Modulation of Peripheral $\mu$-Opioid Analgesia by $\sigma_1$ Receptors

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ABSTRACT

We evaluated the effects of $\sigma_1$-receptor inhibition on $\mu$-opioid-induced mechanical antinociception and constipation. $\sigma_1$-Knockout mice exhibited marked mechanical antinociception in response to several $\mu$-opioid analgesics (fentanyl, oxycodone, morphine, buprenorphine, and tramadol) at systemic (subcutaneous) doses that were inactive in wild-type mice and even unmasked the antinociceptive effects of the peripheral $\mu$-opioid agonist loperamide. Likewise, systemic (subcutaneous) or local (intraplantar) treatment of wild-type mice with the selective $\sigma_1$ antagonists BD-1063 [1-[2-[3,4-dichlorophenyl)ethyl]-4-methylpiperazine dihydrochloride] or S1RA [4-[2-[5-methyl-1-(2-naphthalenyl)1H-pyrazol-3-yl]oxy]ethyl] morpholine hydrochloride] potentiated $\mu$-opioid antinociception; these effects were fully reversed by the $\sigma_1$ agonist PRE-084 [2-(4-morpholinethyl)1-phenylcyclohexanecarboxylate] hydrochloride, showing the selectivity of the pharmacological approach. The $\mu$-opioid antinociception potentiated by $\sigma_1$ inhibition (by $\sigma_1$-receptor knockout or $\sigma_1$-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 $\sigma_1$ receptors cannot account for our results, since the former lacked affinity for $\sigma_1$ receptors (labeled with $[^{3}H]$(+)-pentazocine). A peripheral role for $\sigma_1$ 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 noiception, $\sigma_1$-receptor inhibition did not alter fentanyl- or loperamide-induced constipation, a peripherally mediated nonanalgesic opioid effect. Therefore, $\sigma_1$-receptor inhibition may be used as a systemic or local adjuvant to enhance peripheral $\mu$-opioid analgesia without affecting opioid-induced constipation.

Introduction

Opioid drugs, particularly agonists of the $\mu$-receptor subtype, 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 (dorsal root ganglion [DRG] and peripheral nerve terminals) nervous system (Bigiardi-Qi et al., 2004; Khalefa et al., 2012). The antinoiceptive effect of systemic opioids is thought to be produced mainly at the central (particularly supraspinal) level (Greenwood-Van Meerveld and Sandifer, 2008; Joshi et al., 2008; Thomas et al., 2008; Khalefa et al., 2012), although peripheral opioid receptors might also participate (Craft et al., 1995; Kayser et al., 1995; Shannon and Lutz, 2002).

The $\sigma_1$ receptor has been cloned, and its sequence does not show homology with opioid receptors or any other known mammalian protein; therefore, it is currently considered a unique entity (Cobos et al., 2008; Zamanillo et al., 2013). Inhibition of $\sigma_1$-receptor function either by the systemic administration of $\sigma_1$ antagonists BD-1063, 1-[2-[3,4-dichlorophenyl]ethyl]-4-methylpiperazine dihydrochloride; BLA, basolateral amygdala; DAMGO, [D-Ala$^2$,N-Me-Phe$^4$,Gly-$\alpha$-ol]enkephalin; DMSO, dimethylsulfoxide; dSC, dorsal spinal cord; DRG, dorsal root ganglion; 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; 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.

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antagonists or by \( \sigma_1 \)-receptor knockdown does not influence acute nociception per se (Cendán et al., 2005; De la Puente et al., 2009; Entrena et al., 2009b; Nieto et al., 2012; Romero et al., 2012; Sánchez-Fernández et al., 2013). However, \( \sigma_1 \) inhibition is able to enhance opioid signaling (Kim et al., 2010) and to potentiate the antinociceptive effect of systemic opioids (Chien and Pasternak, 1993, 1994; Marruzzo et al., 2011; Sánchez-Fernández et al., 2013; Vidal-Torres et al., 2013). Opioid antinociception can be potentiated by central \( \sigma_1 \) inhibition (King et al., 1997; Pan et al., 1998; Mei and Pasternak, 2002, 2007; Marruzzo et al., 2006), and we recently reported that the local peripheral coadministration of \( \sigma_1 \) 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 (Gallelli et al., 2010; Layer and Andresen 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 [1-[2-(3,4-dichlorophenyl)ethyl]-4-methylpiperazine dihydrochloride] and S1RA [4-[2-[[5-methyl-1-(2-naphthalenyl)]H]-pyrazol-3-yl][oxylethyl] morpholine hydrochloride]. Moreover, because the data from this 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., 2013). 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 penetrating drug, and loperamide, a peripherally restricted drug.

### Materials 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-hour day/night cycle, constant temperature (22 ± 2°C) with free access to water and food (standard laboratory diet; Harlan Teklad Diet, Madison, WI). Behavioral testing was done during the light phase (from 9:00 AM to 3:00 PM) and randomly throughout the estrous cycle. Animal care was provided in accordance with institutional (Research Ethics Committee of the University of Granada, 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 as follows: 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 Quimica SA, Madrid, Spain); tramadol (supplied by Laboratorios Esteve); and the opioid antagonists naloxone hydrochloride and naloxone methiodide (Sigma-Aldrich Quimica SA). BD-1063 (Tocris Cookson Ltd., Bristol, UK), and S1RA (synthesized and kindly supplied by Laboratorios Esteve) were used as selective \( \sigma_1 \) antagonists (Cobos et al., 2008; Romero et al., 2012). PRE-084 [2-(4-morpholinyl)-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 physiologic saline (0.9% NaCl), with the exception of 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 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 \( \mu l \) with a 1710 TLL Hamilton microsyringe (Teknokroma, Barcelona, Spain) with a 30G \( \times \) 1/2-inch gauge needle. Each control group received the same volume of drug vehicle.

For binding assays, the radioligand was the selective \( \sigma_1 \) agonist \( [\text{H}]^{(+)} \)-pentazocine (Cobos et al., 2006), with a specific activity of 34.8 Ci/mmol (PerkinElmer Life and Analytical Sciences, Boston, MA). Dilutions from the stock \( [\text{H}]^{(+)} \)-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 following 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 \( [\text{H}]^{(+)} \)-pentazocine binding (Cobos et al., 2005, 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 previously described methods (Menéndez et al., 2005; Sánchez-Fernández et al., 2013). In brief, 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 minute between each stimulation, with a 50-second cutoff for each determination. The antinociceptive effects of α1 agonists were evaluated 30 minutes after subcutaneous administration except for buprenorphine, which was administered subcutaneously 1 hour 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 subcutaneously 5 minutes 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 intraplantarly and the animals were evaluated 5 minutes after the injection to minimize possible systemic effects induced by the drug. In experiments to determine the local effects of the σ1 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 μ-opioid antinociception in the presence or absence of σ1 inhibition, these drugs or their solvent were administered subcutaneously 5 minutes before the μ-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). In brief, 8 hours before the experiment, food was withheld and water was available ad libitum. BD-1063 or its solvent was injected subcutaneously, and 5 minutes later fentanyl, loperamide, or their solvent was injected subcutaneously. Thirty minutes after the opioid was given, the mice received 0.3 ml of 0.5% (wt/vol) activated charcoal suspension (2–12 μm of powder; Sigma-Aldrich Química SA) in distilled water. The mice were killed by cervical dislocation 30 minutes after the activated charcoal was administered (i.e., 60 minutes after administration of the opioid). The small intestine was then removed from the pyloric sphincter to the ileocecal junction and straightened to measure the distance traveled by the leading edge of the charocal meal.

**Western Blotting.** The basolateral amygdala (BLA), rostroventral medulla (RVM), periaqueductal gray matter (PAG), dorsal spinal cord of the lumbar enlargement (dSC), and lumbar (L4-L5) DRG were dissected subcutaneously. Thirty minutes after the opioid was given, the mice received 0.3 ml of 0.5% (wt/vol) activated charcoal suspension (2–12 μm of powder; Sigma-Aldrich Química SA) in distilled water. The mice were killed by cervical dislocation 30 minutes after the activated charcoal was administered (i.e., 60 minutes after administration of the opioid). The small intestine was then removed from the pyloric sphincter to the ileocecal junction and straightened to measure the distance traveled by the leading edge of the charocal meal.

**Results**

**Effects of Systemic (Subcutaneous) μ-Opioid Receptor Antagonists on Mechanical Nociception in WT and σ1-KO Mice.** Mechanical antinociception induced by the subcutaneous 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. 1, dose 0).

As expected, the subcutaneous 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-, 13-, and 10-fold, respectively, compared with 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 with 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. 1). 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. 1B, open symbols). The largest antinociceptive effects in σ1-KO mice were recorded after treatment with fentanyl, oxycodone, or morphine, and struggle response latency reached values close to the cutoff time (50 seconds) (Fig. 1A, open symbols). It is noteworthy that σ1-KO animals treated with the highest doses of buprenorphine, tramadol, or even the peripheral μ-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 σ1 receptors clearly potentiated the mechanical antinociception induced by the systemic administration of μ-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 μ-Opioid Analgesics in WT Mice. To shed light on the role of peripheral opioid receptors in the mechanical antinociception induced by μ-opioid analogues in WT mice, we tested the sensitivity of this antinociception to the peripherally restricted opioid antagonist naloxone methiodide.

In WT mice treated subcutaneously 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 compared with 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 analogues tested in WT mice, only oxycodone showed partial sensitivity to peripheral opioid antagonism.

Fig. 1. Mechanical antinociception induced by the systemic administration of several μ-opioid receptor agonists in WT and 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 the following: (A) fentanyl, oxycodone, or morphine; and (B) buprenorphine, loperamide, or tramadol, or their solvents (doses 0). Each point and vertical line represents the mean ± S.E.M. of values obtained in 8 to 10 animals. The morphine dose-response curve for comparison with the other opioids is from Sánchez-Fernández et al. (2013). Two-way analysis of variance followed by the Bonferroni test was used to determine 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).
Contribution of Peripheral Opioid Receptors to Anti-nociception Induced by the Systemic (Subcutaneous) Administration of \(\mu\)-Agonists in \(\sigma_1\)-KO Mice. To explore the contribution of peripheral opioid receptors (sensitive to naloxone methiodide) to the enhanced mechanical antinociception seen in \(\sigma_1\)-KO mice, we used doses of \(\mu\)-agonists that induced little or no antinociception in WT mice but induced maximal antinociception in \(\sigma_1\)-KO mice.

In \(\sigma_1\)-KO mice treated with fentanyl (0.08 mg/kg s.c.), latencies approached the cutoff time (44.97 ± 1.32 seconds). 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 \(\sigma_1\)-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 \(\mu\)-agonists in WT mice but induced maximal antinociception in \(\sigma_1\)-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 with 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 Anti-nociception Induced by μ Agonists in WT 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 subcutaneously in WT mice, but BD-1063 was injected intraplantarly to test for locally induced effects.

In WT mice, fentanyl injected subcutaneously (0.08 mg/kg) and BD-1063 solvent (saline) injected intraplantarly 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 subcutaneous 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 σ1 antagonist BD-1063 synergistically enhanced the antinociception induced by all μ agonists tested when they were given systemically. This effect was observed only in the treated paw and was sensitive to both σ1 agonism and peripheral opioid antagonism.

**Effects of Systemic (Subcutaneous) and Local (Intraplantar) Administration of the Selective σ1 Antagonist S1RA on Mechanical Antinociception Induced by Fentanyl and Loperamide in WT Mice: Involvement of σ1 Agonism and Peripheral Opioid Receptors.** To test whether the effects induced by BD-1063 were replicable by a different σ1 antagonist, we evaluated the effects of S1RA on the antinociceptive effects induced by two different μ 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 subcutaneously with either fentanyl (0.08 mg/kg) or loperamide (4 mg/kg) (Fig. 8A). The intraplantar administration of S1RA (200 μg) was also able to increase the response latency in mice treated with these μ-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 σ1 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. 8).

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.

**σ1-Receptor Expression in the Central and Peripheral Nervous System.** To obtain anatomic 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 mass slightly higher than 25 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, $\sigma_1$-receptor density was much higher in DRG samples than in any of the central areas examined (Fig. 9). To verify the specificity of the anti-$\sigma_1$-receptor antibody, we tested its immunoreactivity in samples from $\sigma_1$-KO mice. We found no immunoreactive $\sigma_1$-receptor bands in PAG, dSC, or DRG samples from $\sigma_1$-KO animals (Fig. 9), or in samples of BLA or RVM from these mice (data not shown). The absence of these bands in $\sigma_1$-KO samples argues for the specificity of the $\sigma_1$ antibody used. In addition, we were unable to detect immunoreactive bands in WT samples when tested in the absence of anti-$\sigma_1$ 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 WT Mice, WT Mice Treated with BD-1063, and $\sigma_1$-KO Mice. Gastrointestinal transit distances did not differ significantly among 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 approximately 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. 10). 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 ± 3.24 nM. This value was similar to that found in previous studies (Entrena et al., 2009a; Cobos et al., 2005, 2006, 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); therefore, their affinity for $[^3H](+)$-pentazocine binding sites was considered negligible (Fig. 11).

Discussion

We found that several centrally penetrant $\mu$-opioid analgesics (fentanyl, oxycodone, morphine, buprenorphine, and tramadol) and the peripheral $\mu$-agonist loperamide at systemic doses that induce little or no antinoiception in control animals have
a marked antinociceptive effect when boosted by $\sigma_1$-receptor inhibition ($\sigma_1$-KO mice or $\sigma_1$-pharmacological blockade). We show that this enhanced opioid antinociception is mediated peripherally, because it can be achieved by local $\sigma_1$-pharmacological blockade and is sensitive to peripheral opioid antagonism. However, the increase in peripheral opioid antinociception by $\sigma_1$-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 $\mu$ opioids used in clinical settings overlap only partially (Ocaña et al., 1995; Pasternak, 2004; Smith, 2008; Pasternak and Pan, 2011; Raehal et al., 2011). Hence, it is important to test whether the widely reported enhancement of morphine antinociception by $\sigma_1$ inhibition (Chien and Pasternak 1993; Mei and Pasternak 2007; Díaz et al., 2009; Sánchez-Fernández et al., 2013) also occurs with other clinically relevant $\mu$ 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 $\mu$-opioid analgesics (fentanyl, oxycodone, buprenorphine, and tramadol) (Vidal-Torres et al., 2013). We now extend those results by showing that this $\sigma_1$ antagonist as well as 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 $\sigma_1$-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., 1985; 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 (Menéndez 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 shows that peripheral $\mu$-opioid

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**Fig. 6.** Contribution of $\sigma_1$- and peripheral opioid receptors to the effects of the local administration of BD-1063 on the antinociception induced by systemically administered fentanyl in WT mice. The results represent the struggle response latency during stimulation with 450 g pressure on the hindpaw of mice treated subcutaneously with the following: (A) fentanyl (0.08 mg/kg) plus intraplantar BD-1063 (50–200 $\mu$g) or its solvent; and (B) fentanyl (0.08 mg/kg s.c.) plus BD-1063 (200 $\mu$g i.pl.) in animals pretreated subcutaneously 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 ± S.E.M. of values obtained in 8 to 10 animal. (A and B) Two-way analysis of variance followed by the Bonferroni test was used to determine 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 ($^{††}P < 0.01$). (B) Two-way analysis of variance followed by the Bonferroni test was used to determine 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 ($^{‡}P < 0.01$).
analgesia is enhanced by $\sigma_1$ inhibition. In fact, the local (intraplantar) administration of $\sigma_1$ antagonists was able to enhance antinociception 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 $\mu$ agonists under normal conditions and during $\sigma_1$ 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 $\sigma_1$ blockade) were sensitive to the centrally penetrant opioid antagonist 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 $\sigma_1$ inhibition. A dose as low as 2 mg/kg naloxone methiodide was enough to completely abolish the opioid antinociception induced by all $\mu$ agonists tested, not only in WT mice treated locally with a $\sigma_1$ antagonist (in which its effects are clearly peripherally mediated), but also in $\sigma_1$-KO mice or WT mice treated systemically with the $\sigma_1$ antagonists. This does not argue against the widely reported potentiation of opioid antinociception by central $\sigma_1$ receptor inhibition (King et al., 1997; Pan et al., 1998; Mei and Pasternak, 2002, 2007; Marrazzo et al., 2006) but indicates that, when both opioid agonism and $\sigma_1$ 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.

Fig. 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 WT 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 µg) or its solvent in combination with the subcutaneous 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 subcutaneous 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 ± S.E.M. of values obtained in 8 to 10 animals. One-way analysis of variance followed by the Bonferroni test was used to determine 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).
Among the opioid drugs tested in this study, only morphine and naloxone had previously been shown to lack affinity for σ₁ receptors (Walker et al., 1990). Here we show that this lack of σ₁ affinity is shared by other opioid drugs (fentanyl, oxycodone, buprenorphine, tramadol, loperamide, and naloxone methiodide). In addition, it is known that the σ₁ drugs tested here (BD-1063, S1RA, and PRE-084) do not bind to μ-opioid receptors (Matsumoto et al., 1995; Sánchez-Fernández et al., 2013). Therefore, our results support a functional link between peripheral σ₁ receptors and the μ-opioid system, rather than interactions of σ₁ ligands with μ-opioid receptors or opioid drugs with σ₁ receptors.

It was recently reported that σ₁ antagonism potentiates μ-opioid signaling (measured as the increase in D-Ala²,N-Me-Phe⁴,Gly-ol⁵-enkephalin (DAMGO)-induced [35S]GTPγS binding), providing a mechanistic explanation for the enhanced opioid antinociception by σ₁ inhibition (Kim et al., 2010). Previous studies of thermal nociception show that σ₁ inhibition potentiates μ, κ, and δ opioid antinociception at central levels (King et al., 1997; Mei and Pasternak, 2002). Therefore, the modulation of peripheral μ-opioid antinociception by σ₁ 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 (e.g., constipation), whereas others are mediated at supraspinal sites (e.g., 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 σ₁ inhibition, the central side effects of morphine (e.g., 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 σ₁ 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 μ opioids, was also not modulated by σ₁ 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

![Fig. 8](https://example.com/fig8.png)

**Fig. 8.** Contribution of σ₁- 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 WT mice. Animals were treated (A) subcutaneously (A) or intraplantarly (B) with S1RA (64 mg/kg and 200 μg, respectively) in combination with fentanyl (0.08 mg/kg s.c.) or loperamide (4 mg/kg s.c.), and its association with the subcutaneous 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 ± S.E.M. of values obtained in 8 to 10 animals. One-way analysis of variance followed by the Bonferroni test was used to determine statistically significant differences between the values obtained in the groups treated with the μ-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 μ agonist with S1RA, and its association with PRE-084 or Nx-M (##P < 0.01).
two additional μ 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 σ1-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 σ1 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 σ1 receptor inhibition enhanced the peripheral opioid antinociception induced by clinically relevant μ-agonists, but did not increase opioid-induced constipation. These data support the conclusion that peripheral σ1 receptors are a biologic brake to μ-opioid antinociception, and that either systemic or local σ1-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.
Fig. 11. Inhibition by unlabeled drugs of [3H]pentazocine binding to brain membranes (P2 fraction) in WT mice. [3H]pentazocine (5 nM) was incubated with 0.8–1 ng/ml brain membrane protein at 30°C, pH 8, for 240 minutes 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.

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