cases, we ensured values meet the ANOVA assumptions. The Student–Newman–Keuls post-test was used in all cases. Differences between means were considered significant when $p < 0.05$.

3. Results

3.1. The effects of sigma-1 antagonists and morphine differ depending on the postoperative pain readout being evaluated

We studied the effects of the sigma-1 antagonists BD-1063 and S1RA, and the opioid drug morphine, used as a standard analgesic, on three aspects of postoperative pain in mice with a transverse laparotomy: tactile allodynia, pain at rest and movement-induced pain.

As shown in Fig. 1A, laparotomized mice treated with vehicle control showed a substantially reduced mechanical threshold (black bar) in comparison to uninjured animals (white bar), denoting the development of tactile allodynia. The administration of BD-1063 (4–16 mg/kg, s.c.) or S1RA (8–32 mg/kg, s.c.) induced a dose-dependent antiallodynic effect, with a full reversion of tactile allodynia at the highest doses tested. Morphine (0.13–0.5 mg/kg, s.c.) also dose-dependently and fully

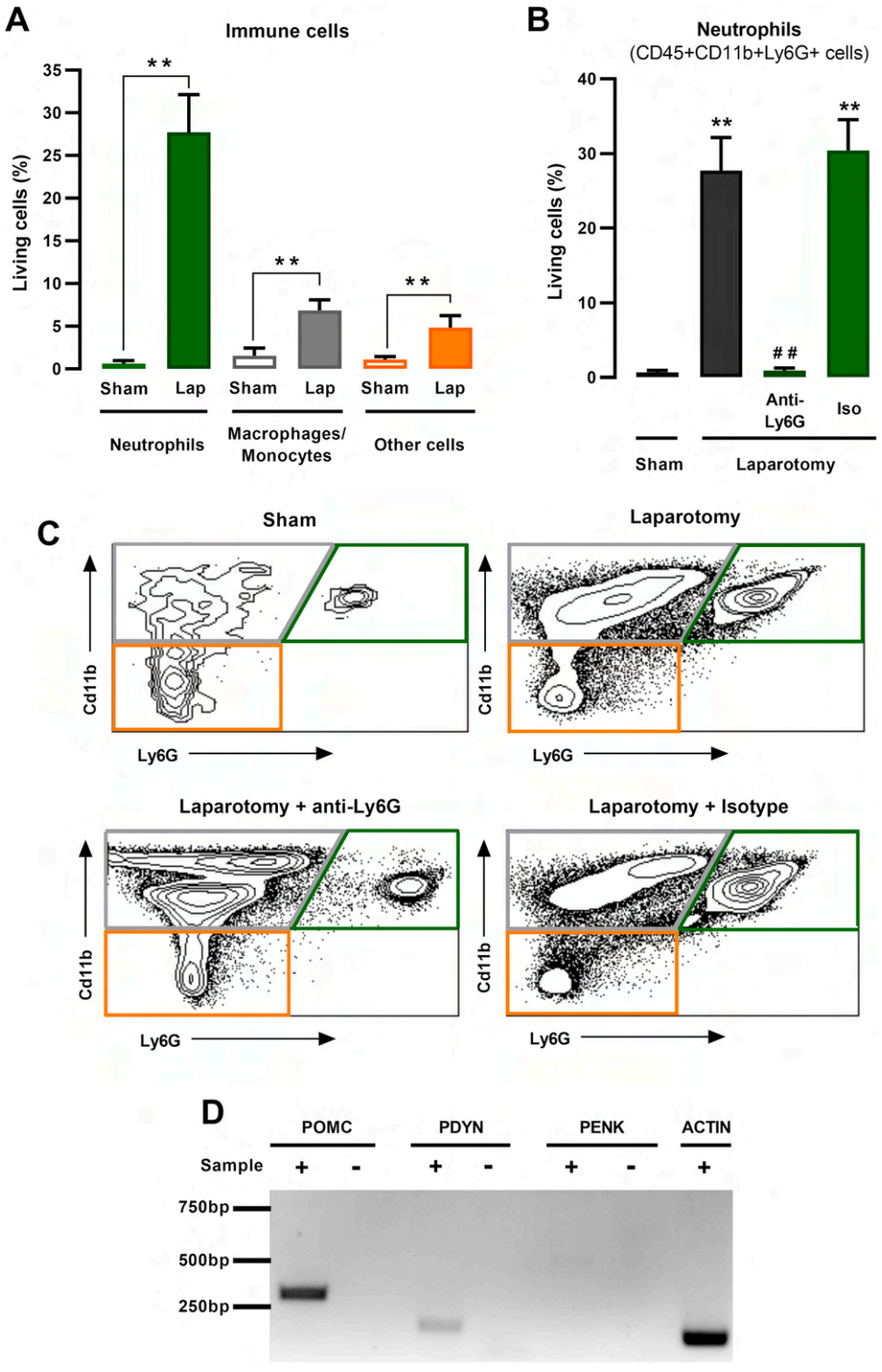

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Fig. 2. Neutrophils are the predominant immune cell at the injured site and harbor endogenous opioid peptides. (A) Quantification of neutrophils (CD45 +CD11b+Ly6G+), macrophages/monocytes (CD45 +CD11b+Ly6G-), and other hematopoietic (CD45 +) cells with respect to number of living cells in abdominal wall samples from naïve and laparotomized mice. (B) Effect of the intraperitoneal (i.p.) administration of saline, anti-Ly6G, or the isotype control (both at 8 μg) on neutrophil load in samples from sham-operated or laparotomized mice. (C) Representative FACS diagrams, with gating from CD45 + cells, showing neutrophils (CD11b+Ly6G+, green), macrophages/monocytes (CD11b+Ly6G–, grey) and other hematopoietic cells (CD11b–Ly6G–, orange) in sham-operated and laparotomized mice treated intraperitoneally (i.p.) with saline, anti-Ly6G, or the isotype control. (D) Electrophoresis of the qPCR products of the transcripts for proopiomelanocortin (POMC), prodynorphin (PYDN) and preproenkephalin (PENK), and β-actin as a housekeeping control from neutrophils sorted after 3.5 h from the abdominal wall of laparotomized animals. Ultrapure water was used as a negative (no template) control. Each bar and vertical line represent the mean ± SEM of the values obtained in (A) 10–12 and (B) 7–12 samples, with each sample taken from a single animal. One-way ANOVA revealed significant differences in A ($F_{5,59}$ = 23.61; $p < 0.001$) and B ($F_{3,37}$ = 71.43; $p < 0.001$). (A and B) Student-Newman-Keuls post hoc test found significant differences between the values obtained in sham-operated mice (white bars) and laparotomized mice (**$p < 0.01$), and (B) statistically significant differences between the values obtained in laparotomized mice treated with saline (black bar) or anti-Ly6G (##$p < 0.01$). The original experimental data shown in A and B were log-transformed to meet the ANOVA assumptions.

reversed tactile allodynia. In fact, the opioid was much more potent than the sigma-1 antagonists tested, reaching a full reversion of allodynia with a dose as low as 0.5 mg/kg. The highest doses tested of these three drugs in laparotomized animals did not alter the mechanical threshold in sham-operated animals (Fig. 1A) and, therefore, these compounds (at the doses tested) induce a full antiallodynic effect without altering nociceptive thresholds to mechanical stimulation.

We also tested the effects of these drugs in pain at rest (see Fig. 1B). Laparotomized animals treated with vehicle control (black bar) displayed a higher proportion of "pain faces" during the observation period compared to uninjured mice (white bar). The administration of either BD-1063 or S1RA, at doses shown above to exert a maximal antiallodynic effect (16 and 32 mg/kg, s.c., respectively) was unable to decrease the percentage of time laparotomized animals were displaying facial pain expressions. In contrast, morphine (0.13–0.25 mg/kg, s.c.) dose-dependently and completely reversed this measure of pain at rest. It is worth noting that morphine needed just 0.25 mg/kg to fully reverse the increase in facial pain expressions induced by laparotomy, indicating a high sensitivity of this pain outcome to the effect of this opioid. The highest doses tested of BD-1063, S1RA or morphine in laparotomized animals did not alter the facial expressions in sham-operated animals (Fig. 1B), and therefore, these drugs do not induce nonspecific alterations of the facial expression that could confound our results.

Finally, we also evaluated the effect of the sigma-1 antagonists and morphine in movement-induced pain (Fig. 1C). Laparotomized mice treated with the vehicle control (black bar) showed a marked decrease in the time spent rearing in comparison to uninjured animals (white bar). The sigma-1 antagonists BD-1063 and S1RA (16 or 32 mg/kg, s.c., respectively) failed to induce a significant reversion of this aspect of postoperative pain. Interestingly, even morphine, at doses equal or higher than those used above to reverse tactile allodynia and pain at rest (0.5 mg/kg, s.c.), was unable to ameliorate the motor impairment in laparotomized mice. None of the drugs (at the doses tested) interfered with locomotor activity in uninjured animals (Fig. 1C), and therefore the lack of drug effect in mice with laparotomy cannot be explained by locomotor alterations masking possible analgesic-like effects.

In summary, although both sigma-1 antagonists and morphine were able to reverse postoperative tactile allodynia, only morphine was able to decrease pain at rest and none of the drugs tested were able to reverse movement-induced pain. Therefore, it is clear each pain outcome examined has a particular drug sensitivity, indicating that tactile allodynia, pain at rest and movement-induced pain are not equivalent evaluation measures of postoperative pain.

3.2. Sigma-1 antagonism reverses postoperative tactile allodynia through the activation of the peripheral opioid system

We aimed to test whether the previously described immune-driven opioid analgesia induced by sigma-1 antagonism could participate in the antiallodynic effects induced by BD-1063 and S1RA in laparotomized animals. Neutrophils are myeloid cells known to express high levels of Ly6G (CD45 +CD11b+Ly6G+), and constitute the majority (about 75 %) of immune cells recruited in the injured abdominal wall of laparotomized animals (3.5 hr after surgery), while macrophages/monocytes (CD45 +CD11b+Ly6G– cells) or other hematopoietic cells (CD45 +CD11b–Ly6G–) were also present but to a lesser extent, as shown in Fig. 2A. Ly6G can be targeted for neutrophil depletion using the *in vivo* administration of an anti-Ly6G antibody (8 μg, i.p.), and produced a very substantial reduction in neutrophils in the abdominal wall from laparotomized animals, while the injection of the same dose of an isotype control antibody was unable to alter neutrophil content of the injured tissue (Fig. 2B). Therefore, this approach is suitable to study the role of neutrophils in the effects of sigma-1 antagonism in subsequent experiments. Representative FACS diagrams illustrating the gating strategy can be found in Fig. 2C. We also sorted neutrophils to evaluate their content by qPCR, assessing the expression of the precursors of the most characteristic endogenous opioid peptides. These included the transcripts for POMC, which is the precursor of ß-endorphin (a μ-opioid agonist), as well as the transcripts for PYDN and PENK, which are the precursors of dynorphin (a δ-opioid agonist) and enkephalins (κ-opioid agonists), respectively. As shown in Fig. 2D, we were able to clearly amplify a ≈ 300-bp band corresponding to POMC, whereas only a weak ≈ 200-bp band for PDYN was detected, and a very faint ≈ 500-bp band was amplified for PENK. Actin (≈150-bp) was used as a reference housekeeping transcript. As a negative (no template) control, ultrapure water was used. We did not find any appreciable band in any lane using water instead of sample. The band sizes are in agreement with the predicted size for each transcript (see Methods for details). The uncropped image of the gel can be found in Fig. S1.

We next investigated the impact of the opioid system in the antiallodynic effects induced by sigma-1 antagonism. We tested whether both sigma-1 and peripheral opioid receptors were involved in the antiallodynic effects of sigma-1 antagonism in laparotomized animals. To do that, we s.c. administered the sigma-1 antagonists BD-1063 or S1RA alone, as well as in combination with PRE-084, a known sigma-1 agonist, or naloxone methiodide, a peripherally-restricted analog of the prototypical opioid antagonist naloxone. The administration of PRE-084 (32 mg/kg, s.c.) completely reversed the effect of both BD-1063 and S1RA on laparotomy-induced tactile allodynia (Fig. 3A). These results support the selectivity of the effects induced by the sigma-1 antagonists on the receptor. The ameliorative effects induced by BD-1063 or S1RA on postoperative tactile allodynia were also reversed by naloxone methiodide (2 mg/kg, s.c.) (Fig. 3A), which suggests the involvement of the peripheral opioid system in the antiallodynic effects induced by sigma-1 antagonism after surgery. To identify which opioid receptor subtype was participating in the antiallodynic effects induced by sigma-1 antagonism, we used antagonists with selectivity for the opioid receptor subtypes. As shown in Fig. 3B, the antiallodynic effect induced by S1RA was abolished by the μ-opioid antagonist cyprodime (15 mg/kg, s.c.), but not the δ-opioid antagonist naltrindole (10 mg/kg, s.c.) or the κ-opioid antagonist nor-binaltorphimine (5 mg/kg, s.c.). Therefore, our results suggest that the effect of sigma-1 antagonism on laparotomy-induced tactile allodynia involves the activation of peripheral μ-opioid receptors, but not other opioid receptor subtypes. We then tested the

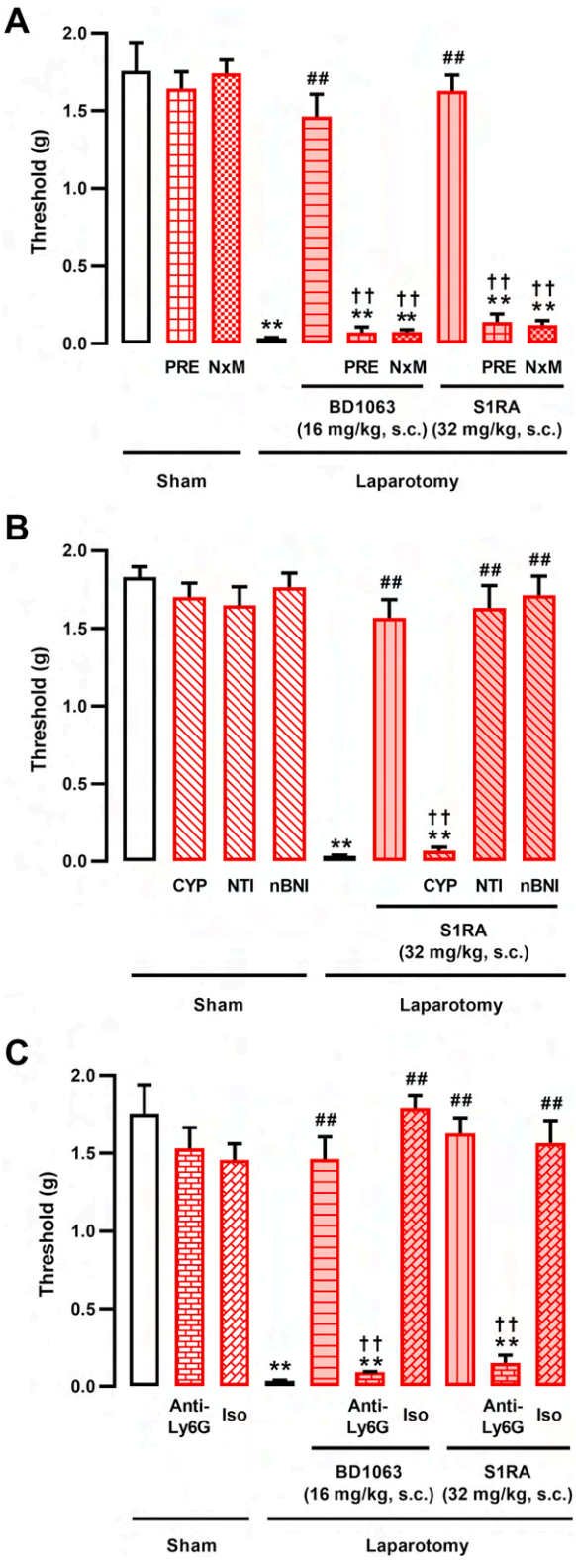

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Fig. 3. The antiallodynic effect of sigma-1 antagonism in laparotomized mice depends on the peripheral opioid system. The results represent the effects on tactile allodynia (reductions in mechanical withdrawal threshold in abdominal area) of the subcutaneous (s.c.) administration of sigma-1 antagonists (S1RA and BD1063) or their vehicle (saline) in combination with (A) PRE-084 (PRE, 32 mg/kg, s.c.), naloxone methiodide (NxM, 2 mg/kg, s.c.) or their vehicle, (B) cyprodime (CYP, 15 mg/kg, s.c.), naltrindole (NTI, 5 mg/kg, s.c.), nor-binaltorphimine (nBNI, 10 mg/kg, s.c.), or their vehicle, and (C) the intraperitoneal (i.p.) administration of saline, anti-Ly6G, or the isotype control antibody (both at 8 μg). Allodynia was evaluated 3.5 h after lapatoromy or sham procedure. (A-C) Each bar and vertical line represent the mean ± SEM of the values obtained in 7–11 mice. One-way ANOVA revealed significant differences in A ($F_{9,76}$ = 205.60; $p < 0.001$), B ($F_{8,66}$ = 130.20; $p < 0.001$) and C ($F_{9,62}$ = 175.40; $p < 0.001$). Student-Newman-Keuls post hoc test found significant differences between the values obtained in sham-operated mice treated with saline (white bars) and the other experimental groups (**$p < 0.01$), between the values obtained in laparotomized mice treated with saline (black bars) or the drugs/antibodies tested (##$p < 0.01$), and between the values obtained in laparotomized mice treated with a sigma-1 antagonist alone or in combination with PRE-084, the opioid antagonists, or the anti-Ly6G (††$P < 0.01$). The original experimental data shown in A-C were square-rooted to meet the ANOVA assumptions.

effects of neutrophil depletion on the antiallodynic effect induced by BD-1063 and S1RA, and found that treatment with the anti-Ly6G antibody (8 μg, i.p.) was able to abolish the effect of sigma-1 antagonism, while treatment with the same dose of an isotype control antibody showed no effect (Fig. 3C).

Finally, we also tested the effect of the sigma-1 agonist PRE-084, the opioid antagonists (naloxone methiodide, cyprodime, naltrindole and nor-binaltorphimine) and the antibodies used for *in vivo* neutrophil depletion experiments (anti-Ly6G and its isotype control antibody) in sham mice, and none of these treatments modified mechanical threshold of uninjured mice (Fig. 3A-C). Therefore, these treatments do not induce nonspecific alterations of the mechanical threshold that could confound our results.

Altogether, our results suggest that sigma-1 antagonism induces antiallodynic effects in laparotomized mice through the actions of endogenous opioid peptides (such as ß-endorphin which is a POMC-derived peptide), produced by neutrophils in the surgical incision site, activating peripheral μ-opioid receptors.

3.3. *Morphine effects in laparotomized mice: sensitivity to peripheral opioid antagonism and sigma-1 agonism*

We examined the combinatory effects of several drugs with doses of morphine able to fully reverse tactile allodynia (0.5 mg/kg, s.c.) and pain at rest (0.25 mg/kg, s.c.) in laparotomized animals. While the peripherally restricted opioid antagonist naloxone methiodide (2 mg/kg, s.c.) was able to fully reverse the antiallodynic effect of morphine (Fig. 4A), it did not alter the effect of the opioid analgesic in the decrease of facial pain expressions (Fig. 4B). However, the centrally penetrant antagonist naloxone (1 mg/kg, s.c.) completely reversed the effect of morphine in this latter aspect of postoperative pain (Fig. 4B). Therefore, peripheral opioid receptors appear to be more relevant for the anti-allodynic effect of morphine than for its effect in pain at rest.

We also tested the combinatory effect of PRE-084 (32 mg/kg, s.c.) with morphine in animals with laparotomy. Although this dose of the sigma-1 agonist was able to reverse the effects of BD-1063 and S1RA (see the preceding section), it was unable to modify the effects of morphine in either tactile allodynia (Fig. 4A) or pain at rest (Fig. 4B), indicating that morphine and the sigma-1 antagonists do not induce their anti-nociceptive effects through the same mechanisms.

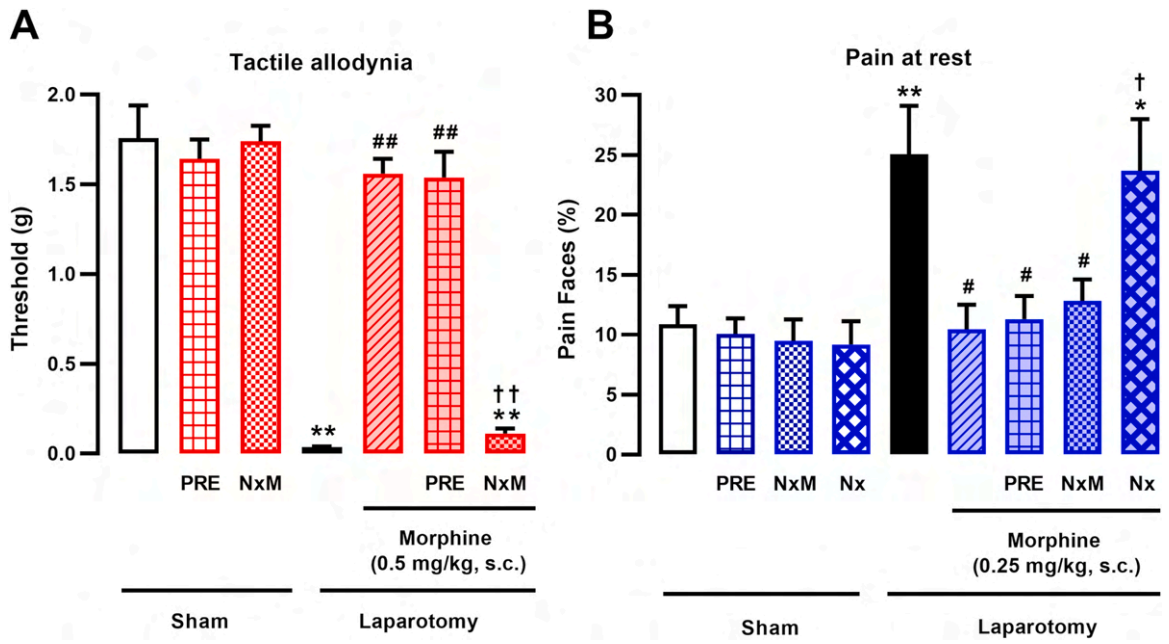

Fig. 4. Contribution of sigma-1 and peripheral opioid receptors to the effects of morphine on tactile allodynia and pain at rest in laparotomized mice. The results represent (A) the effects of subcutaneous (s.c.) administration of morphine (0.5 mg/kg) alone or in combination with PRE-084 (PRE, 32 mg/kg), naloxone methiodide (NxM, 2 mg/kg), or the vehicle on tactile allodynia (reductions in mechanical withdrawal threshold in abdominal area), and (B) the effects of the s.c. administration of morphine (0.25 mg/kg) alone or with PRE (32 mg/kg), NxM (2 mg/kg), naloxone (Nx, 1 mg/kg), or the vehicle in pain at rest (presence of facial pain expressions). Allodynia was evaluated 3.5 h after lapatoromy or sham procedure. Each bar and vertical line represent the mean ± SEM of the values obtained in (A) 8–9 and (B) 7–11 mice. One-way ANOVA revealed significant differences in A ($F_{6,50} = 167.60$; $p < 0.001$) and B ($F_{8,66} = 4.62$; $p < 0.001$). (A and B) Student-Newman-Keuls post hoc test found significant differences between the values obtained in sham mice treated with the solvent of the drugs (white bar) and the other experimental groups (*$p < 0.05$, **$p < 0.01$), between the values obtained in laparotomized mice treated with saline (black bar) or the drugs tested (#$p < 0.05$, ##$p < 0.01$), and between the values obtained in laparotomized mice treated with morphine alone or with the opioid antagonists (†$p < 0.05$; ††$p < 0.01$). The original experimental data shown in A were square-rooted, and data in B were log-transformed to meet the ANOVA assumptions.

Finally, we tested the effect of PRE-084 and the opioid antagonists naloxone or naloxone methiodide in the mechanical threshold and facial expressions of sham mice, and found that drug treatments did not alter behavioral outcomes in uninjured mice (Fig. 4A and B). Therefore, the effects of PRE-084 or the opioid antagonists tested in our experiments are not attributable to nonspecific alterations of mechanical thresholds or facial expressions of the mice.

3.4. *Sigma-1 antagonism enhances the peripheral effects of morphine to reverse postoperative pain*

We investigated the combinatory effects of the sigma-1 antagonist S1RA and morphine on the three pain outcomes examined. Drug doses were selected based on our previous results (Fig. 1A-C), using doses of S1RA and morphine that were ineffective when administered separately against tactile allodynia (S1RA 8 mg/kg + morphine 0.13 mg/kg, Fig. 5A), pain at rest (S1RA 16 mg/kg + morphine 0.13 mg/kg, Fig. 5B) and movement-induced pain (S1RA 16 mg/kg + morphine 0.5 mg/kg, Fig. 5C). We found that the combination of these ineffective doses of both drugs induced a full reversal of all three pain-related outcomes in laparotomized mice, and without altering mechanical threshold, facial expressions, or motor activity in sham-operated animals (Fig. 5A-C).

We next tested the combination of these treatments with PRE-084 (32 mg/kg, s.c.) or naloxone methiodide (2 mg/kg, s.c.), and we found that both sigma-1 agonism and peripheral opioid antagonism were able to fully abolish the effects of the associations of S1RA with morphine in tactile allodynia (Fig. 5A), pain at rest (Fig. 5B) and movement-induced pain (Fig. 5C), without altering normal behaviors in sham mice (Fig. 5A-C).

In summary, S1RA can enhance morphine-dependent effects in the three pain-related outcomes examined in laparatomized animals, and these effects require both sigma-1 and peripheral opioid receptors.

3.5. *Morphine-induced side effects (gastrointestinal transit inhibition and rewarding effects) are not enhanced by S1RA*

Since we observed a marked potentiation of the analgesic effect of morphine when combined with the sigma-1 antagonist S1RA, we aimed to test whether this potentiation of morphine effects was limited to analgesia or could be extended to non-analgesic (side) effects. Specifically, we tested the effects of S1RA in morphine-induced gastrointestinal transit inhibition and in the rewarding properties of the opioid drug.

We used the distance traveled by a charcoal meal in the intestine as an index of gastrointestinal transit. The charcoal meal traveled through approximately 30 cm of small intestine in animals treated with the vehicle control. Morphine (0.5–4 mg/kg, s.c.) administration induced a dose-dependent decrease in gastrointestinal transit (Fig. 6A). We then selected two doses of morphine (0.5 and 4 mg/kg, s.c.) to study the effect of the association with S1RA. The sigma-1 antagonist was administered at 16 mg/kg (s.c.) for these experiments, as this is the maximum dose of the sigma-1 antagonist used in the experiments related to the potentiation of morphine-induced analgesia described in the preceding section. S1RA did not alter gastrointestinal transit in a statistically significant manner when administered alone, and in contradistinction to the clear modulation of morphine-induced analgesia, it did not potentiate the effect of morphine in the inhibition of gastrointestinal transit (Fig. 6B).

We studied the rewarding effect of several doses of morphine (4–16 mg/kg, i.p.) or its vehicle (saline). To explore the rewarding properties of the treatments, a CPP paradigm was used. We ensured that mice did not show a basal (preconditioning) preference for any of the two compartments, as the time spent in each compartment was equivalent in each experimental group tested (Fig. S2A). Administration of 4 mg/kg morphine did not induce any conditioning, as mice showed a

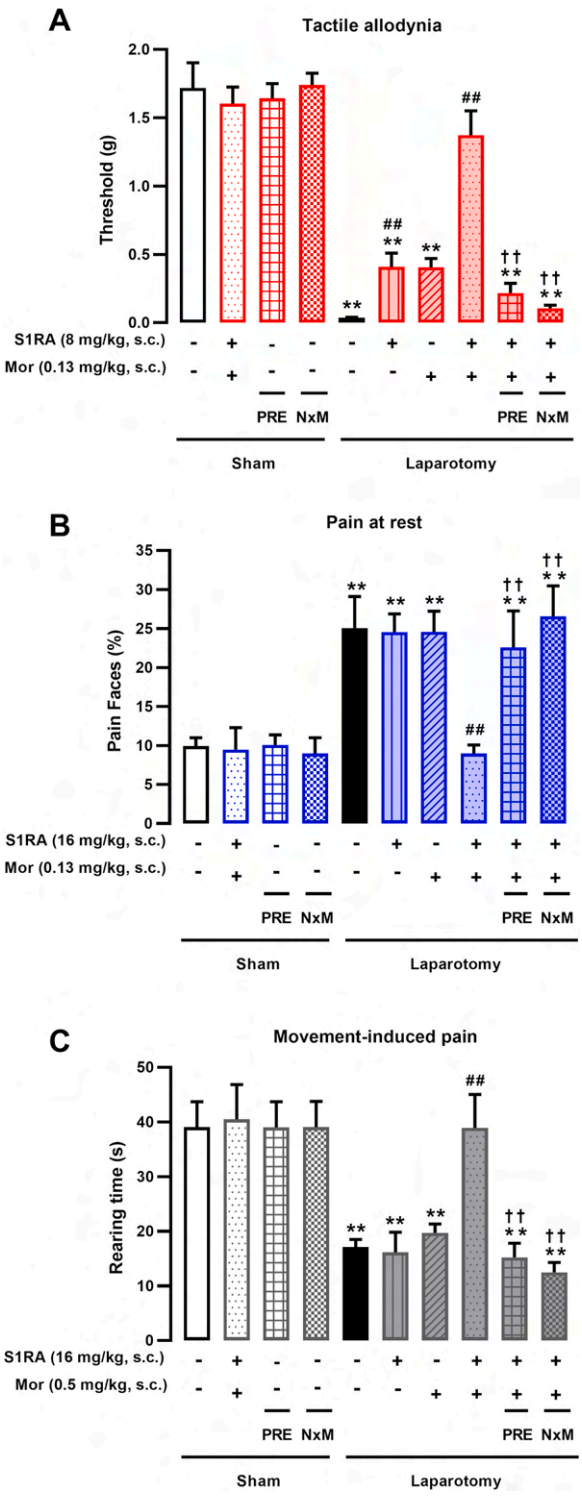

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Fig. 5. Effect of the association of S1RA and morphine on the different aspects of postoperative pain in laparotomized mice. The results represent the effects of the subcutaneous (s.c.) administration of low (inactive) doses of S1RA and morphine (Mor) individually or in combination and combined with PRE-084 (PRE, 32 mg/kg), naloxone methiodide (NxM, 2 mg/kg), or saline (A) on tactile allodynia (reductions in mechanical withdrawal threshold in abdominal area), (B) pain at rest (presence of facial pain expressions), and (C) movement-induced pain (reductions in time spent rearing). Read-outs were evaluated 3.5 h after laparotomy or sham procedure. Each bar and vertical line represent the mean ± SEM of the values obtained in (A) 7–9, (B) 7–11 and (C) 8–9 mice. One-way ANOVA revealed significant differences in A ($F_{9,68} = 72.03$; $p < 0.001$), B ($F_{9,82} = 10.74$; $p < 0.001$) and C ($F_{9,76} = 10.64$; $p < 0.001$). (A-C) Student-Newman-Keuls post hoc test found significant differences between the values obtained in sham mice treated with the vehicle (white bars) and the other experimental groups (**$p < 0.01$), between the values obtained in laparotomized mice treated with saline (black bars) or the drugs tested (##$p < 0.01$), and between the values obtained in laparotomized mice treated with S1RA + morphine alone or in combination with PRE or NxM (††$p < 0.01$). The original experimental data shown in A and C were square-rooted, and data in B were log-transformed to meet the ANOVA assumptions.

similar value for the time spent in the morphine-paired (white) or vehicle-paired (black) compartments. However, after the administration of morphine 8 mg/kg, animals clearly increased the time spent in the morphine-paired compartment, concomitantly decreasing the time spent in the vehicle-paired compartment (Fig. S2B), i.e. animals showed a clear preference for the morphine-paired side. This increase in the preference for the morphine-paired compartment after conditioning can be better presented as the difference in the time spent between both compartments at baseline and after the conditioning procedure (Fig. S2C). It is interesting to note that morphine produced a bell curve-shaped effect, as 16 mg/kg of the drug induced no preference for the drug-paired compartment, yielding preference values similar to those of saline-treated animals (Fig. S2B and C).

We then aimed to test whether S1RA 16 mg/kg (s.c.) was able to modify the reward-seeking effects of morphine 8 mg/kg (i.p.). Mice of the different experimental groups did not show a basal (preconditioning) preference for either one of the two compartments, as the time spent in each of them was equivalent for every experimental group tested (Fig. 7A). Treatment with S1RA did not alter the preference of the mice for any compartment, which remained neutral (i.e. time spent in the black and white compartments was similar after treatment) (Fig. 7B and C). Finally, we tested the effect of the combination of S1RA (or its solvent) with morphine and found that the sigma-1 antagonist did not alter the conditioning induced by the opioid, which induced a similar preference for the drug-paired (white) compartment in mice treated with S1RA or its solvent (Fig. 7B and C).

In summary, our data suggest that S1RA does not modify the inhibition of gastrointestinal transit or the reward-seeking behaviors induced by morphine.

4. Discussion

We have provided a comprehensive examination of the effects of sigma-1 antagonism, alone or in combination with morphine, on postoperative pain in mice. In addition, we have evaluated the effects of sigma-1 antagonism on relevant morphine-induced non-analgesic (side) effects: gastrointestinal transit inhibition and reward-related behavior.

Bench to bedside translation requires not only research models that successfully mimic the pathological condition in humans, but also translational outcome measures [34]. We have used three different

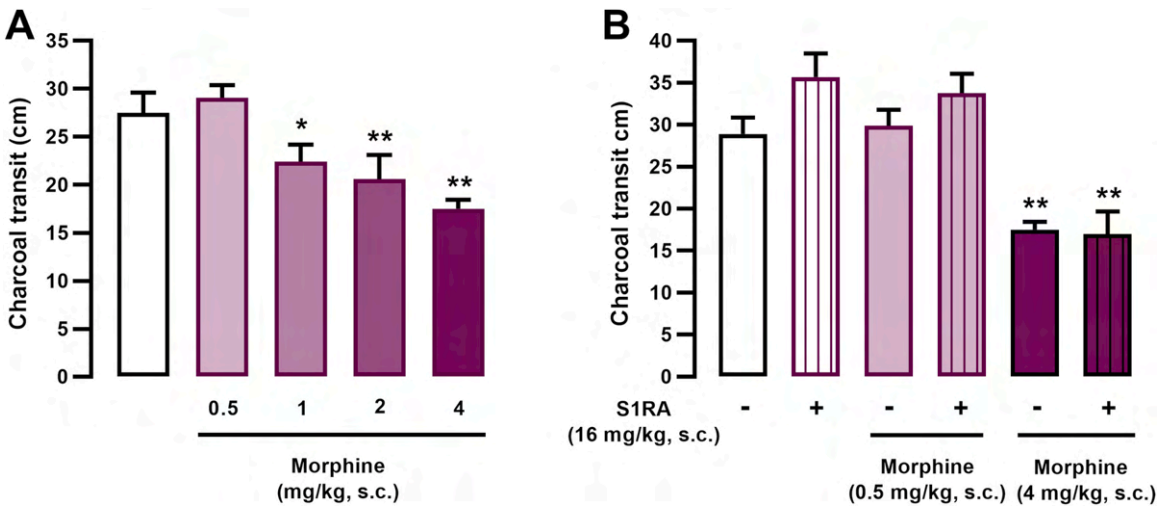

Fig. 6. S1RA does not alter morphine-induced gastrointestinal transit inhibition. The results represent the transit of a charcoal suspension (0.5 %) administered to mice treated subcutaneously (s.c.) with (A) different doses of morphine (0.5–4 mg/kg) or the vehicle (saline), and with (B) S1RA (16 mg/kg) alone and in combination with morphine (0.5 or 4 mg/kg) or saline. (A, B) Each bar and vertical line represent the mean ± SEM of values obtained in 7–9 mice. One-way ANOVA revealed significant differences in A ($F_{4,34} = 7.15$; $p < 0.001$) and B ($F_{4,39} = 9.08$; $p < 0.001$). Student-Newman-Keuls post hoc test found significant differences between the values obtained in saline- and morphine-treated groups (*$p < 0.05$, **$p < 0.01$). No statistically significant differences were found between animals treated with saline or morphine alone or with S1RA. The experimental data met the assumptions of ANOVA and were therefore not transformed before performing the statistical analysis.

measures to provide a complete and realistic view of postoperative pain in laparotomized mice: tactile allodynia (measured using the von Frey threshold), pain at rest (facial expressions evaluated using an artificial intelligence algorithm), and movement-induced pain (decrease in exploratory behavior).

The ameliorative effects of sigma-1 inhibition on cutaneous hypersensitivity are well described in a variety of clinically relevant pain models in rodents, which include inflammatory [9,24,37] and neuropathic pain [15,38–40]. Here we show for the first time that sigma-1 antagonism by BD-1063 or S1RA can decrease cutaneous hypersensitivity after surgical injury. The doses of the sigma-1 antagonists with a significant reversion of tactile allodynia after surgery (BD-1063 at 8–16 mg/kg and S1RA at 16–32 mg/kg) are in full agreement with the doses with known activity in sensory hypersensitivity in other mouse pain models [9,24,39]. The higher potency of BD-1063 is also consistent with a higher affinity of this compound for sigma-1 receptors, which is in fact twice that of S1RA [21,41]. Morphine also has a robust antiallodynic effect on mice with postoperative pain. This result is expected, considering that this pain type is one of the major clinical indications for opioid drugs. The antiallodynic effect of the sigma-1 antagonists, but not the effect of morphine, was reversed by the sigma-1 agonist PRE-084. These results indicate that both BD-1063 and S1RA, but not the opioid drug, were inducing their effects through sigma-1 receptors.

Postoperative pain is caused by a multitude of factors produced or released by both the injured cells at the surgical site and immune cells accumulated during the inflammatory process triggered by tissue injury [42]. Both sensory neurons and immune cells can produce endogenous opioid peptides with analgesic potential [43,44]. It has been described that sigma-1 receptors are chaperone proteins [15] able to bind to other receptors, such as μ-opioid receptors, to modulate their actions [27,45]. We have previously described that sigma-1 antagonism can enhance the actions of endogenous opioid peptides from either immune cells [9] or neurons [27] to ameliorate sensory hypersensitivity. Here we show that not only the antiallodynic effect of morphine, but also that of sigma-1 antagonists, was reversed by the peripheral opioid antagonist naloxone methiodide, suggesting that the effect of sigma-1 antagonism was mediated by endogenous opioid peptides produced peripherally. Our results using a neutrophil-depletion strategy indicate that the effect of sigma-1 antagonists is fully dependent on the presence of these immune cells accumulated at the injured site, consistent with previous descriptions of immune cells being the main source of endogenous opioid peptides in inflamed tissues. [46,47]. We also show that neutrophils in the injured abdominal wall express high levels of POMC (the precursor of β-endorphin) but low levels of PDY (the precursor of dynorphin) and nearly no PENK (the precursor of enkephalins). Virtually identical results were also obtained from samples of injured skin (data not shown). These results are in agreement with previous reports showing that β-endorphin is the predominant endogenous opioid peptide in inflamed tissue [48]. In fact, we show that the antiallodynic effect induced by sigma-1 antagonists is fully dependent on μ-opioid receptor activation, in line with β-endorphin being a potent endogenous agonist for μ-receptors [49,50]. Altogether, our results suggest that the antiallodynic effect induced by sigma-1 antagonism during postoperative pain is due to the potentiation of the actions of the μ-opioid agonist β-endorphin produced by neutrophils.

Despite the prominent effect of the sigma-1 antagonists on tactile allodynia, they failed to significantly alleviate pain at rest after surgical injury. Thus, the immune-driven endogenous opioid effects induced by sigma-1 inhibition, while effective in controlling cutaneous hypersensitivity, do not translate into overall relief of the postoperative pain phenotype. In contrast, morphine showed a complete reversion of pain at rest and even at lower doses than those needed to abolish allodynia. Therefore, pain at rest does not have a low sensitivity to analgesics but rather has a different pharmacology than tactile allodynia. Interestingly, peripheral opioid receptors do not appear to participate in the effect of morphine in pain at rest (as it was resistant to naloxone methiodide), suggesting that this is a centrally mediated morphine effect. In fact, peripheral opioid antagonism is currently used to treat opioid-induced constipation in human patients, and it is thought to have little to no effect on opioid-induced analgesia [51]. The fact that morphine's amelioration of pain at rest is not sensitive to peripheral opioid antagonism is in line with the clinical use of opioid antagonists and suggests that pain at rest might be more relevant to clinical pain than tactile allodynia. This is important since tactile allodynia is currently the most used read-out in rodent pain studies [34] but represents a very limited aspect of the pain experience which does not predict drug effects on other relevant aspects of the pain phenotype, as exemplified here by a marked difference between tactile allodynia and pain at rest when

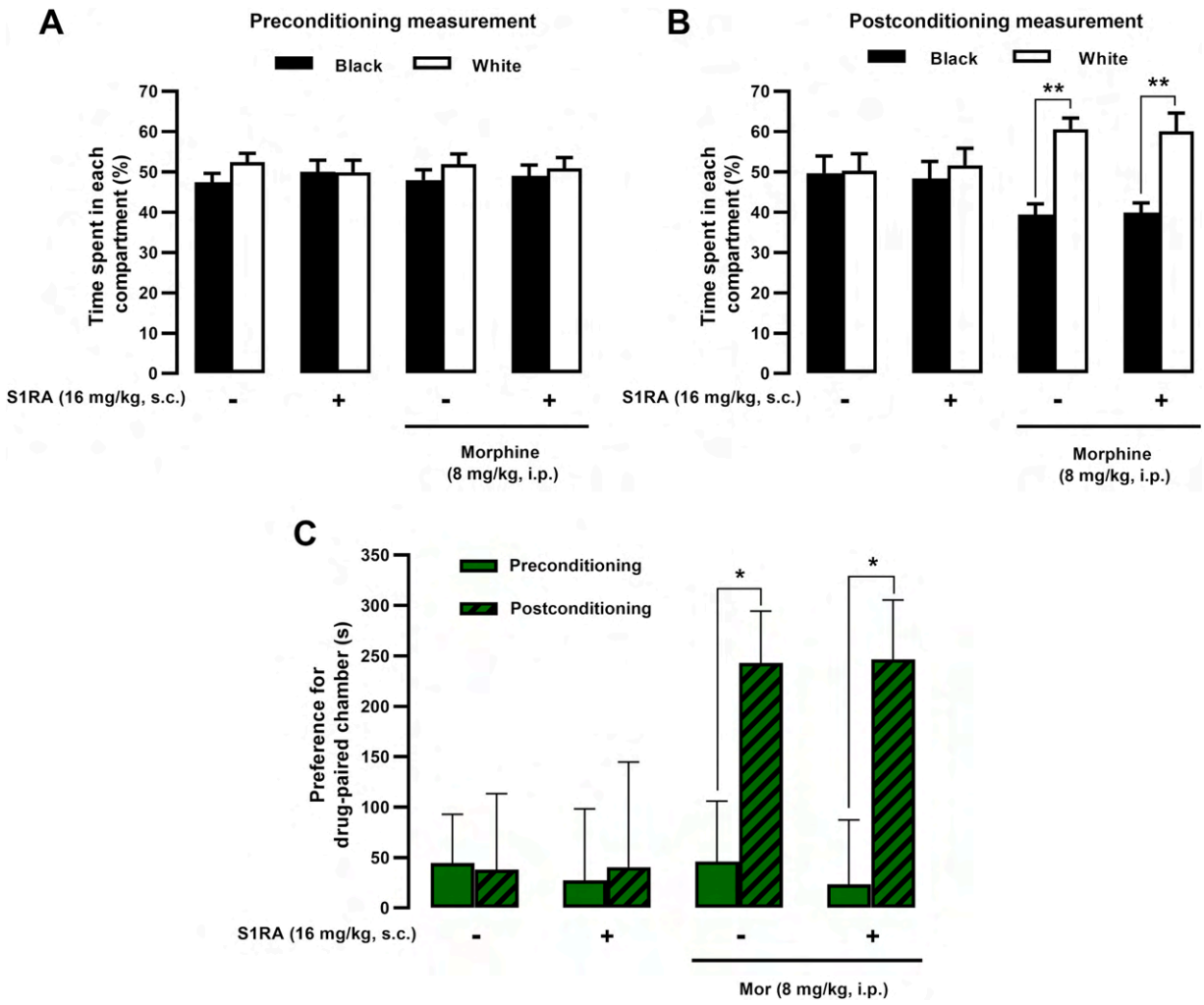

Fig. 7. S1RA does not alter morphine-induced reward-seeking behavioral effects. The reward-seeking behavioral effects of treatments were measured in a conditioned place preference (CPP) paradigm, where the apparatus is composed of two distinct compartments: black and white. The intraperitoneal (i.p.) administration of morphine (Mor) (8 mg/kg) was paired with the white compartment, and the administration of its vehicle (saline) with the black compartment. S1RA (16 mg/kg) was injected subcutaneously (s.c.) and always paired with the white compartment. The results represent time spent in each compartment (expressed in %) by the mice during (A) the baseline (preconditioning measurement), and (B) after repeated injection with morphine alone or in combination with S1RA or the vehicle (postconditioning measurement). (C) Preference for the drug-paired compartment was calculated as the difference in the time spent in the drug-paired *versus* the vehicle-paired compartment. (A-C) Each bar and vertical line represents the mean ± SEM of the values obtained in 13–18 mice. One-way ANOVA revealed no statistically significant differences in A ($F_{7116} = 0.56$; $p = 0.785$), but significant differences in B ($F_{7,98} = 5.24$; $p < 0.001$), and Student-Newman-Keuls post hoc test found significant differences between the values obtained in saline- and morphine-treated groups (**$p < 0.01$). Two-way repeated measures ANOVA revealed significant differences in C ($F_{1,58} = 5.90$; $p < 0.05$), and Student-Newman-Keuls post hoc test found significant differences between baseline and postconditioning measures in animals treated with morphine (*$p < 0.05$). The experimental data met the assumptions of ANOVA and were therefore not transformed before performing the statistical analysis.

testing sigma-1 antagonists and naloxone methiodide on morphine-induced effects.

We also tested movement-induced pain. Morphine failed to improve this outcome, which is consistent with the known lack of analgesic effect of opioid drugs on movement-induced pain after surgery in both mice [11,52] and human patients [3,4]. Sigma-1 antagonism also failed to improve this aspect of the postoperative pain phenotype. Taken together, our results show that sigma-1 antagonism induces a limited effect on postoperative pain, exclusively alleviating tactile allodynia without ameliorating pain at rest and movement-induced pain, whereas morphine can robustly reverse allodynia and pain at rest, although it does not improve movement-induced pain. Therefore, none of these treatments, when administered alone, appear to be optimal for the management of postoperative pain. We also evaluated the combination of both the selective sigma-1 antagonist S1RA and morphine, and found that when given at doses which lack an effect when administered alone, drug combination was able to induce a full reversal of not only tactile allodynia and pain at rest but also movement-induced pain, in a manner dependent on the activation of peripheral opioid receptors. These robust effects fall within the definition of cooperative effect synergy (a response to a drug combination that is greater than the responses to the drugs individually) [53]. Hence, combined drug administration is clearly advantageous when compared with the effects of sigma-1 antagonism or morphine alone, as it covers all postoperative pain aspects examined. Taking our results together, sigma-1 antagonism can boost the actions of peripheral endogenous opioid peptides from immune cells to partially reverse postoperative pain, but in combination with morphine, a potent pharmacological agonist of μ-opioid receptors, the enhancement of peripheral opioid actions by sigma-1 antagonism can achieve complete reversal of postoperative pain. Sigma-1 receptors interact with μ-opioid receptors but also with additional proteins, such as transient receptor potential vanilloid-1 (TRPV1) [27]. Indeed, sigma-1 receptors

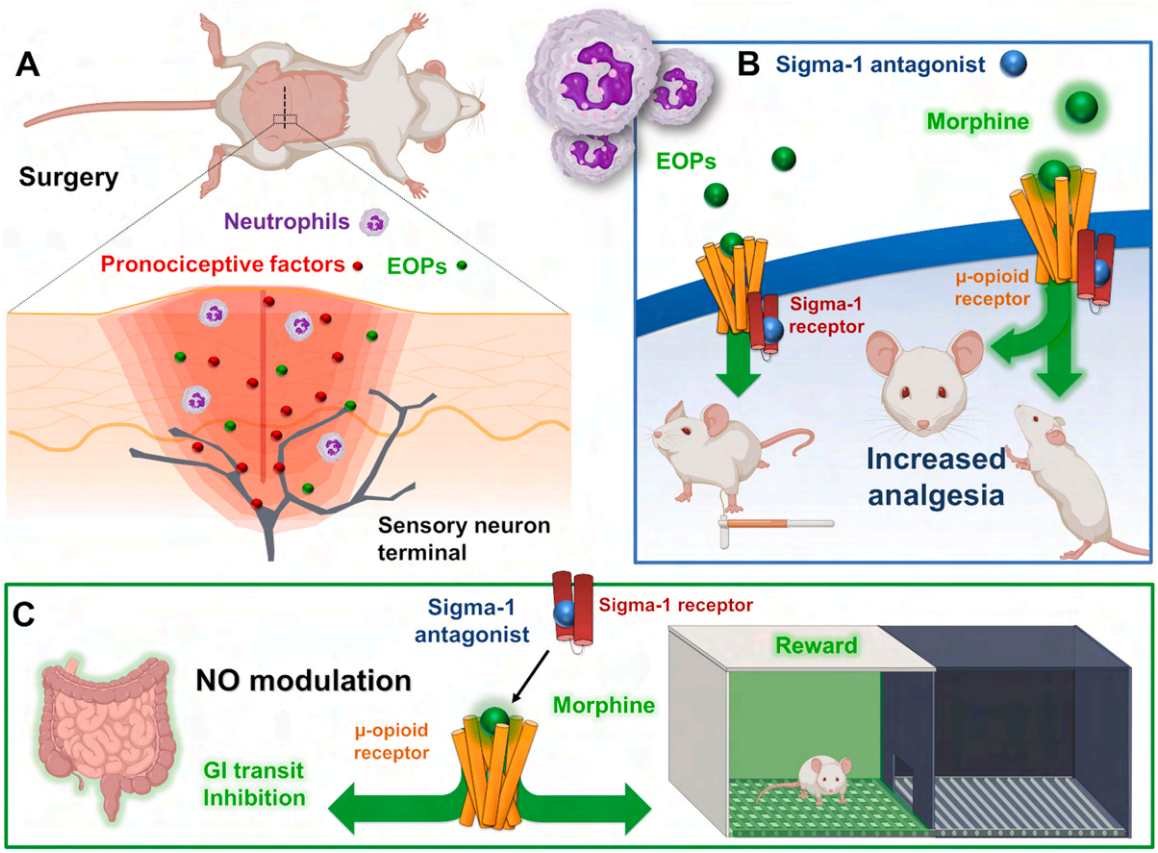

Fig. 8. Summary of the actions of sigma-1 antagonism during postoperative pain. (A) Surgery induces recruitment of neutrophils, which produce endogenous opioid peptides (EOPs), as well as pronociceptive factors. (B) Sigma-1 antagonism enhances the actions of neutrophil-derived EOPs to relieve tactile allodynia but not pain at rest or movement-induce pain. Sigma-1 antagonism, through the potentiation of the effect of morphine, can also reverse these two latter aspects of postoperative pain. (C) Sigma-1 antagonism does not modify some key non-analgesic (side) effects of morphine, including the inhibition of gastrointestinal transit and morphine-induced reward-seeking behavior. Some images were created with BioRender.com.

participate in the crosstalk between both μ-opioid receptors and TRPV1, and sigma-1 antagonism decreases the sensitization of TRPV1-expressing nociceptors through opioid mechanisms [27]. Considering the known importance of this type of nociceptors for post-operative pain [54], it is tempting to speculate that TRPV1 and its communication with sigma-1 and μ-opioid receptors would participate in the effects observed.

Opioid-induced constipation is the most worrisome peripheral side effect of opioids [55,56], and one of the main reasons for patients' voluntary withdrawal from opioid medication [57]. We found that S1RA, at doses able to increase the peripheral opioid analgesia induced by morphine in mice with postoperative pain, did not induce any significant effect on gastrointestinal transit alone or when administered with morphine. These results agree with a previous report [13]. Opioid analgesics can also produce limiting side effects at central levels, such as their abuse potential [58], which is responsible for the opioid overdose epidemic in the USA which has caused the death of countless people in the past years[6]. For many people, postoperative pain treatment represents the first contact with opioids, and postsurgical patients are at increased risk for chronic opioid use [7]. Therefore, there is a need to reduce the use of opioids for the treatment of postoperative pain without compromising the analgesic needs of patients. We found that S1RA, at doses able to increase morphine analgesia in mice with postoperative pain, did not alter the rewarding properties of morphine. It has even been reported that S1RA is able to decrease morphine-induced reward-seeking behavior, although it was tested at a higher dose than what we used in our study (40 *vs* 16 mg/kg) [13]. In addition, the morphine dose used to induce reward-seeking behavior in the previous study was lower than the dose used here (5 *vs* 8 mg/kg/day) [13]. Therefore, our experimental conditions may have been too stringent to allow detection of a possible decrease in the reward-seeking effects of morphine by S1RA. It has been shown that while sigma-1 antagonists decrease the reward-seeking effects of commonly abused drugs, sigma-1 agonists promote drug-seeking behavior [59]. Some commonly prescribed drugs, including antipsychotic, antidepressant, or antitussive drugs, show high affinity for sigma-1 receptors [60,61]. These drugs could affect both postoperative pain and/or reward-seeking behaviors induced by opioid medication through sigma-1 receptors, warranting further investigation.

Our data, using comparable doses of S1RA in the testing of analgesia and side effects, suggest that sigma-1 antagonism could allow a reduction in the dose of morphine while achieving a more effective control of postoperative pain without increasing the risk of adverse events. In fact, morphine-induced adverse events could be lower due to the reduced opioid dose, thereby increasing the therapeutic range of morphine.

5. Conclusion

In summary, our study used several read-outs to provide a detailed view of postoperative pain in laparotomized mice and demonstrates that sigma-1 antagonism holds the potential to alleviate this type of pain, particularly when given in combination with morphine. Sigma-1 antagonism boosts the actions of endogenous opioid peptides from neutrophils to reverse tactile allodynia, and, when associated with morphine, it enhances peripheral opioid analgesia to also reverse pain at rest and movement-induced pain (Fig. 8A and B). Enhancement of

opioid-induced analgesia by sigma-1 antagonism is not accompanied by an increase in two key opioid-related adverse events; gastrointestinal transit inhibition and reward-seeking behavior (Fig. 8C). Modulation of opioid analgesia by sigma-1 receptor antagonists might have potential clinical applications to increase the therapeutic range of opioids in the treatment of postoperative pain and may warrant further clinical investigation.

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CRediT authorship contribution statement

Hannah K. Mayr: Methodology, Investigation, Writing – review & editing. **M. Carmen Ruiz-Cantero:** Writing – review & editing, Investigation. **Makeya A. Hasoun:** Investigation. **Miguel Á. Huerta:** Investigation. **Cobos Enrique J:** Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Project administration, Methodology, Funding acquisition. **Rafael González-Cano:** Writing – review & editing, Writing – original draft, Supervision, Software, Methodology. **Miriam Santos-Caballero:** Writing – review & editing, Writing – original draft, Methodology, Investigation. **Cronin Shane J.F.:** Writing – review & editing, Methodology, Investigation, Funding acquisition. **Amada Puerto-Moya:** Investigation. **María Robles-Funes:** Investigation.

Declaration of Competing Interest

The authors declare no competing interests.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2025.118298.

Data availability

Data will be made available on request.

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