Modulation of peripheral µ-opioid analgesia by σ₁ receptors

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Abbreviations
ANOVA: analysis of variance; BD-1063: (1-[(2-(3,4-dichlorophenyl)ethyl]-4-methylpiperazine dihydrochloride); BLA: basolateral amygdala; DMSO: dimethylsulfoxide; dSC: dorsal spinal cord; DRG: dorsal root ganglion; IC_{50}: concentration of unlabeled drug that inhibited 50% of radioligand-specific binding; i.pl.: intraplantar; KO: knockout; L4-L5: lumbar vertebrae 4 and 5; Nx: naloxone; Nx-M: naloxone methiodide P_{2}: crude synaptosomal fraction; PAG: periaqueductal grey
matter; PRE-084: ([2-(4-morpholinethyl)1-phenylcyclohexanecarboxylate) hydrochloride]); RVM: rostroventral medulla; σ₁ receptor: sigma-1 receptor; S1RA: (4-[2-[[5-methyl-1-(2-naphthalenyl)1H-pyrazol-3-yl]oxy]ethyl] morpholine hydrochloride); T-TBS: Tween 20 in Tris-buffered saline; WT: wild-type

**Recommended section:** Behavioral Pharmacology
Abstract

We evaluated the effects of σ₁ receptor inhibition on µ-opioid-induced mechanical antinociception and constipation. σ₁-knockout mice exhibited marked mechanical antinociception in response to several µ-opioid analgesics (fentanyl, oxycodone, morphine, buprenorphine and tramadol) at systemic (subcutaneous, s.c.) doses that were inactive in wild-type mice, and even unmasked the antinociceptive effects of the peripheral µ-opioid agonist loperamide. Similarly, systemic (s.c.) or local (intraplantar) treatment of wild-type mice with the selective σ₁ antagonists BD-1063 or S1RA potentiated µ-opioid antinociception; these effects were fully reversed by the σ₁ agonist PRE-084, showing the selectivity of the pharmacological approach. The µ-opioid antinociception potentiated by σ₁ inhibition (by σ₁ receptor knockout or σ₁ pharmacological antagonism) was more sensitive to the peripherally-restricted opioid antagonist naloxone methiodide than opioid antinociception under normal conditions, indicating a key role for peripheral opioid receptors in the enhanced antinociception. Direct interaction between the opioid drugs and σ₁ receptor cannot account for our results, since the former lacked affinity for σ₁ receptors (labeled with [³H](+)-pentazocine). A peripheral role for σ₁ receptors was also supported by their higher density (western blot results) in peripheral nervous tissue (dorsal root ganglia) than in several central areas involved in opioid antinociception (dorsal spinal cord, basolateral amygdala, periaqueductal gray and rostroventral medulla). In contrast to its effects on nociception, σ₁ receptor inhibition did not alter fentanyl- or loperamide-induced constipation, a peripherally-mediated nonanalgesic opioid effect. Therefore, σ₁-receptor inhibition may be used as a systemic or local adjuvant to enhance peripheral µ-opioid analgesia without affecting opioid-induced constipation.
Introduction

Opioid drugs, particularly agonists of the μ-receptor subtype, are widely used to treat moderate-to-severe pain (Pasternak and Pan, 2011; Al-Hasani and Bruchas, 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 (dorsal root ganglion [DRG] and peripheral nerve terminals) nervous system (Khalefa et al., 2012, Bigliardi-Qi et al., 2004). The antinociceptive effect of systemic opioids is thought to be produced mainly at the central (particularly supraspinal) level (Greenwood-Van Meerveld and Standifer, 2008; Thomas et al., 2008; Joshi et al., 2008; Khalefa et al., 2012), although peripheral opioid receptors might also participate (Kayser et al., 1995; Craft et al., 1995; Shannon and Lutz, 2002).

The sigma-1 (σ₁) receptor has been cloned and its sequence does not show homology with opioid receptors or any other known mammalian protein; it is therefore currently considered a unique entity (Cobos et al., 2008; Zamanillo et al., 2013). Inhibition of σ₁ receptor function either by the systemic administration of σ₁ antagonists or by σ₁ receptor knockdown does not influence acute nociception per se (Cendán et al., 2005; Entrena et al., 2009b; De la Puente et al., 2009; Nieto et al., 2012; Romero et al., 2012; Sánchez-Fernández et al., 2013). However, σ₁ inhibition is able to enhance opioid signaling (Kim et al., 2010) and to potentiate the antinociceptive effect of systemic opioids (e.g. Chien and Pasternak, 1993 and 1994; Marrazzo et al., 2011; Sánchez-Fernández et al., 2013; Vidal-Torres et al., 2013). Opioid antinociception can be potentiated by central σ₁ inhibition (King et al., 1997; Pan et al., 1998; Mei and Pasternak 2002 and 2007; Marrazzo et al., 2006), and we recently reported that the local peripheral coadministration of σ₁ antagonists and morphine also resulted in markedly enhanced antinociception (Sánchez-Fernández et al., 2013). However, it is unknown...
whether peripheral mechanisms are involved in the antinociception induced by systemic opioids when \( \sigma_1 \) receptors are inhibited. We hypothesize that the contribution of peripheral opioid receptors to overall antinociception induced by the combination of systemic opioids and \( \sigma_1 \) inhibition might be more relevant than the contribution of these receptors to the effect of systemic opioids under normal conditions. The main goal of this study was to test this hypothesis.

To do so, we compared the effect of the peripherally restricted opioid antagonist naloxone methiodide (Menéndez et al., 2005; Sevostianova et al., 2005; Parenti et al., 2012) on the mechanical antinociception induced by systemic \( \mu \)-opioids in the presence or absence of \( \sigma_1 \) receptor inhibition. We used several clinically relevant \( \mu \)-opioids with different intrinsic activities and blood–brain barrier permeabilities. These opioid drugs include the centrally active analgesics fentanyl, morphine, oxycodone, tramadol and buprenorphine (Pergolizzi et al., 2008; Schäfer 2010), and the peripherally restricted \( \mu \)-agonist loperamide, used clinically as an antidiarrheal drug (Layer et al., 2010; Gallelli et al., 2010). To inhibit \( \sigma_1 \) receptors we used \( \sigma_1 \)-knockout (\( \sigma_1 \)-KO) mice and systemic and local treatments of wild-type (WT) mice with the selective \( \sigma_1 \) antagonists BD-1063 and S1RA. Moreover, because the data from the present study support the importance of peripheral \( \sigma_1 \) receptors as modulators of opioid antinociception, we compared the expression of \( \sigma_1 \) receptors in peripheral (DRG) and central areas (dorsal spinal cord, basolateral amygdala, periaqueductal gray and rostroventral medulla) known to be involved in opioid antinociception (Millan, 2002). Finally, to rule out possible direct interactions between the opioid drugs tested here and \( \sigma_1 \) receptors, we determined their affinity for \( \sigma_1 \) receptors.

Opioid-induced constipation is the most clinically relevant peripheral side effect of \( \mu \)-opioids (Benyamin et al 2008; Al-Hasani and Bruchas, 2011; Ringkamp and Raja,
2012), and it is one of the main reasons for patients’ voluntary withdrawal from opioid medication (Dhingra et al., 2012). We recently showed that although morphine-induced antinociception was potentiated in \( \sigma_1 \)-KO mice, morphine-induced constipation remained unaltered (Sánchez-Fernández et al., 2013; Vidal-Torres et al., 2013). However, it is unknown whether these differential effects of \( \sigma_1 \) inhibition also occur with other \( \mu \)-opioids. Therefore, an additional goal of this study was to test the effects of \( \sigma_1 \) inhibition on constipation induced by two very different \( \mu \)-opioids: fentanyl, a centrally penetrant drug, and loperamide, a peripherally restricted drug.
Material and methods

Experimental animals

Most experiments were performed in female WT (Charles River, Barcelona, Spain) and $\sigma_1$-KO CD-1 mice (Laboratorios Esteve, Barcelona, Spain) weighing 25-30 g. The knockout mice were backcrossed for 10 generations to a CD-1 genetic background as described previously (Entrena et al., 2009a). Some experiments were also performed in male WT mice to ensure that sex differences did not affect our results. All animals were kept in our animal facilities for a minimum of 7 days before the experiments. Animals were housed under controlled environmental conditions: 12/12 h day/night cycle, constant temperature (22 ± 2 °C) with free access to water and food (standard laboratory diet, Harlan Teklad Research Diet, Madison, WI, USA). Behavioral testing was done during the light phase (from 9.00 h to 15.00 h) and randomly throughout the estrous cycle. Animal care was provided in accordance with institutional (Research Ethics Committee of the University of Granada, Spain) and international standards (European Communities Council directive 86/609), and with the guidelines for the investigation of experimental pain in conscious animals (Zimmermann 1983).

Radioligand, drugs and drug administration

The opioid drugs and their suppliers were: the $\mu$-opioid agonists morphine hydrochloride (from the General Directorate of Pharmacy and Drugs, Spanish Ministry of Health), fentanyl citrate, oxycodone hydrochloride, buprenorphine hydrochloride and loperamide (all from Sigma-Aldrich Química SA, Madrid, Spain), tramadol (supplied by Laboratorios Esteve, Barcelona, Spain) and the opioid antagonists naloxone hydrochloride and naloxone methiodide (Sigma-Aldrich Química SA). BD-1063 (1-[2-(3,4-dichlorophenyl)ethyl]-4-methylpiperazine dihydrochloride) (Tocris Cookson Ltd.,
Bristol, UK), and S1RA (4-[2-[[5-methyl-1-(2-naphthalenyl)1H-pyrazol-3-yl]oxy]ethyl] morpholine hydrochloride) (synthesized and kindly supplied by Laboratorios Esteve, Barcelona, Spain) were used as selective $\sigma_1$ antagonists (Cobos et al., 2008; Romero et al., 2012). PRE-084 ([2-(4-morpholinethyl)1-phenylcyclohexanecarboxylate] hydrochloride) (Tocris Cookson Ltd.) was used as a selective $\sigma_1$ agonist (Hayashi and Su, 2004; Cobos et al., 2008).

All drugs used for the in vivo studies were dissolved in sterile physiological saline (NaCl 0.9%) except loperamide, which was dissolved in 1% dimethylsulfoxide (DMSO, Merck KGaA, Darmstadt, Germany) in ultrapure water. The solutions of the $\sigma_1$ ligands BD-1063 and PRE-084 were appropriately alkalinized with NaOH. To evaluate the effects of systemic treatments, drugs or their solvents were injected subcutaneously (s.c.) into the interscapular zone in a volume of 5 mL/kg. When the effect of the association of several drugs was tested, each drug was injected into a different area of the interscapular zone. To study the local effects of BD-1063 or S1RA, these drugs were injected intraplantarly (i.pl.) into the right hindpaw at a volume of 20 µL with a 1710 TLL Hamilton microsyringe (Teknokroma, Barcelona, Spain) with a 30 1/2–gauge needle. Each control group received the same volume of drug vehicle.

For binding assays, the radioligand was the selective $\sigma_1$ agonist $[^3]H$ (+)-pentazocine (Cobos et al., 2006), with a specific activity of 34.8 Ci/mmol (PerkinElmer Life Sciences, Boston, MA, USA). Dilutions from the stock $[^3]H$ (+)-pentazocine solution were prepared with ice-cold incubation buffer (50 mM Tris–HCl buffer, pH 8 at 30 °C). The cold drugs tested were the opioids: fentanyl, oxycodone, morphine, buprenorphine, loperamide, tramadol, naloxone and naloxone methiodide. BD-1063 was used as a control with known high-affinity for $\sigma_1$ receptors (Cobos et al., 2007; Entrena et al., 2009a). All drugs were dissolved at a concentration of 1 mM in ultrapure water with the
exception of loperamide, which was dissolved in absolute ethanol. Further dilutions were prepared with incubation buffer. The final maximal concentration of ethanol in the incubation medium was 0.1% (vol/vol), which did not affect [3H](+)-pentazocine binding (Cobos et al., 2005 and 2006).

**Evaluation of mechanical nociception (paw pressure)**

The effects of the drugs on mechanical nociception were evaluated with an Analgesimeter (Model 37215, Ugo-Basile, Varese, Italy) according to methods described previously (Menéndez et al., 2005; Sánchez-Fernández et al., 2013). Briefly, the hindpaw of the mice was stimulated with a constant pressure of 450 g using a cone-shaped paw-presser with a rounded tip until the animal showed a struggle reaction. Immediately thereafter, the stimulus was stopped and the response latency (in seconds) was recorded. The test was done twice alternately to each hindpaw at intervals of 1 min between each stimulation, with a 50-s cutoff for each determination. The antinociceptive effects of µ-agonists were evaluated 30 min after s.c. administration except for buprenorphine, which was administered s.c. 1 h before the evaluation, since the onset of its antinociceptive effect is known to be much slower than for other opioids (Yassen et al., 2005). To study the effects of the systemic administration of BD-1063 or S1RA on µ-opioid antinociception, these drugs (or its solvent) were administered s.c. 5 min before the µ-agonists. When drugs were administered systemically the struggle response latency was calculated as the mean of the two averaged times in each hindpaw, since no differences between sides were seen in the response of each hindpaw. To study the local effects of σ₁ inhibition on µ-opioid antinociception, BD-1063, S1RA or their solvent were administered i.pl. and the animals were evaluated 5 min after the injection to minimize possible systemic effects induced by the drug. In experiments to determine
the local effects of the $\sigma_1$ antagonists, we calculated the average of the two values from the injected and noninjected hindpaws independently. To test the effects of PRE-084, naloxone or naloxone methiodide on $\mu$-opioid antinociception in the presence or absence of $\sigma_1$ inhibition, these drugs or their solvent were administered s.c. 5 min before the $\mu$-agonist solution, when appropriate.

The experimenters who evaluated the behavioral responses were blinded to the treatment group and genotype of each experimental animal.

**Evaluation of opioid-induced inhibition of gastrointestinal transit**

Gastrointestinal transit was estimated as previously described (Sánchez-Fernández et al., 2013). Briefly, 8 h before the experiment, food was withheld and water was available *ad libitum*. BD-1063 or its solvent was injected s.c., and 5 min later fentanyl, loperamide or their solvent was injected s.c. Thirty minutes after the opioid was given, the mice received 0.3 mL of a 0.5% (wt/vol) activated charcoal suspension (2–12 $\mu$m powder, Sigma-Aldrich Química SA) in distilled water. The mice were killed by cervical dislocation 30 min after the activated charcoal was administered (i.e. 60 min after administration of the opioid); then the small intestine was removed from the pyloric sphincter to the ileocecal junction and straightened to measure the distance travelled by the leading edge of the charcoal meal.

**Western blotting**

The basolateral amygdala (BLA), rostroventral medulla (RVM), periaqueductal grey matter (PAG), dorsal spinal cord of the lumbar enlargement (dSC) and lumbar (L4-L5) root ganglia (DRG) were carefully removed from naïve WT and $\sigma_1$-KO mice. The tissue was homogenized by sonication in a buffer solution (50 mM Tris, 150 mM NaCl, 2 mM
EDTA, 0.5% Triton X-100, 0.1% SDS, 1% NP-40, 1mM phenylmethylsulfonyl fluoride, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 25 mM NaF, 0.5% protease inhibitor cocktail, 1% phosphatase inhibitor cocktail, all from Sigma-Aldrich Química SA). Protein concentration in the tissue homogenate was measured with the Bradford assay. The samples were stored at −80 °C until use.

Twenty-five micrograms of protein was loaded on 12% (wt/vol) SDS-polyacrylamide gels and transferred electrophoretically to nitrocellulose membranes (Bio-Rad, Madrid, Spain). The membranes were blocked at room temperature for 1 h with blocking buffer containing 5% dry skim milk in T-TBS (10 mM Tris, 150 mM NaCl, 0.1% Tween-20, pH 7.5). Then the membranes were incubated overnight at 4 °C with a mouse monoclonal antibody that recognized σ<sub>1</sub> receptor (1:1,000, sc-137075, Santa Cruz Biotechnology, Dallas, TX, USA). Mouse monoclonal anti-β-actin antibody (1:2,500, sc-81178, Santa Cruz Biotechnology) was used as a loading control. Both primary antibodies were diluted in T-TBS containing 0.5% dry skim milk. The membranes were washed (3 × 10 min) with T-TBS and incubated for 1 h at room temperature with horseradish peroxidase-linked goat anti-mouse IgG (sc-2005, Santa Cruz Biotechnology), diluted to 1:2,500 in T-TBS containing 0.5% dry skim milk. The membranes were washed (6 × 10 min) with T-TBS, and the bands were visualized with an enhanced chemiluminescence method (ECL Prime Western Blotting Detection Reagents, Amersham Biosciences, Buckinghamshire, UK) according to the manufacturer’s instructions (5 min incubation at room temperature). Densitometric analysis of immunoreactive bands was done with Quantity One software (Bio-Rad). The data are presented as the ratio of the intensity of the σ<sub>1</sub> receptor bands to the β-actin bands. To estimate the molecular weight of the resulting immunoreactive bands, when the samples were loaded we included in the same gel a mixture of 10 blue-stained...
recombinant proteins of different molecular weights (Precision Plus Protein All Blue Standards, Bio-Rad).

[3H](+)-Pentazocine competition binding assays

Binding assays were done in WT mouse brain membranes (P2 fraction) as previously described (Entrena et al., 2009a and b; Sánchez-Fernández et al., 2013). Briefly, membrane solutions (460 μL) were incubated at a final protein concentration of 0.8 mg/mL with 20 μL of several concentrations of the cold drug or its solvent and 20 μL [3H](+)-pentazocine (final concentration of 5 nM) for 240 min at 30 °C, pH 8. Five milliliters of ice-cold filtration buffer (Tris 10 mM, pH 7.4) was added to the tubes, and the solutions were rapidly filtered (Brandel cell harvester Model M-12 T, Brandel Instruments, SEMAT Technical, St. Albans, Hertfordshire, UK) over Whatman GF/B glass fiber filters (SEMAT Technical) pre-soaked with 0.5% polyethylenimine in Tris 10 mM, pH 7.4. The filters were washed twice with 5 mL of ice-cold filtration buffer. Then 4 mL of liquid scintillation cocktail (CytoScint scintillation counting solution, MP Biomedicals, Irvine, CA, USA) was added to each filter. On the next day, their radioactivity level was measured by liquid scintillation spectrometry (Beckman Coulter España SA, Madrid, Spain). Nonspecific binding was defined as the binding retained in the presence of a high concentration of BD-1063 (1 μM), and was always less than 20% of total binding.

Data analysis

The data were analyzed with the SigmaPlot 12.0 program (Systat Software Inc., San Jose, CA, USA). In behavioral assays, the dose-response curves of the drugs were estimated with equations for a sigmoid plot. For binding assays, the concentration of
unlabeled BD-1063 that inhibited 50% of $[^3H](+)$-pentazocine specific binding (IC$_{50}$) and its standard error were calculated from the inhibition curve with nonlinear regression analysis from the equation for a sigmoid plot, assuming one-site competition. To compare different mean values from in vivo studies, one-way or two-way analysis of variance (ANOVA) was used depending on the experiment; a Bonferroni post hoc test was done in both cases. $P < 0.05$ was considered significant in all tests.
Results

Effects of systemic (subcutaneous) μ-opioid receptor agonists on mechanical nociception in wild-type and σ₁ knockout mice

Mechanical antinociception induced by the s.c. administration of several opioid drugs was measured as the increase in struggle response latency in drug-treated mice with respect to control (solvent-treated) mice when constant pressure (450 g) was applied to the hindpaw. The struggle response latency was similarly short in WT and σ₁-KO solvent-treated mice (Fig. 1A and B, dose 0).

As expected, the s.c. administration of the opioids fentanyl (0.04 – 0.32 mg/kg), oxycodone (0.75 – 4 mg/kg) or morphine (0.5 – 16 mg/kg) induced a dose-dependent mechanical antinociceptive effect in WT mice. The highest doses tested of fentanyl, oxycodone and morphine increased the latency values 20-fold, 13-fold and 10-fold, respectively, compared to solvent-treated WT mice (Fig. 1A, closed symbols). Buprenorphine (0.06 – 0.48 mg/kg, s.c.) evoked a significant mechanical antinociceptive effect only at the highest dose tested, increasing the latency 6-fold compared to solvent-treated WT mice. However, tramadol (5 – 40 mg/kg, s.c.) and the peripheral μ-agonist loperamide (1 – 4 mg/kg, s.c.) did not induce a significant increase in struggle response latency in WT mice at the doses used here (Fig. 1B, closed symbols).

In σ₁-KO mice the antinociceptive effect in response to fentanyl, oxycodone, morphine or buprenorphine was greater than in WT mice. The doses of these drugs needed to induce significant mechanical antinociception in σ₁-KO mice were much lower than in WT mice, resulting in a displacement to the left of the dose-response curves (Fig. 1A and B). In addition, tramadol or loperamide treatments, which had no effect in WT mice under our experimental conditions, evoked marked antinociception in σ₁-KO mice (Fig.
The largest antinociceptive effects in \( \sigma_1 \)-KO mice were recorded after treatment with fentanyl, oxycodone or morphine, and struggle response latency reached values close to the cutoff time (50 s) (Fig. 1A, open symbols). Interestingly, \( \sigma_1 \)-KO animals treated with the highest doses of buprenorphine, tramadol or even the peripheral \( \mu \)-agonist loperamide showed latencies higher than those in WT mice treated with fentanyl, oxycodone or morphine (Fig. 1B, open symbols). Therefore, the genetic inactivation of \( \sigma_1 \) receptors clearly potentiated the mechanical antinociception induced by the systemic administration of \( \mu \)-opioid agonists, including antinociception induced by the peripherally restricted opioid loperamide.

**Contribution of peripheral opioid receptors to antinociception induced by the systemic (subcutaneous) administration of \( \mu \)-opioid analgesics in wild-type mice**

To shed light on the role of peripheral opioid receptors in the mechanical antinociception induced by \( \mu \)-opioid analgesics in WT mice, we tested the sensitivity of this antinociception to the peripherally restricted opioid antagonist naloxone methiodide.

In WT mice treated s.c. with fentanyl (0.16 mg/kg), oxycodone (4 mg/kg), morphine (16 mg/kg) or buprenorphine (0.48 mg/kg) and pretreated with the naloxone methiodide solvent, struggle response latency increased markedly in comparison to untreated mice (Fig. 2). Tramadol and loperamide were not evaluated in this experiment because they did not induce any antinociceptive effect at any dose tested in WT mice (Fig. 1B). Pretreatment with naloxone methiodide (2 – 8 mg/kg) did not alter the increase in struggle response latency induced by fentanyl, morphine or buprenorphine (Fig. 2). However, the antinociception induced by oxycodone was partially reversed by the highest dose of this peripheral opioid antagonist (Fig. 2). As expected, the centrally-
penetrant opioid antagonist naloxone (0.5 mg/kg, s.c.) was able to completely reverse the antinociceptive effect induced by all μ-agonists tested: latencies were undistinguishable from those in untreated mice (Fig. 2).

Therefore, among the opioid analgesics tested in WT mice, only oxycodone showed partial sensitivity to peripheral opioid antagonism.

**Contribution of peripheral opioid receptors to antinociception induced by the systemic (subcutaneous) administration of μ-agonists in σ₁ knockout mice**

To explore the contribution of peripheral opioid receptors (sensitive to naloxone methiodide) to the enhanced mechanical antinociception seen in σ₁-KO mice, we used doses of μ-agonists that induced little or no antinociception in WT mice but induced maximal antinociception in σ₁-KO mice.

In σ₁-KO mice treated with fentanyl (0.08 mg/kg, s.c.), latencies approached the cutoff time (44.97 ± 1.32 s). This effect was reversed by naloxone methiodide in a dose-dependent manner (0.5 – 2 mg/kg, s.c), and a dose as low as 2 mg/kg completely reversed the potentiation of fentanyl antinociception seen in σ₁-KO mice (Fig. 3A). This dose of naloxone methiodide was chosen to test the involvement of peripheral opioid receptors in the mechanical antinociception induced by several other μ-agonists in σ₁-KO mice. The mechanical antinociception induced by the s.c. administration of oxycodone (2 mg/kg), morphine (4 mg/kg), buprenorphine (0.24 mg/kg), loperamide (4 mg/kg) or tramadol (40 mg/kg) in σ₁-KO mice was completely abolished by naloxone methiodide (Fig. 3B).

Therefore, the peripheral opioid antagonist naloxone methiodide was able to fully reverse the enhanced mechanical antinociception induced by several μ-agonists in σ₁-KO mice.
Effects of systemic (subcutaneous) administration of the selective $\sigma_1$ antagonist BD-1063 on mechanical antinociception induced by $\mu$-agonists in WT mice: involvement of $\sigma_1$ and peripheral opioid receptors

We tested whether the pharmacological blockade of $\sigma_1$ receptors in WT mice would replicate the phenotype seen in $\sigma_1$-KO mice, potentiating $\mu$-opioid analgesia.

BD-1063 (8 – 32 mg/kg) increased the antinociception induced by a low dose of fentanyl (0.08 mg/kg, s.c.) in a dose-dependent manner (Fig. 4A). To confirm the selectivity of this effect, we tested its sensitivity to treatment with PRE-084, a $\sigma_1$ agonist. PRE-084 (8 – 32 mg/kg) completely reversed, in a dose-dependent manner, the potentiation of fentanyl antinociception induced by BD-1063 (Fig. 4B).

To test the involvement of peripheral opioid receptors in the potentiation of fentanyl-induced antinociception by systemic treatment with BD-1063, we assayed the sensitivity of this effect to naloxone methiodide. A dose of 2 mg/kg (s.c.) of this peripheral opioid antagonist completely reversed the antinociception induced by the association of BD-1063 with fentanyl (Fig. 4B). BD-1063, PRE-084 and naloxone methiodide did not modify struggle response latency per se (data not shown).

Our results were not affected by sex differences, since as described above for female mice, the fentanyl-induced antinociception in response to the systemic administration of BD-1063 (32 mg/kg, s.c.) was also greatly increased in male mice. This enhanced antinociception was also abolished by PRE-084 (32 mg/kg, s.c.) or Nx-M (2 mg/kg, s.c.) in male mice (data not shown).

We also studied the effects of the association of BD-1063 (32 mg/kg, s.c.) with other $\mu$-opioid receptor agonists administered at doses that induce little or no significant antinociceptive activity in WT mice. The association of BD-1063 with oxycodone (2
mg/kg, s.c.), morphine (4 mg/kg, s.c.), buprenorphine (0.24 mg/kg, s.c.), tramadol (40 mg/kg, s.c.) or even loperamide (4 mg/kg, s.c.) induced a statistically significant increase in struggle response latency compared to animals treated with the opioid drugs alone (Fig. 5). This increase was completely reversed by PRE-084 (32 mg/kg, s.c.) (Fig. 5). Naloxone methiodide (2 mg/kg, s.c.) decreased the effect of the association of BD-1063 and the μ-agonists to the same extent as the effect of μ-opioids administered alone, with the exception of the combination of BD-1063 and oxycodone: the effect of this combination was decreased more markedly than the effect of oxycodone alone (Fig. 5).

Therefore, systemic treatment with the σ₁ antagonist BD-1063 synergistically enhanced the antinociception induced by the systemic administration of all μ-agonists tested. This effect was sensitive to both the σ₁ receptor agonist PRE-084 and the peripheral opioid antagonist naloxone methiodide.

Effects of local (intraplantar) administration of the selective σ₁ antagonist BD-1063 on mechanical antinociception induced by μ-agonists in wild-type mice: involvement of σ₁- and peripheral opioid receptors

We also tested whether the local administration of BD-1063 into the hindpaw was sufficient to enhance μ-opioid-induced mechanical antinociception. As in the experiments described in the preceding section, low doses of systemic μ-agonists were injected s.c. in WT mice, but BD-1063 was injected i.pl. to test for locally-induced effects.

In WT mice, fentanyl injected s.c. (0.08 mg/kg) and BD-1063 solvent (saline) injected i.pl. led to similarly short struggle response latencies in the treated paw and the contralateral (untreated) paw (Fig. 6A). However, when this dose of fentanyl was
associated to BD-1063 (50-200 µg, i.pl.), a dose-dependent increase in latency was seen only in the treated hindpaw (black bars in Fig. 6A).

To test the σ₁ specificity of the effects induced by local treatment with BD-1063, we used the σ₁ agonist PRE-084, and also evaluated the effects of naloxone methiodide to determine whether peripheral opioid receptors were involved in the enhanced antinociception induced by the combination of fentanyl and local σ₁ pharmacological blockade. PRE-084 (8-32 mg, s.c.) completely reversed, and in a dose-dependent manner, the mechanical antinociceptive effect induced by the combination of fentanyl (0.08 mg/kg, s.c.) and BD-1063 (200 µg, i.pl.) (Fig. 6B). Treatment with naloxone methiodide (2 mg/kg, s.c.) was also able to completely abolish the local potentiation of fentanyl-induced antinociception by BD-1063 (200 µg, i.pl.) (Fig. 6B). Neither PRE-084 nor naloxone methiodide induced any change in latencies of the noninjected paw in animals treated with systemic fentanyl (white bars in Fig. 6B).

We also tested the association of local treatment of BD-1063 (200 µg, i.pl.) with low doses of the other µ-agonists. Local treatment with BD-1063 was also able to increase struggle response latencies in animals that were given (s.c.) oxycodone (2 mg/kg), morphine (4 mg/kg), buprenorphine (0.24 mg/kg), loperamide (4 mg/kg) or tramadol (40 mg/kg) (Fig. 7). This effect was only seen in the treated hindpaw, whereas latencies in the untreated hindpaw remained unaltered (data not shown). This increased latency in the hindpaw treated with BD-1063 in mice that were given µ-agonists was abolished by treatment with either PRE-084 (32 mg/kg, s.c.) or naloxone methiodide (2 mg/kg, s.c.) (Fig. 7). As in the experiments described in the preceding section, PRE-084 reversed the effects of the local administration of BD-1063, but not beyond the level of the effects of the µ-agonists alone (Fig. 7). Naloxone methiodide decreased latencies in animals treated with BD-1063 together with µ-agonists to the same extent as the effect of the
opioids administered alone, with the exception of the combination of BD-1063 and oxycodone: the effect of this combination was decreased more markedly than the effect of oxycodone alone (Fig. 7).

Therefore, local treatment with the $\sigma_1$ antagonist BD-1063 synergistically enhanced the antinociception induced by all $\mu$-agonists tested when they were given systemically. This effect was observed only in the treated paw and was sensitive to both $\sigma_1$ agonism and peripheral opioid antagonism.

**Effects of systemic (subcutaneous) and local (intraplantar) administration of the selective $\sigma_1$ antagonist S1RA on mechanical antinociception induced by fentanyl and loperamide in wild-type mice: involvement of $\sigma_1$- and peripheral opioid receptors**

To test whether the effects induced by BD-1063 were replicable by a different $\sigma_1$ antagonist, we evaluated the effects of S1RA on the antinociceptive effects induced by two different $\mu$-opioids: the centrally active fentanyl and the peripherally restricted loperamide.

The systemic administration of S1RA (64 mg/kg, s.c.) increased the struggle response latencies in animals treated s.c. with either fentanyl (0.08 mg/kg) or loperamide (4 mg/kg) (Fig. 8A). The i.pl. administration of S1RA (200 µg) was also able to increase the response latency in mice treated with these $\mu$-opioid agonists (Fig. 8B), and this enhanced antinociception was detectable in the injected but not in the contralateral paw (data not shown). As described in the preceding sections regarding the effects of BD-1063, treatment with either the $\sigma_1$ agonist PRE-084 (32 mg/kg, s.c.) or the peripheral opioid antagonist naloxone methiodide (2 mg/kg, s.c.) abolished the effects on opioid
antinociception of S1RA administered either systemically or locally (Fig. 8A and B, respectively).

Therefore, systemic or local treatment with the σ₁ antagonist S1RA synergistically enhanced the antinociception induced by the systemic administration of fentanyl or loperamide, and these effects were fully reversed by either σ₁ agonism or peripheral opioid antagonism.

**Sigma-1 receptor expression in the central and peripheral nervous system**

To obtain anatomical support for the marked behavioral effects of local σ₁ receptor blockade on opioid antinociception, we compared the expression of σ₁ receptors in different areas of the nervous system involved in opioid analgesia, including supraspinal (BLA, RVM and PAG), spinal (dSC) and peripheral nervous locations (DRG). All samples from WT mice yielded immunoreactive bands at a molecular weight slightly higher than 25 kDa (Fig. 9A), which is consistent with the molecular weight of the cloned σ₁ receptor from the mouse (~28 kDa) (Pan et al., 1998). We found no significant differences in σ₁ receptor band intensities among all central areas in WT mice (Fig. 9B); however, σ₁ receptor density was much higher in DRG samples than in any of the central areas examined (Fig. 9A and B). To verify the specificity of the anti-σ₁ receptor antibody, we tested its immunoreactivity in samples from σ₁-KO mice. We found no immunoreactive σ₁ receptor bands in PAG, dSC or DRG samples from σ₁-KO animals (Fig. 9A and B), or in samples of BLA or RVM from these mice (data not shown). The absence of these bands in σ₁-KO samples argues for the specificity of the σ₁ antibody used. In addition, we were unable to detect immunoreactive bands in WT samples when tested in the absence of anti-σ₁ antibody (data not shown), which further confirms the specificity of the procedure.
The high level of $\sigma_1$ receptor expression in peripheral nervous tissue argues for a possible major role of peripheral $\sigma_1$ receptors in nociception.

Effects of fentanyl and loperamide on gastrointestinal transit in wild-type mice, wild-type mice treated with BD-1063, and $\sigma_1$ knockout mice

Gastrointestinal transit distances did not differ significantly between WT mice, WT mice treated with BD-1063 (32 mg/kg, s.c.) and $\sigma_1$-KO mice that had been given the fentanyl (Fig. 10A) or loperamide solvents (Fig. 10B). In all cases the charcoal meal traversed about 30 cm of the small intestine. Both fentanyl (0.04 – 0.16 mg/kg, s.c.) and loperamide (0.125 – 1 mg/kg, s.c.) induced a dose-dependent decrease in gastrointestinal transit in WT mice (white bars in Fig. 10A and 10B). For each dose of fentanyl (Fig. 10A) or loperamide (Fig. 10B), this decrease was similar in WT mice treated with BD-1063 and in $\sigma_1$-KO mice. Therefore, $\sigma_1$ inhibition alone did not alter gastrointestinal transit and did not modify the effects of fentanyl or loperamide on this outcome.

Affinity of $\mu$-opioid drugs for $[^3H](+)$-pentazocine binding sites

We used competition binding assays to test the affinity of the opioid drugs assayed $in$ $vivo$ for $[^3H](+)$-pentazocine-labeled $\sigma_1$ receptors in brain membranes from WT mice. As expected, the known $\sigma_1$ antagonist BD-1063 inhibited $[^3H](+)$-pentazocine specific binding in a concentration-dependent manner, with an $IC_{50}$ value of $40.21 \pm 3.24$ nM. This value was similar to that found in previous studies (Entrena 2009a; Cobos et al., 2005, 2006 and 2007). However, none of the opioid drugs (fentanyl, oxycodone, morphine, buprenorphine, tramadol, loperamide, naloxone or naloxone methiodide) significantly inhibited $[^3H](+)$-pentazocine binding at any concentration tested (ranging
from $10^{-10}$ to $10^{-6}$ M), and therefore their affinity for $[^3H](+)$-pentazocine binding sites was considered negligible (Fig. 11).
Discussion

We found that several centrally penetrant μ-opioid analgesics (fentanyl, oxycodone, morphine, buprenorphine and tramadol) and the peripheral μ-agonist loperamide, at systemic doses which induce little or no antinociception in control animals, have a marked antinociceptive effect when boosted by σ₁ receptor inhibition (σ₁–KO mice or σ₁ pharmacological blockade). We show that this enhanced opioid antinociception is mediated peripherally, since it can be achieved by local σ₁ pharmacological blockade, and is sensitive to peripheral opioid antagonism. However, the increase in peripheral opioid antinociception by σ₁ receptor inhibition is not accompanied by increased inhibition of opioid-induced gastrointestinal transit, a known peripherally–mediated side effect of opioids.

It has been proposed that the analgesic mechanisms of different μ-opioids used in clinical settings overlap only partially (Ocaña et al., 1995; Pasternak, 2004; Pasternak and Pan 2011; Smith, 2008; Raehal et al., 2011). Hence the importance of testing whether the widely reported enhancement of morphine antinociception by σ₁ inhibition (e.g. Chien and Pasternak 1993; Mei and Pasternak 2007; Díaz et al., 2009; Sanchez-Fernandez et al., 2013) also occurs with other clinically relevant μ-opioids. It was recently reported that the systemic (subcutaneous) administration of S1RA enhanced antinociception against a thermal stimulus induced by morphine and other systemically administered centrally-penetrant μ-opioid analgesics (fentanyl, oxycodone, buprenorphine and tramadol) (Vidal-Torres et al., 2013). We now extend those results by showing that this σ₁ antagonist and also BD-1063 enhance opioid antinociception against a different type of nociceptive (mechanical) stimulus, and more importantly, that the enhanced antinociception is mediated peripherally (see below). Moreover, we show that opioid-induced mechanical antinociception is clearly potentiated in σ₁-KO mice,
which contrasts with the previously reported absence of modulation of opioid thermal antinociception in \( \sigma_1 \)-KO mice (Díaz et al., 2009; Vidal-Torres et al., 2013). These apparently contradictory results seem to be related to the type of sensory stimulation used, and may be attributable to the known differences in the neurochemical mechanisms of thermal and mechanical opioid antinociception (Kuraishi et al., 1995; Wegert et al., 1997). These mechanisms may be affected differentially by possible compensatory mechanisms in \( \sigma_1 \)-KO mice.

In addition to centrally-acting \( \mu \)-opioid analgesics, we also tested the effects of the peripheral \( \mu \)-opioid agonist loperamide under normal conditions and during \( \sigma_1 \) inhibition. In agreement with previous reports, loperamide had no antinociceptive effects in WT mice (Menendez et al., 2005; Sevostianova et al., 2005). However, in \( \sigma_1 \)-KO mice or WT mice treated systemically with BD-1063 or S1RA, we observed a profound antinociceptive effect in response to loperamide. Therefore, \( \sigma_1 \) inhibition is sufficient to unmask the strong antinociceptive effects of this peripherally acting \( \mu \)-opioid. This finding evidences that peripheral \( \mu \)-opioid analgesia is enhanced by \( \sigma_1 \) inhibition. In fact, the local (intraplantar) administration of \( \sigma_1 \) antagonists was able to enhance \( \mu \)-antinociception in response to all \( \mu \)-agonists tested. This enhanced antinociception occurred only in the paw injected with the \( \sigma_1 \) antagonist, but not in the contralateral paw, clearly indicating that the effects of these \( \sigma_1 \) antagonists occurred locally. The selective \( \sigma_1 \) agonist PRE-084 reversed the effects of BD-1063 or S1RA (administered either systemically or locally), arguing for a \( \sigma_1 \)-mediated action. Interestingly, PRE-084 was unable to reverse the enhanced antinociception seen in \( \sigma_1 \)-KO mice treated with fentanyl, supporting on-target mechanisms in the effects induced by this \( \sigma_1 \) agonist (data not shown).
To investigate the role of peripheral opioid receptors in the antinociceptive effect of systemically administered μ-agonists under normal conditions and during σ₁ inhibition, we tested the sensitivity of antinociception to peripheral opioid antagonism in both situations. The antinociceptive effects of the opioids fentanyl, morphine and buprenorphine in WT mice (in the absence of σ₁ blockade) were sensitive to the centrally penetrant opioid antagonist naloxone. However, they were resistant to the peripherally restricted antagonist naloxone methiodide. These findings support a preferential location of the antinociceptive effects of μ-opioids at central levels under our experimental conditions, and are consistent with previous studies (Greenwood-Van Meerveld and Standifer, 2008; Thomas et al., 2008; Joshi et al., 2008; Khalefa et al., 2012; Ringkamp and Raja, 2012). Among the opioids tested in WT mice (in the absence of σ₁ blockade), only oxycodone had an antinociceptive effect that was partially reversible by naloxone methiodide, and this only occurs at the highest dose (8 mg/kg) of the antagonist. Our findings are consistent with clinical data suggesting that part of the analgesic effects of oxycodone may be mediated peripherally (Olesen et al., 2010). The sensitivity to peripheral opioid antagonism is diametrically different when opioid antinociception is enhanced by σ₁ inhibition. A dose as low as 2 mg/kg of naloxone methiodide was enough to completely abolish the opioid antinociception induced by all μ-agonists tested, not only in WT mice treated locally with a σ₁ antagonist (in which its effects are clearly peripherally mediated), but also in σ₁–KO mice or WT mice treated systemically with the σ₁ antagonists. This does not argue against the widely reported potentiation of opioid antinociception by central σ₁ receptor inhibition (King et al., 1997; Pan et al., 1998; Mei and Pasternak 2002 and 2007; Marrazzo et al., 2006), but indicates that when both opioid agonism and σ₁ inhibition are induced systemically, the increase in antinociception occurs mainly at the peripheral level. In support of the
peripheral location of these modulatory effects of $\sigma_1$ receptors, we found much higher levels of these receptors in the DRG than in several areas of the central nervous system that play a key role in opioid antinociception.

Among the opioid drugs tested in the present study, only morphine and naloxone had previously been shown to lack affinity for $\sigma_1$ receptors (Walker et al., 1990). Here we show that this lack of $\sigma_1$ affinity is shared by other opioid drugs (fentanyl, oxycodone, buprenorphine, tramadol, loperamide and naloxone methiodide). In addition, it is known that the $\sigma_1$ drugs tested here (BD-1063, S1RA and PRE-084) do not bind to $\mu$-opioid receptors (Matsumoto et al., 1995; Sánchez-Fernández et al., 2013). Therefore, our results support a functional link between peripheral $\sigma_1$ receptors and the $\mu$-opioid system rather than interactions of $\sigma_1$ ligands with $\mu$ opioid receptors or opioid drugs with $\sigma_1$ receptors.

It was recently reported that $\sigma_1$ antagonism potentiates $\mu$-opioid signaling (measured as the increase in DAMGO-induced $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding), providing a mechanistic explanation for the enhanced opioid antinociception by $\sigma_1$ inhibition (Kim et al. 2010).

Previous studies of thermal nociception show that $\sigma_1$ inhibition potentiates $\mu$, $\kappa$ and $\delta$ opioid antinociception at central levels (King et al., 1997; Mei and Pasternak, 2002). Therefore, the modulation of peripheral $\mu$ opioid antinociception by $\sigma_1$ inhibition that we report here might occur with other subtypes of opioid receptors, although this hypothesis remains to be tested.

 Clinically, a dose-limiting factor in obtaining maximal analgesia with systemic opioids is the risk of adverse side effects. Some of these effects are mediated through peripheral opioid receptors (constipation), whereas others are mediated at supraspinal sites (addiction, dependency, nausea, somnolence, respiratory depression) (Benyamin et al 2008; Greenwood-Van Meerveld and Standifer, 2008; Al-Hasani and Bruchas, 2011;
Ringkamp and Raja, 2012). Targeting peripheral opioid receptors has been proposed as a strategy to minimize opioid-induced side effects that are produced centrally (Sehgal et al., 2011; Ringkamp and Raja, 2012). However, since most of the analgesia from systemic opioids is produced normally at central sites, it seems difficult to dissociate antinociceptive effects from centrally-induced side effects. In this regard, we previously reported that despite the potentiation of antinociception by \( \sigma_1 \) inhibition, the central side effects of morphine (such as hyperlocomotion, physical dependence or mydriasis) were not altered (Sánchez-Fernández et al., 2013; Vidal-Torres et al., 2013). Here we show that the enhancement of opioid antinociception by systemic \( \sigma_1 \) inhibition occurs primarily at peripheral levels, which might explain the lack of potentiation of morphine-induced central side effects. Interestingly, morphine-induced constipation, which is the most clinically relevant peripheral side effect of \( \mu \)-opioids, was also not modulated by \( \sigma_1 \) receptors (Chien and Pasternak, 1994; Sánchez-Fernández et al., 2013; Vidal-Torres et al., 2013); however, it is unknown whether this characteristic is shared by other opioids. To find out, we tested two additional \( \mu \)-opioids of very different characteristics: the central analgesic fentanyl and the peripherally-acting loperamide. Importantly, the gastrointestinal transit inhibition induced by these opioids was unaffected in either \( \sigma_1 \)–KO mice or WT mice treated with BD-1063. Regardless of the exact mechanistic nature of the differential modulation of opioid antinociception and adverse events by \( \sigma_1 \) inhibition, our findings point to a potentially beneficial avenue of research aimed at improving the safety profile of opioid drugs.

In summary, we found that \( \sigma_1 \) receptor inhibition enhanced the peripheral opioid antinociception induced by clinically relevant \( \mu \)-agonists, but did not increase opioid-induced constipation. These data support the conclusion that peripheral \( \sigma_1 \) receptors are a biological brake to \( \mu \)-opioid antinociception, and that either systemic or local \( \sigma_1 \)
receptor inhibition is potentially useful as an adjuvant to enhance peripheral μ-opioid analgesia. Combinations of σ₁ antagonists and μ-opioid agonists may be of therapeutic interest in terms of both efficacy and safety, and merit clinical studies.
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Authorship Contributions

Participated in research design: Cobos, Baeyens, Entrena, Sánchez-Fernández, Fernández-Pastor.


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Wrote or contributed to the writing of the manuscript: Cobos, Baeyens, Entrena, Sánchez-Fernández, Merlos

All authors declare that they have no conflict of interest.
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Footnotes

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Figure Legends

Figure 1. Mechanical antinociception induced by the systemic administration of several μ-opioid receptor agonists in wild-type (WT) and σ₁ knockout (σ₁-KO) mice. The results represent the struggle response latency during stimulation with 450 g pressure on the hindpaws of mice treated subcutaneously with several doses of: (A) fentanyl, oxycodone, or morphine; and (B) buprenorphine, loperamide or tramadol, or their solvents (doses 0). Each point and vertical line represents the mean ± SEM of values obtained in 8–10 animals. The morphine dose-response curve for comparison with the other opioids is from Sánchez-Fernández et al., 2013. Statistically significant differences between the values obtained in solvent- and opioid-treated groups: *P < 0.05; **P < 0.01, and between the values obtained in WT and σ₁-KO mice at the same dose of a given opioid: ##P < 0.01 (two-way ANOVA followed by Bonferroni test).

Figure 2. Contribution of peripheral opioid receptors to the antinociception induced by the systemic administration of several μ-agonists in wild-type mice. The results represent the struggle response latency during stimulation with 450 g pressure on the hindpaw of naïve mice, and animals treated subcutaneously with fentanyl (0.16 mg/kg), oxycodone (4 mg/kg), morphine (16 mg/kg) or buprenorphine (0.48 mg/kg), associated with naloxone (Nx, 0.5 mg/kg), naloxone methiodide (Nx-M, 2-8 mg/kg) or their solvent. Each bar and vertical line represents the mean ± SEM of values obtained in 8–10 animals. Statistically significant differences between the values obtained in naïve mice and animals treated with the μ-opioid analgesics: *P < 0.05, **P < 0.01; and between the groups treated with the different μ-opioid agonists alone and associated with Nx or Nx-M: ##P < 0.01 (one-way ANOVA followed by Bonferroni test).
**Figure 3.** Contribution of peripheral opioid receptors to the antinociception induced by the systemic administration of low doses of several μ-agonists in σ₁ knockout mice. The results represent the struggle response latency during stimulation with 450 g pressure on the hindpaws of (A) mice treated subcutaneously with the combination of fentanyl (0.08 mg/kg), and naloxone methiodide (Nx-M, 0.5-2 mg/kg) or its solvent, and mice treated with the solvent of both drugs (white bar), or (B) naïve mice and mice treated subcutaneously with oxycodone (2 mg/kg), morphine (4 mg/kg), buprenorphine (0.24 mg/kg), loperamide (4 mg/kg) or tramadol (40 mg/kg), associated with Nx-M (2 mg/kg) or its solvent. Each bar and vertical line represents the mean ± SEM of values obtained in 8–10 animals. Statistically significant differences between the values obtained in mice treated and not treated with the μ-opioid analgesics: **P < 0.01; and between the groups treated with different μ-opioid agonists associated with Nx-M or its solvent: ##P < 0.01 (one-way ANOVA followed by Bonferroni test).

**Figure 4.** Contribution of σ₁ and peripheral opioid receptors to the antinociception induced by the association of fentanyl (0.08 mg/kg) and BD-1063, both administered systemically to wild-type mice. The results represent the struggle response latency during stimulation with 450 g pressure on the hindpaws of (A) mice treated subcutaneously (s.c.) with fentanyl and BD-1063 (8-32 mg/kg) or its solvent, and (B) mice s.c. given fentanyl (0.08 mg/kg) or fentanyl (0.08 mg/kg) + BD-1063 (32 mg/kg) associated to PRE-084 (8-32 mg/kg, s.c.), naloxone methiodide (Nx-M, 2 mg/kg, s.c.) or their solvent. Each bar and vertical line represents the mean ± SEM of values obtained in 8–10 animals. Statistically significant differences between the values obtained in the groups treated with fentanyl associated with BD-1063 or its solvent: **P < 0.01; and between the values obtained in mice treated with the combination of
fentanyl and BD-1063, and its association with PRE-084 or Nx-M: **P < 0.01 (one-way ANOVA followed by Bonferroni test).

**Figure 5.** Contribution of σ₁ and peripheral opioid receptors to the antinociception induced by the association of several μ-agonists and BD-1063, both administered systemically to wild-type mice. The results represent the struggle response latency during stimulation with 450 g pressure on the hindpaw of mice treated subcutaneously (s.c.) with BD-1063 (32 mg/kg) or its solvent in combination with oxycodone (2 mg/kg), morphine (4 mg/kg), buprenorphine (0.24 mg/kg), loperamide (4 mg/kg) or tramadol (40 mg/kg), and its association with the s.c. administration of PRE-084 (32 mg/kg), naloxone methiodide (Nx-M, 2 mg/kg) or their solvent. Each bar and vertical line represents the mean ± SEM of values obtained in 8–10 animals. Statistically significant differences between the values obtained in the groups treated with different μ-opioid agonists associated with BD-1063 or its solvent: **P < 0.01; and between the values obtained in mice treated with the combination of a given μ-agonist with BD-1063, and its association with PRE-084 or Nx-M: ##P < 0.01 (one-way ANOVA followed by Bonferroni test).

**Figure 6.** Contribution of σ₁ and peripheral opioid receptors to the effects of the local administration of BD-1063 on the antinociception induced by systemically administered fentanyl in wild-type mice. The results represent the struggle response latency during stimulation with 450 g pressure on the hindpaw of mice treated subcutaneously (s.c.) with: (A) fentanyl (0.08 mg/kg) + intraplantar (i.pl.) BD-1063 (50-200 μg) or its solvent, and (B) fentanyl (0.08 mg/kg, s.c.) + BD-1063 (200 μg, i.pl.) in animals pretreated s.c. with PRE-084 (8-32 mg/kg), naloxone methiodide (Nx-M, 2 mg/kg) or
their solvent. Each bar and vertical line represents the mean ± SEM of values obtained in 8–10 animal. (A and B) Statistically significant differences between the values obtained upon stimulation after the injection of BD-1063 or its solvent in the hindpaw: *$P < 0.05$, **$P < 0.01$; and between the values obtained from the injected and noninjected hindpaws: #$P < 0.01$ (two-way ANOVA followed by Bonferroni test). (B) Statistically significant differences between the values obtained in mice treated with the combination of fentanyl with BD-1063, and its association with PRE-084 or Nx-M: †† $P < 0.01$ (two-way ANOVA followed by Bonferroni test).

**Figure 7.** Contribution of $\sigma_1$ and peripheral opioid receptors to the effects of the local administration of BD-1063 on the antinociception induced by several $\mu$-agonists administered systemically to wild-type mice. The results represent the struggle response latency during stimulation with 450 g pressure on the injected hindpaw in mice treated intraplantarly with BD-1063 (200 $\mu$g) or its solvent in combination with the subcutaneous (s.c.) administration of oxycodone (2 mg/kg), morphine (4 mg/kg), buprenorphine (0.24 mg/kg), loperamide (4 mg/kg) or tramadol (40 mg/kg), and its association with the s.c. administration of PRE-084 (32 mg/kg), naloxone methiodide (Nx-M, 2 mg/kg) or their solvent. Each bar and vertical line represents the mean ± SEM of values obtained in 8–10 animals. Statistically significant differences between the values obtained in the groups treated with the different $\mu$-opioid agonists alone and associated with BD-1063: **$P < 0.01$; and between the values obtained in mice treated with the combination of a given $\mu$-agonist with BD-1063, and its association with PRE-084 or Nx-M: #$P < 0.01$ (one-way ANOVA followed by Bonferroni test).
Figure 8. Contribution of $\sigma_1$ and peripheral opioid receptors to the effects of the systemic and local administration of S1RA on the antinociception induced by systemically administered fentanyl and loperamide in wild-type mice. Animals were treated (A) subcutaneously (s.c.) or (B) intraplantarly (i.pl.) with S1RA (64 mg/kg and 200 $\mu$g, respectively) in combination with fentanyl (0.08 mg/kg, s.c.) or loperamide (4 mg/kg, s.c.), and its association with the s.c. administration of PRE-084 (32 mg/kg), naloxone methiodide (Nx-M, 2 mg/kg) or their solvent. The results represent the struggle response latency in mice during stimulation with 450 g pressure on the hindpaws (A) or on the injected hindpaw (B). Each bar and vertical line represents the mean ± SEM of values obtained in 8–10 animals. Statistically significant differences between the values obtained in the groups treated with the $\mu$-opioid agonists associated with S1RA or its solvent: **$P < 0.01$; and between the values obtained in mice treated with the combination of a given $\mu$-agonist with S1RA, and its association with PRE-084 or Nx-M: ##$P < 0.01$ (one-way ANOVA followed by Bonferroni test).

Figure 9. Expression of sigma-1 ($\sigma_1$) receptors in the basolateral amygdala (BLA), rostroventral medulla (RVM), periaqueductal grey matter (PAG), dorsal spinal cord from the lumbar enlargement (dSC), and lumbar dorsal root ganglia (DRGs) in wild-type (WT) and $\sigma_1$ knockout ($\sigma_1$-KO) mice. (A) Representative immunoblots for $\sigma_1$ receptors in WT and $\sigma_1$-KO mice. $\beta$-actin was used as the loading control. The migration positions of molecular weight standards (in kDa) are shown to the left of the gel. (B) Quantification of immunoblotting for the $\sigma_1$ receptor in WT and $\sigma_1$-KO mice. Each bar and vertical line represents the mean ± SEM of the densitometric values obtained in 8 animals. The $\sigma_1$ receptor band intensities were relativized to those of their corresponding $\beta$-actin loading control bands. Statistically significant differences
between the values obtained in samples of central nervous system regions (BLA, RVM, PAG, dSC) and the lumbar dorsal root ganglia (DRGs) from WT mice \( **P < 0.01 \). No \( \sigma_1 \) receptor expression was found in samples from \( \sigma_1 \)-KO mice.

**Figure 10.** Effects of the systemic (subcutaneous, s.c.) administration of several doses of fentanyl (A) and loperamide (B) on gastrointestinal transit of wild-type mice (WT), WT mice treated with BD-1063 (32 mg/kg, s.c.), and \( \sigma_1 \) knockout mice (\( \sigma_1 \)-KO). BD-1063 or its solvent was injected, and 5 min later the animals were treated with fentanyl, loperamide or their solvents. 30 minutes after the administration of the opioid drug, the mice were given a 0.5% charcoal suspension intragastrically. Transit of the charcoal was measured 30 min after administration. Each bar and vertical line represents the mean ± SEM of values obtained in 8-12 mice. Statistically significant differences between the values obtained in saline- and opioid-treated groups: \( **P < 0.01 \). No statistically significant differences were found between genotypes with the same treatment, or between animals treated and not treated with BD-1063 (two-way ANOVA followed by Bonferroni test).

**Figure 11.** Inhibition by unlabeled drugs of \([^{3}\text{H}]\,(+)-\text{pentazocine}\) binding to brain membranes (P2 fraction) in wild-type mice. \([^{3}\text{H}]\,(+)-\text{pentazocine}\) (5 nM) was incubated with 0.8–1 mg/mL brain membrane protein at 30 °C, pH 8, for 240 min and increasing concentrations of BD-1063, fentanyl, oxycodone, morphine, buprenorphine, loperamide, tramadol, naloxone or naloxone methiodide. The data shown are the averages of two experiments carried out in triplicate.
Figure 3

A

B

Struggle response latency (s)

Fentanyl (0.08 mg/kg, s.c.)

Naïve

Oxycodone (2 mg/kg, s.c.)

Morphine (4 mg/kg, s.c.)

Buprenorphine (0.24 mg/kg, s.c.)

Loperamide (4 mg/kg, s.c.)

Tramadol (40 mg/kg, s.c.)

0.5  1  2

Nx-M (mg/kg, s.c.)

0  10  20  30  40  50

Struggle response latency (s)

Nx-M (2 mg/kg, s.c.)
Figure 4

A

Struggle response latency (s)

BD-1063 (mg/kg, s.c.)

Fentanyl (0.08 mg/kg, s.c.)

B

Struggle response latency (s)

PRE-084 (mg/kg, s.c.)

Nx-M (mg/kg, s.c.)

BD-1063 (32 mg/kg, s.c.)

Fentanyl (0.08 mg/kg, s.c.)
Figure 5

- **Vehicle**
- **BD-1063 (32 mg/kg, s.c.)**
- **BD-1063 (32 mg/kg, s.c.) + PRE-084 (32 mg/kg, s.c.)**
- **BD-1063 (32 mg/kg, s.c.) + Nx-M (2 mg/kg, s.c.)**

**Struggle response latency (s)**

- **Oxycodone (2 mg/kg, s.c.)**
- **Morphine (4 mg/kg, s.c.)**
- **Buprenorphine (0.24 mg/kg, s.c.)**
- **Loperamide (4 mg/kg, s.c.)**
- **Tramadol (40 mg/kg, s.c.)**
Figure 6

A

- **Injected hind-paw**
- **Non-injected hind-paw**

![Graph showing struggle response latency for BD-1063 (µg, i.pl.)](image)

<table>
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<tr>
<th>Dose (µg, i.pl.)</th>
<th>Struggle response latency (s)</th>
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<tr>
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B

- **Fentanyl (0.08 mg/kg, s.c.)**

![Graph showing struggle response latency for PRE-084 and Nx-M](image)

<table>
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<th>Struggle response latency (s)</th>
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**BD-1063 (200 µg, i.pl.)**

- **Fentanyl (0.08 mg/kg, s.c.)**
Figure 7

Struggle response latency (s)

- **Vehicle**
- **BD-1063 (200 μg, i.pl.)**
- **BD-1063 (200 μg, i.pl.) + PRE-084 (32 mg/kg, s.c.)**
- **BD-1063 (200 μg, i.pl.) + Nx-M (2 mg/kg, s.c.)**

**Oxycodone**
- (2 mg/kg, s.c.)

**Morphine**
- (4 mg/kg, s.c.)

**Buprenorphine**
- (0.24 mg/kg, s.c.)

**Loperamide**
- (4 mg/kg, s.c.)

**Tramadol**
- (40 mg/kg, s.c.)
Figure 8

A

- Vehicle
- S1RA (64 mg/kg, s.c.)
- S1RA (64 mg/kg, s.c.) + PRE-084 (32 mg/kg, s.c.)
- S1RA (64 mg/kg, s.c.) + Nx-M (2 mg/kg, s.c.)

Struggle response latency (s)

Fentanyl (0.08 mg/kg, s.c.)

Loperamide (4 mg/kg, s.c.)

B

- Vehicle
- S1RA (200 μg, i.pl.)
- S1RA (200 μg, i.pl.) + PRE-084 (32 mg/kg, s.c.)
- S1RA (200 μg, i.pl.) + Nx-M (2 mg/kg, s.c.)

Struggle response latency (s)

Fentanyl (0.08 mg/kg, s.c.)

Loperamide (4 mg/kg, s.c.)
Figure 9

A

WT

σ₁-KO

BLA RVM PAG dSC DRG

37

β-actin

25

σ₁

B

Relative levels (σ₁/β-actin)

**

BLA RVM PAG dSC DRG

WT

σ₁-KO
Figure 11

A graph showing the concentration of various drugs on binding. The y-axis represents binding as a percentage, while the x-axis represents the log concentration of the drug in Molar (M). The graph includes data points for BD-1063, Fentanyl, Oxycodone, Morphine, Buprenorphine, Loperamide, Tramadol, Naloxone, and Naloxone Methiodide. The concentration ranges from 10^-10 to 10^-6 Molar.