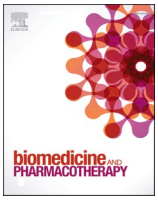
Biomedicine & Pharmacotherapy 180 (2024) 117459

Contents lists available at ScienceDirect

Biomedicine & Pharmacotherapy

journal homepage: www.elsevier.com/locate/biopha



Pharmacological differences in postoperative cutaneous sensitivity, pain at rest, and movement-induced pain in laparotomized mice

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ARTICLE INFO

Keywords:
Postoperative pain
Morphine
Ibuprofen
Neutrophil
Gefapixant
Olcegepant

ABSTRACT

Postoperative pain management is challenging. We used mice with a transverse laparotomy to study tactile allodynia measured by the von Frey test, pain at rest measured by facial pain expressions detected by an artificial intelligence algorithm, and movement-induced pain measured by reductions in exploratory activity. The standard analgesics morphine and ibuprofen induced distinct patterns of outcome-dependent effects. Whereas morphine was more effective in reversing pain at rest compared to tactile allodynia, it was unable to alter movement-induced pain. Ibuprofen showed comparable effects across the three outcomes. Administered together, the compounds induced synergistic effects in the three aspects of postoperative pain, mirroring the known advantages of multimodal analgesia used in clinical practice. We explored the impact of neuroimmune interactions using a neutrophil depletion strategy. This reversed pain at rest and movement-induced pain, but did not alter cutaneous sensitivity. Non-peptidergic (IB4+) and peptidergic (CGRP+) nociceptors are segregated neuronal populations in the mouse. We tested the effects of gefapixant, an antitussive drug targeting non-peptidergic nociceptors through P2X3 antagonism, and olcegepant, an antimigraine drug acting as a CGRP antagonist. Both compounds reversed tactile allodynia, while only gefapixant reversed pain at rest, and none of them reversed movement-induced pain. In conclusion, tactile allodynia, pain at rest, and movement-induced pain after surgery have different pharmacological profiles, and none of the three aspects of postoperative pain can predict the effects of a given intervention on the other two. Combining these measures provides a more realistic view of postoperative pain and has the potential to benefit analgesic development.

1. Introduction

Postoperative pain is inadequately managed. Three-quarters of patients experience moderate, severe, or even extreme pain in the immediate postoperative period despite pharmacological treatment [1], which mostly consists of nonsteroidal anti-inflammatory drugs (NSAIDs) and opioids [2].

Postoperative pain comprises distinct aspects, or components, including tactile allodynia (cutaneous sensitivity around the surgical injury), pain at rest (stimulus-independent pain), and movement-induced pain (pain provoked or aggravated by movement) [3]. Clinical studies have shown differential pharmacological effects on these

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https://doi.org/10.1016/j.biopha.2024.117459
Received 3 July 2024; Received in revised form 9 September 2024; Accepted 19 September 2024
Available online 20 September 2024

three aspects of pain. Opioid drugs, for instance, are highly effective in relieving pain at rest, but provide relatively little relief from movement-induced pain [3,4]. NSAIDs, by contrast, provide comparable relief from both pain at rest and movement-induced pain [5,6]. Different responses might reflect distinct mechanisms of pain production, but our understanding of the basic mechanisms involved is limited, in part because preclinical pain studies have relied almost exclusively on measures of cutaneous sensitivity [7]. There has, however, been a recent increase in the use of other measures, such as facial pain expressions (to assess pain at rest) and reductions in exploratory locomotor activity (to assess movement-induced pain). Although these measures have been evaluated in animal models of postoperative pain [8–10], studies have typically focused on single measures. It is still unknown whether standard analgesics have differential effects on different aspects of postoperative pain in rodents, as occurs in humans.

The most widely used rodent model of postoperative pain is the plantar incision model, which consists of a surgical incision in the plantar hindpaw [11], but pain can also be studied after other types of surgery, including laparotomy [9,10,12,13]. A laparotomy is a surgical incision made through the abdominal wall to gain access to the abdominal cavity. It is the initial step for open abdominal surgeries. In the United States alone, 2 million people undergo open abdominal surgery every year [14]. Modeling postoperative pain using a laparotomy rather than a plantar incision model, therefore, could be more clinically relevant.

Algogenic chemicals produced by immune cells participate in pain development [15,16]. Immune cell recruitment is a natural response to tissue injury and inflammation. How immune cells contribute to tactile allodynia, pain at rest, and movement-induced pain after laparotomy, however, is not known. Chemicals released by injured tissue might also play a role in postoperative pain. ATP, for instance, which is part of the cytoplasmic contents of lysed cells [17], is known to activate P2X3 receptors contributing to pain [18]. Considering that surgery necessarily involves tissue injury and hence P2X3 activation, it would be interesting to test the effects of P2X3 antagonism on postoperative pain. Gefapixant is a first-in-class P2X3 receptor antagonist approved as an antitussive drug in the European Union, Switzerland, and Japan [19]. Its effects on postoperative pain are unknown. P2X3 receptors are expressed by non-peptidergic nociceptors, while peptide neurotransmitters, such as calcitonin gene-related peptide (CGRP), are expressed by peptidergic nociceptors [20–22]. A new class of drugs, CGRP receptor antagonists, called gepants, have been developed for migraine treatment [23]. CGRP is known to play an important role in several types of pain [24], but the effects of CGRP antagonism on postoperative pain have not yet been studied.

We performed a comprehensive assessment of the effects of a variety of interventions on tactile allodynia, pain at rest, and movement-induced pain in laparotomized mice with the aim of improving our understanding of key aspects of postoperative pain. These interventions included treatment with standard analgesics, immune cell depletion, and administration of gefapixant (a P2X3 antagonist) and olcegepant (a CGRP receptor antagonist).

2. Material and methods

2.1. Experimental animals

The experiments were performed in 8- to 10-week-old CD-1 mice (Charles River, Barcelona, Spain) weighing 25–32 g. Since open abdominal surgeries are much more common in women than in men (~90 % vs 10 %) [14], this study was performed in female animals. The mice were housed in colony cages (10 mice per cage) in a temperature-controlled room (22 ± 2ºC) with an automatic 12-h light/dark cycle (08:00–20:00). An igloo and plastic tunnel were 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. To minimize the number of animals used, when possible, the same mice used for behavioral experiments were used for hematoxylin-eosin staining, immunostaining, or fluorescence-activated cell sorting (FACS).

2.2. Surgical procedures

We used an experimental laparotomy model to mimic postoperative pain. The mice were anesthetized in an induction chamber with 3.5 % isoflurane (IsoVet®, B. Braun, Barcelona, Spain) in oxygen. The 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 % alcohol solution followed by 10 % povidone-iodine solution. Most experiments were performed with a transverse laparotomy, which consisted of a 1.5-cm incision made 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 used single knot sutures to close the muscle and horizontal mattress sutures to close the skin. The sutures were done with Supramid® 5/0 non-absorbable polyamide multifilament using a TB15-CT 19-mm needle (Laboratorio Aragó, Barcelona, Spain). Some mice underwent a longitudinal (midline) laparotomy, which consisted of a midline incision made through the skin and linea alba, with closure of the muscle and skin as described above. An additional set of mice underwent the same longitudinal laparotomy procedure, but in this case the skin was closed using surgical staples (EZ clips, Stoelting, Illinois, USA), as this is a common closure method used in surgical procedures [13]. The sham procedure involved anesthesia, shaving, and sterile preparation of the abdomen, with no incision.

A previously described plantar incision model [22] was used as a control for some experiments. Briefly, the mice were anesthetized in an induction chamber with 3.5 % isoflurane in oxygen. Anesthesia was maintained with 3 % isoflurane delivered via a nose cone during the procedure. The left hindpaw was prepared with 10 % povidone-iodine and a 5-mm longitudinal incision made through the skin with a single stroke of a number 11 blade. The skin was opposed with two single sutures of Supramid® 5/0 non-absorbable polyamide multifilament thread using a TB15-CT 19-mm needle. The sham procedure comprised anesthesia and antiseptic preparation of the hindpaw, with no incision.

2.3. Administration of drugs and antibodies for in vivo use

The standard analgesics tested were the NSAID ibuprofen (8, 16, 32 mg/kg; Sigma-Aldrich, Madrid, Spain) and the opioid agonist morphine hydrochloride (0.13, 0.25, 0.5, 1 mg/kg; Biogen, Madrid, Spain). The other drugs tested were the P2X3 receptor antagonist gefapixant (8, 16 mg/kg; Merck KGaA, Darmstadt, Germany) and the CGRP1 receptor antagonist olcegepant (8, 16 mg/kg; Sigma-Aldrich). All the 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 combination of ibuprofen and morphine. In these experiments, ibuprofen was administered immediately before morphine and 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.

Drug effects were evaluated in the immediate postoperative period, specifically 3.5 h after surgery to allow inflammation to develop [25].

All drugs and drug combinations were injected 1 h before the behavioral evaluations, that is, 2.5 h after surgery.

To inhibit neutrophil infiltration, we administered an anti-Ly6G antibody (BE0075–1; Bio X Cell, Lebanon, NH, USA). The antibody was dissolved in physiological saline and administered intraperitoneally (i.p.) at a standard dose (8 μg/0.2 mL) [22,26]. Saline injections and a non-reactive isotype antibody (BE0089; Bio X Cell) were used as controls. The antibodies or saline were injected 24 h before the laparotomy or sham procedure. The behavioral effects were determined 3.5 h after surgery.

2.4. Behavioral tests

Laparotomized and sham mice were placed in the experimental room for a 1-h acclimation period before the behavioral tests. We evaluated mechanical withdrawal thresholds as measures of tactile allodynia (cutaneous hypersensitivity), facial pain expressions as measures of pain at rest, and reductions in exploratory locomotor activity as measures of movement-induced pain. Each mouse was used in just one test. The evaluators were blinded to the interventions in all cases.

2.4.1. Mechanical threshold assessment

Mechanical withdrawal thresholds were tested following a previously described protocol [27]. Briefly, the mice were placed in individual opaque plastic boxes (5 × 9 × 13 cm) on an elevated platform with a wire mesh floor. After a 1-h acclimation period, the thresholds were 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 0.02–2 g (0.19–19.6 mN) applied to the abdomen, approximately 2 mm from the surgical incision site. The filaments were applied three times for 1–2 seconds. Testing was initiated with the 0.4-g (3.92-mN) von Frey filament. The test 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 applied, and if there was a negative response, a stronger filament was applied. The 50 % withdrawal threshold was determined using the up-down method and calculated in the Up-Down Reader software [28].

To elucidate the time course of laparotomy-induced mechanical allodynia, the behavioral responses of each animal were tested the day before the surgical procedure (naïve condition) and 3.5, 24, 48, 144, and 240 h after surgery or sham procedure. To test for the effects of drugs or antibodies on the mechanical threshold, mice were evaluated only once, 3.5 h after surgery or sham procedure (see “Administration of drugs and antibodies for in vivo use”).

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; the procedure has been previously described [29,30]. The mice were placed individually in custom-made, black-walled test compartments (50 × 120 × 60 mm) on an elevated (1.1-m drop) mesh-bottomed platform with a 0.5-cm^2 grid. The compartments were arranged in arrays of four and positioned at the edge of the platform, with the fourth wall opened and facing high-resolution (1440 × 1024 pixels) infrared video cameras (Kuman RPi camera, USA), each connected to two infrared light-emitting diodes. The test compartments were arranged to encourage the mice to look towards the visual cliff, hence facing the cameras, positioned 25 cm from the test compartments. Each camera can simultaneously record two mice and is controlled by a raspberry Pi zero single-board computer (Kubii, France), which stores the recordings on USB sticks for subsequent transfer to a computer for analysis. This custom-made device is described in detail elsewhere [30]. No experimenters were present in the testing room during evaluations.

Before facial pain expressions could be analyzed, the DeepLabCut [31] network was trained to recognize the animals’ ears, eyes, and nose. Only frames in which the mice were facing the camera (simultaneous detection of all these body parts with high confidence [>0.9]) were used to analyze facial pain expressions, as previously described [30]. Using DeepLabCut we obtained 4204 images of laparotomized mice from 23 new video recordings and 2683 images of control mice from 13 new video recordings. An interval of at least 5 seconds was left between images to avoid using consecutive images with virtually identical content. The images were manually classified by an experienced pain researcher, who studied the characteristics of the ears, eyes, cheeks, and nose and selected 244 images that clearly exemplified "pain" and 1042 images that clearly exemplified “no pain”. These images were then used to train a temporal classifier, used to search for pain-like (false-positive) facial expressions in video recordings of the control mice. The 183 resulting images were used to expand the “no pain” group. The full set of images was used to train a convolutional neural network based on Google’s InceptionV3 model for over 25,000 iterations, as previously described [29,30]. The trained neural network showed a predicted discrimination accuracy of 89.6 %. After training was completed, the software was able to examine each frame from new video recordings of laparotomized mice and assign 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 on an Ubuntu Linux computer equipped with an NVIDIA 2080Ti graphics processing unit (GPU).

All recordings of laparotomized and sham mice were programmed to last 15 min. To elucidate the time course of laparotomy-induced facial pain expressions, different groups of mice were tested in the naïve condition and 3.5, 5, 24, and 48 h after surgery, since repeated exposure to the test compartments induced habituation that interfered with the intepretation of facial pain expressions. For instance, sleep-related features (partially closed eyes or related expressions) can be interpreted as pain by the neural network, increasing the number of false-positive frames. To test for the effects of drugs or antibodies on facial pain expressions, the mice were evaluated only once (3.5 h after surgery or sham procedure) (see “Administration of drugs and antibodies for in vivo use”).

2.4.3. Assessment of exploratory locomotor activity

Reductions in exploratory locomotor activity were used as a measure of movement-induced pain after surgery. This is an instinctive behavior known to be depressed by several painful circumstances, and it is not prone to the same confounders as standard reflex responses, such as the von Frey test. Substances producing sedation or muscle weakness, although able to attenuate reflex responses and thereby induce false analgesic-like effects, will exacerbate rather than ameliorate deficits in exploratory locomotor activity [7]. Therefore, this measure is a useful complement to other pain-related outcomes. We measured vertical activity (time spent rearing) rather than horizontal activity, as it is a more sensitive measure of pain-induced alterations [7]. Vertical activity was determined using an infrared detector (Med Associates Inc., St. Albans, VT, USA) equipped with 48 infrared photocell emitters and detectors. We used a previously described method [32] with slight modifications. The animals were placed individually in transparent evaluation chambers (27.5 cm wide × 27.5 cm long × 20 cm high), and vertical activity was recorded for 30 min. No experimenters were present in the testing room during the evaluation. The mice were tested just once to avoid habituation to the evaluation chambers, as this would have markedly decreased their exploratory behavior. To elucidate the time course of movement-induced pain, different groups of mice were tested in the naïve condition and 3.5, 5, 24, and 48 h after surgery. To test for the effects of drugs or antibodies on reductions in exploratory locomotor activity, mice were evaluated only once (3.5 h after surgery or sham procedure) (see “Administration of drugs and antibodies for in vivo use”).

2.5. FACS analysis

Samples containing the incision site and surrounding tissue in the abdominal wall were harvested 3.5, 24, 48, 144, and 240 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 37ºC 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).

2.6. Histology

Samples containing the surgical wound, which included both the injured skin and abdominal wall, were obtained from laparotomized animals 3.5, 48 and 240 h after surgery. Control samples from an equivalent anatomical location were obtained from uninjured animals 3.5 h after the sham procedure. The tissue samples were obtained and fixed with 10 % buffered formalin for 24 h at room temperature. Next, they were sectioned transversally, dehydrated with alcohol, and embedded in paraffin. Tissue sections measuring 5–7 μm were stained with hematoxylin and eosin. Images were acquired with a Nikon Eclipse 50i microscope equipped with a DS-Ri1 camera.

2.7. Inmunohistochemistry

All dorsal root ganglion (DRG) neurons were labeled using the pan-neuronal marker NeuN [33]. Peptidergic and non-peptidergic nociceptors were identified by immunostaining for CGRP and bandeiraea simplicifolia lectin I (isolectin B4, IB4), respectively [20]. ATF3 staining was used as a stress marker to identify injured neurons [34,35].

Briefly, the mice were anesthetized with 4 % isoflurane in oxygen and perfused transcardially with 0.9 % saline solution followed by 4 % paraformaldehyde (Sigma-Aldrich). The DRG were dissected and post-fixed for 1 h in the same paraformaldehyde solution. Embedding procedures differed depending on the staining to be performed, as not all the antibodies showed optimal results in all the embedding media. Samples for CGRP immunostaining were dehydrated and embedded in paraffin. Tissue sections were cut to a thickness of 5 μm on a sliding microtome, mounted on microscope slides (Sigma-Aldrich), deparaffinized in xylol (Panreac Quimica, Castellar del Vàlles, Spain), and rehydrated before antigen retrieval (steam heating for 22 min with 1 % citrate buffer, pH 8). Samples for ATF3 staining were incubated for 48 h in 30 % sucrose (Sigma-Aldrich) at 4º C, and embedded in O.C.T Tissue-Tek medium (Sakura Finetek, Barcelona, Spain); they were then frozen and stored at −80 ºC until the immunohistochemical study. DRG sections were cut to a thickness of 15 μm with a cryostat and thaw-mounted onto Superfrost Plus microscope slides (Thermo Fisher Scientific).

Tissue sections were incubated for 1 h in blocking solution with 5 % normal goat serum, 0.3 % Triton X-100, and 0.1 % Tween 20 in Tris buffer solution. The slides were then incubated with the primary antibody rabbit anti-CGRP (1:800, T-4032, BMA Biomedicals) or rabbit anti-ATF3 (1:200, HPA001562, Sigma-Aldrich) in blocking solution for 1 h at room temperature. The sections were washed again three times for 10 min and incubated with the mouse anti-NeuN (neuronal nuclei) conjugated with Alexa Fluor 488 (1:500, MAB377X, Merck Millipore, MA, USA), the secondary antibody Alexa Fluor-647 goat anti-rabbit (1:500, A-21245,Thermo Fisher Scientific), and IB4 conjugated with Dylight-594 (1:100, DL-1207, Lot ZG0123; Vector Laboratories Ltd., Peterborough, UK) for 1 h at room temperature. The slides were incubated for 5 min with Hoechst 33342 for nucleic acid staining (1:1000, Life Technologies, Carlsbad, CA, USA) and washed three times before mounting. Finally, they were coverslipped with ProLong Gold Antifade mounting medium (Thermo Fisher Scientific). Images were acquired with a confocal laser-scanning microscope (Model ZEISS LSM 900, Carl Zeiss Microscopy, NY, USA).

2.8. Data Analysis

Data were analyzed using GraphPad Prism 8 (GraphPad Software, Boston, USA). Results are shown as the mean ± SEM of three or more independent experiments. To calculate analgesic effect as a percentage, values from saline-treated sham animals were considered to represent a 100 % effect (maximum pain relief), while those from saline-treated laparotomized animals were considered to a 0 % effect (no pain relief). Most statistical analyses were performed using one-way analysis of variance (ANOVA). Two-way repeated-measures ANOVA was used to analyze the time course of variations in mechanical thresholds after the laparotomy or sham procedure. 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. Laparotomized mice show tactile allodynia, pain at rest, and movement-induced pain

We studied tactile allodynia, pain at rest, and movement-induced pain to gain a better understanding of the pain phenotype in mice following a laparotomy.

We first compared the effects of midline and transverse laparotomies on tactile allodynia and pain at rest 3.5 h after surgery. The midline incision had been closed using surgical staples or sutures. Representative pictures of the midline laparotomy (with the two closure methods) and the transverse laparotomy (with suture closure only) can be found in Supplemental Figure S1A. Regardless of the procedure used, the mice displayed a marked decrease in the von Frey threshold after laparotomy. The values were close to the minimum possible value (0.02 g), indicating significant tactile allodynia (Supplemental Figure S1B). Choice of surgical procedure, therefore, does not appear to have a marked impact on the development of tactile allodynia, as this was of a similar intensity after all three procedures.

To test for pain at rest after laparotomy we used a convolutional neural network–based artificial intelligence algorithm trained with facial pain images obtained in our laboratory (see Material and Methods for details). When we tested the effects of the three surgical procedures on pain at rest, we found that mice with midline laparotomy wounds closed with staples were much more likely to exhibit facial pain expressions than sham-operated animals (Supplemental Figure S1C). When the midline laparotomy wounds were closed with sutures, no differences in facial pain expressions were observed between the

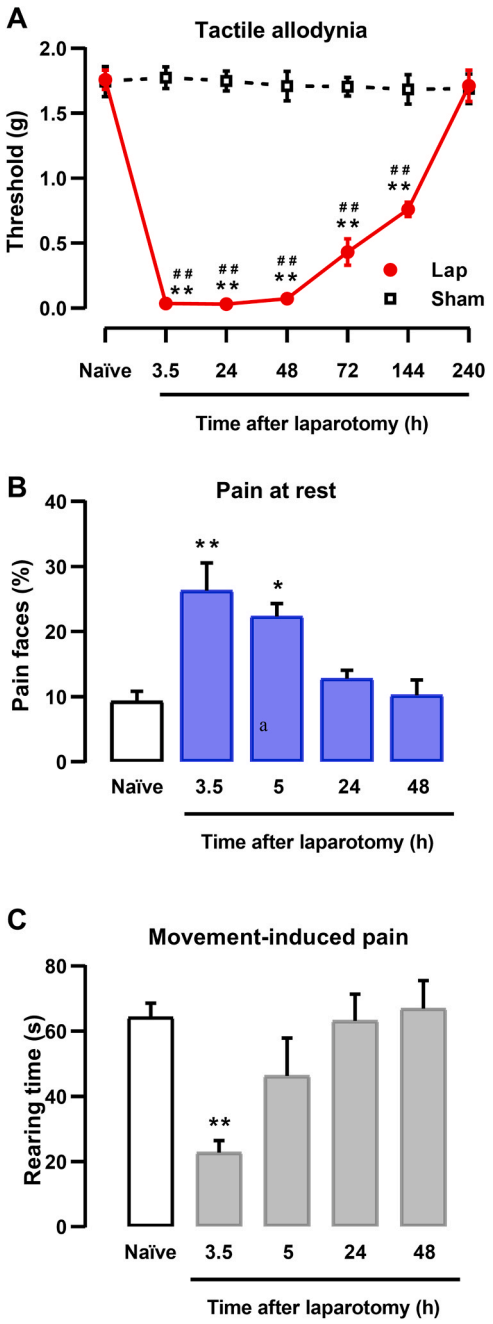

Fig. 1. Time course of three aspects of postoperative pain in mice with a transverse laparotomy: tactile allodynia, pain at rest, and movement-induced pain. (A) Tactile allodynia was demonstrated by a reduction in the mechanical threshold in the abdominal area. Mechanical threshold was measured using von Frey filaments before and 3.5, 24, 48, 72 and 240 h after laparotomy or sham procedure. Each point and vertical line represent the mean ± SEM of the values obtained in 7–8 mice. Statistically significant differences between baseline and post-surgery values (**p < 0.01) and between values from sham and injured mice evaluated at the same time points after the procedure (##p < 0.01) (two-way repeated-measures ANOVA followed by Student-Newman-Keuls test). (B) Time course of facial pain expressions as a measure of pain at rest and (C) reduction in time spent rearing as a measure of movement-induced pain after laparotomy. Pain faces and rearing time were determined in naïve animals and 3.5, 5, 24 and 48 h after laparotomy. (B and C) Each bar and vertical line represents the mean ± SEM of the values obtained in 7–11 mice. Statistically significant differences between naïve control animals and laparotomized animals (**p < 0.01) (one-way ANOVA followed by Student-Newman-Keuls test).

laparotomy and sham groups (Supplemental Figure S1C). We therefore decided not to use midline laparotomy or staples in subsequent experiments, since the former does not appear to induce pain at rest, while the latter might cause pain that is difficult to distinguish from true postoperative pain. The mice with a transverse laparotomy closed with surgical sutures showed a measurable increase in facial pain expressions (equivalent to those detected in mice with a midline incision closed with staples) (Supplemental Figure S1C). We decided to use a transverse laparotomy with surgical sutures for all subsequent experiments, as this seemed to yield results compatible with the development of postoperative pain at rest.

Supplemental Figure S2 shows the probability ratings given by the neural network for facial pain expressions in each video frame for three representative sham mice (left panels) and three representative laparotomized mice (transverse procedure) (right panels). There was a marked increase in the number of frames with probability values close to 1 after laparotomy. There are some gaps in the recordings (as can be seen in Supplemental Figure S2), as only the frames in which the animals were facing the camera could be used for the analysis (see Material and Methods section for details). A typical recording had approximately 60–70 % usable frames, which was considered sufficient to obtain appropriate averaged values over the evaluated period.

We then evaluated the time course of tactile allodynia in mice with a transverse laparotomy and found that the marked sensory hypersensitivity seen at 3.5 h post-surgery persisted up to 48 h. After 72 h, the mechanical threshold progressively increased and returned to naïve levels by 240 h (Fig. 1A). Sham-operated mice did not show any significant alterations to mechanical thresholds at any of the time points evaluated (Fig. 1A).

The time course of pain at rest differed. The laparotomized mice showed an increase in facial pain expressions (relative to the naïve group) at the first time point evaluated (3.5 h), but this increase subsided progressively and rapidly, and was no longer evident at 24 h (Fig. 1B). Mice tested 3.5 and 5 h after the sham procedure showed no significant increase in facial pain expressions compared with naïve animals (data not shown).

Finally, we evaluated the time course of movement-induced pain, measured by reductions in exploratory activity after laparotomy (see Material and Methods for details). Mice analyzed 3.5 h after laparotomy spent significantly less time rearing than naïve mice. The reduction in vertical activity, however, disappeared rapidly, with no changes relative to naïve values observed at 24 h (Fig. 1C). Compared with the naïve group, sham mice did not show any alterations in exploratory behavior at 3.5 or 5 h (data not shown).

The above time-course analyses offer insights into different aspects of postoperative pain: tactile allodynia lasted longer than both pain at rest and movement-induced pain, which subsided much faster and at a similar rate.

3.2. Effects of ibuprofen and morphine on the different aspects of postoperative pain

We studied the effects of ibuprofen and morphine, two standard analgesics, on tactile allodynia, pain at rest, and movement-induced pain in mice after a transverse laparotomy.

As shown in Fig. 2A, laparotomized mice treated with the solvents of the drugs showed a very low mechanical threshold (indicative of tactile allodynia) (black bar) in comparison to uninjured animals (white bar). Ibuprofen (8–32 mg/kg, s.c.) induced a dose-dependent antiallodynic effect, although it was not able to achieve the threshold values observed in uninjured animals, even at the highest dose tested. Morphine (0.13–0.5 mg/kg, s.c.), by contrast, fully reversed tactile allodynia at a dose as low as 0.5 mg/kg. The highest doses of ibuprofen and morphine tested in laparotomized animals did not alter the mechanical threshold when administered to sham-operated animals (Fig. 2A). Accordingly, both compounds (at the doses tested) induced an antiallodynic effect

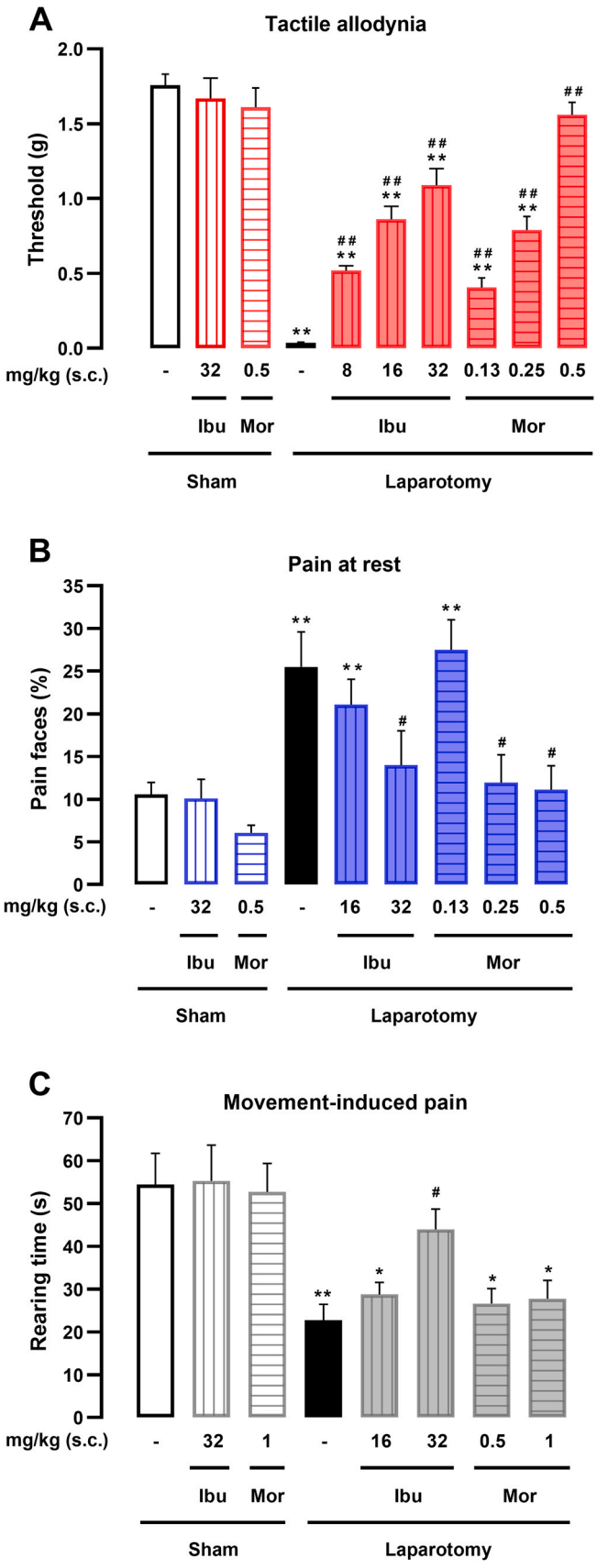

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Fig. 2. Effects of ibuprofen and morphine on the different aspects of postoperative pain in mice with a transverse laparotomy. The results represent the effects of the subcutaneous (s.c.) administration of ibuprofen, morphine, or their solvent (saline) on (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) after laparotomy. Behavioral evaluations were performed 3.5 h after laparotomy or sham procedure. (A-C) Each bar and vertical line represents the mean ± SEM of the values obtained in 7–11 mice. Statistically significant differences between the values obtained in sham mice treated with the solvent of the drugs (white bars) and the other experimental groups (*p < 0.05; **p < 0.01), and between the values obtained in laparotomized mice treated with saline (black bars) or the drugs tested (#p < 0.05; ##p < 0.01) (one-way ANOVA followed by Student-Newman-Keuls test).

without altering the nociceptive threshold to mechanical stimulation.

The effects of ibuprofen and morphine on pain at rest are shown in Fig. 2B. Laparotomized mice treated with the solvents of the drugs (black bar) showed higher rates of facial pain expressions than sham-operated mice (white bar). Both ibuprofen (16–32 mg/kg, s.c.) and morphine (0.13–0.5 mg/kg, s.c.) markedly decreased the detection of painful expressions in laparotomized mice, but at very different doses. For instance, 32 mg/kg of ibuprofen but just 0.25 mg/kg of morphine were needed to fully reverse the increase in facial pain expressions. The highest doses tested in laparotomized animals did not alter the facial expressions in sham-operated animals in either case (Fig. 2B), indicating that the effects of ibuprofen and morphine are not attributable to nonspecific facial expression alterations.

The effects of ibuprofen and morphine on movement-induced pain are shown in Fig. 2C. Laparotomized mice treated with the solvents of the drugs (black bar) spent considerably less time rearing in comparison to uninjured animals (white bar). Ibuprofen (16–32 mg/kg, s.c.) induced a dose-dependent increase in vertical activity in laparotomized mice, but the 32-mg/kg dose did not alter the exploratory behavior of uninjured animals. Therefore, the effects observed for this dose in laparotomized animals cannot be attributed to non-specific increases in motor behavior. We were unable to reliably evaluate ibuprofen 64 mg/kg (s.c.) in laparotomized mice, as in the uninjured animals, it produced a marked reduction in vertical activity (data not shown). Interestingly, despite the high potency and efficacy observed for morphine in the tactile allodynia and pain at rest tests, the administration of relatively high doses (0.5–1 mg/kg, s.c.) in laparotomized mice did not alter motor impairment.

To compare the effects of ibuprofen and morphine on each of the three aspects of postoperative pain, we expressed the data as a percentage of the analgesic effect. A 100 % effect was defined by the values from the saline-treated sham mice, while a 0 % effect was defined by the values from the saline-treated laparotomized animals. The comparison is shown in Fig. 3. Ibuprofen (8–16 mg/kg, s.c.) had a similar effect on tactile allodynia, pain at rest, and movement-induced pain. The analgesic effect was clearly dose dependent; its maximum pain relief (~70 %) was achieved with 16 mg/kg (Fig. 3A). As noted above, higher doses were not tested because they induced measurable motor impairment. When we compared the effects of morphine (0.13–1 mg/kg, s.c.), we found that a dose as low as 0.25 mg/kg was able to fully reverse pain at rest. A higher dose, 0.5 mg/kg, was needed to completely reverse tactile allodynia, while doses of up to 1 mg/kg had no effect on movement-induced pain (Fig. 3B).

Since NSAIDs and opioids are frequently used in combination to treat postoperative pain, we tested their combined effects on tactile allodynia, pain at rest, and movement-induced pain. For each of the drugs and scenarios, we chose the doses that had been ineffective in the above experiments: ibuprofen 8 mg/kg + morphine 0.13 mg/kg (Fig. 4A) for tactile allodynia, ibuprofen 16 mg/kg + morphine 0.13 mg/kg (Fig. 4B) for pain at rest, and ibuprofen 16 mg/kg + morphine 0.5 mg/kg (Fig. 4C) for movement-induced pain. The combination of inactive doses

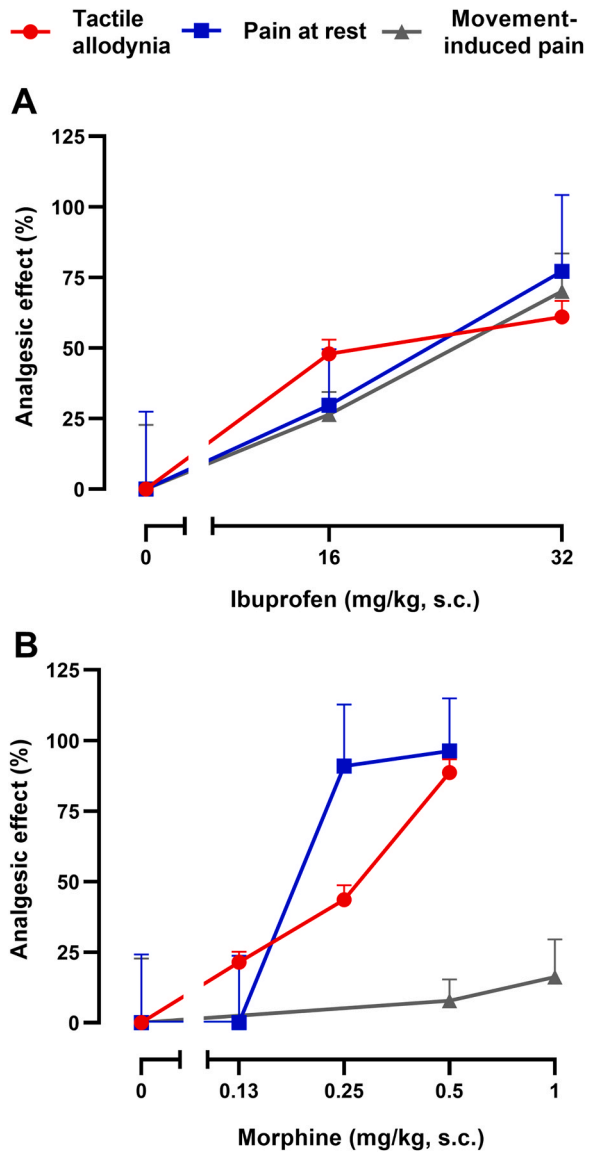

Fig. 3. Differences in sensitivity to ibuprofen and morphine for each aspect of postoperative pain evaluated. The results represent the effects of the subcutaneous (s.c.) administration of ibuprofen (A), morphine (B), or their solvent (saline, dose 0) on tactile allodynia (red), pain at rest (blue), and movement-induced pain (gray). Analgesic effects (%) were calculated from data shown in Fig. 3; the values from saline-treated sham animals and saline-treated laparotomized animals were considered to represent maximum pain relief (100 % effect) and zero pain relief (0 % effect), respectively. Behavioral evaluations were performed 3.5 h after laparotomy or sham procedure. Each point and vertical line represent the mean ± SEM of the values obtained in 7–11 mice.

of ibuprofen and morphine fully reversed the three pain-related outcomes in laparotomized mice and did not alter mechanical thresholds, facial expressions, or locomotor activity in sham-operated animals (Fig. 4A-C).

In summary, ibuprofen and morphine relieved postoperative pain in mice. The sensitivity of the drugs, and morphine in particular, varied according to the pain outcome examined. This indicates that tactile allodynia, pain at rest, and movement-induced pain are not equivalent measures of postoperative pain. Finally, the combined use of ibuprofen and morphine induced an enhanced analgesic effect on postoperative pain, mirroring the effects of NSAIDs and opioids in human patients.

3.3. Histological findings and immune cell recruitment in the surgical wound after laparotomy

We used hematoxylin-eosin staining to study the time course of histological changes in abdominal tissue after a transverse laparotomy.

Samples from laparotomized mice and sham-operated controls showed marked differences in tissue structure and integrity. In the control sham animals, the abdominal wall showed a normal histological architecture, with no signs of disruption of the epidermis, dermis, subcutaneous tissue, underlying muscle tissue, or peritoneum (Fig. 5A, first panel). A macroscopic view of the surgical wound at 3.5 h post-laparotomy shows a fresh wound with apparent swelling (second upper panel, Fig. 5A). Histological sections revealed a pronounced disruption of all tissue layers due to the surgical incision, with prominent edema in the subcutaneous tissue (second middle and lower panels, Fig. 5A). The macroscopic view from 48 h post-surgery shows some persistent swelling around the incision (third upper panel, Fig. 5A). The histological sections showed no signs of recovery from the incision in the dermis and epidermis; edema persisted in the subcutaneous tissue, although it was substantially less pronounced than at 3.5 h. There was evidence of initial muscle tissue regeneration (third middle and lower panels, Fig. 5A). At 240 h (10 days) after the laparotomy, there was macroscopically observable skin repair (last upper panel, Fig. 5A). The tissue samples showed almost complete regeneration of the injury, with near-full restoration of the normal structure and morphology of all skin layers, muscle tissue, and peritoneum (last middle and lower panels, Fig. 5A). The healing process at the macroscopic and microscopic levels suggests a regenerative response to the surgical insult within the studied time frame.

We then used FACS to investigate the dynamics of immune cell recruitment to the injury site at different time points. Naïve mice had almost negligible levels of immune (CD45+) cells, which is consistent with an undisturbed physiological state. Mice that had undergone laparotomy, by contrast, showed significant neutrophil infiltration (CD45+ CD11b+ Ly6G+), in particular at early time points (3.5 and 24 h post-surgery). This infiltration had subsided by later time points and returned to naïve levels by day 10 (240 h after laparotomy) (Fig. 5B and C). Macrophages/monocytes infiltrated the tissue later, to peak at 48 hours post-laparotomy. This cell population was still present 10 days after surgery (Fig. 5B and C). Other immune cells (CD45+ CD11b- Ly6G-) were virtually absent at all time points explored (Fig. 5B and C). In summary, immune cell recruitment after laparotomy showed a biphasic pattern characterized by an initial acute neutrophilic response followed by a sustained presence of macrophages/monocytes. This pattern is consistent with the tissue repair processes observed histologically.

3.4. Neutrophil depletion does not affect all aspects of postoperative pain equally

As neutrophils were the predominant immune cell in the immediate postoperative period (3.5 h after laparotomy), we administered anti-Ly6G in vivo to selectively deplete neutrophils to study their role in postoperative tactile allodynia, pain at rest, and movement-induced pain.

Treatment with anti-Ly6G (2–8 μg, i.p.) induced a dose-dependent decrease in neutrophil load in the surgical wound and fully prevented significant neutrophil infiltration after laparotomy at the highest dose tested (Fig. 6A). Notably, an equivalent dose (8 μg, i.p.) of an isotype non-reactive antibody did not induce any significant effects on neutrophil load in the surgical wound (Fig. 6A), indicating the selectivity of the neutrophil depletion strategy used. Representative FACS diagrams illustrating the gating strategy can be found in Fig. 6B.

We then tested the effects of anti-Ly6G and its isotype control (both at 8 μg, i.p.) on postoperative tactile allodynia, pain at rest, and movement-induced pain. Administration of these antibodies did not increase the mechanical threshold of laparotomized mice; this threshold

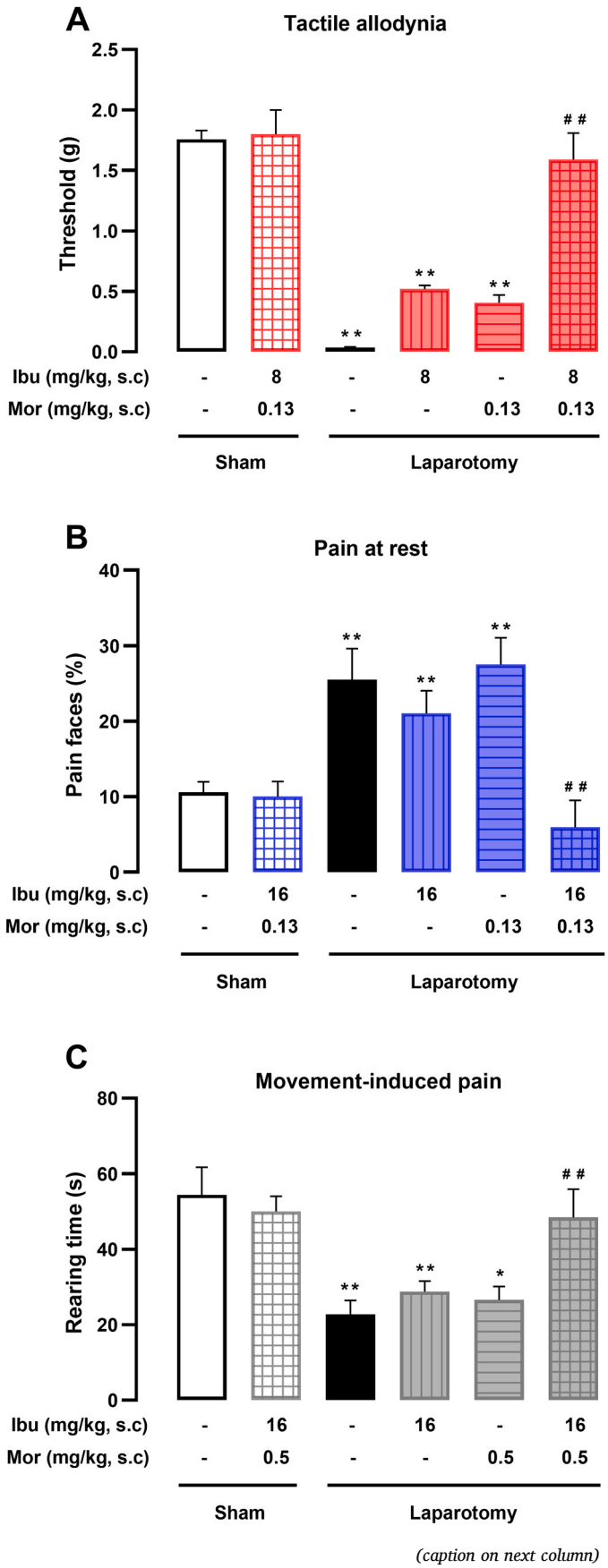

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Fig. 4. Effect of combined use of ibuprofen and morphine on the different aspects of postoperative pain in mice with a transverse laparotomy. The results represent the effects of the subcutaneous (s.c.) administration of low (inactive) doses of ibuprofen, morphine, and ibuprofen and morphine combined, and the administration of their solvent (saline) on (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). Behavioral evaluations were performed 3.5 h after laparotomy or sham procedure. (A-C) Each bar and vertical line represents the mean ± SEM of the values obtained in 7–11 mice. Statistically significant differences between the values obtained in sham mice treated with the solvent of the drugs (white bars) and the other experimental groups (*$p < 0.05$; **$p < 0.01$), and between the values obtained in laparotomized mice treated with saline (black bars) or the drugs tested (#$p < 0.05$; ##$p < 0.01$) (one-way ANOVA followed by Student-Newman-Keuls test).

remained markedly lower than those observed for sham-operated animals treated with the antibody solvents (Fig. 6C). Neutrophil depletion did not, therefore, alter tactile allodynia induced by laparotomy. However, laparotomized mice administered anti-Ly6G showed no apparent increase in facial pain expressions (Fig. 6C) and were able to stand on their hindlimbs, just like the uninjured mice (Fig. 6D). The isotype control did not alter any of the pain outcomes studied (Fig. 6C and D). These latter results suggest that neutrophils play an important role in the development of pain at rest and movement-induced pain after laparotomy.

In summary, the role of neutrophils in postoperative pain varied according to the aspect of pain evaluated. Whereas neutrophils seem to be relevant for pain at rest and movement-induced pain, they do not appear to have a role in tactile allodynia.

3.5. *The abdominal wall is innervated by thoracic DRG*

To determine the location of neurons innervating the site injured during transverse laparotomy, we used immunofluorescence double labeling for ATF3, a marker of neuronal stress, and NeuN, a general neuronal marker. We tested DRG from levels T6 to S2 in laparotomized animals and found that ATF3 expression was largely absent in most of the ganglia examined; the exception was T11-T13 DRG, which had a notable concentration of ATF3+ neurons, with a clear peak in the DRG at T12 (Fig. 7A). Sham-operated animals did not show appreciable numbers of ATF3+ cells in T11-T13 DRG (Fig. 7A) or T6-S2 DRG (data not shown). Fig. 7B shows representative images of ATF3 and NeuN staining in T12 DRG samples obtained from sham-operated (upper panels) and laparotomized animals (lower panels). The images clearly show an absence of ATF3 staining for uninjured animals and intense staining for laparotomized mice.

We also compared ATF3 expression in L3 and T12 DRG between laparotomized mice and mice with a plantar incision. Transverse laparotomy triggered ATF3 expression in T12 but not lumbar DRG, whereas plantar incision induced ATF3 expression in L3 DRG but not T12 DRG (Supplemental Figure S3). These results serve as a control as they demonstrate the specificity of ATF3 expression in DRG corresponding to neurons innervating the injured area.

3.6. *Differential effects of pharmacological inhibition of nociceptive neuron subtypes on the different aspects of postoperative pain*

We first studied the distribution of peptidergic and non-peptidergic nociceptors in T12 DRG, which innervate the surgical site, as described in the preceding section. We stained DRG samples with NeuN to identify all sensory neurons, IB4 to identify non-peptidergic nociceptors, and the neuropeptide CGRP to identify peptidergic nociceptors. There was no overlap between IB4 and CGRP staining, reflecting the ability of these markers to label two distinct populations within NeuN+ cells (see Fig. 8A).

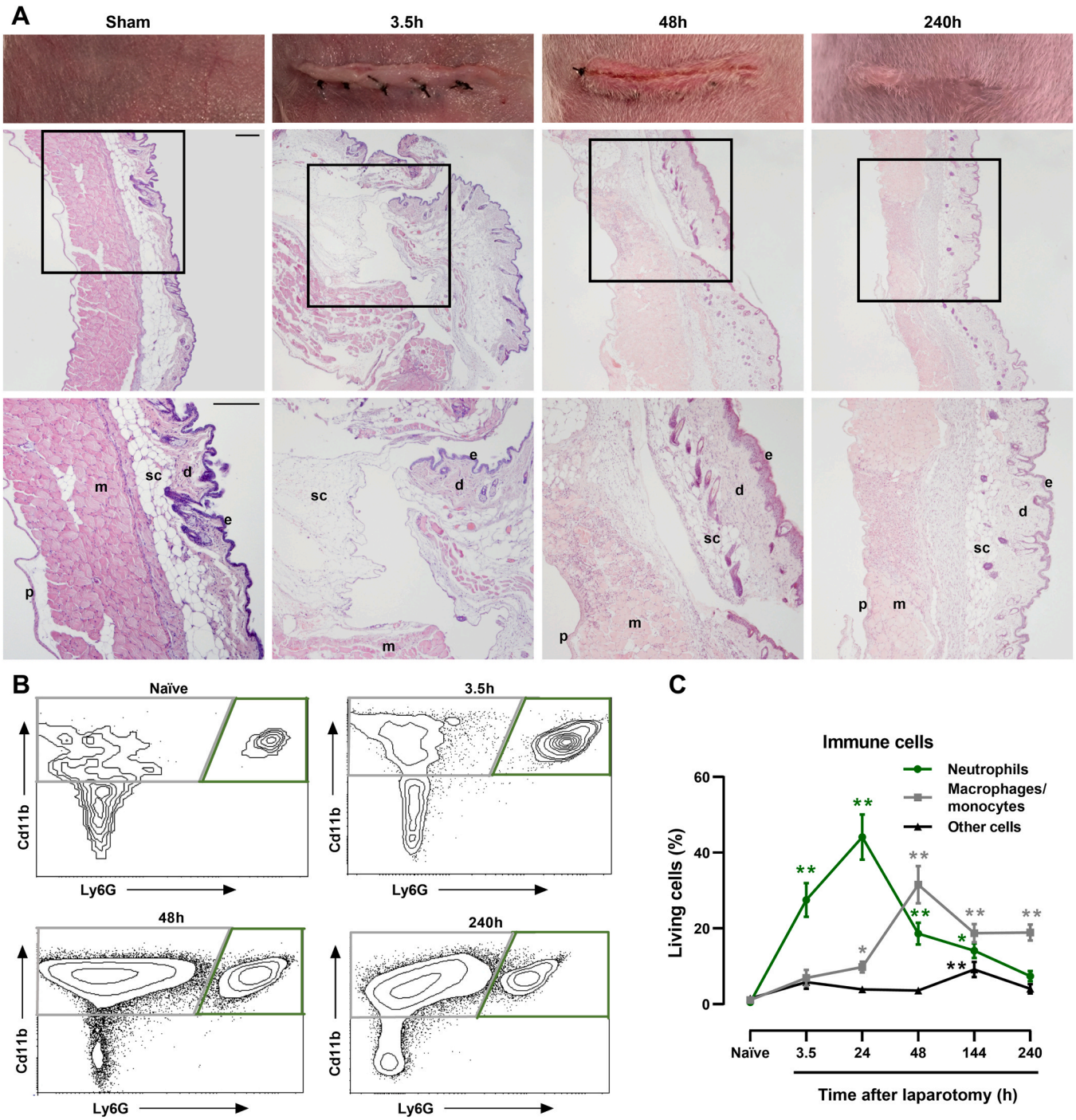

Fig. 5. Time course of changes in histological findings and immune cell recruitment in the surgical wound after a transverse laparotomy. (A) Representative pictures of the lower abdominal area from sham mice 3.5 h after shaving the abdominal area under anesthesia and from injured mice 3.5, 48 and 240 h after laparotomy, and corresponding photomicrographs of hematoxylin eosin–stained abdominal sections. The relevant structures are labeled for clarity (e, epidermis; d, dermis; sc, subcutaneous tissue; m, muscle tissue; p, peritoneum). Scale bar is 200 μm. The bottom panels show details of the boxed areas in the middle panels. (B) Representative FACS diagrams with gating from CD45+ cells showing neutrophils (CD11b+Ly6G+), macrophages/monocytes (CD11b+Ly6G-) and other hematopoietic cells (CD11b-) from samples of the abdominal wall of naïve animals and from laparotomized mice 3.5, 48 and 240 h after surgery. Gating for neutrophil and macrophage/monocyte quantification is shown separately from the rest of hematopoietic cells by green and gray polygons. (C) Time course of quantification of neutrophils, macrophages/monocytes, and other hematopoietic cells with respect to number of living cells in abdominal wall samples from naïve and laparotomized mice. Each point and vertical line represent the mean ± SEM of the values obtained in 6–10 samples per group, with each sample taken from a single animal. Statistically significant differences between the values obtained for each cell type in samples from naïve mice and the other experimental groups (*$p < 0.05$; **$p < 0.01$) (one-way ANOVA followed by Student-Newman-Keuls test).

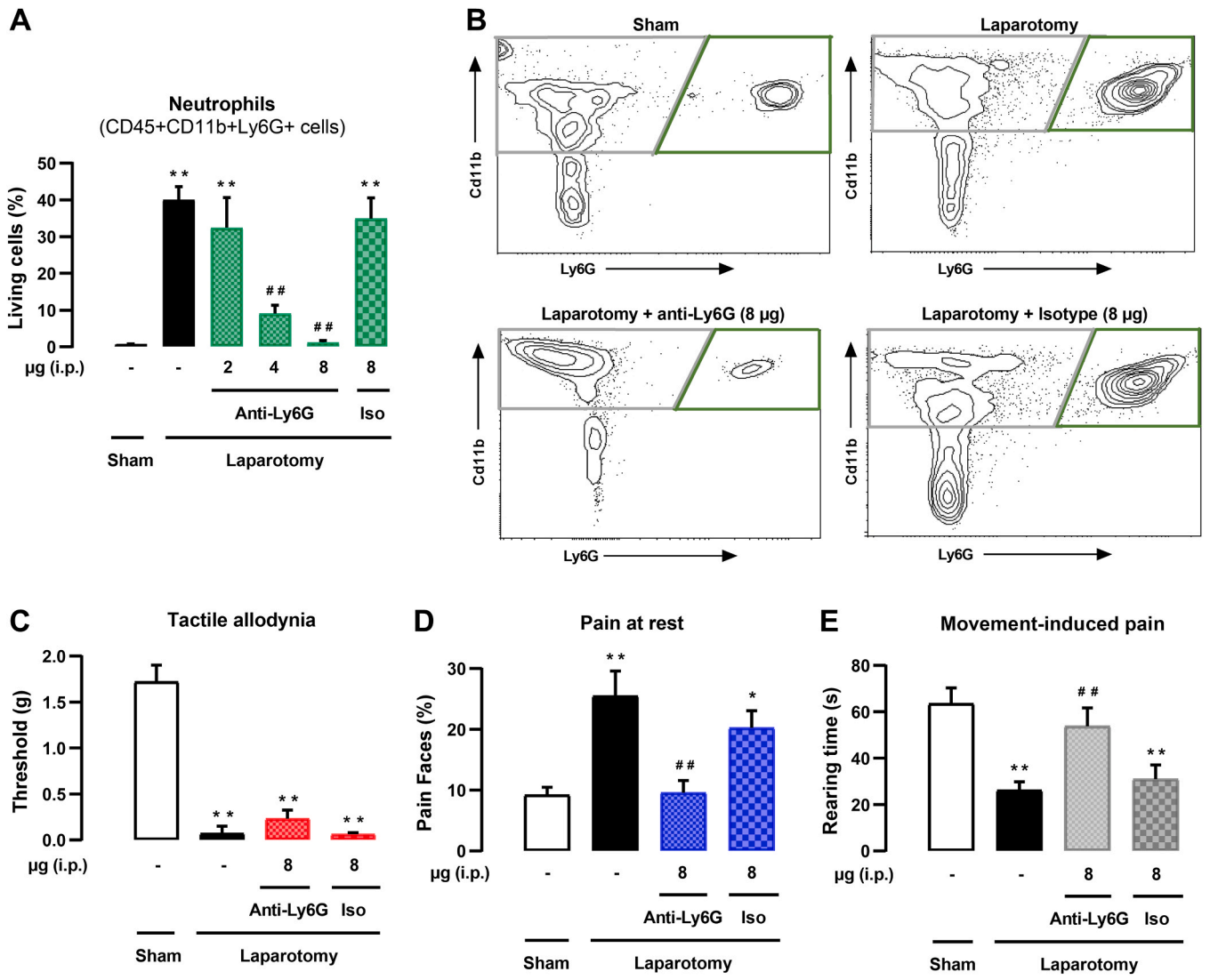

Fig. 6. Effect of neutrophil depletion on the different aspects of postoperative pain in mice with a transverse laparotomy. (A) Quantification of neutrophils (CD45+CD11b+Ly6G+ cells) with respect to number of living cells in samples of sham and laparotomized mice treated with saline, anti-Ly6G, or the isotype control. Each bar and vertical line represent the mean ± SEM of the values obtained in 7–8 samples per group, with each sample taken from a single animal. (B) Representative FACS diagrams, with gating from CD45+ cells, showing neutrophils (CD11b+Ly6G+) and macrophages/monocytes (CD11b+Ly6G-) in uninjured (sham) mice and laparotomized mice treated intraperitoneally (i.p.) with saline, anti-Ly6G, or the isotype control. Gating for neutrophils and macrophage/monocytes is shown in green and gray rectangles, respectively. Samples were obtained 3.5 h after laparotomy or sham procedure (C-E) The results represent the effects of the administration of saline, anti-Ly6G, or the isotype control on (C) tactile allodynia (reductions in mechanical withdrawal threshold in abdominal area), (D) pain at rest (presence of facial pain expressions), and (E) movement-induced pain (reductions in time spent rearing). Behavioral evaluations were performed 3.5 h after laparotomy or sham procedure. Each bar and vertical line represent the mean ± SEM of the values obtained in 8–11 mice. (B-E) Statistically significant differences between the values obtained in sham mice treated with the solvent of the drugs (white bars) and the other experimental groups (*$p < 0.05$; **$p < 0.01$), and between the values obtained in laparotomized mice treated with saline (black bars) or anti-Ly6G (#$p < 0.05$; ##$p < 0.01$) (one-way ANOVA followed by Student-Newman-Keuls test).

Next, we studied the effects of gefapixant and olcegepant (both at 8–16 mg/kg, s.c.) on the different aspects of postoperative pain. Gefapixant is a selective P2X3 antagonist that inhibits non-peptidergic nociceptors, while olcegepant is a selective antagonist of the CGRP receptor (see Introduction for references). Gefapixant induced a dose-dependent near-complete reversal of tactile allodynia in laparotomized mice. The antiallodynic effect of olcegepant was also dose dependent, although slightly lower than that of gefapixant (Fig. 8B). Neither of these drugs, at the highest dose tested, modified the mechanical threshold in uninjured animals (Fig. 8B).

On testing the effects of gefapixant and olcegepant on pain at rest, we found that gefapixant fully reversed, in a dose-dependent fashion, the post-laparotomy increase in facial pain expressions observed (Fig. 8C). Notably, gefapixant at a dose of 8 mg/kg, which had a ~50 % antiallodynic effect, almost completely reversed pain at rest (compare Fig. 8B and C). The opposite was observed for olcegepant (16 mg/kg), which had a clear antiallodynic effect but was unable to relieve pain at rest in laparotomized animals (Fig. 8C). Neither of these drugs, at the highest dose tested, modified facial expressions in sham-operated mice (Fig. 8C).

Finally, neither gefapixant nor olcegepant (16 mg/kg, s.c., in both cases) reversed movement-induced pain in laparotomized animals (Fig. 8C) or altered exploratory activity in uninjured animals (Fig. 8C). Therefore, their lack of efficacy against this aspect of the postoperative pain phenotype cannot be explained by non-specific effects on exploratory locomotor activity that might have interfered with the analgesic

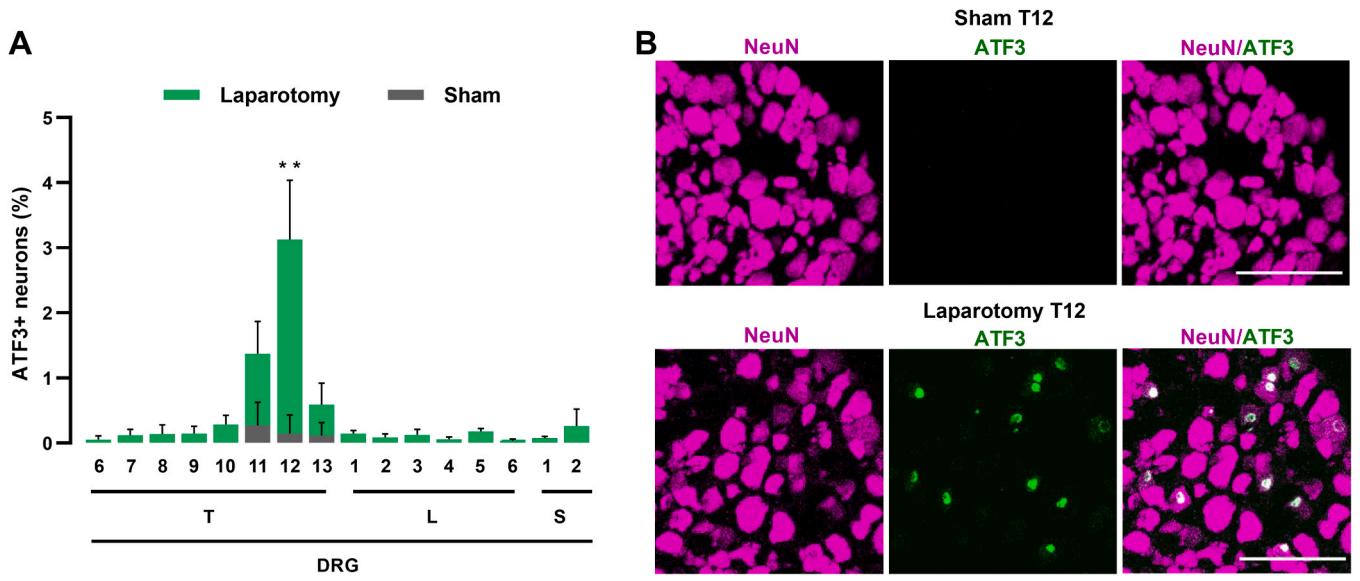

Fig. 7. Most neurons innervating the surgical wound in mice with a transverse laparotomy are located in T12 dorsal root ganglion (DRG). (A) Percentage of ATF3-expressing neurons in thoracic (T6-T13), lumbar (L1–6), and sacral (S1–2) DRG from sham and laparotomized mice. Since AFT3 staining was virtually absent in samples from sham mice at all the levels explored, only values from T11–13 are shown for clarity. Each bar and vertical line represent the mean ± SEM of the values obtained in four DRG samples each obtained from independent mice. Statistically significant differences between values from sham and injured mice obtained at the same DRG level (*$p < 0.05$, **$p < 0.01$) (one-way ANOVA followed by Student-Newman-Keuls test). (B) Representative images from double labeling of pan-neuronal marker NeuN (magenta) and ATF3 (green) in T12 DRG from sham and laparotomized animals. Scale bar is 50 μm.

effect. On testing higher doses (32 mg/kg), we found that whereas gefapixant remained inactive, olcegepant induced a significant reduction in time spent rearing by uninjured animals (data not shown). We therefore could not reliably evaluate its effects on laparotomized animals.

These results highlight differential pharmacological effects on the various aspects of postoperative pain.

4. Discussion

Our study provides a comprehensive examination of postoperative pain in laparotomized mice and reveals fundamental differences in pharmacological and basic mechanisms underlying tactile allodynia, pain at rest, and movement-induced pain.

The mechanical withdrawal threshold returned to baseline levels 10 days after laparotomy, when the histological architecture of the abdominal wall and levels of infiltrated immune cells had returned to near-normal levels. These findings suggest that the healing process and coordinated immune-mediated resolution of inflammation and edema have an impact on cutaneous sensitivity. However, pain at rest and movement-induced pain were much less sensitive markers of pain-induced alterations, as they were of a much shorter duration. The persistence of tactile allodynia over other measures of pain is a common feature of pain models [7], and it might suggest that the mechanisms driving cutaneous hypersensitivity might not fully overlap with those involved in pain at rest or movement-induced pain.

We show here that morphine was able to fully reverse both tactile allodynia and pain at rest in laparotomized mice, an unsurprising finding considering the effectiveness of this drug in the management of moderate to severe pain [36]. Notably, it provided greater relief against pain at rest, as assessed by facial pain expressions. Facial expressions are thought to be social signals that convey emotional states in both humans and rodents [37]. In mouse models, damage to the insula, an area involved in emotional processing, has been found to reduce facial expressions of pain but not other pain measures [9]. As the insula and other areas related to emotional processing are major contributors to opioid antinociception [38], the strong effect exerted by morphine on pain at rest might be explained by its actions on the emotional component of pain. Supporting previous findings from rodent studies [8], morphine did not improve the exploratory locomotor activity of laparotomized mice [8]. This finding is also consistent with the known lack of analgesic effect of opioid drugs on movement-induced pain in postsurgical patients [3,4]. Although it had limited efficacy, ibuprofen showed a consistent effect across all three pain outcomes, even movement-induced pain, in laparotomized mice. These data are consistent with effects observed for NSAIDs in post-surgical patients, showing that, unlike opioids, NSAIDs have comparable efficacy in providing relief from pain at rest and movement-induced pain [5,6].

We also evaluated the combined use of morphine and ibuprofen, and found that at doses that were ineffective when the drugs were administered separately, the drugs combined fully reversed tactile allodynia, pain at rest, and movement-induced pain. This effect could fall within the definition of cooperative effect synergy [39]. Our results are in agreement with the known synergistic analgesic effects induced by the combination of NSAIDs and opioids in both rodents [8] and post-surgical patients [40]. They also support guideline recommendations to use multimodal analgesia to treat postoperative pain [2]. Altogether, our data on the use of ibuprofen and morphine in laparotomized animals closely reproduce the pharmacological effects observed in human patients, validating the behavioral outcomes selected in this study to assess the different aspects of postoperative pain.

The mechanism underlying the analgesic actions of NSAIDs involves inhibiting the synthesis of prostaglandins, particularly prostaglandin E2 (PGE2). This compound is a major algogenic chemical released during inflammation, such as that triggered by tissue injury. Infiltrating inflammatory cells constitute a major source of PGE2 [41] and are therefore a prominent target for NSAIDs. We sought to determine the contribution of infiltrating immune cells to postoperative pain. Supporting previous reports [42], neutrophils constituted the largest cell population in the early immune infiltrate, while macrophages became prominent several days after injury. We then used a selective depletion strategy to study the contribution of neutrophils to postoperative pain. Neutrophil depletion in the surgical wound did not alter postoperative tactile allodynia in laparotomized mice, but it completely reversed pain at rest and movement-induced pain. If we consider that NSAIDs mainly reduce allodynia by decreasing PGE2 production by inflammatory cells,

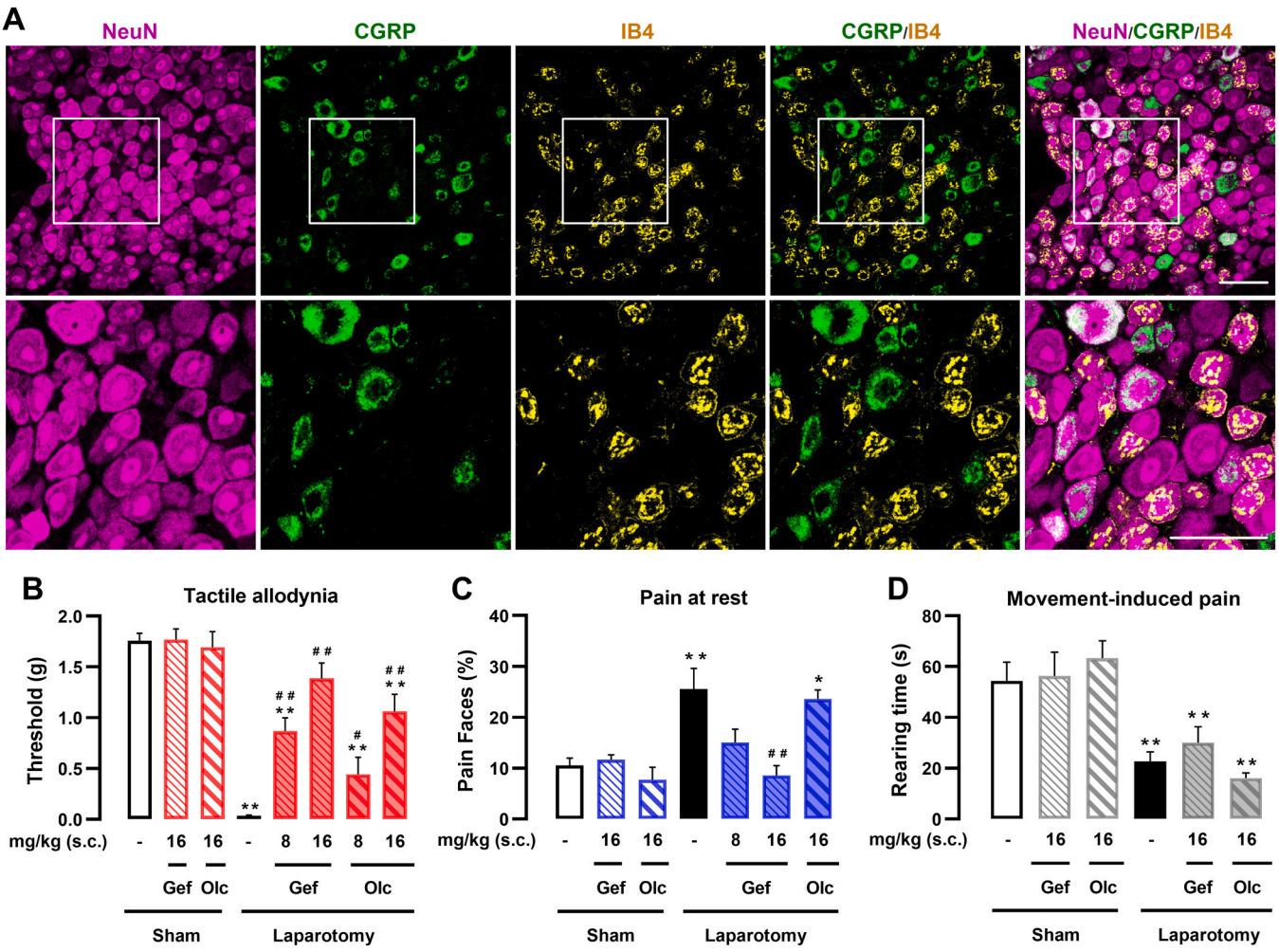

Fig. 8. Nociceptors and the different aspects of postoperative pain in mice with a transverse laparotomy. (A) Representative images showing triple labeling of pan-neuronal marker NeuN (magenta), CGRP (green), and isolectin B4 (IB4, yellow) in T12 dorsal root ganglion (DRG). The bottom panels show a higher-magnification view of the areas squared in the top panels. Scale bar is 50 μm. (B-D) The results represent the effects of the subcutaneous (s.c.) administration of the P2X3 antagonist gefapixant (Gef), the CGRP receptor antagonist olcegepant (Olc), or their solvent (saline) in (B) tactile allodynia (reductions in mechanical withdrawal threshold in abdominal area), (C) pain at rest (presence of facial pain expressions), and (D) movement-induced pain (reductions in time spent rearing). Behavioral evaluations were performed 3.5 h after laparotomy or sham procedure. (B-D) Each bar and vertical line represents the mean ± SEM of the values obtained in 6–11 mice. Statistically significant differences between the values obtained in sham mice treated with the solvent of the drugs (white bars) and the other experimental groups (*$p < 0.05$; **$p < 0.01$), and between the values obtained in laparotomized mice treated with saline (black bars) or the drugs tested (#$p < 0.05$; ##$p < 0.01$) (one-way ANOVA followed by Student-Newman-Keuls test).

then neutrophil depletion should have a similar effect on this particular aspect of pain. Actions of NSAIDs in DRG [43] and the spinal cord [44] might partly explain why ibuprofen exerts antiallodynic effects without necessarily targeting neutrophils. Regardless of the exact mechanisms involved, our neutrophil depletion results clearly show that tactile allodynia does not contribute to pain at rest or movement-induced pain.

The absence of effect of neutrophil depletion on tactile allodynia in laparotomized mice in our study is fully consistent with previous reports in mice with a plantar incision [42,45,46], and suggests that neutrophils do not play a determining role in this aspect of postoperative pain. Only one study has evaluated the effects of neutrophil depletion on post-operative pain at rest and movement-induced pain in a plantar incision mouse model [46]. Unlike us, the authors found that inhibition of these immune cells had no significant effects on pain at rest (comparison of weight borne by injured and uninjured paws) and movement-induced pain (gait analysis). Considering that measurement of both outcomes requires contact between the injured paw and the floor, it may be that tactile allodynia, which consistently remained unaltered by neutrophil depletion, might have inadvertently influenced measures of pain at rest and movement-induced pain. This confounder was absent from our study of laparotomized animals, enabling us to unambiguously measure these aspects of postoperative pain.

We also tested the effects of gefapixant, a P2X3 antagonist. Gefapixant has been approved by several regulatory agencies as a non-narcotic antitussive drug [19], but it has also been tested in preclinical pain models of inflammatory and neuropathic pain, mainly using measures of cutaneous hypersensitivity [18]. ATP released from the cytoplasmic contents of lysed cells is known to be an important driver of tissue injury-induced pain through P2X3 receptors [17]. Surprisingly, even though tissue injury is unavoidable during surgical procedures, no studies to date have analyzed the effects of gefapixant on postoperative pain. In our study of laparotomized mice, gefapixant reversed cutaneous hypersensitivity and pain at rest, but had no effect on movement-induced pain. P2X3 receptors are expressed by non-peptidergic nociceptors, which can be labeled with IB4 [22]. We have demonstrated here that IB4+ neurons constitute a distinct population to nociceptors expressing the neuropeptide CGRP in thoracic DRG, which are known to innervate visceral organs (such as the peritoneum) [47] and according to our data also innervate the abdominal wall. The segregation of these neuronal populations is consistent with

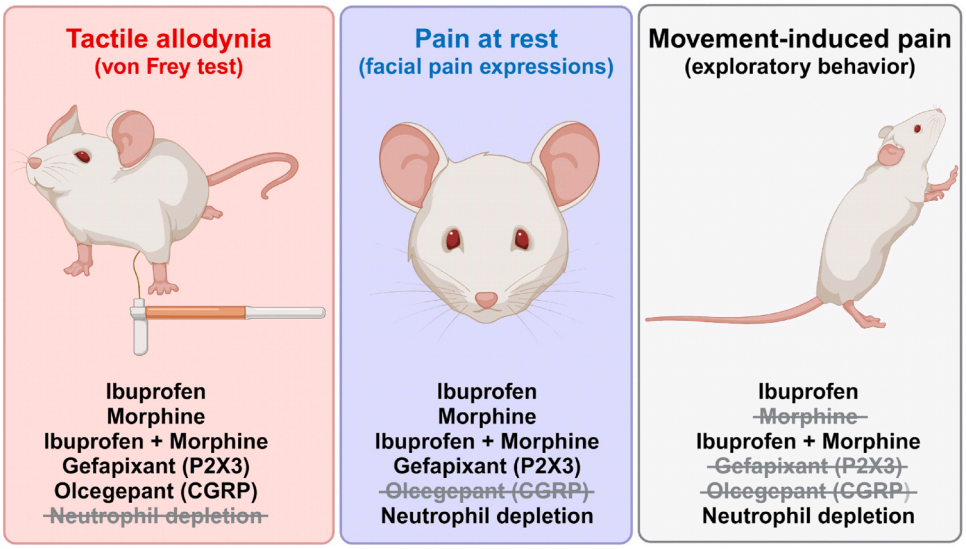

Fig. 9. Summary of the differences observed for the three aspects of postoperative pain in mice with a transverse laparotomy. The picture shows the results for tactile allodynia, (reductions in mechanical withdrawal threshold [von Frey test] in abdominal area) (red), pain at rest (detection of facial pain expressions) (blue), and movement-induced pain (reductions in exploratory behavior) after laparotomy (gray). Treatments with an effect on each outcome are shown in black; treatments with no effect are shown crossed out and in gray. Images were created with BioRender.com.

previous findings from studies of lumbar DRGs innervating hind paws [20–22]. We also tested the effects of olcegepant, a CGRP receptor antagonist belonging to a new class of antimigraine drugs called gepants [23]. Although their effects on postoperative pain have not been studied, gepants have been investigated in other preclinical pain models, such as pain sensitization induced by chemical algogens or chronic arthritis, but always with cutaneous sensitivity as the readout [48,49]. In our study, olcegepant decreased postoperative tactile allodynia, but did not alter pain at rest or movement-induced pain. Our results suggest that while ATP signaling through P2X3 receptors participates in both tactile allodynia and pain at rest, the effects of CGRP appear to be limited to tactile hypersensitivity, and that neither of the compounds has an effect on movement-induced pain. Once again, these findings highlight the differences in pharmacological responses across distinct aspects of postoperative pain.

5. Conclusion

This study shows that none of the three aspects of postoperative pain explored—tactile allodynia, pain at rest, and movement-induced pain—can predict the effects of a given intervention on the other two. This is exemplified by the results observed for morphine, gefapixant, olcegepant, and immune cell depletion, which reversed some aspects of postoperative pain but had no effects on others (see Fig. 9 for a summary). Our results indicate that the mechanisms underlying the different aspects of postoperative pain are not fully overlapping and merit further investigation. Combinations of pain measures provide a more complete and realistic view of postoperative pain and have the potential to benefit analgesic development by providing nonoverlapping information that extends and complements findings provided by standard pain measures.

Funding

This study was partially supported by the Spanish State Research Agency (10.13039/501100011033) under the auspices of the Spanish Ministry of Science, Innovation and Universities (grant number PID2019-108691RB-I00), the Andalusian Regional Government (grant CTS-109), and "la Caixa" Foundation (CaixaResearch Consolidate 2022-CC22-10176). The authors also thank the support of the Unit of Excellence 'UNETE' from the University of Granada (reference UCE-PP2017-05). M. Santos-Caballero was supported by the Research Personnel Training Program (FPI grant PRE2020-096203) and M.Á. Huerta by the Training University Lecturers program (FPU21/02736), both from the Spanish Ministry of Science, Innovation and Universities.

CRediT authorship contribution statement

Francisco J. Cañizares: Writing – review & editing, Methodology, Investigation. **Rafael González-Cano:** Writing – review & editing, Writing – original draft, Supervision, Software, Methodology. **Enrique J Cobos:** Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Project administration, Methodology, Funding acquisition. **Miriam Santos-Caballero:** Writing – review & editing, Writing – original draft, Methodology, Investigation. **Makeya A. Hasoun:** Investigation. **Miguel Á. Huerta:** Investigation. **M. Carmen Ruiz-Cantero:** Writing – review & editing, Investigation. **Miguel Á. Tejada:** Investigation. **María Robles-Funes:** Investigation. **Eduardo Fernández-Segura:** Writing – review & editing, Methodology, Investigation.

Declaration of Competing Interest

The authors declare no competing interests.

Data availability

Data will be made available on request.

Acknowledgments

We thank Gustavo Ortiz Ferrón for his technical support with the fluorescence-activated cell sorting and Sara Moreno Sanjuan with confocal microscopic image acquisition. We also thank A. Murray for improving the use of English in a version of this manuscript. This research was done in partial fulfilment of the requirements for the doctoral thesis of Miriam Santos-Caballero.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the

online version at doi:10.1016/j.biopha.2024.117459.

References

[1] T.J. Gan, Poorly controlled postoperative pain: prevalence, consequences, and prevention, J. Pain. Res 10 (2017) 2287–2298, https://doi.org/10.2147/JPR.S144066.

[2] R. Chou, D.B. Gordon, O.A. de Leon-Casasola, et al., Management of Postoperative Pain: A Clinical Practice Guideline From the American Pain Society, the American Society of Regional Anesthesia and Pain Medicine, and the American Society of Anesthesiologists' Committee on Regional Anesthesia, Executive Committee, and Administrative Council, J. Pain. 17 (2016) 131–157, https://doi.org/10.1016/j.jpain.2015.12.008.

[3] S. Srikandarajah, I. Gilron, Systematic review of movement-evoked pain versus pain at rest in postsurgical clinical trials and meta-analyses: a fundamental distinction requiring standardized measurement, Pain 152 (2011) 1734–1739, https://doi.org/10.1016/j.pain.2011.02.008.

[4] J.F. Fiore, C. El-Kefraoui, M. Chay, et al., Opioid versus opioid-free analgesia after surgical discharge: a systematic review and meta-analysis of randomised trials, Lancet 399 (2022) 2280–2293, https://doi.org/10.1016/S0140-6736(22)00582-7.

[5] I. Gilron, B. Milne, M. Hong, Cyclooxygenase-2 inhibitors in postoperative pain management: current evidence and future directions, Anesthesiology 99 (2003) 1198–1208, https://doi.org/10.1097/00000542-200311000-00029.

[6] A.M. Zeng, N.F. Nami, C.L. Wu, J.D. Murphy, The analgesic efficacy of nonsteroidal anti-inflammatory agents (NSAIDs) in patients undergoing cesarean deliveries: a meta-analysis, Reg. Anesth. Pain. Med 41 (2016) 763–772, https://doi.org/10.1097/AAP.0000000000000460.

[7] R. González-Cano, Á. Montilla-García, M.C. Ruiz-Cantero, et al., The search for translational pain outcomes to refine analgesic development: where did we come from and where are we going? Neurosci. Biobehav Rev. 113 (2020) 238–261, https://doi.org/10.1016/j.neubiorev.2020.03.004.

[8] T.J. Martin, N.L. Buechler, W. Kahn, et al., Effects of laparotomy on spontaneous exploratory activity and conditioned operant responding in the rat: a model for postoperative pain, Anesthesiology 101 (2004) 191–203, https://doi.org/10.1097/00000542-200407000-00030.

[9] D.J. Langford, A.L. Bailey, M.L. Chanda, et al., Coding of facial expressions of pain in the laboratory mouse, Nat. Methods 7 (2010) 447–449, https://doi.org/10.1038/nmeth.1455.

[10] D. Bree, O. Moriarty, C.M. O'Mahony, et al., Development and characterization of a novel, anatomically relevant rat model of acute postoperative pain, J. Pain. 16 (2015) 421–426, https://doi.org/10.1016/j.jpain.2015.01.010.

[11] T.J. Brennan, E.P. Vandermeulen, G.F. Gebhart, Characterization of a rat model of incisional pain, Pain 64 (1996) 493–502, https://doi.org/10.1016/0304-3959(95)01441-1.

[12] P. Jirkof, N. Cesarovic, A. Rettich, et al., Burrowing behavior as an indicator of post-laparotomy pain in mice, Front Behav. Neurosci. 4 (2010) 165, https://doi.org/10.3389/fnbeh.2010.00165.

[13] L.V. Kendall, D.J. Wegenast, B.J. Smith, et al., Efficacy of sustained-release buprenorphine in an experimental laparotomy model in female mice, J. Am. Assoc. Lab Anim. Sci. 55 (2016) 66–73. ⟨https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4747013/⟩.

[14] M.J. Carney, J.M. Weissler, J.P. Fox, et al., Trends in open abdominal surgery in the United States-Observations from 9,950,759 discharges using the 2009-2013 National Inpatient Sample (NIS) datasets, Am. J. Surg. 214 (2017) 287–292, https://doi.org/10.1016/j.amjsurg.2017.01.001.

[15] R. Ji, A. Chamessian, Y. Zhang, Pain Regulation by Non-neuronal Cells and Inflammation, Science 354 (2016) 572–577, https://doi.org/10.1126/science.aaf8924.

[16] M.C. Ruiz-Cantero, R. González-Cano, M.Á. Tejada, et al., Sigma-1 receptor: a drug target for the modulation of neuroimmune and neuroglial interactions during chronic pain, Pharm. Res 163 (2021) 105339, https://doi.org/10.1016/j.phrs.2020.105339.

[17] S.P. Cook, E.W. McCleskey, Cell damage excites nociceptors through release of cytosolic ATP, Pain 95 (2002) 41–47, https://doi.org/10.1016/s0304-3959(01)00372-4.

[18] J.L. Krajewski, P2X3-containing receptors as targets for the treatment of chronic pain, Neurotherapeutics 17 (2020) 826–838, https://doi.org/10.1007/s13311-020-00934-2.

[19] A. Markham, Gefapixant: first approval, Drugs 82 (2022) 691–695, https://doi.org/10.1007/s40265-022-01700-8.

[20] M. Zwick, B.M. Davis, C.J. Woodbury, et al., Glial cell line-derived neurotrophic factor is a survival factor for isolectin B4-positive, but not vanilloid receptor 1-positive, neurons in the mouse, J. Neurosci. 22 (2002) 4057–4065, https://doi.org/10.1523/JNEUROSCI.22-10-04057.2002.

[21] D.J. Cavanaugh, H. Lee, L. Lo, et al., Distinct subsets of unmyelinated primary sensory fibers mediate behavioral responses to noxious thermal and mechanical stimuli, Proc. Natl. Acad. Sci. USA 106 (2009) 9075–9080, https://doi.org/10.1073/pnas.0901507106.

[22] M.C. Ruiz-Cantero, M.Á. Huerta, M.Á. Tejada, et al., Sigma-1 receptor agonism exacerbates immune-driven nociception: Role of TRPV1 + nociceptors, Biomed. Pharm. 167 (2023) 115534, https://doi.org/10.1016/j.biopha.2023.115534.

[23] S. Younis, N.V. Latysheva, A.B. Danilov, M. Ashina, CGRP receptor antagonists (gepants), Handb. Clin. Neurol. 199 (2024) 51–66, https://doi.org/10.1016/B978-0-12-823357-3.00033-1.

[24] S. Iyengar, M.H. Ossipov, K.W. Johnson, The role of calcitonin gene-related peptide in peripheral and central pain mechanisms including migraine, Pain 158 (2017) 543–559, https://doi.org/10.1097/j.pain.0000000000000831.

[25] J.V. Roughan, H.G.M.J. Bertrand, H.M. Isles, Meloxicam prevents COX-2-mediated post-surgical inflammation but not pain following laparotomy in mice, Eur. J. Pain. 20 (2016) 231–240, https://doi.org/10.1002/ejp.712.

[26] M.A. Tejada, A. Montilla-García, S.J. Cronin, et al., Sigma-1 receptors control immune-driven peripheral opioid analgesia during inflammation in mice, Proc. Natl. Acad. Sci. USA 114 (2017) 8396–8401, https://doi.org/10.1073/pnas.1620068114.

[27] R. González-Cano, M. Merlos, J.M. Baeyens, C.M. Cendán, σ1 receptors are involved in the visceral pain induced by intracolonic administration of capsaicin in mice, Anesthesiology 118 (2013) 691–700, https://doi.org/10.1097/ALN.0b013e318280a60a.

[28] R. Gonzalez-Cano, B. Boivin, D. Bullock, et al., Up-down reader: an open source program for efficiently processing 50% von frey thresholds, Front Pharm. 9 (2018) 433, https://doi.org/10.3389/fphar.2018.00433.

[29] A.H. Tuttle, M.J. Molinaro, J.F. Jethwa, et al., A deep neural network to assess spontaneous pain from mouse facial expressions, Mol. Pain. 14 (2018) 1744806918763658, https://doi.org/10.1177/1744806918763658.

[30] T. Vezza, J.A. Molina-Tijeras, R. González-Cano, et al., Minocycline prevents the development of key features of inflammation and pain in DSS-induced colitis in mice, J. Pain. 24 (2023) 304–319, https://doi.org/10.1016/j.jpain.2022.09.016.

[31] A. Mathis, P. Mamidanna, K.M. Cury, et al., DeepLabCut: markerless pose estimation of user-defined body parts with deep learning, Nat. Neurosci. 21 (9) (2018) 1281, https://doi.org/10.1038/s41593-018-0209-y.

[32] Á. Montilla-García, M.Á. Tejada, G. Perazzoli, et al., Grip strength in mice with joint inflammation: a rheumatology function test sensitive to pain and analgesia, Neuropharmacology 125 (2017) 231–242, https://doi.org/10.1016/j.neuropharm.2017.07.029.

[33] H.K. Wolf, R. Buslei, R. Schmidt-Kastner, et al., NeuN: a useful neuronal marker for diagnostic histopathology, J. Histochem Cytochem 44 (1996) 1167–1171, https://doi.org/10.1177/44.10.8813082.

[34] C.J. Laedermann, M. Pertin, M.R. Suter, I. Decosterd, Voltage-gated sodium channel expression in mouse DRG after SNI leads to re-evaluation of projections of injured fibers, Mol. Pain. 10 (2014) 19, https://doi.org/10.1186/1744-8069-10-19.

[35] I. Bravo-Caparrós, M.C. Ruiz-Cantero, G. Perazzoli, et al., Sigma-1 receptors control neuropathic pain and macrophage infiltration into the dorsal root ganglion after peripheral nerve injury, FASEB J. 34 (2020) 5951–5966, https://doi.org/10.1096/fj.201901921R.

[36] G.W. Pasternak, Mu opioid pharmacology: 40 years to the promised land, Adv. Pharm. 82 (2018) 261–291, https://doi.org/10.1016/bs.apha.2017.09.006.

[37] S.F. Nakashima, M. Ukezono, H. Nishida, et al., Receiving of emotional signal of pain from conspecifics in laboratory rats, R. Soc. Open Sci. 2 (2015) 140381, https://doi.org/10.1098/rsos.140381.

[38] C. Labrakakis, The Role of the Insular Cortex in Pain, Int J. Mol. Sci. 24 (2023) 5736, https://doi.org/10.3390/ijms24065736.

[39] N. Geary, Understanding synergy, Am. J. Physiol. Endocrinol. Metab. 304 (3) (2013) E237–E253, https://doi.org/10.1152/ajpendo.00308.2012.

[40] E. Marret, O. Kurdi, P. Zufferey, F. Bonnet, Effects of nonsteroidal antiinflammatory drugs on patient-controlled analgesia morphine side effects: meta-analysis of randomized controlled trials, Anesthesiology 102 (2005) 1249–1260, https://doi.org/10.1097/00000542-200506000-00027.

[41] P. Kalinski, Regulation of immune responses by prostaglandin E2, J. Immunol. 188 (2012) 21–28, https://doi.org/10.4049/jimmunol.1101029.

[42] N. Ghasemlou, I.M. Chiu, J. Julien, C.J. Woolf, CD11b+Ly6G− myeloid cells mediate mechanical inflammatory pain hypersensitivity, Proc. Natl. Acad. Sci. USA 112 (2015), https://doi.org/10.1073/pnas.1501372112.

[43] D. Araldi, L.F. Ferrari, C.M. Lotufo, et al., Peripheral inflammatory hyperalgesia depends on the COX increase in the dorsal root ganglion, Proc. Natl. Acad. Sci. USA 110 (2013) 3603–3608, https://doi.org/10.1073/pnas.1220668110.

[44] T.A. Samad, K.A. Moore, A. Sapirstein, et al., Interleukin-1beta-mediated induction of Cox-2 in the CNS contributes to inflammatory pain hypersensitivity. Nature 410 (2001) 471–475, https://doi.org/10.1038/35068566.

[45] P. Sahbaie, X. Li, X. Shi, J.D. Clark, Roles of Gr-1+ leukocytes in postincisional nociceptive sensitization and inflammation, Anesthesiology 117 (2012) 602–612, https://doi.org/10.1097/ALN.0b013e3182655f9f.

[46] D. Segelcke, B. Pradier, S. Reichl, et al., Investigating the Role of Ly6G+ neutrophils in incisional and inflammatory pain by multidimensional pain-related behavioral assessments: bridging the translational gap, Front Pain. Re. 2 (2021) 735838, https://doi.org/10.3389/fpain.2021.735838.

[47] D.R. Robinson, G.F. Gebhart, Inside information: the unique features of visceral sensation, Mol. Inter. 8 (2008) 242–253, https://doi.org/10.1124/mi.8.5.9.

[48] S. Oggero, C. Cecconello, R. Silva, et al., Dorsal root ganglia CX3CR1 expressing monocytes/macrophages contribute to arthritis pain, Brain Behav. Immun. 106 (2022) 289–306, https://doi.org/10.1016/j.bbi.2022.09.008.

[49] C. Paige, I. Plasencia-Fernandez, M. Kume, et al., A female-specific role for calcitonin gene-related peptide (CGRP) in rodent pain models, J. Neurosci. 42 (2022) 1930–1944, https://doi.org/10.153/JNEUROSCI.1137-21.202.