Modality-specific peripheral antinociceptive effects of μ-opioid agonists on heat and mechanical stimuli: Contribution of sigma-1 receptors

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Abstract

Morphine induces peripherally μ-opioid-mediated antinociception to heat but not to mechanical stimulation. Peripheral sigma-1 receptors tonically inhibit μ-opioid antinociception to mechanical stimuli, but it is unknown whether they modulate μ-opioid heat antinociception. We hypothesized that sigma-1 receptors might play a role in the modality-specific peripheral antinociceptive effects of morphine and other clinically relevant μ-opioid agonists. Mechanical nociception was assessed in mice with the paw pressure test (450 g), and heat nociception with the unilateral hot plate (55 °C) test. Local peripheral (intraplantar) administration of morphine, buprenorphine or oxycodone did not induce antinociception to mechanical stimulation but had dose-dependent antinociceptive effects on heat stimuli. Local sigma-1 antagonism unmasked peripheral antinociception by μ-opioid agonists to mechanical stimuli, but did not modify their effects on heat stimulation. TRPV1+ and IB4+ cells are segregated populations of small neurons in the dorsal root ganglion (DRG) and the density of sigma-1 receptors was higher in IB4+ than in the rest of small nociceptive neurons. The in vivo ablation of TRPV1-expressing neurons with resiniferatoxin did not alter IB4+ neurons in the DRG, mechanical nociception, or the effects of sigma-1 antagonism on local mechanical antinociception in this type of stimulus. However, it impaired the responses to heat stimuli and the effect of local morphine on heat nociception. In conclusion, peripheral opioid antinociception to mechanical stimuli is limited by sigma-1 tonic inhibitory actions, whereas peripheral opioid antinociception to heat stimuli (produced in TRPV1-expressing neurons) is not. Therefore, sigma-1 receptors contribute to the modality-specific peripheral effects of opioid analgesics.

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1. Introduction

Opioid agonists, particularly those of the μ-receptor subtype such as morphine, oxycodone and buprenorphine, are widely used to treat moderate to severe pain (Al-Hasani and Bruchas, 2011; Pasternak and Pan, 2011). Opioid receptors are located at different sites along the pain-processing pathway, including both the central (spinal cord and different supraspinal nuclei) and peripheral nervous system (dorsal root ganglion (DRG) and peripheral nerve...
Although opioid analgesia is thought to be stronger at central levels (Porreca et al., 2002), peripherally administered opioid agonists can undoubtedly exert analgesic effects under some circumstances. In fact, morphine induces robust topical antinociceptive effects to heat stimulation in rodents (Kolesnikov and Pasternak, 1999), whereas this drug was unable to induce antinociception to a mechanical stimulus when administered locally (Sánchez-Fernández et al., 2013). These preclinical results are consistent with the preferential peripheral actions of morphine on heat rather than mechanical nociception in human volunteers (Koppert et al., 1999). However, it remains unknown whether this modality-specific peripheral effect of morphine also occurs in other clinically relevant μ-agonists. Therefore, the first aim of this research was to compare peripheral antinociceptive actions in response to heat and mechanical stimuli induced by clinically relevant μ-agonists of different intrinsic efficacy, to test whether the modality-specific effects of morphine are a general characteristic of μ-opioid drugs.

Sigma-1 (σ1) receptors are located in several areas of the central nervous system of great relevance for pain control (Alonso et al., 2000; Kitaichi et al., 2000; Roh et al., 2008; Sánchez-Fernández et al., 2014; Rodríguez-Muñoz et al., 2015a); nonetheless, the greatest concentration of σ1 receptors is found in the DRG (Sánchez-Fernández et al., 2014), pointing to a prominent role for these peripheral receptors in pain modulation. In fact, we recently reported that peripheral σ1 antagonism was able to greatly potentiate the peripheral opioid antinociception induced by a variety of μ-agonists in response to mechanical stimulation (Sánchez-Fernández et al., 2013, 2014) — findings which suggests that peripheral σ1 receptors constantly inhibit opioid functioning and thus constitute a tonically active biological brake to opioid mechanical analgesia (Sánchez-Fernández et al., 2017). However, it is unknown whether peripheral σ1 receptors also modulate μ-opioid antinociception to thermal stimulation. Hence, the second aim of this research was to compare the effect of pharmacological and genetic inactivation of σ1 receptors on peripherally-mediated μ-opioid-induced heat and mechanical antinociception.

Distinct subsets of high-threshold peripheral sensory neurons are activated by mechanical or thermal stimuli. Heat-sensitive nociceptors correspond mainly to peptidergic C-type neurons that express transient receptor potential vanilloid 1 (TRPV1), whereas the C non-peptidergic subtype (which can be labelled with resiniferatoxin, a drug able to selectively ablate TRPV1-expressing peptidergic C-type peripheral sensory neurons (Hsieh et al., 2008) and homozgyous σ1 receptor knockout mice (σ1-KO) (Laboratorios Esteve, Barcelona, Spain) were used in all experiments. Knockout mice were generated on a CD-1 background as previously described (Entrena et al., 2009). Animals weighing 25–30 g were tested randomly throughout the estrous cycle. They were housed in colony cages with free access to food and water prior to the experiments, and were maintained in temperature- and light-controlled rooms (22 ± 2 °C, lights on at 08:00 h and off at 20:00 h). The experiments were performed during the light phase (from 9:00 h to 15:00 h). The number of animals and the intensity of noxious stimuli used were the minimum necessary to demonstrate consistent effects of the drug treatments. For behavioral experiments, after habituation the mice were randomized to treatment groups. Animal care was provided in accordance with institutional (Research Ethics Committee of the University of Granada, Granada, Spain), regional (Junta de Andalucía, Spain) and international standards (European Communities Council directive 2010/63).

2.2. Drugs and drug administration

We used the μ-opioid agonists morphine hydrochloride (General Directorate of Pharmacy and Drugs, Spanish Ministry of Health), oxymorphone hydrochloride and buprenorphine hydrochloride (Sigma-Aldrich Química SA, Madrid, Spain). We also tested the effects of the opioid antagonists naloxone hydrochloride and naltoxone methiodide (Sigma-Aldrich Química SA, Madrid, Spain). Naloxone is a centrally-penetrant opioid antagonist, whereas its quaternary derivative naltrexone methiodide is unable to cross the blood–brain barrier and is therefore a useful tool to determine peripheral opioid effects (e.g. Menéndez et al., 2005; Sánchez-Fernández et al., 2014). S1RA ([4-2-[[5-methyl-1-(2-naphthalenyl)1H-pyraol-3-yl]oxy]ethyl]morpholine hydrochloride) was used as a selective σ1 antagonist (Romero et al., 2012) (DC Chemicals, Shanghai, China). PRE-084 ([2-(4-morpholinyl)-1-phenylcyclohexane(carboxylate) hydrochloride]) was used as a selective σ1 agonist (Hayashi and Su, 2004; Cobos et al., 2008) (Tocris Cookson Ltd., Bristol, United Kingdom). All drugs were dissolved in sterile physiologic saline (0.9% NaCl); the solution of PRE-084 was alkalinized as appropriate with NaOH. To evaluate the effects of systemic treatments, drugs or their solvents were administered i.p. to the interscapular area in a volume of 5 mL/kg. When the effect of the association of two drugs administered i.p. was tested, each drug was injected into different parts of the interscapular area. To test for local effects of treatments, drugs or their solvents were administered i.p. in a volume of 20 μL with a 1710 TLL Hamilton micro-syringe (Tecnokroma, Barcelona, Spain) and a 30G × 1/2-inch gauge needle. When opioids and S1RA were associated i.p., they were dissolved in the same solution and injected (in a volume of 20 μL) together to avoid paw lesions due to multiple injections in the same paw, except in experiments in which opioids were injected in one hindpaw and S1RA in the other.

2.3. Evaluation of mechanical nociception (paw pressure)

Mechanical stimulation was applied to the hindpaw of animals with a pressure algometer (Model 37215, Ugo-Basile, Varese, Italy) as previously described (Menéndez et al., 2005; Sánchez-Fernández et al., 2013, 2014). Briefly, mice were gently pincer grasped between the thumb and index fingers by the skin above the interscapular area. Then a blunt cone-shaped paw-pressor exerting a pressure of 450 g was applied to the dorsal surface of the hindpaw until the animal showed a struggle response (see Supplementary Video 1, which demonstrates the procedure used to measure mechanical nociception). The latency in seconds from paw stimulation to the struggle response was measured with a digital chronometer.

Supplementary video related to this article can be found at...
2.4. Evaluation of heat nociception (unilateral hot plate)

Heat nociception was assessed as previously described (Menéndez et al., 2002). To obtain comparable results between mechanical and thermal nociception, the mice were held gently in the same manner as described above. Then the plantar side of the stimulated hindpaw was placed on the surface of a thermal analgesiometer (Model PE34, Series 8, IITC Life Science Inc., Los Angeles, USA) previously set at 55 ± 1 °C until the animal showed a paw withdrawal response. The latency in seconds from paw stimulation to the behavioral response was measured with a digital chronometer. Only a clear unilateral withdrawal of the paw was recorded as the nociceptive response. We avoided simultaneous heat stimulations in both hindpaws by placing the plantar side of the tested hindpaw on the hot plate while the other hindpaw was placed on filter paper (off the hot plate) during observations (see Supplementary Video 2, which demonstrates the procedure used to measure heat nociception).

Supplementary video related to this article can be found at https://doi.org/10.1016/j.neuropharm.2018.03.025.

2.5. General procedures for behavioral assays and drug treatments

All handling of the rodents before and during the evaluation, as well as the test environment, were identical for evaluations of mechanical and heat nociception with the exception of the sensory stimulus used (see Supplementary Videos 1 and 2). The animals were placed in the experimental room for a 1-h acclimation period before starting the experiments. The behavioral responses of untreated mice tested with 55 °C and 450 g pressure force were very similar (non-significantly different) (See Fig. 1, dose 0). The similarities between the evaluation procedures and baseline responses of the mice to both types of sensory stimulation facilitated comparisons of drug effects on mechanical and heat nociception. To study the effects of systemic opioids, morphine and oxycodone were administered s.c. 30 min before paw stimulation; buprenorphine was administered s.c. 1 h before the evaluation, because its antinociceptive effect is much slower than the other opioids (Yassen et al., 2005). To explore the contribution of peripheral opioid receptors to μ-opioid antinociception, the effects of naloxone and naloxone methiodide (or saline) were compared. These opioid antagonists were administered s.c. 5 min before the μ-agonist. To test the effect of local treatments (S1RA, μ-agonists or the association of both), the animals were evaluated 5 min after i.pl. injection to minimize possible systemic effects induced by drug treatments. To study the contribution of σ1 receptors to the effects induced by the local administration of opioids alone or in association with S1RA, PRE-084 was administered s.c. 30 min before i.pl. administration. This σ1 agonist was administered at a dose of 32 mg/kg, since it was shown in previous studies to reverse the effect of the local administration of σ1 antagonists (Sánchez-Fernández et al., 2014). Control groups received the same volume of drug vehicle.

All mice were used in only one experimental procedure (mechanical or thermal nociception). Evaluations were done twice alternately in each hindpaw at intervals of 1 min between each stimulation. A 50-s cut-off was used for each measurement to prevent tissue damage. The mean value of the two averaged measurements for each hindpaw was used to analyze the effects of systemic (s.c.) treatment, because animals showed similar response latencies during stimulation in each hindpaw. However, in local (i.pl.) drug treatment experiments, the average of the two values was considered independently for each paw (ipsi- and contralateral). The experimenters who evaluated the behavioral responses were blinded to the treatment group and genotype of each experimental animal.

2.6. In vivo ablation of TRPV1-expressing nociceptors

To investigate the effects of the ablation of TRPV1-expressing neurons on mechanical and thermal nociception, we administered resiniferatoxin (RTX) (Tocris Cookson Ltd.) dissolved in vehicle (10% Tween 80 and 10% ethanol in normal saline). Each animal received a single dose of RTX (50 μg/kg) via i.p. injection (Hsieh et al., 2008; Montilla-García et al., 2017). The control group received an equal volume of vehicle. To minimize suffering, RTX or vehicle was injected under isoflurane anaesthesia (IsoVet®, B. Braun, Barcelona, Spain). After the i.p. injection, the mice were returned to their home cages for 5 days before behavioral testing and sample collection for immunostaining assays.

2.7. Immunohistochemistry

Mice were anesthetized with isoflurane and perfused transcardially with formaldehyde solution 4.0% wt/wt (Scharlab,
Tissue sections 5 μm thick were cut with a sliding microtome, deparaffinized in xylol (Panreac Quimica, Castellar del Vallés, Spain) and rehydrated before antigen retrieval (steam heating for 22 min with 1% citrate buffer, pH 8). Sections were incubated for 1 h in blocking solution (5% normal donkey serum or goat serum, depending on the experiment, 0.3% Triton X-100, 0.1% Tween 20 in Tris buffer solution). After blocking, the slides were incubated for 1 h at room temperature with the primary antibodies in blocking solution. The primary antibodies used were: TRPV1 goat anti-mouse (sc-12498, 1:100, Santa Cruz Biotechnology, Heidelberg, Germany), σ1 mouse anti-mouse (sc-137075, 1:200, Santa Cruz Biotechnology) or NeuN rabbit anti-mouse (ABN78, 1:500, Merck Millipore, Madrid, Spain). NeuN was used as a pan-neuronal marker which labels neuronal nuclei, perikarya, and to a lesser extent the cytoplasm (Mullen et al., 1992; Wolf et al., 1996). After incubation with the primary antibodies, tissue sections were washed three times for 10 min and incubated for 1 h with the secondary antibodies Alexa Fluor-488 donkey anti-goat (A11055, 1:500), Alexa Fluor-488 goat anti-mouse (A11029, 1:500) or Alexa Fluor-594 goat anti-rabbit (A11012, 1:500) (all from Life Technologies, Alcobendas, Spain). We also stained tissue sections with Bandeiraea simplicifolia lectin I, isoelectin B4 (IB4) conjugated with Dylight-594 (DL-1207, Merck Millipore, Madrid, Spain). When several means were compared, statistical analysis was performed in DRG slices from 5 mice treated with all three μ-opioid agonists morphine and oxycodone were particularly robust, and at the highest doses tested the response latencies were close to the cut-off time of 50 s for heat antinociception and about 20 s for the mechanical stimulus (Fig. 1). The partial μ-opioid agonist buprenorphine showed more limited antinociceptive effects, but as observed for the other opioid drugs tested, its effects were more prominent for heat than for mechanical antinociception (Fig. 1).

We compared the sensitivity of the effects induced by systemic opioids to the peripherally restricted opioid antagonist naloxone methiodide. Animals were treated s.c. with morphine (16 mg/kg), oxycodone (3 mg/kg), buprenorphine (0.5 mg/kg) or their solvent in association with naloxone methiodide (2–6 mg/kg, s.c.) or its solvent. Naloxone methiodide at any dose tested did not alter the increase in struggle response latency induced by morphine or buprenorphine to mechanical stimulation (Fig. 2A). However, the antinociceptive effect induced by oxycodone was slightly reversed by the highest dose of this peripheral opioid antagonist (Fig. 2A). In contrast, the antinociceptive effect on thermal stimulation in animals treated with all three μ-opioid agonists was markedly and dose-dependently reversed by naloxone methiodide (Fig. 2B). The centrally-penetrant opioid antagonist naloxone (0.5 mg/kg, s.c.) completely reversed the antinociceptive effect induced by all μ-opioids in mice exposed to the mechanical or thermal stimulus (Fig. 2A and B, respectively). Therefore the contribution of peripheral opioid receptors to the antinociceptive effects induced by systemic μ-opioid analgesics differed depending on the type of stimulus, and was more prominent for heat than for mechanical antinociception.

2.8. Data and statistical analysis

When several means were compared, statistical analysis was done with one- or two-way ANOVA depending on the experiment; a Bonferroni post-hoc test was done in both cases. To compare fluorescence intensity of σ1 receptor staining in IB4+ neurons and other small DRG neurons (soma size similar to or smaller than IB4+ neurons) was quantified with ImageJ 1.50 g image analysis software (National Institutes of Health, Bethesda, MD, USA). Quantification was performed in DRG slices from 5 different animals. Fluorescence intensity was measured in 30 random small neurons in each slice by an experimenter blinded to the IB4 labelling.

3.2. Effects of local (intraplantar) μ-opioid agonists on mechanical and thermal nociception in wild-type and σ1−/-knockout mice

In contrast to the antinociceptive effects induced by systemic opioids to mechanical stimulation in WT mice described above, the i.pl. administration of buprenorphine (3.125–25 μg), oxycodone (12.5–50 μg) or morphine (25–200 μg) did not induce significant mechanical antinociceptive effects (Fig. 3A, closed symbols). However, the same doses of i.pl. opioids induced marked and dose-dependent antinociceptive effects to heat stimulation (Fig. 3B closed symbols). These results highlight the modality-specific effects of peripheral opioid antinociception.

We then compared the antinociceptive effects induced by the i.pl. administration of buprenorphine, oxycodone or morphine in WT and σ1−/-KO mice. The response latency to mechanical and heat stimuli was similar in WT and σ1−/-KO mice treated with the solvent of the opioid analogs (Fig. 3A and B, dose 0). Despite the absence of local antinociceptive effects of i.pl. opioids to mechanical stimulation in WT mice, σ1−/-KO animals showed robust dose-dependent antinociceptive effects with each of the three μ-opioid agonists tested. The antinociceptive effects after mechanical stimulation in σ1−/-KO mice were particularly prominent for oxycodone and morphine, but weaker for buprenorphine (Fig. 3A). In marked contrast, the antinociceptive effects of i.pl. opioid agonists after heat stimulation were similar in WT and σ1−/-KO mice (Fig. 3B). None of the treatments tested here modified the response latency when the stimulus was applied to the hindpaw contralateral to the injection, with the exception of the highest dose of oxycodone, which induced a significant antinociceptive effect after mechanical stimulation in σ1−/-KO mice, and an antinociceptive effect after heat stimulation in mice of both genotypes (Suppl. Fig. 1A and 1B, respectively).
Fig. 2. Contribution of peripheral opioid receptors to the antinociception induced by the systemic administration of different \(\mu\)-opioid agonists on pain induced by mechanical and heat stimulation in wild-type mice. The results represent the response latency during stimulation of the hindpaws with (A) 450 g pressure or (B) 55 °C. Animals were treated s.c. with an opioid agonist (morphine 16 mg/kg, oxycodone 3 mg/kg, or buprenorphine 0.5 mg/kg) or its solvent (saline), associated with the s.c. injection of naloxone (Nx, 0.5 mg/kg), naloxone methiodide (Nx-M, 2–6 mg/kg) or their solvent (saline). Each animal was evaluated in only one pain test and received only one treatment. Each bar and vertical line represents the mean ± SEM of values obtained in both hindpaws in 8 animals. Statistically significant differences between the values obtained in animals treated with saline and the \(\mu\)-opioid agonists: **\(P < 0.01\); and between the groups treated with the different \(\mu\)-opioid agonists alone and associated with Nx or Nx-M: ##\(P < 0.01\) (one-way ANOVA followed by Bonferroni test).

Fig. 3. Mechanical and heat antinociception induced by the local administration of different \(\mu\)-opioid receptor agonists in wild-type (WT) and \(\sigma_1\)-knockout (\(\sigma_1\)-KO) mice. The results represent the response latency during stimulation with (A) 450 g pressure or (B) 55 °C of the injected hindpaw in mice treated intraplantarly (i.pl.) with the \(\mu\)-opioid agonists. Each animal was evaluated in only one pain test and received only one dose of drug or saline. Each point and vertical line represents the mean ± SEM of values obtained in the injected hindpaw in 8 animals. Statistically significant differences between the values obtained in saline- and opioid-treated groups: **\(P < 0.01\); and between the values obtained in WT and \(\sigma_1\)-KO mice at the same dose of a given opioid: ***\(P < 0.01\) (two-way ANOVA followed by Bonferroni test).
3.3. Effects of σ1 drugs on mechanical antinociception induced by local (intraplantar) administration of μ-opioid agonists

To test whether the local pharmacological blockade of σ1 receptors in WT mice replicated the phenotype seen in σ1−KO mice exposed to mechanical stimulation, we studied the effects of the co-administration of S1RA with the opioids (or their solvents) to the hindpaw. The response latency to mechanical stimulation was similarly short in WT mice stimulated in the paw treated with morphine 200 μg alone or in the contralateral (untreated) hindpaw, indicating the absence of antinociceptive effects of morphine to mechanical stimulation (Fig. 4A, dose 0 of S1RA). The co-administration of S1RA (6.25–100 μg) with this dose of morphine induced a dose-dependent increase in struggle response latency after mechanical stimulation in the injected hindpaw (Fig. 4A, closed symbols), without altering the behavioral response in mice stimulated in the hindpaw contralateral to the site of drug administration (Fig. 4A, open symbols), suggesting that these effects were not produced systemically. Treatment with S1RA (100 μg) alone was devoid of antinociceptive effects (1.9 ± 0.3 s in controls vs 1.3 ± 0.2 s in S1RA-treated mice).

We also compared the effects of the i.pl. administration of oxycodone (50 μg) and buprenorphine (25 μg) alone or associated with S1RA (100 μg). As observed for the results with morphine, the local administration of oxycodone or buprenorphine alone was devoid of antinociceptive effects in response to mechanical stimulation, but the co-administration of S1RA (at a dose which had no effect) with these opioids induced a robust antinociceptive effect (Fig. 4B). This enhanced antinociceptive effect was weaker for buprenorphine compared to morphine or oxycodone (Fig. 4B).

None of the treatments produced any effect in the non-injected paw (Suppl. Fig. 2A), and importantly, when S1RA was administered i.pl. in the hindpaw contralateral to the morphine, oxycodone or buprenorphine injection, there was no increase in the struggle response latency in the paw in which the opioids were injected (Fig. 4B). These results rule out any possible systemic effect of the i.pl. administration of S1RA and demonstrate that this σ1 agonist is able to locally potentiate opioid-induced mechanical antinociception at peripheral sites.

We also evaluated the effects of the σ1 agonist PRE-084 (32 mg/kg, s.c.) on the mechanical antinociception induced by the local co-administration of S1RA with the opioid agonists, and found that treatment with the σ1 agonist abolished the S1RA-induced potentiation of μ-opioid antinociception to mechanical stimulation (Fig. 5). These results support the selectivity of the effects induced by S1RA.

3.4. Effects of σ1 drugs on heat antinociception induced by local (intraplantar) administration of μ-opioid agonists

We tested the effects on heat nociception of the co-administration of S1RA (25–100 μg) with morphine, oxycodone or buprenorphine (or their solvent) to the hindpaw. The i.pl. administration of morphine (200 μg) induced an increase in the response latency to heat stimulation applied to the injected paw, whereas the latency in the hindpaw contralateral to the site of drug administration remained short (Fig. 6A, dose 0 of S1RA). In contrast to the marked potentiation by the co-administration of S1RA of the local mechanical antinociceptive effect induced by morphine 200 μg as reported above in the previous section, S1RA did not increase the antinociceptive effect to heat stimulation induced by the same dose of morphine (Fig. 6A). Because this i.pl. dose of morphine exerted a marked antinociceptive effect to heat stimulation, we also tested the effects of S1RA co-administration with a lower dose of morphine (100 μg) to avoid a possible ceiling effect which would make it difficult to detect an increase in morphine antinociception induced by S1RA. Although the antinociceptive effect of morphine 100 μg was lower than morphine 200 μg, co-administration with S1RA was still unable to modify the effect of this opioid (Fig. 6A). The co-administration of S1RA with morphine did not modify the response latency in mice stimulated in the hindpaw contralateral to the drug-treated paw (Fig. 6A, open symbols).

As seen regarding the absence of effects of S1RA on heat antinociception induced by local morphine, this σ1 agonist did not increase the local antinociceptive effect to heat stimulation induced by submaximal doses of oxycodone (25 μg) or buprenorphine (9 μg) (Fig. 6B). None of the treatments had any effect in the non-injected paw (Suppl. Fig. 2B). These results clearly showed that peripheral σ1 antagonism was not able to modulate heat antinociception induced by peripheral μ-opioid agonism.

We also tested whether σ1 agonism was able to decrease peripheral μ-opioid antinociception in response to heat stimulation. The administration of PRE-084 (32 mg/kg, s.c.) did not result in alterations in the antinociceptive effects induced by i.pl. morphine (200 μg), oxycodone (50 μg) or buprenorphine (12.5 μg) (Fig. 7). Therefore, neither σ1 antagonism nor agonism led to modifications in peripheral μ-opioid antinociception to heat stimulation.

3.5. Contribution of TRPV1-expressing neurons to mechanical and heat nociception, and to the modulatory effects of σ1 receptors on peripheral μ-opioid antinociception

We then tested whether the behavioral effects seen were mediated by TRPV1-expressing neurons. Staining for IB4 and TRPV1 showed minimal overlap among DRG neurons from intact mice (Fig. 8A, top panels). Treatment with RTX completely abolished TRPV1 labelling although IB4 staining remained (Fig. 8A, bottom panels), confirming the specificity of the ablation procedure. In addition, RTX treatment did not alter response latency to nociceptive mechanical stimulation in either of the two hindpaws (Fig. 8B). The i.pl. co-administration of S1RA and morphine induced a marked increase in the response latency to mechanical stimulation in the injected paw compared to solvent-treated mice, and this increase was similar in RTX-treated and RTX-untreated mice (Fig. 8B, black bars). Latency values in the contralateral non-injected paw remained unchanged and indistinguishable from control values in all experimental groups (Fig. 8B, white bars). Therefore, TRPV1-expressing neurons did not participate in the response to nociceptive mechanical stimuli or in the modulatory effect induced by σ1 receptors on peripheral μ-opioid antinociception to mechanical stimulation. In contrast, TRPV1 neuron ablation induced a bilateral increase in the response latency to the nociceptive heat stimulus (Fig. 8C).

The i.pl. administration of morphine induced a significant increase in the time to response to heat stimulation in the injected paw (but not in the contralateral hindpaw) in mice treated with the RTX solvent. When we tested the effects of i.pl. morphine in RTX-treated mice, we found that the response latency was equally high in the injected and the non-injected hindpaw and non-significantly different from that obtained in animals treated with RTX and the solvent of morphine (Fig. 8C), which indicates that local peripheral morphine administration was not able to produce antinociception in RTX-treated mice. The lack of effect of i.pl. morphine was not due to the high latency values observed in animals treated with RTX, since the systemic administration of morphine (16 mg/kg, s.c.) induced a marked increase in the response latency after heat stimulation despite ablation of the TRPV1 neurons (see Suppl. Fig. 3). This indicated that successful analgesic treatment was able to increase the response latency in TRPV1-ablated mice.
Fig. 4. Effects of the local administration of the \( \alpha_1 \) receptor antagonist S1RA on mechanical antinociception induced by the local administration of different \( \mu \)-opioid agonists in wild-type mice. The results represent the struggle response latency during mechanical stimulation in: (A) mice treated intraplantarly (i.pl.) with morphine (200 \( \mu \)g) co-administered with S1RA (6.25–100 \( \mu \)g) or its solvent (saline, dose 0), and stimulated in the injected (ipsilateral, ipsi) and non-injected (contralateral, contra) hindpaw; (B) the hindpaw ipsilateral to the site of \( \mu \)-opioid agonist injection (morphine 200 \( \mu \)g, oxycodone 50 \( \mu \)g or buprenorphine 25 \( \mu \)g) alone or associated with S1RA (100 \( \mu \)g) in the same or in the contralateral paw. (A and B) Each animal received only one treatment. Each point or bar and vertical line represents the mean ± SEM of values obtained in 8 animals. (A) Statistically significant differences between the values obtained in the groups treated with S1RA and its solvent: ** \( P < 0.01 \), and between the values obtained in the ipsilateral and contralateral hindpaw at the same dose of S1RA (or saline): ## \( P < 0.01 \) (two-way ANOVA followed by Bonferroni test). (B) Statistically significant differences between the values obtained in the groups treated with S1RA or its solvent in the paw ipsilateral to the opioid injection: ** \( P < 0.01 \); and between the groups treated with S1RA in the paw ipsilateral or contralateral to the opioid injection: #\# \( P < 0.01 \) (one-way ANOVA followed by Bonferroni test).
Increased response latency was thus achieved by systemic morphine, which is able to act both centrally and peripherally, but not by the i.pl. administration of this opioid. These results suggested that morphine acted on TRPV1 neurons to induce a local peripheral antinociceptive effect on the response to heat stimulation.

### 3.6. Expression of $\sigma_1$ receptors in the dorsal root ganglion of wild-type and $\sigma_1$-knockout mice

To study the expression of $\sigma_1$ receptors in the DRG, we used immunohistochemical double labelling for the neuronal marker NeuN and $\sigma_1$ receptors (Fig. 9). The pan-neuronal marker NeuN was located in the somas of all neurons from WT mice. $\sigma_1$ receptor immunoreactivity was also found in DRG cells. Merging the two images showed that cells that expressed NeuN and $\sigma_1$ receptor formed completely overlapping cell populations (Fig. 9, top panels). These results indicated that all sensory neurons expressed $\sigma_1$ receptors. In $\sigma_1$-KO mice, NeuN staining was preserved but $\sigma_1$ receptor labelling was completely absent (Fig. 9, bottom panels), supporting the specificity of the $\sigma_1$ receptor antibody used.

Higher magnification photomicrographs showed that NeuN staining was more intense within a rounded area in DRG neurons, which overlapped with Hoechst 33342 staining, indicating the location of neuronal nuclei. This area was devoid of $\sigma_1$ immunostaining, clearly indicating that $\sigma_1$ receptors were not detected inside neuronal nuclei (see Fig. 10). Interestingly, other Hoechst-stained cell nuclei were seen in non-neuronal cells (NeuN-negative cells) in close proximity to NeuN-labelled neurons, and these cells were devoid of $\sigma_1$ receptor staining, as this was completely restricted to NeuN-labelled cells (see Fig. 10).

Although we found that $\sigma_1$ receptors were expressed in all DRG neurons, the intensity of $\sigma_1$ receptor labelling appeared to be much higher in IB4+ neurons than in other small nociceptive neurons in the DRG (Fig. 11A). In fact, when we quantified signal intensity, we found that it was 2.3-fold higher in IB4+ neurons than in other small DRG neurons (Fig. 11B). These distinct expression levels of $\sigma_1$ receptors in subsets of nociceptive neurons may account for the differential modulation by $\sigma_1$ receptor inhibition of $\mu$-opioid agonist-induced mechanical and heat antinociception.

### 4. Discussion

In this study we show that the clinically relevant $\mu$-opioid analgesics morphine, buprenorphine and oxycodone induced prominent peripheral antinociceptive effects to a heat stimulus but little (oxycodone) or no peripheral antinociceptive effects to a mechanical stimulus under control conditions (in the absence of $\sigma_1$ receptor inhibition). However, $\sigma_1$ inhibition markedly boosted peripherally-mediated mechanical antinociception induced by $\mu$-opioid agonists without affecting peripheral $\mu$-opioid effects on heat nociception.

We found that peripheral opioid receptor antagonism (with naltrexone methiodide) did not alter mechanical antinociception induced by systemic buprenorphine or morphine and only slightly inhibited oxycodone effect. These results are consistent with our previous findings (Sanchez-Fernández et al., 2014), and are compatible with the presumed central analgesic effects of opioids.
Fig. 6. Effects of the local administration of the σ1 receptor antagonist S1RA on heat antinociception induced by the local administration of different μ-opioid agonists in wild-type mice. The results represent the paw withdrawal latency during heat stimulation in: (A) mice treated intraplantarly (i.pl.) with morphine (100 or 200 μg) associated with S1RA (6.25–100 μg) or its solvent (saline, dose 0), and stimulated in the injected (ipsilateral, ipsi) and non-injected (contralateral, contra) hindpaw; (B) the hindpaw ipsilateral to the site of μ-opioid agonist injection (morphine 100 μg, oxycodone 25 μg or buprenorphine 9 μg) alone or co-administered with S1RA (100 μg). (A and B) Each animal received only one treatment. Each point or bar and vertical line represents the mean ± SEM of values obtained in 8 animals. (A) Statistically significant differences between the values obtained in the hindpaw ipsilateral and contralateral to the same dose of morphine: ##P < 0.01. There were no statistically significant differences between the values obtained in the groups treated with S1RA or its solvent (two-way ANOVA followed by Bonferroni test). (B) Statistically significant differences between the values obtained in the groups treated with the opioids or their solvent: **P < 0.01. There were no statistically significant differences between the values obtained in the groups treated with the opioid agonists injected alone or with S1RA (one-way ANOVA followed by Bonferroni test).
In marked contrast to the results for mechanical nociceptive pain, the local peripheral administration of buprenorphine, morphine or oxycodone exhibited a similar level of thermal antinociception in WT and σ1-KO mice, and the heat antinociception induced by the local peripheral administration of these μ-opioid agonists was not modified by their co-administration with S1RA.

These results differ from those obtained when σ1 antagonists were administered systemically: both the non-selective σ1 drug haloperidol and the selective σ1 antagonist S1RA clearly enhanced opioid antinociception to heat stimulation (Chien and Pasternak, 1994, 1995; Marrazzo et al., 2006; Vidal-Torres et al., 2013). In addition, central σ1 inhibition is known to markedly increase opioid antinociception to a heat stimulus (Pan et al., 1998; Mei and Pasternak, 2002, 2007). Together, these results show that although σ1 receptors tonically inhibit opioid antinociception to heat stimulation, this must occur at the central but not at the peripheral level.

It was reported that σ1 agonism is able to decrease heat antinociception induced by systemic or central opioids (Chien and Pasternak, 1994). We therefore hypothesized that although the peripheral tonic inhibitory activity of the σ1 system might be too weak to allow σ1 antagonism to enhance opioid antinociception to heat, treatment with the σ1 agonist PRE-084 might be able to decrease heat antinociception induced by peripherally administered opioids. However, we show here that σ1 agonism did not modify peripheral μ-opioid-induced heat antinociception. Our
Effect of the ablation of TRPV1-expressing neurons on mechanical and heat nociception, and on the modulatory effects of σ₁ receptors on peripheral μ-opioid antinociception. Animals were treated i.p. with resiniferatoxin (RTX, 50 μg/kg) or its solvent five days before obtaining samples or performing the behavioral experiments. (A) Double labelling of isoelectric B4 (IB4, red) and TRPV1 (green) in the L4 dorsal root ganglion. Top panels: samples from control (solvent-treated) wild-type (WT) mice. White arrow indicates co-localization of both markers. Bottom panels: samples from WT-mice treated with RTX. Scale bar, 100 μm. (B and C) The behavioral results represent: (B) the struggle response latency during stimulation with 450 g pressure in the hindpaw of WT-mice pretreated with RTX or its solvent, and treated with an intraplantar (i.pl.) injection of morphine (200 μg) co-administered with S1RA (100 μg) or their solvent; (C) the paw withdrawal latency during stimulation with 55 °C in the hindpaw of WT-mice pretreated with RTX or its solvent, and i.pl. injected with morphine (200 μg) or its solvent. (B and C) Mice were stimulated in the paw ipsilateral (ipsi) or contralateral (contra) to the opioid injection. Each animal was evaluated in only one pain test and received only one treatment. Each bar and vertical line represents the mean ± SEM of 8 animals. Statistically significant differences compared to mice treated with solvent only: **P < 0.01 (one-way ANOVA followed by Bonferroni test). Statistically significant differences between the values obtained in the hindpaw ipsilateral and contralateral to the opioid injection for each group: **P < 0.01 (one-way ANOVA followed by Bonferroni test).
findings thus show for the first time that peripherally-mediated μ-opioid antinociception to heat stimuli is clearly dissociated from the actions of σ₁ receptors.

In the mouse TRPV1+ and IB4+ cells constitute separate neuronal populations, as shown here and in previous studies (Zwick et al., 2002; Woodbury et al., 2004), and correspond to peptidergic and non-peptidergic C-nociceptors, respectively (Priestley, 2009). Heat-sensitive nociceptors correspond mainly to TRPV1-expressing C-nociceptors (Scherrer et al., 2009; Bardoni et al., 2014), whereas non-peptidergic C-nociceptors are mecha-nosensitive (Cavanaugh et al., 2009; Scherrer et al., 2009). The ablation of TRPV1-expressing neurons by RTX in our mice resulted in marked impairment of the nociceptive responses to heat stimulation, as also reported in other studies (Menéndez et al., 2006; Hsieh et al., 2012; Zhang et al., 2013). Moreover, we show that although the local peripheral administration of morphine induced marked peripheral antinociception in response to heat stimulation in intact mice, it was unable to induce further antinociceptive effects in TRPV1-ablated mice, indicating that this opioid acts on TRPV1 neurons to induce a local antinociceptive effect on this type of stimulus. It is known that there is heavy crosstalk between TRPV1 and μ-opioid receptors (reviewed in Bao et al., 2015), so the prominent effects induced by the local administration of morphine in the heat nociception assays reported here may be due to

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**Fig. 9.** σ₁ receptors are expressed in all dorsal root ganglion neurons. Double labelling of NeuN (red) and σ₁ receptors (green) in the L4 dorsal root ganglion. Top panels: samples from wild-type (WT) mice. Bottom panels: samples from σ₁-knockout (σ₁-KO) mice. Scale bar, 100 μm.

**Fig. 10.** σ₁ receptors are expressed selectively in dorsal root ganglion neurons, with wide distribution in the cytoplasm and plasma membrane, but not inside the neuronal nuclei. Hoechst 33342 (Hoechst) staining (blue), and NeuN (red) and σ₁ receptor (green) labelling in the L4 dorsal root ganglion. Scale bar, 50 μm.
modulation of the action of TRPV1 receptors in peptidergic C-nociceptors.

In marked contrast to the results discussed above, the ablation of TRPV1-expressing neurons by RTX in our mice left IB4+ cells unaltered and did not affect the responses to mechanical stimulation, indicating that the TRPV1-expressing neuronal population is dispensable for mechanical nociception, as shown here and in previous studies (Mishra and Hoon, 2010; Zhang et al., 2013). In addition, we show that RTX treatment did not modify the effect of the local administration of morphine injected alone or with SIRA on mechanical stimulation, indicating that σ1 receptors tonically inhibit peripheral μ-opioid antinociception in sensory neurons other than TRPV1-expressing afferents. Therefore, we hypothesized that differential expression of the σ1 receptor in subsets of peripheral nociceptive neurons might underpin the differential modulation by σ1 receptor antagonism of μ-opioid peripherally mediated heat- and mechanical-antinociception.

We found that σ1 receptors were located in the somas of all DRG neurons, with a neuron-specific distribution. These results are in agreement with a recent study of the location of σ1 receptors in the DRG (Mavlyutov et al., 2016). Although the antibody used in our study (sc-137075) was recently reported to intensely stain tissue from σ1-KO mice (Mavlyutov et al., 2016), we show that σ1 receptor immunostaining was completely absent in our mutant mice, supporting the specificity of the antibody. The marked differences between our findings and previous studies may be due to the use of frozen sections embedded in optimal cutting temperature (OCT) compound by Mavlyutov and colleagues vs paraffin-embedded samples in the present study, and to a possible effect of our antigen retrieval procedure, which might improve antibody selectivity. It is worth pointing out that the knockout mice used in the two studies were generated with different procedures, which may also account for the differences. However, staining with the sc-137075 antibody was also seen in our σ1 knockout mice when the procedure was performed with frozen sections embedded in OCT (data not shown). These results suggest that the differences in antibody selectivity may be attributable to the experimental procedures rather than to the type of σ1 knockout mouse used.

Although σ1 receptors were located in all DRG neurons, the distribution of these receptors was not homogeneous: we show that the expression levels of σ1 receptors were much higher in IB4+ neurons than in all other small nociceptive neurons in the DRG. As previously noted, IB4+ neurons are well known to code for mechanical nociception. Therefore, the enrichment of σ1 receptor expression in this type of sensory neuron may account for our finding that σ1 antagonism acted preferentially on opioid-induced peripheral antinociception in response to mechanical stimuli.

Our results on nociceptive pain may seem to contradict a recent study showing that σ1 antagonism fully ameliorated both mechanical and thermal hyperalgesia in mice with carrageenan-induced inflammation, by enhancing the peripheral actions of endogenous opioid peptides (Tejada et al., 2017). The interaction between σ1 receptors and their protein targets is known to be Ca2+-dependent (Rodriguez-Munoz et al., 2015b), and inflammatory mediators can induce a marked increase in intracellular Ca2+ concentration, leading to nociceptor sensitization (Linhart et al., 2003; Richardson and Vasko, 2002). Therefore, inflammation might trigger plastic changes in heat-sensing peripheral neurons that enable opioid modulation by σ1 receptors, although further studies are needed to clarify this issue. It is worth noting that the potency of σ1 antagonists in ameliorating inflammatory mechanical hyperalgesia is much higher than for heat hyperalgesia (Tejada et al., 2010). This suggests that even under inflammatory conditions, σ1 receptors play a more prominent role in the modulation of peripheral opioid effects in response to mechanical than to heat stimulation, which is consistent with our results.

5. Conclusions

In conclusion, we found that peripheral opioid antinociception in response to mechanical stimulation is limited by peripheral σ1 tonic inhibitory actions, whereas peripheral opioid antinociception in response to heat stimulation (produced in TRPV1-expressing neurons) is exempt from this control. Therefore, σ1 receptors contribute to the modality-specific peripheral effects of opioid analgesics, and this effect may be dependent on differential σ1 actions in neurons coding for different types of sensory stimuli.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.neuropharm.2018.03.025.

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