Neuropharmacology 70 (2013) 348–358

Contents lists available at SciVerse ScienceDirect

Neuropharmacology

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

Potentiation of morphine-induced mechanical antinociception by σ_1 receptor inhibition: Role of peripheral σ_1 receptors

Cristina Sánchez-Fernández [a,b], Francisco Rafael Nieto [a,b], Rafael González-Cano [a,b], Antonia Artacho-Cordón [a], Lucía Romero [a], Ángeles Montilla-García [a], Daniel Zamanillo [d], José Manuel Baeyens [a,b], José Manuel Entrena [b,c], Enrique José Cobos [a,b,*]

[a] Department of Pharmacology, School of Medicine, University of Granada, Avenida de Madrid 11, 18012 Granada, Spain
[b] Institute of Neuroscience, Biomedical Research Center, University of Granada, Parque Tecnológico de Ciencias de la Salud, 18100 Armilla, Granada, Spain
[c] Animal Behavior Research Unit, Scientific Instrumentation Center, University of Granada, Parque Tecnológico de Ciencias de la Salud, 18100 Armilla, Granada, Spain
[d] Drug Discovery and Preclinical Development, Esteve, Avenida Mare de Déu de Montserrat 221, 08041 Barcelona, Spain

ARTICLE INFO

Article history:
Received 3 October 2012
Received in revised form
25 February 2013
Accepted 4 March 2013

Keywords:
Sigma-1 (σ_1) receptors
Morphine
Pain
Peripheral antinociception
Hyperlocomotion
Gastrointestinal transit

ABSTRACT

We studied the modulation of morphine-induced mechanical antinociception and side effects by σ_1 receptor inhibition. Both wild-type (WT) and σ_1 receptor knockout (σ_1-KO) mice showed similar responses to paw pressure (100–600 g). The systemic (subcutaneous) or local (intraplantar) administration of σ_1 antagonists (BD-1063, BD-1047, NE-100 and S1RA) was devoid of antinociceptive effects in WT mice. However, σ_1-KO mice exhibited an enhanced mechanical antinociception in response to systemic morphine (1–16 mg/kg). Similarly, systemic treatment of WT mice with σ_1 antagonists markedly potentiated morphine-induced antinociception, and its effects were reversed by the selective σ_1 agonist PRE-084. Although the local administration of morphine (50–200 μg) was devoid of antinociceptive effects in WT mice, it induced dose-dependent antinociception in σ_1-KO mice. This effect was limited to the injected paw. Enhancement of peripheral morphine antinociception was replicated in WT mice locally co-administered with σ_1 antagonists and the opioid. None of the σ_1 antagonists tested enhanced morphine-antinociception in σ_1-KO mice, confirming a σ_1-mediated action. Morphine-induced side-effects (hyperlocomotion and inhibition of gastrointestinal transit) were unaltered in σ_1-KO mice. These results cannot be explained by a direct interaction of σ_1 ligands with μ-opioid receptors or adaptive changes of μ-receptors in σ_1-KO mice, given that [^{3}H]DAMGO binding in forebrain, spinal cord, and hind-paw skin membranes was unaltered in mutant mice, and none of the σ_1 drugs tested bound to μ-opioid receptors. These results show that σ_1 receptor inhibition potentiates morphine-induced mechanical analgesia but not its acute side effects, and that this enhanced analgesia can be induced at peripheral level.

1. Introduction

Although sigma (σ) receptors were initially confused with opioid receptors, they are now considered a distinct entity. In contradistinction to the seven transmembrane domains of opioid receptors, typical of the G-protein coupled receptor family, the sigma-1 (σ_1) receptor only has two transmembrane domains. Furthermore, its sequence does not resemble any other known mammalian protein, underscoring its uniqueness (see Cobos et al., 2008; Guitart et al., 2004; Hayashi and Su, 2004; for reviews). The σ_1 receptor has been pharmacologically characterized and is known to exert neuromodulatory actions (Cobos et al., 2008), which may be attributable, at least in part, to its physical association with other receptors and channels (Aydar et al., 2002; Kim et al., 2010). σ_1 receptors are widely distributed in both the central and peripheral nervous system, including important pain control areas such as the

Abbreviations: ANOVA, analysis of variance; BD-1063, (1-[2-(3,4-dichlorophenyl)ethyl]-4-methylpiperazine dihydrochloride); BD-1047, (*N*-[2-(3,4-dichlorophenyl)ethyl]-*N*-methyl-2-(dimethylamino)ethylamine dihydrobromide); B_{max}, maximum number of binding sites; [^{3}H]DAMGO, [^{3}H][D-Ala^2,*N*-Me-Phe^4,-Gly-ol^5]enkephalin; IC_{50}, concentration of unlabeled drug that inhibited 50% of radioligand-specific binding; i.pl., intraplantar; K_D, equilibrium dissociation constant; K_i, inhibition constant; KO, knockout; *L*, concentration of radioligand; NE-100, (*N*,*N*-dipropyl-2-[4-methoxy-3-(2-phenylethoxy)phenyl]-ethylamine monohydrochloride); P_1, crude nuclear fraction; P_2, crude synaptosomal fraction; PRE-084, ([2-(4-morpholinethyl)1-phenylcyclohexanecarboxylate) hydrochloride]); s.c., subcutaneous; S1RA, (4-[2-[[5-methyl-1-(2-naphtalenyl)1H-pyraol-3-yl]oxy]ethyl] morpholine hydrochloride); σ receptor, sigma receptor; σ_1 receptor, sigma-1 receptor; WT, wild-type.

* Corresponding author. Department of Pharmacology and Institute of Neuroscience, School of Medicine, University of Granada, Avenida de Madrid 11, E-18012 Granada, Spain. Tel.: +34 958 243538, +34 610 763392; fax: +34 958 243537.
E-mail address: ejcobos@ugr.es (E.J. Cobos).

0028-3908/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.neuropharm.2013.03.002

spinal cord dorsal horn, periaqueductal gray matter, and dorsal root ganglia (Alonso et al., 2000; Kitaichi et al., 2000; Roh et al., 2008; Ueda et al., 2001).

σ_1 receptor inhibition does not influence acute pain induced by thermal or punctate mechanical stimuli (e.g. Chien and Pasternak, 1994; De la Puente et al., 2009; Entrena et al., 2009b; Marrazzo et al., 2011; Romero et al., 2012). However, it induces a marked decrease in pain responses in experimental models of tonic/chronic pain involving central sensitization, such as formalin-induced pain (Cendán et al., 2005a, 2005b; Kim et al., 2006; Romero et al., 2012), capsaicin-induced mechanical hypersensitivity (Entrena et al., 2009a, 2009b; Romero et al., 2012), and neuropathic pain (De la Puente et al., 2009; Nieto et al., 2012; Roh et al., 2008; Romero et al., 2012). Although σ_1 inhibition appears to have no effect on acute pain, it has been widely reported that σ_1 antagonism or downregulation (by antisense oligodeoxynucleotides) in the central nervous system greatly increases opioid-induced thermal antinociception (King et al., 1997; Marrazzo et al., 2006; Mei and Pasternak, 2002, 2007; Pan et al., 1998). These results indicate that the σ_1 system at central levels modulates both pain hypersensitivity and the effects of opioids in acute thermal nociception.

Most of the antinociceptive effects of morphine and other opioids have been attributed to their action on the central nervous system (e.g. Christie et al., 2000; Khalefa et al., 2012), but they can also have local antinociceptive effects under some circumstances (Sehgal et al., 2011; Stein et al., 2003). However, the possible modulatory role of σ_1 receptors on peripherally-mediated opioid antinociception is not known. Moreover, the ontogenesis and neurochemical mechanisms underlying morphine-induced mechanical and thermal antinociception differ (Kuraishi et al., 1985; Sato et al., 1999; Tseng et al., 1995; Wegert et al., 1997). Therefore, the previously described modulation of morphine thermal antinociception by σ_1 receptors is not necessarily expected to be applicable to mechanical stimulation, which remains unexplored. Consequently, the main aim of this study was to evaluate the role of σ_1 receptors in the modulation of morphine-induced mechanical antinociception, particularly in the periphery. To this end, we studied the effects of σ_1 receptor inactivation (σ_1-knockout) and of the systemic (subcutaneous) and local (intraplantar) administration of the known σ_1 receptor antagonists BD-1063, BD-1047, NE-100, and S1RA (Cobos et al., 2008; Díaz et al., 2012; Hayashi and Su, 2004; Romero et al., 2012) on the mechanical antinociception induced by the systemic and local administration of morphine. We also assessed the specificity of the effects induced by the σ_1 antagonists by attempting to reverse them with the selective σ_1 agonist PRE-084 (Cobos et al., 2008; Su et al., 1991) and by testing their expected inactivity in σ_1-knockout (σ_1-KO) mice.

Morphine is widely used to treat moderate-to-severe pain, despite its clinically relevant side effects, which are mediated at both central (e.g. nausea, respiratory depression) and peripheral (e.g. constipation) levels (Al-Hasani and Bruchas, 2011; Waldhoer et al., 2004). An additional goal of the present study was to determine the possible influence of σ_1 receptor inhibition on some adverse effects of morphine. For this purpose, we compared the responses of wild-type (WT) and σ_1-KO mice to morphine-induced hyperlocomotion, an acute centrally-mediated side effect of morphine in rodents (Hnasko et al., 2005), and their responses to morphine-induced gastrointestinal transit inhibition, a major peripherally-mediated adverse effect of this opioid (Al-Hasani and Bruchas, 2011).

Finally, in order to rule out any direct effects of σ_1 ligands on μ-opioid receptors or any adaptive changes in μ-receptors that could account for effects observed in σ_1-KO animals, we compared [^{3}H]DAMGO binding properties between WT and σ_1-KO mice and studied the affinity of all σ_1 ligands used in the behavioral experiments for μ-opioid receptors in WT mice.

2. Material and methods

2.1. Experimental animals

Experiments were performed in female wild-type (Charles River, Barcelona, Spain) and σ_1-KO CD-1 mice (Laboratorios Esteve, Barcelona, Spain) weighing 25–30 g. The knockout mice were generated on a CD-1 background as previously described (Entrena et al., 2009b). Animals were housed under a 12/12 h day/night cycle in temperature-controlled rooms (22 ± 2 °C) and were fed a standard laboratory diet (Harlan Teklad Research Diet, Madison, WI, USA) and tap water *ad libitum*. After arrival at our vivarium, animals were allowed to acclimatize for at least 4 days before experimental handling. Testing was performed during the light phase (from 9.00 h to 15.00 h) randomly throughout the estrous cycle. Animal care was carried out in accordance with institutional (Research Ethics Committee of the University of Granada, Spain) and international standards (European Communities Council directive 86/609).

2.2. Radioligand, drugs, and drug administration

The σ_1 receptor antagonists used were: BD-1063 (1-[2-(3,4-dichlorophenyl) ethyl]-4-methylpiperazine dihydrochloride), BD-1047 (*N*-[2-(3,4-dichlorophenyl) ethyl]-N-methyl-2-(dimethylamino)ethylamine dihydrobromide), NE-100 (*N*,*N*-dipropyl-2-[4-methoxy-3-(2-phenylethoxy)phenyl]-ethylamine monohydrochloride), and the new selective σ_1 antagonist S1RA (4-[2-[[5-methyl-1-(2-naphtalenyl)1H-pyraol-3-yl]oxy]ethyl] morpholine hydrochloride) (Cobos et al., 2008; Díaz et al., 2012; Hayashi and Su, 2004; Romero et al., 2012). BD-1047 and BD-1063 were purchased from Tocris Cookson Ltd. (Bristol, United Kingdom), NE-100 was synthesized as previously described (Nakazato et al., 1999), and S1RA was synthesized and kindly supplied by Laboratorios Esteve. As σ_1 receptor agonist, we used PRE-084 ([2-(4-morpholinethyl)1-phenylcyclohexanecarboxylate) hydrochloride]) provided by Tocris Cookson Ltd. The μ-opioid receptor agonist morphine hydrochloride was obtained from the General Directorate of Pharmacy and Drugs, Spanish Ministry of Health (Madrid, Spain).

For *in vivo* studies, all drugs were dissolved in sterile physiological saline (0.9% NaCl). To evaluate the effects of systemic treatments, 5 ml/kg of the drugs or their solvent were administered by subcutaneous injection (s.c.) into the interscapular zone. When the systemic effect of the association of two drugs was assessed, each injection was performed in different areas of the interscapular zone. The local effect of treatments was evaluated through the intraplantar (i.pl.) injection of 20 μL of the drug into the right hind-paw using a 1710 TLL Hamilton microsyringe (Teknokroma, Barcelona, Spain) with a $30^{1/2}$-gage needle. The control group received the same volume of sterile saline in the same manner. When morphine and a σ_1 receptor antagonist were associated, they were dissolved in the same solution and injected together to avoid paw lesions from multiple injections. In one experiment, morphine was injected in the right hind-paw and BD-1063 in the left hind-paw of the same animals.

For binding assays, the radioligand used to label μ-opioid receptors was [^{3}H]DAMGO (D-Ala^2,Me-Phe^4,Gly-ol^5]enkephalin), with a specific activity of 50.0 Ci/mmol (PerkinElmer Life Sciences, Boston, MA, USA). Naloxone HCl (Sigma–Aldrich, Madrid, Spain) was used to measure the non-specific binding. Dilutions from the stock [^{3}H]DAMGO solution were prepared with incubation buffer (50 mM HCl–Tris buffer, pH 7.44 at 25 °C). Naloxone, morphine, BD-1063, BD-1047, NE-100, S1RA, and PRE-084 were dissolved in ultrapure water to make up a 1 mM stock solution, from which further dilutions were prepared with incubation buffer.

2.3. Evaluation of the behavioral response to paw pressure

Animals were placed in the experimental room for a 1-h acclimation period before starting the experiments. Then, after gently restraining the animals, blunt mechanical stimulation was applied to the hind-paws with an Analgesimeter (Model 37215, Ugo-Basile, Varese, Italy), as previously described by Menéndez et al. (2005) with slight modifications. Briefly, a cone-shaped paw-presser with a rounded tip was applied carefully (to avoid alarming the animal) to the dorsal surface of the hind-paw. The analgesimeter allowed a constant pressure (of varied intensity depending on the type of experiment, see below) to be applied to the paw until the animal showed a struggle reaction. A chronometer was manually activated at the start of the pressure application and stopped at the onset of the struggle reaction, and the response latency was recorded in seconds. The test was performed twice alternately in each hind-paw, with a 1-min interval between each stimulation. A 50-sec cut-off was established for each measurement to prevent tissue damage. In the systemic drug treatment experiments, animals showed a similar struggle response latency during the stimulation of each hind-paw, and the mean value of the two averaged measurements for each hind-paw was considered in the analyses. In the local (i.pl.) treatment experiments, the average of the two values was independently considered for each paw (injected and non-injected).

To test whether the lack of σ_1 receptor alters the response to paw pressure stimulation, we compared the responses of naïve WT and σ_1-KO mice, applying a wide range of pressures (100–600 g) to the hind-paws and recording the struggle response latency at each pressure, as described above. Each pressure was tested in a

different group of animals to avoid paw sensitization from repeated stimulation. Based on these data, a pressure–response curve (stimulus pressure *vs.* latency time) was constructed for each genotype and was used to determine the optimal pressure for the subsequent experiments. A pressure of 450 g was always used as nociceptive stimulus to test the effect of the drugs because the response latency was markedly reduced at this pressure, offering a wide window to observe an increase in the latency up to the cut-off time (see Fig. 1). Furthermore, this pressure was used in previous research on the analgesic effects of opioids in mice (Menéndez et al., 2005).

In experiments on the effects of the systemic administration of morphine or its solvent, these were s.c injected at 30 min before application of the mechanical stimulus to the hind-paws; in those on the effects of systemic administration of σ_1 drugs, these were s.c. injected at 5 min before injection of the opioid or solvent. When PRE-084 was used to reverse the effects of σ_1 receptor antagonists, it was s.c. injected immediately before the σ_1 antagonist solution. In the study of the local antinociceptive effects of morphine, σ_1 antagonist, or their combination, these were i.pl. injected at 5 min before application of the mechanical stimulus to the hind-paw to minimize their systemic absorption.

2.4. Assessment of morphine-induced hyperlocomotion

Ambulatory locomotion was monitored by using an infrared detector (Med associated Inc., St Albans, VT, USA) equipped with 48 infrared photocell emitters and detectors (12 photo sensors on front and back walls and 12 on each side wall). Mice were habituated to the evaluation chambers (27.5 cm wide × 27.5 cm long × 20 cm high) for 90 min before the s.c. administration of morphine or its solvent. After the injection, we immediately returned the animal to its evaluation cage and recorded the distance it traveled between 30 and 60 min post-injection. To avoid distracting factors that could interfere with the locomotor activity, the evaluation chambers were always in a sound-isolated testing room, and no experimenters were present at any time during the habituation or evaluation period except for the time needed to inject the drug.

2.5. Assessment of morphine-induced inhibition of gastrointestinal transit

Evaluation of gastrointestinal transit was performed following a previously published protocol (Chien and Pasternak, 1994) with modifications. Briefly, mice were fasted for 8 h with water available *ad libitum* before evaluation of the morphine effects. At 30 min after the s.c. administration of morphine or its solvent (saline), 0.3 ml of 0.5% (w/v) activated charcoal (2–12 μm powder, Sigma-Aldrich) suspended in distilled water was intragastrically administered. At 30 min after ingestion of the activated charcoal, mice were killed by cervical dislocation, and the small intestine from the pyloric sphincter to the ileocecal junction was isolated. The distance traveled by the leading edge of the charcoal meal was measured with a ruler for calculation of the gastrointestinal transit.

2.6. Membrane preparations for binding assays

Experiments were performed in crude synaptosomal membranes (P_2 fraction) obtained as previously described (Cobos et al., 2005, 2006) with slight modifications.

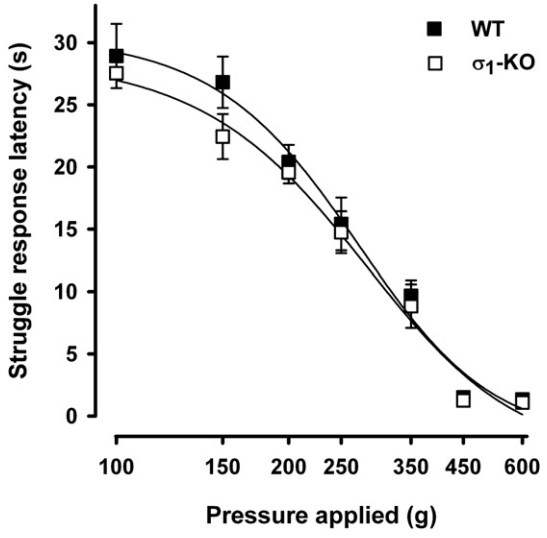

Fig. 1. Latency to struggle response evoked by blunt mechanical stimulation (100–600 g pressure) of the hind-paws of wild-type (WT) and σ_1 knockout (σ_1-KO) mice. Each point and vertical line represents the mean ± SEM of values obtained in 8–10 animals. Each group was stimulated with only one pressure. There were no statistically significant differences between the values obtained in WT and σ_1-KO mice at any pressure applied (two-way ANOVA followed by Bonferroni test).

Mice were killed by cervical dislocation, and the forebrain, spinal cord, and hind-paw plantar skin were rapidly removed. Forebrains and spinal cords were homogenized in 15 volumes (w/v) of 0.32 M sucrose-10 mM Tris–HCl, pH 7.4, with a Polytron homogenizer (model PT10-35, Kinematica AG, Basel, Switzerland). The same procedure was followed with the paw plantar skin except that it was frozen with liquid nitrogen before homogenization, as previously described (Baamonde et al., 2007). All homogenates were centrifuged (Avanti 30, Beckman Coulter España S.A., Madrid, Spain) at 1000 × *g* for 13 min, discarding the resulting pellets (P_1 pellets) and then centrifuging the supernatants at 21,000 × *g* for 15 min to obtain the P_2 pellets. Each P_2 pellet (obtained from 2 forebrains, 5 spinal cords or the plantar skin of 20 hind-paws) was washed by resuspension in 15 ml of 10 mM Tris–HCl, pH 7.4, and centrifuged again at 21,000 × *g* for 15 min. The entire process was performed at 4 °C. Finally, each pellet was resuspended in 1 ml of 10 mM Tris–HCl, pH 7.4 and frozen in aliquots at −80 °C. The binding characteristics of the tissue were stable for at least 1 month when stored at −80 °C.

2.7. [^{3}H]DAMGO binding assays

Binding assays were performed as previously described (Narita et al., 2001) with slight modifications. Membrane aliquots, obtained as reported in the previous section, were slowly thawed and resuspended in fresh incubation buffer to obtain a final protein concentration of 400–500 μg/ml. Protein concentration was measured by the method of Lowry et al. (1951) with some modifications, using bovine serum albumin as the standard. Membrane solutions were incubated with 20 μl of [^{3}H]DAMGO solution and 20 μl of the cold ligand solution or its solvent at 25 °C for 120 min, at a final volume of 500 μl. The final concentration of [^{3}H]DAMGO was 5 nM in competition assays and 0.25–45 nM in saturation assays. Because the amount of plantar skin sample was limited, a single saturating concentration (20 nM) of [^{3}H]DAMGO was used to estimate the maximum number of binding sites (B_{max}) labeled by the radioligand, as reported in other binding assays (Barturen and Garcia-Sevilla, 1992; Cobos et al., 2007). The same procedure was also used with forebrain and spinal cord samples to permit comparison of the results. In all experiments, non-specific binding was defined as the binding retained on the filter and membranes in the presence of 10 μM naloxone.

To stop the [^{3}H]DAMGO binding, 5 ml ice-cold filtration buffer (10 mM Tris pH 7.4) was added to the membrane solution. The bound and free radioligand were separated by rapid filtration under a vacuum using a Brandel cell harvester (Model M-12 T Brandel Instruments; SEMAT Technical Ltd., St Albans, Hertfordshire, UK) on Whatman GF/B glass fiber filters (SEMAT Technical Ltd.), which were pre-soaked with 0.5% polyethylenimine (Sigma–Aldrich) in 10 mM Tris pH 7.4, for at least 1 h prior to their utilization to reduce non-specific binding. The filters were washed twice with 5 ml volumes of the ice-cold filtration buffer and transferred to scintillation counting vials; then, a 4 ml liquid scintillation cocktail (Opti-phase Hisafe II; PerkinElmer Wallac, Loughborough, UK) was added and the mixture was equilibrated for at least 20 h. The radioactivity retained in the filter was measured by using a liquid scintillation spectrometer (Beckman Coulter España S.A.) with an efficiency of 52%. Each assay was conducted in triplicate.

2.8. Data analysis

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 and the pressure–response curves were estimated by using the equation for a sigmoid plot. In the binding experiments, the equilibrium dissociation constant (K_D) and the maximum number of binding sites (B_{max}) from saturation assays were calculated by non-linear regression analysis of the results fitted to a rectangular hyperbola equation. The IC_{50} value (concentration of morphine that inhibited 50% of [^{3}H]DAMGO-specific binding) was calculated from competition assays using non-linear regression analysis, assuming one-site competition. The K_i value of morphine in forebrain membranes (indicating the affinity of the inhibitor for the receptor) was calculated with the Cheng–Prussoff equation: $K_i = IC_{50}/(1 + [L]/K_D)$, where $[L]$ is the concentration of radioligand used, and K_D is the value obtained by nonlinear regression analysis from the saturation experiment. Parameters obtained from non-linear regressions of binding assays were compared with Snedecor's *F* test to check the goodness-of-fit of different models that shared one or more parameters. When several means were compared, the statistical analysis was carried out using a one-way or two-way analysis of variance (ANOVA), depending on the experiment, followed by a Bonferroni *post-hoc* test. $P < 0.05$ was considered significant in all tests.

3. Results

3.1. Comparison of mechanical sensitivity in wild-type and σ_1 knockout mice

The cone-shaped paw-presser was applied at different intensities (100–600 g) on the dorsal hind-paw of the animals, and response latency values were compared between WT and σ_1-KO

mice. The struggle response latency decreased as the mechanical pressure on the dorsal hind-paw increased in both WT and σ_1-KO mice, which did not significantly differ in response latency at any pressure applied (Fig. 1); i.e., the WT and σ_1-KO mice showed equivalent responses to noxious paw pressure.

3.2. Effects of systemic (subcutaneous) morphine on mechanical nociception in wild-type and σ_1 knockout mice

The effects of s.c. morphine on mechanical nociception were evaluated as the increased response latency with respect to solvent-treated mice when noxious pressure (450 g) was applied to the hind-paw. The mechanical stimulus produced a fast and similar response in both WT (1.50 ± 0.14 s) and σ_1-KO (1.25 ± 0.25 s) mice treated with saline (Fig. 2, dose 0). Morphine administration (0.5–16 mg/kg, s.c.) induced a dose-dependent increase in response latency in WT mice, i.e., exerted mechanical antinociception, and this effect was significant at doses of 4 mg/kg ($P < 0.05$) or higher (Fig. 2, closed circles). We were not able to accurately test the effects on nociception of doses higher than 16 mg/kg of morphine, because the mice exhibited behavioral abnormalities (nervousness and stiffness) that hampered the behavioral evaluation. Morphine also induced a dose-dependent antinociceptive effect in σ_1-KO mice; however, in contrast to WT mice, they showed a strong and highly significant ($P < 0.001$) antinociception from a dose of 2 mg/kg, and latency values close to the cut-off time were obtained at doses of 4 mg/kg and higher (Fig. 2, open circles). Hence, the genetic inactivation of σ_1 receptors induced a clear and marked potentiation of systemic morphine-induced mechanical antinociception.

3.3. Effects of systemic (subcutaneous) administration of selective σ_1 drugs on mechanical antinociception induced by systemic morphine

As reported above, 4 mg/kg (s.c.) of morphine elicited a slight but statistically significant increase in struggle response latency in WT mice submitted to noxious pressure (450 g) on the paw (see Fig. 2). When this morphine dose was associated with systemic administration of the selective σ_1 receptor antagonist BD-1063 in WT mice, a dose-dependent potentiation of the morphine-induced increase in response latency was observed (Fig. 3). WT mice treated with systemic morphine (4 mg/kg, s.c.) and with the highest tested dose of BD-1063 (32 mg/kg, s.c.) showed similar response latency values (42.87 ± 2.71 s, Fig. 3) to those observed in σ_1-KO mice treated with the same dose of morphine (44.6 ± 2.15 s, Fig. 2).

We evaluated the specificity of the effects of systemic BD-1063 on morphine-induced mechanical antinociception by testing whether the selective σ_1 agonist PRE-084 was able to reverse the effect of the σ_1 antagonist. In contrast to the effects induced by BD-1063, treatment with the selective σ_1 agonist PRE-084 (16 mg/kg, s.c.) did not significantly modify the antinociceptive effect of morphine, either at 4 mg/kg (Fig. 4) or at 16 mg/kg (data not shown). However, when PRE-084 (4–16 mg/kg, s.c.) was co-administered with BD-1063 (32 mg/kg, s.c.), it completely reversed the potentiation of morphine-induced mechanical antinociception by the σ_1 antagonist in a dose-dependent manner (Fig. 4). We confirmed the effects of the pharmacological antagonism of σ_1 receptors on morphine-induced antinociception by using a panel of selective σ_1 antagonists: BD-1047 (32 mg/kg, s.c.), NE-100 (4 mg/kg, s.c.) and SR1A (32 mg/kg, s.c.). All of these drugs mimicked the effects of BD-1063 on morphine-induced mechanical antinociception, increasing the response latency of morphine-treated WT mice, and their effects were dose-dependently reversed by PRE-084 (Fig. 4).

We further evaluated the selectivity of the effects induced by the σ_1 antagonists by testing their action on morphine-induced mechanical antinociception in σ_1-KO mice. A slightly lower morphine dose (3 mg/kg, s.c.) was used in this experiment in order to facilitate the detection of possible non-specific increases in struggle response latency induced by the σ_1 antagonists. Response

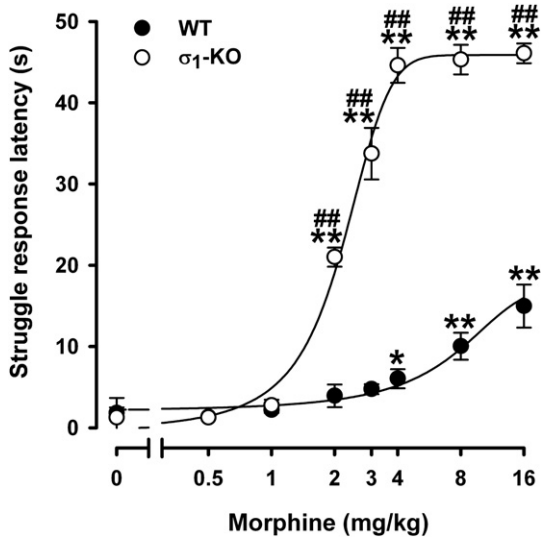

Fig. 2. Effects of the systemic (subcutaneous) administration of morphine on mechanical nociceptive pain in wild-type (WT) and σ_1 knockout (σ_1-KO) mice. The results represent the struggle response latency during stimulation with 450 g pressure of the hind-paws of mice treated with several doses of morphine (0.5–16 mg/kg) or its solvent (saline, dose 0). Each point and vertical line represents the mean ± SEM of values obtained in 8–10 animals. Statistically significant differences between the values obtained in saline- and morphine-treated groups: $^{*}P < 0.05$; $^{**}P < 0.01$, and between the values obtained in WT and σ_1-KO mice at the same dose of morphine: $^{\#\#}P < 0.01$ (two-way ANOVA followed by Bonferroni test).

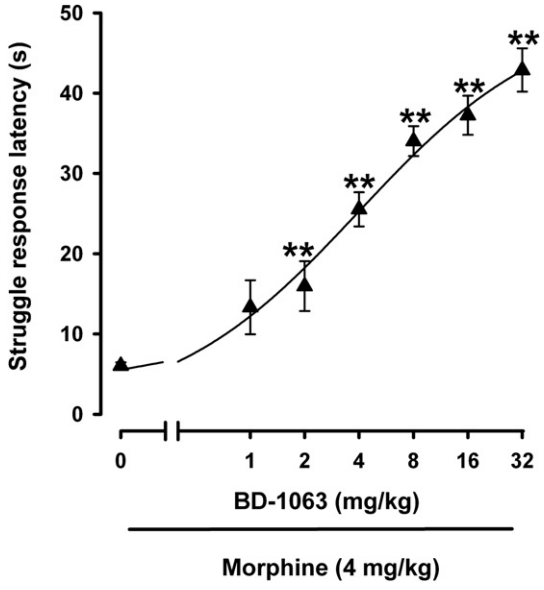

Fig. 3. Effects of systemic (subcutaneous) administration of BD-1063 on mechanical antinociception induced by systemic morphine in wild-type mice. The results represent the struggle response latency during stimulation with 450 g pressure of the hind-paws of mice treated with several doses of BD-1063 (1–32 mg/kg) or its solvent (dose 0) associated with morphine (4 mg/kg). Each point 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 BD-1063 and its solvent (dose 0): $^{**}P < 0.01$ (one way ANOVA followed by Bonferroni test).

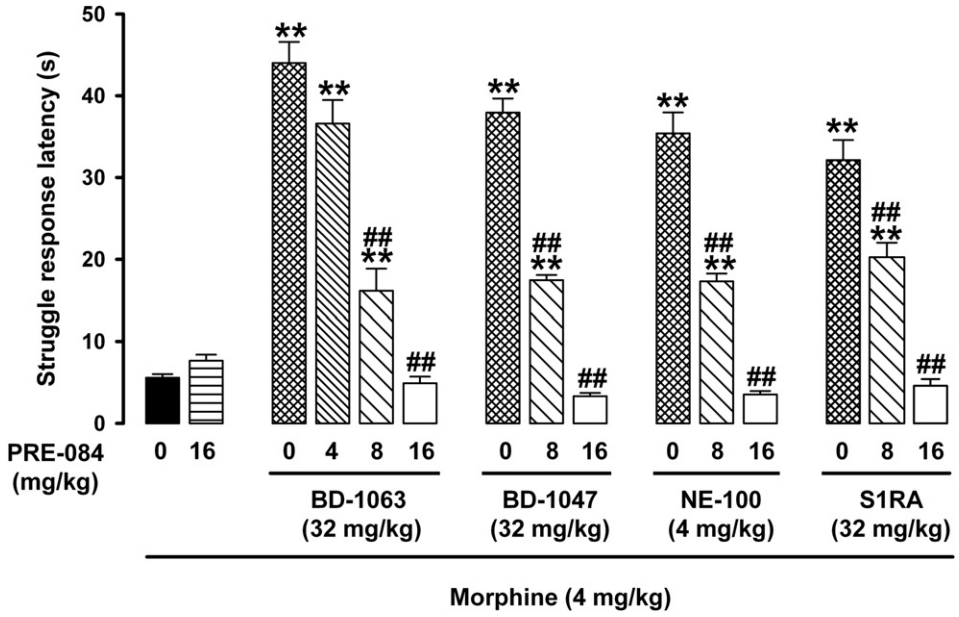

Fig. 4. Effect of the systemic (subcutaneous) administration of the σ_1 agonist PRE-084 on the mechanical antinociception induced by the systemic administration of morphine associated with several σ_1 antagonists in wild-type mice. The results represent the struggle response latency during stimulation with 450 g pressure of the hind-paws of mice treated with PRE-084 (4–16 mg/kg) or its solvent and the σ_1 antagonists BD-1063 (32 mg/kg), BD-1047 (32 mg/kg), NE-100 (4 mg/kg), or S1RA (32 mg/kg) or their solvent, associated with morphine (4 mg/kg). 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 morphine alone and associated with the σ_1 drugs: $^{**}P < 0.01$, and between the values obtained in mice given each σ_1 antagonist associated with PRE-084, with respect to each σ_1 antagonist associated with PRE-084 solvent (dose 0): $^{\#\#}P < 0.01$ (one-way ANOVA followed by Bonferroni test).

latencies were significantly higher in WT mice co-administered s.c. with 3 mg/kg morphine and σ_1 antagonist than in those treated with morphine alone (Fig. 5, middle panel). However, none of the σ_1 antagonists further increased the morphine-induced mechanical antinociception in σ_1-KO mice (Fig. 5, right panel), suggesting that off-target effects do not significantly contribute to the potentiation of morphine-induced antinociception by these drugs in our experimental conditions. Response latency values did not significantly differ between those of WT mice treated by any of the σ_1 antagonists tested in combination with morphine and those of σ_1-KO mice treated with morphine alone (Fig. 5, middle and right panel), indicating that similar levels of enhanced morphine

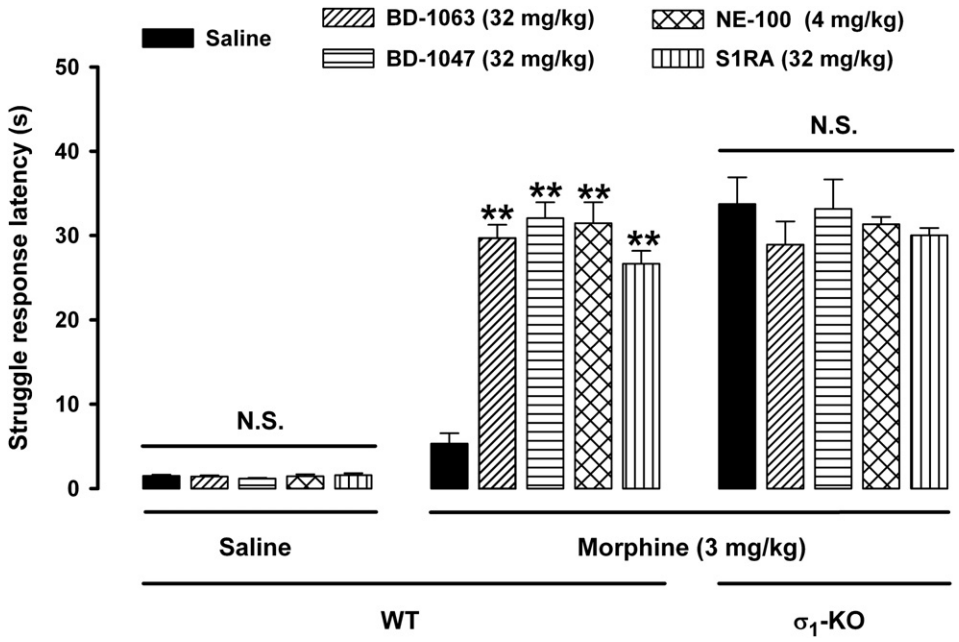

Fig. 5. Effects of the systemic (subcutaneous) administration of several σ_1 antagonists on the mechanical antinociceptive effect induced by systemic morphine in wild-type (WT) and σ_1 knockout (σ_1-KO) mice. The results represent the struggle response latency during stimulation with 450 g pressure of the hind-paws of mice treated with the σ_1 antagonists BD-1063 (32 mg/kg), BD-1047 (32 mg/kg), NE-100 (4 mg/kg), or S1RA (32 mg/kg) or their solvent (saline), associated to morphine (3 mg/kg) or its solvent (saline). 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 each σ_1 antagonist and their solvent in morphine-treated WT mice: $^{**}P < 0.01$. N.S.: no statistically significant differences between the values (two-way ANOVA followed by Bonferroni test).

analgesia were induced by the systemic pharmacological blockade of σ_1 receptors and by their genetic inactivation.

None of the σ_1 antagonists administered alone was able to modify the struggle response latency in WT mice, i.e., they did not affect the responses to noxious pressure in the absence of morphine (Fig. 5, left panel). Therefore, the antinociceptive effects observed in WT mice treated with the association of morphine and σ_1 antagonist were synergistic rather than merely additive.

3.4. Local antinociceptive effects induced by intraplantar administration of morphine in wild-type and σ_1 knockout mice

We tested whether σ_1 receptors are able to modulate the antinociceptive effects of morphine in the periphery by the i.pl. administration of this opioid in WT and σ_1-KO mice. The i.pl. administration of morphine (50–200 μg) did not significantly modify the struggle response latency in WT mice in either the injected or non-injected paw (Fig. 6), indicating that these doses were unable to locally induce a significant mechanical antinociceptive effect in this genotype. In contrast, σ_1-KO mice i.pl injected with the same doses of morphine showed a marked dose-dependent increase in response latency in the injected paw, reaching values close to the cut-off time at the highest dose (200 μg) (Fig. 6). Latency values in the contralateral non-injected paw of σ_1-KO mice remained unchanged and undistinguishable from control values in mice treated with the solvent of morphine (Fig. 6), indicating that the antinociceptive effect of morphine in σ_1-KO mice was locally produced. Hence, the local effect of morphine against noxious pressure was potentiated in mice globally lacking functional σ_1 receptors.

3.5. Potentiation of the local antinociceptive effect of morphine by pharmacological blockade of σ_1 receptors

We investigated whether local pharmacological antagonism of σ_1 receptors could also potentiate morphine-induced peripheral mechanical antinociception in WT mice by testing the effects of the i.pl. co-administration of the selective σ_1 receptor antagonist BD-1063 (12.5–200 μg) with 200 μg morphine in WT mice.

The intraplantar injection of BD-1063 alone did not produce antinociception (Fig. 7). The co-administration of BD-1063 with morphine in WT mice produced a dose-dependent increase in struggle response latency in the injected paw but not in the non-injected paw (Fig. 7). In addition, mice i.pl. administered with BD-1063 in the contralateral hind-paw to the morphine injection (200 μg each) showed no increase in struggle response latency (data not shown). These results rule out any possible systemic effect of the i.pl. administration of BD-1063 and demonstrate that BD-1063 is able to locally potentiate morphine-induced mechanical antinociception in the periphery. In order to establish the specificity of these effects, we tested the effect of BD-1063 in σ_1-KO mice. For this experiment, we used a lower dose of morphine (100 μg) to facilitate detection of any non-specific increases in morphine-antinociception attributable to the i.pl. injection of the σ_1 antagonist. BD-1063 (100 μg) enhanced the effects of morphine in WT mice but did not significantly alter the response of morphine-treated σ_1-KO mice (Fig. 8, middle and right panel).

Likewise, the i.pl. administration of BD-1047 (50 μg), NE-100 (50 μg), or S1RA (100 μg) had no effects on the behavioral response in the absence of morphine (Fig. 8, left panel). However, these drugs increased the response latency in the injected paw of morphine-treated (100 μg, i.pl.) WT mice (Fig. 8, middle panel), although not in their non-injected paw (data not shown). In

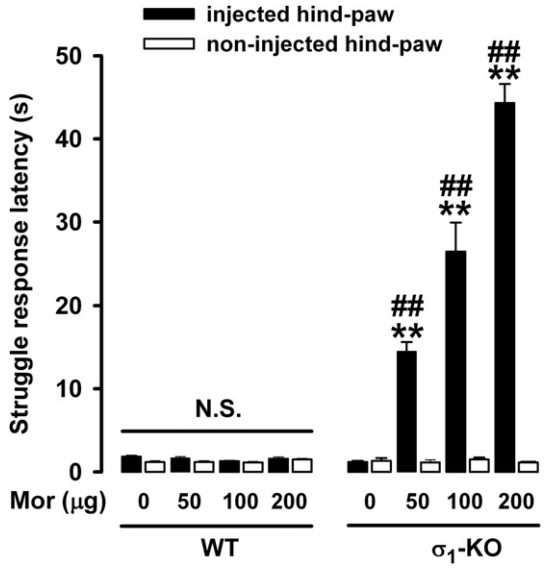

Fig. 6. Effect of the local (intraplantar) injection of morphine (Mor) on mechanical nociceptive pain in wild-type (WT) and σ_1 knockout (σ_1-KO) mice. The results represent the struggle response latency during stimulation with 450 g pressure of the mice hind-paw injected with morphine (50–200 μg) or its solvent (dose 0), in comparison to that obtained during stimulation of the non-injected hind-paw. Each bar and vertical line represents the mean ± SEM of values obtained in 8–10 animals. Statistically significant differences between the values obtained stimulating the morphine-injected hind-paw versus those obtained stimulating the solvent-treated hind-paw (dose 0) in σ_1-KO mice: $^{**}P < 0.01$, and between the values obtained in the injected and non-injected hind-paws in σ_1-KO mice: $^{\#\#}P < 0.01$ (two-way ANOVA followed by Bonferroni test). No statistically significant differences (N.S.) were found in the values from WT groups between the injected and non-injected hind-paws or between any dose of morphine with respect to its solvent (two-way ANOVA followed by Bonferroni test).

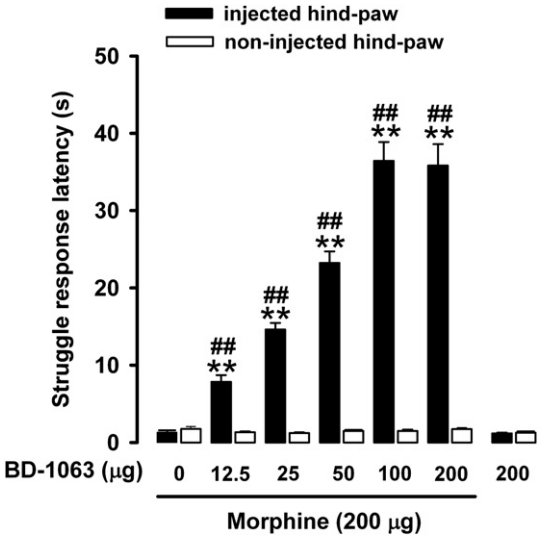

Fig. 7. Effect of the local (intraplantar) administration of BD-1063 associated with morphine on mechanical nociceptive pain in wild-type mice. The results represent the struggle response latency during stimulation with 450 g pressure of the mice hind-paw injected with morphine (200 μg) or its solvent associated with BD-1063 (12.5–200 μg) or its solvent in comparison to values obtained during stimulation of the non-injected hind-paw. Statistically significant differences between the values obtained stimulating hind-paws injected with morphine and BD-1063 with respect to those treated with morphine alone (BD-1063 dose 0): $^{**}P < 0.01$, and between the values obtained from the injected and non-injected hind-paws: $^{\#\#}P < 0.01$ (two-way ANOVA followed by Bonferroni test). No statistically significant differences were found among the values in the non-injected hind-paws with any treatment or between the values obtained in the injected and non-injected paw in the group treated with BD-1063 alone (two-way ANOVA).

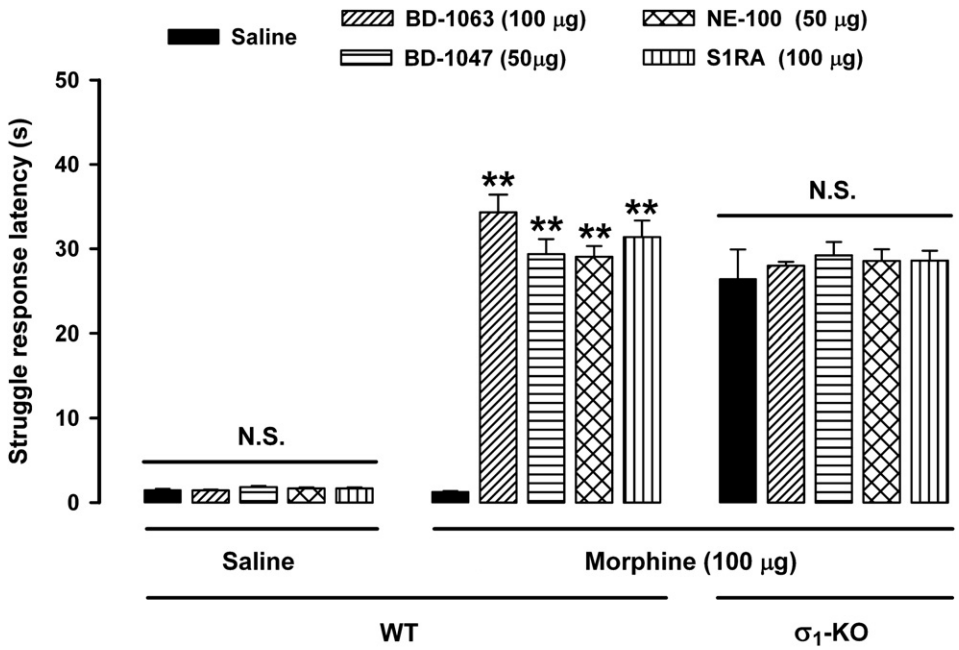

Fig. 8. Effects of the local (intraplantar) administration of several σ_1 antagonists on the mechanical antinociceptive effect induced by intraplantar morphine in wild-type (WT) and σ_1 knockout (σ_1-KO) mice. The results represent the struggle response latency during stimulation with 450 g pressure of the hind-paws of mice treated with the σ_1 antagonists BD-1063 (100 μg), BD-1047 (50 μg), NE-100 (50 μg), or S1RA (100 μg) or their solvent (saline) associated with morphine (100 μg) or its solvent (saline). Each bar and vertical line represents the mean ± SEM of values obtained in 8–10 animals. Statistically significant differences in values between morphine-injected WT mice treated with each σ_1 antagonist and those treated with its solvent: $^{**}P < 0.01$. N.S.: no statistically significant differences between the values (two-way ANOVA followed by Bonferroni test). None of the treatments produced any effect in the non-injected paw (data not represented in order to simplify the figure).

contrast, the co-administration of these σ_1 antagonists with morphine did not potentiate the effect of the opioid in σ_1-KO mice (Fig. 8, right panel). These results support that the enhancement of locally-induced morphine antinociception produced by the σ_1 antagonists is mediated by their interaction with σ_1 receptors, and that no additional effects of these drugs are participating in the effects observed. In addition, the response latency of WT mice locally co-administered with σ_1 antagonist and morphine did not significantly differ from that obtained in σ_1-KO mice treated with morphine alone (Fig. 8, middle and right panel). This finding indicates that a similar potentiation of local morphine antinociceptive effects was produced by local σ_1 pharmacological blockade and by genetic inactivation.

According to our results, the local pharmacological blockade of σ_1 receptors potentiates the mechanical antinociception induced locally by morphine but does not alter nociceptive responses in the absence of the opioid.

3.6. Morphine-induced side effects (hyperlocomotion and inhibition of gastrointestinal transit) in wild-type and σ_1 knockout mice

To determine whether the increased morphine-induced antinociception observed in σ_1-KO mice was accompanied by an increase in the non-analgesic effects of morphine, we tested morphine-induced hyperlocomotion and gastrointestinal transit inhibition. WT and σ_1-KO mice showed a similar ambulatory locomotion when injected with saline (Fig. 9A). Systemic administration of morphine (4–16 mg/kg, s.c.) induced a marked and dose-dependent increase in the distance traveled by both WT mice and morphine-injected σ_1-KO mice, with no statistically significant differences between them (Fig. 9A).

Gastrointestinal transit values did not significantly differ between saline-treated WT and σ_1-KO mice, with the charcoal meal traveling about 30 cm of the small intestine in both genotypes (Fig. 9B). Morphine (1–8 mg/kg, s.c.)-treated mice of both genotypes showed a dose-dependent decrease in gastrointestinal transit of a similar magnitude (Fig. 9B).

Therefore, two different non-analgesic effects of morphine were unaltered by the genetic inactivation of σ_1 receptors.

3.7. [^{3}H]DAMGO saturation binding assays in spinal cord, forebrain, and hind-paw skin membranes from wild-type and σ_1 knockout mice

Saturation assays showed that the selective μ-opioid receptor radioligand [^{3}H]DAMGO bound in a saturable manner to forebrain and spinal cord membranes (P_2 fraction) from both WT and σ_1-KO mice (Fig. 10A). Replicates were fitted by nonlinear regression analysis to hyperbolic equations. In the case of the forebrain membranes, no significant differences were found between WT and σ_1-KO mice in [^{3}H]DAMGO equilibrium dissociation constant (K_D) values (1.530 ± 0.212 nM *vs.* 1.172 ± 0.166 nM, respectively; $P > 0.05$) or in the maximal number of receptors (B_{max}) (0.137 ± 0.004 *vs.* 0.133 + 0.004 pmol/mg of protein, respectively; $P > 0.05$). Likewise, in the spinal cord membranes, no significant differences between WT and σ_1-KO mice were found in K_D (2.028 ± 0.269 *vs.* 1.861 ± 0.226 nM, respectively; $P > 0.05$) or B_{max} (0.179 ± 0.005 *vs.* 0.180 ± 0.004 pmol/mg of protein, respectively; $P > 0.05$) values.

The [^{3}H]DAMGO B_{max} in paw skin membranes was estimated by using a single saturating concentration (20 nM) of this radioligand. For a better comparison, we used the same concentration of the radioligand to determine the estimated B_{max} in both spinal cord and forebrain membranes. Using this approach, we obtained very similar [^{3}H]DAMGO-specific binding values to those obtained with the full saturation assay in both WT (0.180 ± 0.012 and 0.1266 ± 0.003 pmol/mg of protein for spinal cord and forebrain membranes, respectively) and σ_1-KO (0.183 ± 0.013 and 0.122 ± 0.07 pmol/mg of protein for spinal cord and forebrain membranes, respectively) mice (Fig. 10B). [^{3}H]DAMGO binding sites were much less abundant in the paw skin membranes than in the tissues from the central nervous

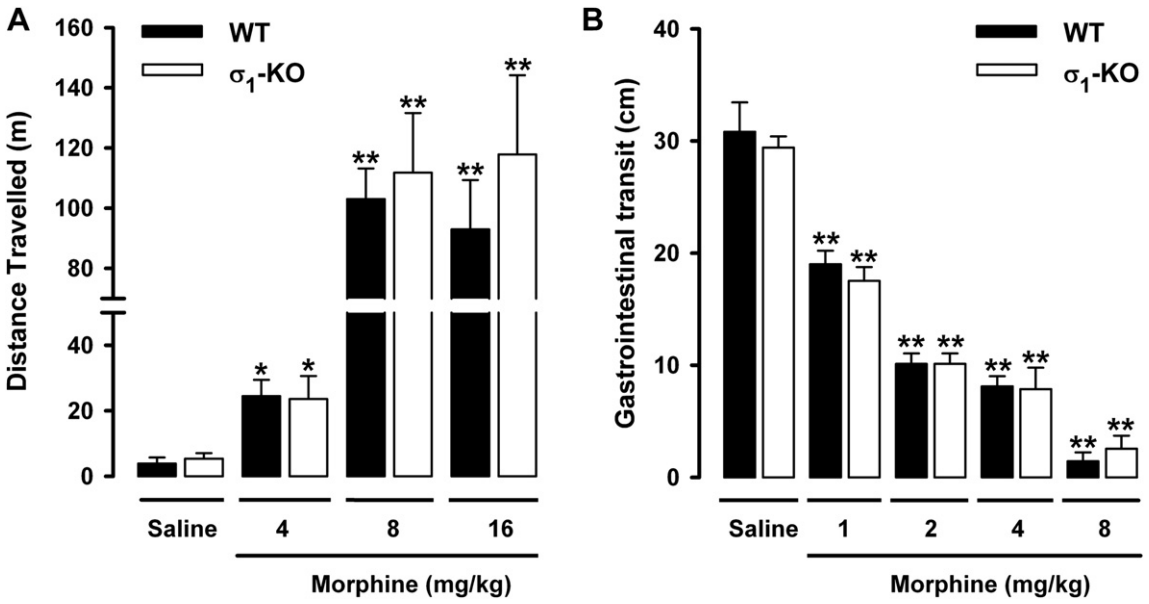

Fig. 9. Effects of the systemic (subcutaneous) administration of morphine or saline on (A) locomotor activity and (B) gastrointestinal transit of wild-type (WT) and σ_1 knockout (σ_1-KO) mice. (A) Mice were injected with morphine or saline, and the distance that they traveled between 30 and 60 min post-injection was recorded. Each bar and vertical line represents the mean ± SEM of values obtained in 8–13 mice. (B) At 30 min after the injection of morphine or saline, mice were intragastrically administered with 0.5% charcoal suspension. Transit of the charcoal was measured at 30 min after its ingestion. Each bar and vertical line represents the mean ± SEM of values obtained in 6 mice. Statistically significant differences between the values obtained in saline- and morphine-treated groups: $^{**}P < 0.01$. No statistically significant differences were found between genotypes under the same treatment (two-way ANOVA followed by Bonferroni test).

system, with no significant differences between the genotypes (0.030 ± 0.002 and 0.030 ± 0.001 pmol/mg of protein for WT and σ_1-KO mice, respectively) (Fig. 10B).

3.8. Affinity of selective σ_1 ligands and morphine for [^{3}H]DAMGO binding sites in forebrain membranes from wild-type mice

We used competition binding assays to test the binding of the studied drugs to [^{3}H]DAMGO-labeled μ-receptors in forebrain membranes from WT mice. As expected, the [^{3}H]-DAMGO-specific binding was concentration-dependently inhibited by morphine, which showed an affinity (K_i) value of 3.746 ± 0.319 nM. However, the specific binding of [^{3}H]DAMGO was not inhibited by any of the selective σ_1-ligands tested (BD-1063, BD-1047, NE-100, S1RA, or PRE-084), therefore demonstrating negligible affinity of these drugs for [^{3}H]DAMGO binding sites (Fig. 11).

4. Discussion

In this study, pharmacological antagonism or genetic inactivation of σ_1 receptors induced a strong functional synergism with the mechanical antinociceptive effect of morphine, without altering

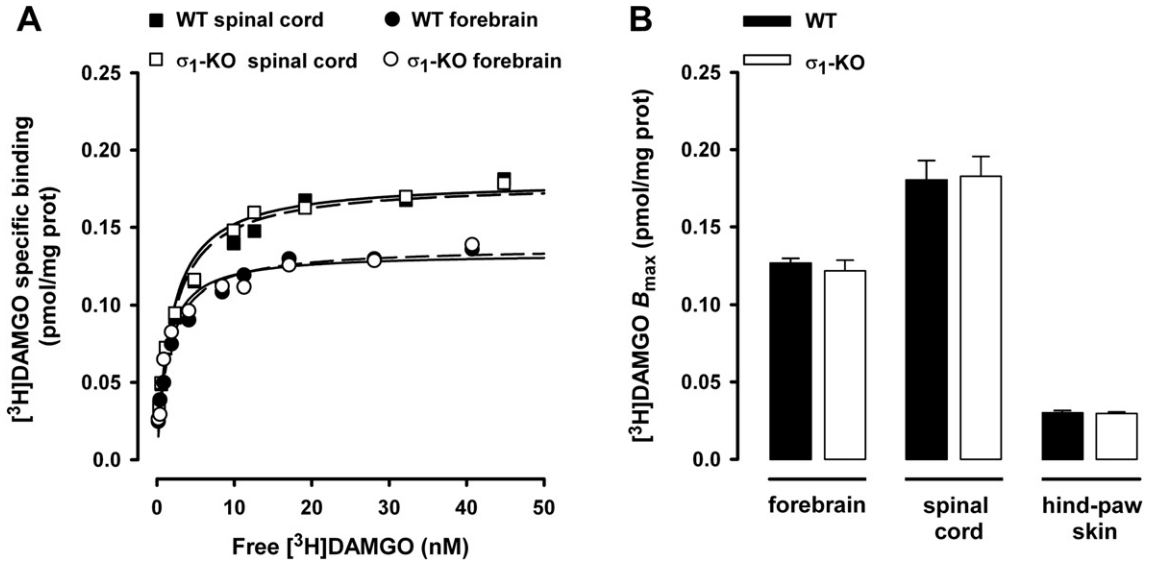

Fig. 10. [^{3}H]DAMGO binding assays in wild-type (WT) and σ_1 knockout (σ_1-KO) mice. (A) [^{3}H]DAMGO saturation assays in forebrain and spinal cord membranes (P_2 fraction) from WT (closed symbols) or σ_1-KO (open symbols) mice. Experiments were performed by incubating the membranes for 120 min at 25 °C with several concentrations of [^{3}H]DAMGO (0.25–45 nM). (B) [^{3}H]DAMGO B_{max} values in forebrain, spinal cord, and hind-paw skin membranes. B_{max} values were estimated by incubating the membranes with a single saturating concentration of [^{3}H]DAMGO (20 nM) under the experimental conditions described above. All experiments were carried out in the presence of 10 μM naloxone (to define non-specific binding) or its solvent. The data shown represent three experiments carried out in triplicate.

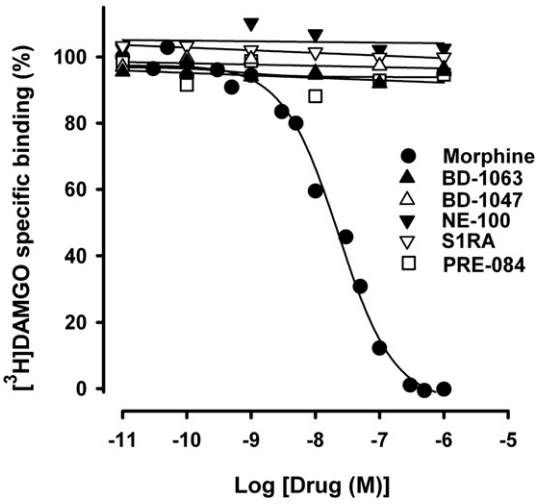

Fig. 11. Inhibition by unlabeled drugs of [^{3}H]DAMGO binding to forebrain membranes (P_2 fraction) in wild-type mice. Membranes were incubated for 120 min at 25 °C with 5 nM [^{3}H]DAMGO and increasing concentrations of morphine or the σ_1 ligands BD-1063, BD-1047, NE-100, S1RA, or PRE-084. Naloxone (10 μM) was used to define the non-specific binding. Data are the average of three experiments carried out in triplicate.

its non-analgesic effects (hyperlocomotion and inhibition of gastrointestinal transit). Furthermore, this synergistic interaction occurred at the peripheral level. None of these findings have been previously reported.

We found that the pain-like responses evoked by a blunt mechanical stimulus were virtually identical between σ_1-KO and WT mice over a wide range of pressure intensities. Neither were they altered by the local or systemic administration of σ_1 antagonists. These findings expand the results found in previous studies reporting that σ_1-KO mice or WT mice treated with σ_1 antagonists showed unaltered responses against different sensory modalities of acute nociceptive pain, including mechanical (punctate) and thermal (cold and heat) stimulation (e.g. Chien and Pasternak, 1994; De la Puente et al., 2009; Entrena et al., 2009a,b; Marrazzo et al., 2011; Nieto et al., 2012; Romero et al., 2012). These results suggest that the basic mechanisms for perceiving these stimuli and the motor response for producing the pain-like responses are intact in σ_1-KO mice and are not altered by the pharmacological blockade of σ_1 receptors in WT mice. However, despite the apparent absence of σ_1 receptor involvement in acute nociception, we found that the mechanical antinociceptive effect of morphine was markedly enhanced in σ_1-KO mice and that this effect was mimicked by the systemic administration of several prototypic σ_1 antagonists in WT mice. Our results extend previous reports on the potentiation of opioid-induced thermal antinociception by σ_1 inhibition (Chien and Pasternak, 1993, 1994; Marrazzo et al., 2011, 2006; Mei and Pasternak, 2002, 2007; Pan et al., 1998; Ronsisvalle et al., 2001). Taken together, these findings suggest that a tonically active anti-opioid σ_1 system modulates both the mechanical and thermal antinociception induced by morphine.

We found that the i.pl. administration of morphine had no analgesic effect against mechanical stimuli in naïve mice over a wide range of doses. This finding is in contrast to previous reports showing that local morphine is effective against thermal stimuli (e.g. Kolesnikov et al., 1996, 2000), highlighting the differences between the effects of this opioid against thermal and mechanical stimuli. The lack of effect of local morphine demonstrated here is in agreement with clinical reports showing that the local application of opioid agonists (including morphine) to uninjured tissue does not reliably produce analgesic effects (reviewed by Stein et al., 2003), and it is consistent with the preferentially central action of opioids to induce analgesia in either humans or rodents (e.g. Christie et al., 2000; Thomas et al., 2008; Khalefa et al., 2012). Because of this preferentially central localization of opioid-induced analgesia, previous studies focused on the role of σ_1 receptors at central levels in modulating this opioid-mediated effect, demonstrating that the central administration of either σ_1 antagonists or antisense oligodeoxynucleotides enhances morphine-induced thermal antinociception (Mei and Pasternak, 2002, 2007; Pan et al., 1998). In the present study, we show that σ_1-KO mice locally treated with morphine and WT mice locally co-administered with this opioid and σ_1 receptor antagonist exhibit a strong synergistic mechanical antinociceptive effect at the site of the administration of the combined drug solution but not at a site distant from its injection (contralateral paw) which suggests that the interaction is produced locally. This view is further supported by the fact that WT mice treated with morphine (200 μg) in one paw and BD-1063 (200 μg) in the contralateral one evidenced no antinociception in either paw (data not shown). Hence, our data reveal for the first time that the tonic inhibition of morphine analgesia by σ_1 receptors is also present at the periphery and is strongly involved in mechanical nociceptive pain.

We found similar levels of ([^{3}H]DAMGO-labeled) μ-opioid receptors in σ_1-KO and WT mice in all tissues examined, and the affinity of this radioligand for its binding site was not altered in σ_1-KO mice. In addition, all σ_1 ligands tested showed a negligible affinity for μ-opioid receptors from mouse forebrain membranes, as previously reported for some of these σ_1-ligands in other tissues or species (Kim et al., 2010; Matsumoto et al., 1995; Romero et al., 2012). Hence, our results cannot be explained by abnormal μ-opioid receptors in the σ_1-KO mice or by a direct interaction of the σ_1 drugs used in this study with μ-opioid receptors.

Several of our findings indicate that a selective σ_1 receptor action is involved in the modulation of morphine-induced mechanical antinociception. Firstly, the enhancement of morphine-induced mechanical antinociception in σ_1-KO mice was replicated, at a similar magnitude, by all of the selective σ_1 antagonists tested in WT mice. Secondly, the selective σ_1 agonist PRE-084, which had no effect on morphine-induced antinociception, was able to reverse the effects of the systemic σ_1 antagonists. Finally, none of the σ_1 antagonists tested (administered either systemically or locally) further enhanced morphine-induced antinociception in σ_1-KO mice, indicating that off-target effects do not account for the effects observed. The similarities in the antinociceptive effects of morphine between σ_1-KO mice and σ_1 antagonist-treated WT mice, together with the clear σ_1 pharmacology of these effects, strongly suggest that the effects observed are mediated by σ_1 receptor inhibition. A recent study indicates a possible mechanism for these effects. Thus, the prototypic σ_1 receptor antagonist BD-1047 was found to increase DAMGO-induced G-protein couple receptor signaling (measured as the increase in [^{35}S]GTPγS binding) without altering opioid receptor binding, and σ_1 receptors and μ-opioid receptor were shown to physically interact (Kim et al., 2010). Therefore, basal σ_1 receptor activity may tonically reduce μ-opioid receptor signaling, explaining the increase in morphine analgesia by σ_1 receptor inhibition.

Previous research on opioid-induced thermal antinociception showed that the selective σ_1 agonist (+)-pentazocine could decrease morphine-induced thermal antinociception, indicating that tonic inhibition of this analgesic effect of morphine can be enhanced by further σ_1 activation (Chien and Pasternak, 1993, 1994; Mei and Pasternak, 2002, 2007). However, under our conditions, PRE-084 did not influence the effect of morphine on mechanical stimuli, suggesting that the tonic inhibition of morphine-induced mechanical antinociception by σ_1 receptors was already maximal and could not be further increased by exogenous

σ_1 activation. The role of σ_1 receptors in animal models of tonic/chronic pain has been thoroughly studied over the past decade (Cendán et al., 2005a, 2005b; De la Puente et al., 2009; Entrena et al., 2009a, 2009b; Kim et al., 2006; Nieto et al., 2012; Roh et al., 2008; Romero et al., 2012). Modulation of these pain behaviors by σ_1 receptors is thought to be located at central levels, because σ_1 receptor activation in the spinal cord triggers central sensitization to induce mechanical allodynia (Kim et al., 2008; Roh et al., 2010, 2011; Ohsawa et al., 2011), likely contributing through this mechanism to the modulation of activity-induced spinal sensitization (De la Puente et al., 2009; Romero et al., 2012). We previously demonstrated that the effect of σ_1 antagonists on some of those behavioral outcomes (formalin-induced pain and capsaicin-induced mechanical hypersensitivity) was insensitive to naloxone treatment (Cendán et al., 2005a; Entrena et al., 2009a). Therefore, these effects of σ_1 antagonists do not appear to depend on modulation of the opioid system.

Opioids, and particularly morphine, are considered to be the "gold standard" for pain management (Christie et al., 2000; Waldhoer et al., 2004). However, several serious side effects associated with acutely administered morphine, including constipation, sedation, respiratory depression, and nausea, represent substantial drawbacks to its use (reviewed in Al-Hasani and Bruchas, 2011; Waldhoer et al., 2004). Two strategies have been proposed to minimize the adverse effects of opioids. One of these approaches is to administer an adjuvant drug with synergistic analgesic effects in order to minimize the dose of the opioid (and hence its side effects) while maintaining acceptable levels of analgesia. One example is the combination of non-steroidal anti-inflammatory drugs or anticonvulsants with opioids (Christie et al., 2000; Mao et al., 2011). In the present study, we show that, despite the strong potentiation of the analgesic effects of morphine observed in σ_1-KO mice, σ_1 receptors do not appear to influence two morphine-induced side effects, i.e., hyperlocomotion and gastrointestinal transit inhibition. Hyperlocomotion is a common side effect of opioids in rodents (e.g. Cobos et al., 2012; Elhabazi et al., 2012) and reflects an increase in supraspinal dopamine release (Hnasko et al., 2005), whereas the inhibition of gastrointestinal transit mainly results from opioid action at peripheral level (Al-Hasani and Bruchas, 2011). In agreement with our data, a previous study found that the selective σ_1 agonist (+)-pentazocine did not modify the morphine-induced decrease in gastrointestinal transit or lethality (Chien and Pasternak, 1994). The differential impact of σ_1 receptors on the modulation of opioid analgesia and side effects may suggest that σ_1 receptors are present in a specific subset of opioid receptor-expressing neurons that are more involved in pain pathways or, alternatively, that further mechanisms besides the direct modulation of opioid signaling may participate in the analgesia enhancement.

A different approach to reducing the side effects of opioids is to target peripheral opioid antinociception in order to minimize undesirable centrally mediated effects (reviewed by Sehgal et al., 2011; Stein et al., 2003). Here we show that σ_1 inhibition is able to potentiate local morphine analgesia, producing an even greater antinociceptive effect than is induced by systemic morphine at the highest dose administered in this study (16 mg/kg). Consequently, the potentiation of peripherally mediated morphine analgesia by σ_1 receptor antagonists may offer safer and improved therapeutic outcomes in pain management.

5. Conclusions

In summary, we found that systemic σ_1 receptor inhibition synergistically enhances morphine-induced mechanical antinociception without modifying a centrally-induced (hyperlocomotion) or peripherally-induced (constipation) side effect of morphine. Enhancement of morphine analgesia by σ_1 inhibition can be produced locally, indicating that σ_1 receptors tonically inhibit peripheral opioid functioning. The local combination of morphine with σ_1 receptor blockade may represent a strategy to minimize the adverse effects of morphine by differentially potentiating its therapeutic analgesic actions.

Acknowledgments

C. Sánchez-Fernández and R González-Cano were supported by FPU grants from the Spanish Ministerio de Educación y Ciencia (MEC), and E. J. Cobos by the Research Program of the University of Granada. This research was done in partial fulfillment of the requirements for the doctoral thesis of C. Sánchez-Fernández. The authors thank Richard Davies for revising the English style of the manuscript. This study was partially supported by the Spanish Ministry of Education and Science (MEC) [grant SAF2006-06122], Junta de Andalucía [grant CTS 109], Laboratorios Esteve, and The Centre for Industrial Technological Development (Spanish Government) [Genius Pharma project].

References

Al-Hasani, R., Bruchas, M.R., 2011. Molecular mechanisms of opioid receptor-dependent signaling and behavior. Anesthesiology 115, 1363–1381.

Alonso, G., Phan, V., Guillemain, I., Saunier, M., Legrand, A., Anoal, M., Maurice, T., 2000. Immunocytochemical localization of the sigma$_1$ receptor in the adult rat central nervous system. Neuroscience 97, 155–170.

Aydar, E., Palmer, C.P., Klyachko, V.A., Jackson, M.B., 2002. The sigma receptor as a ligand-regulated auxiliary potassium channel subunit. Neuron 34, 399–410.

Baamonde, A., Curto-Reyes, V., Juárez, L., Meana, A., Hidalgo, A., Menéndez, L., 2007. Antihyperalgesic effects induced by the IL-1 receptor antagonist anakinra and increased IL-1beta levels in inflamed and osteosarcoma-bearing mice. Life Sci. 81, 673–682.

Barturen, F., Garcia-Sevilla, J.A., 1992. Long term treatment with desipramine increases the turnover of α_2-adrenoceptors in the rat brain. Mol. Pharmacol. 42, 846–855.

Cendán, C.M., Pujalte, J.M., Portillo-Salido, E., Baeyens, J.M., 2005a. Antinociceptive effects of haloperidol and its metabolites in the formalin test in mice. Psychopharmacology (Berl.) 182, 485–493.

Cendán, C.M., Pujalte, J.M., Portillo-Salido, E., Montoliu, L., Baeyens, J.M., 2005b. Formalin-induced pain is reduced in σ_1 receptor knockout mice. Eur. J. Pharmacol. 511, 73–74.

Chien, C.C., Pasternak, G.W., 1993. Functional antagonism of morphine analgesia by (+)-pentazocine: evidence for an anti-opioid σ_1 system. Eur. J. Pharmacol. 250, R7–R8.

Chien, C.C., Pasternak, G.W., 1994. Selective antagonism of opioid analgesia by a sigma system. J. Pharmacol. Exp. Ther. 271, 1583–1590.

Christie, M.J., Connor, M., Vaughan, C.W., Ingram, S.L., Bagley, E.E., 2000. Cellular actions of opioids and other analgesics: implications for synergism in pain relief. Clin. Exp. Pharmacol. Physiol. 27, 520–523.

Cobos, E.J., Baeyens, J.M., Del Pozo, E., 2005. Phenytoin differentially modulates the affinity of agonist and antagonist ligands for σ_1 receptors of guinea pig brain. Synapse 55, 192–195.

Cobos, E.J., del Pozo, E., Baeyens, J.M., 2007. Irreversible blockade of sigma-1 receptors by haloperidol and its metabolites in guinea pig brain and SH-SY5Y human neuroblastoma cells. J. Neurochem. 102, 812–825.

Cobos, E.J., Entrena, J.M., Nieto, F.R., Cendán, C.M., Del Pozo, E., 2008. Pharmacology and therapeutic potential of sigma$_1$ receptor ligands. Curr. Neuropharmacol. 6, 344–366.

Cobos, E.J., Ghasemlou, N., Araldi, D., Segal, D., Duong, K., Woolf, C.J., 2012. Inflammation-induced decrease in voluntary wheel running in mice: a non-reflexive test for evaluating inflammatory pain and analgesia. Pain 153, 876–884.

Cobos, E.J., Lucena, G., Baeyens, J.M., Del Pozo, E., 2006. Differences in the allosteric modulation by phenytoin of the binding properties of the σ_1 ligands [^{3}H](+)-pentazocine and [^{3}H]NE-100. Synapse 59, 152–161.

De la Puente, B., Nadal, X., Portillo-Salido, E., Sánchez-Arroyos, R., Ovalle, S., Palacios, G., Muro, A., Romero, L., Entrena, J.M., Baeyens, J.M., López-García, J.A., Maldonado, R., Zamanillo, D., Vela, J.M., 2009. Sigma-1 receptors regulate activity-induced spinal sensitization and neuropathic pain after peripheral nerve injury. Pain 145, 294–303.

Díaz, J.L., Cuberes, R., Berrocal, J., Contijoch, M., Christmann, U., Fernández, A., Port, A., Holenz, J., Buschmann, H., Laggner, C., Serafini, M.T., Burgueño, J., Zamanillo, D., Merlos, M., Vela, J.M., Almansa, C., 2012. Synthesis and biological evaluation of the 1-Arylpyrazole class of σ_1 receptor antagonists: identification of 4-{2-[5-methyl-1-(naphthalen-2-yl)-1H-pyrazol-3-yloxy]ethyl}morpholine (S1RA, E-52862). J. Med. Chem. 55, 8211–8224.

Elhabazi, K., Trigo, J.M., Mollereau, C., Moulédous, L., Zajac, J.M., Bihel, F., Schmitt, M., Bourguignon, J.J., Meziane, H., Petit-demoulière, B., Bockel, F., Maldonado, R., Simonin, F., 2012. Involvement of neuropeptide FF receptors in neuroadaptive responses to acute and chronic opiate treatments. Br. J. Pharmacol. 165, 424–435.

Entrena, J.M., Cobos, E.J., Nieto, F.R., Cendán, C.M., Baeyens, J.M., Del Pozo, E., 2009a. Antagonism by haloperidol and its metabolites of mechanical hypersensitivity induced by intraplantar capsaicin in mice: role of sigma-1 receptors. Psychopharmacology (Berl.) 205, 21–33.

Entrena, J.M., Cobos, E.J., Nieto, F.R., Cendán, C.M., Gris, G., Del Pozo, E., Zamanillo, D., Baeyens, J.M., 2009b. Sigma-1 receptors are essential for capsaicin-induced mechanical hypersensitivity: studies with selective sigma-1 ligands and sigma-1 knockout mice. Pain 143, 252–261.

Guitart, X., Codony, X., Monroy, X., 2004. Sigma receptors: biology and therapeutic potential. Psychopharmacology (Berl.) 174, 301–319.

Hayashi, T., Su, T.P., 2004. Sigma-1 receptor ligands: potential in the treatment of neuropsychiatric disorders. CNS Drugs 18, 269–284.

Hnasko, T.S., Sotak, B.N., Palmiter, R.D., 2005. Morphine reward in dopamine-deficient mice. Nature 438, 854–857.

Khalefa, B.I., Shaqura, M., Al-Khrasani, M., Fürst, S., Mousa, S.A., Schäfer, M., 2012. Relative contributions of peripheral versus supraspinal or spinal opioid receptors to the antinociception of systemic opioids. Eur. J. Pain 16, 690–705.

Kim, F.J., Kovalyshyn, I., Burgman, M., Neilan, C., Chien, C.C., Pasternak, G.W., 2010. σ_1 receptor modulation of G-protein-coupled receptor signaling: potentiation of opioid transduction independent from receptor binding. Mol. Pharmacol. 77, 695–703.

Kim, H.W., Kwon, Y.B., Roh, D.H., Yoon, S.Y., Han, H.J., Kim, K.W., Beitz, A.J., Lee, J.H., 2006. Intrathecal treatment with σ_1 receptor antagonists reduces formalin-induced phosphorylation of NMDA receptor subunit 1 and the second phase of formalin test in mice. Br. J. Pharmacol. 148, 490–498.

Kim, H.W., Roh, D.H., Yoon, S.Y., Seo, H.S., Kwon, Y.B., Han, H.J., Kim, K.W., Beitz, A.J., Lee, J.H., 2008. Activation of the spinal sigma-1 receptor enhances NMDA-induced pain via PKC- and PKA-dependent phosphorylation of the NR1 subunit in mice. Br. J. Pharmacol. 154, 1125–1134.

King, M., Pan, Y.X., Mei, J., Chang, A., Xu, J., Pasternak, G.W., 1997. Enhanced κ-opioid receptor-mediated analgesia by antisense targeting the σ_1 receptor. Eur. J. Pharmacol. 331, R5–R6.

Kitaichi, K., Chabot, J.G., Moebius, F.F., Flandorfer, A., Glossmann, H., Quirion, R., 2000. Expression of the purported sigma$_1$ (σ_1) receptor in the mammalian brain and its possible relevance in deficits induced by antagonism of the NMDA receptor complex as revealed using an antisense strategy. J. Chem. Neuroanat. 20, 375–387.

Kolesnikov, Y.A., Chereshnev, I., Pasternak, G.W., 2000. Analgesic synergy between topical lidocaine and topical opioids. J. Pharmacol. Exp. Ther. 295, 546–551.

Kolesnikov, Y.A., Jain, S., Wilson, R., Pasternak, G.W., 1996. Peripheral morphine analgesia: synergy with central sites and a target of morphine tolerance. J. Pharmacol. Exp. Ther. 279, 502–506.

Kuraishi, Y., Hirota, N., Satoh, M., Takagi, H., 1985. Antinociceptive effects of intrathecal opioids, noradrenaline and serotonin in rats: mechanical and thermal algesic tests. Brain Res. 326, 168–171.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurements with the folin phenol reagent. J. Biol. Chem. 193, 265–271.

Mao, J., Gold, M.S., Backonja, M.M., 2011. Combination drug therapy for chronic pain: a call for more clinical studies. J. Pain 12, 157–166.

Marrazzo, A., Cobos, E.J., Parenti, C., Aricò, G., Marrazzo, G., Ronsisvalle, S., Pasquinucci, L., Prezzavento, O., Colabufo, N.A., Contino, M., González, L.G., Scoto, G.M., Ronsisvalle, G., 2011. Novel potent and selective σ ligands: evaluation of their agonist and antagonist properties. J. Med. Chem. 54, 3669–3673.

Marrazzo, A., Parenti, C., Scavo, V., Ronsisvalle, S., Scoto, G.M., Ronsisvalle, G., 2006. In vivo evaluation of (+)-MR200 as a new selective sigma ligand modulating MOP, DOP and KOP supraspinal analgesia. Life Sci. 78, 2449–2453.

Matsumoto, R.R., Bowen, W.D., Tom, M.A., Vo, V.N., Truong, D.D., De Costa, B.R., 1995. Characterization of two novel σ receptor ligands: antidystonic effects in rats suggest σ receptor antagonism. Eur. J. Pharmacol. 280, 301–310.

Mei, J., Pasternak, G.W., 2002. σ_1 receptor modulation of opioid analgesia in the mouse. J. Pharmacol. Exp. Ther. 300, 1070–1074.

Mei, J., Pasternak, G.W., 2007. Modulation of brainstem opiate analgesia in the rat by σ_1 receptors: a microinjection study. J. Pharmacol. Exp. Ther. 322, 1278–1285.

Menéndez, L., Lastra, A., Meana, A., Hidalgo, A., Baamonde, A., 2005. Analgesic effects of loperamide in bone cancer pain in mice. Pharmacol. Biochem. Behav. 81, 114–121.

Nakazato, A., Kumagai, T., Ohta, K., Chaki, S., Okuyama, S., Tomisawa, K., 1999. Synthesis and SAR of 1-alkyl-2-phenylethylamine derivatives designed from *N, N*-dipropyl-4-methoxy-3-(2-phenylethoxy)phenylethylamine to discover σ_1 ligands. J. Med. Chem. 42, 3965–3970.

Narita, M., Ozaki, S., Ioka, M., Mizoguchi, H., Nagase, H., Tseng, L., Suzuki, T., 2001. Different motivational effects induced by the endogenous μ-opioid receptor ligands endomorphin-1 and -2 in the mouse. Neuroscience 105, 213–218.

Nieto, F.R., Cendán, C.M., Sánchez-Fernández, C., Cobos, E.J., Entrena, J.M., Tejada, M.,A., Zamanillo, D., Vela, J.M., Baeyens, J.M., 2012. Role of sigma-1 receptors in paclitaxel-induced neuropathic pain in mice. J. Pain 13, 1107–1121.

Ohsawa, M., Carlsson, A., Asato, M., Koizumi, T., Nakanishi, Y., Fransson, R., Sandström, A., Hallberg, M., Nyberg, F., Kamei, J., 2011. The dipeptide Phe-Phe amide attenuates signs of hyperalgesia, allodynia and nociception in diabetic mice using a mechanism involving the sigma receptor system. Mol. Pain 31, 7–11.

Pan, Y.X., Mei, J., Xu, J., Wan, B.L., Zuckerman, A., Pasternak, G.W., 1998. Cloning and characterization of a mouse σ_1 receptor. J. Neurochem. 70, 2279–2285.

Roh, D.H., Choi, S.R., Yoon, S.Y., Kang, S.Y., Moon, J.Y., Kwon, S.G., Han, H.J., Beitz, A.J., Lee, J.H., 2011. Spinal neuronal NOS activation mediates sigma-1 receptor-induced mechanical and thermal hypersensitivity in mice: involvement of PKC-dependent GluN1 phosphorylation. Br. J. Pharmacol. 163, 1707–1720.

Roh, D.H., Kim, H.W., Yoon, S.Y., Seo, H.S., Kwon, Y.B., Kim, K.W., Han, H.J., Beitz, A.J., Na, H.S., Lee, J.H., 2008. Intrathecal injection of the σ_1 receptor antagonist BD1047 blocks both mechanical allodynia and increases in spinal NR1 expression during the induction phase of rodent neuropathic pain. Anesthesiology 109, 879–889.

Roh, D.H., Yoon, S.Y., Seo, H.S., Kang, S.Y., Moon, J.Y., Song, S., Beitz, A.J., Lee, J.H., 2010. Sigma-1 receptor-induced increase in murine spinal NR1 phosphorylation is mediated by the PKCα and ε, but not the PKCξ, isoforms. Neurosci. Lett. 477, 95–99.

Romero, R., Zamanillo, D., Nadal, X., Sánchez-Arroyos, R., Rivera-Arconada, I., Dordal, A., Montero, A., Muro, A., Bura, A., Segalés, C., Laloya, M., Hernández, E., Portillo-Salido, E., Escriche, M., Codony, X., Encina, G., Burgueño, J., Merlos, M., Baeyens, J.M., Giraldo, J., López-García, J.A., Maldonado, R., Plata-Salamán, C.R., Vela, J.M., 2012. Pharmacological properties of S1RA, a new sigma-1 receptor antagonist that inhibits neuropathic pain and activity-induced spinal sensitization. Br. J. Pharmacol. 166, 2289–2306.

Ronsisvalle, G., Marrazzo, A., Prezzavento, O., Cagnotto, A., Mennini, T., Parenti, C., Scoto, G.M., 2001. Opioid and sigma receptor studies. New developments in the design of selective sigma ligands. Pure Appl. Chem. 73, 1499–1509.

Sato, T., Sakurada, S., Takahashi, N., Sakurada, T., Tan-No, K., Wako, K., Kisara, K., 1999. Contribution of spinal μ_1-opioid receptors to morphine-induced antinociception. Eur. J. Pharmacol. 369, 183–187.

Sehgal, N., Smith, H.S., Manchikanti, L., 2011. Peripherally acting opioids and clinical implications for pain control. Pain Physician 14, 249–258.

Stein, C., Schäfer, M., Machelska, H., 2003. Attacking pain at its source: new perspectives on opioids. Nat. Med. 9, 1003–1008.

Su, T.P., Wu, X.Z., Cone, E.J., Shukla, K., Gund, T.M., Dodge, A.L., Parish, D.W., 1991. *Sigma* compounds derived from phencyclidine: identification of PRE-084, a new, selective *sigma* ligand. J. Pharmacol. Exp. Ther. 259, 543–550.

Thomas, J., Karver, S., Cooney, G.A., Chamberlain, B.H., Watt, C.K., Slatkin, N.E., Stambler, N., Kremer, A.B., Israel, R.J., 2008. Methylnaltrexone for opioid-induced constipation in advanced illness. N. Engl. J. Med. 358, 2332–2343.

Tseng, L.F., Collins, K.A., Wang, Q., 1995. Differential ontogenesis of thermal and mechanical antinociception induced by morphine and β-endorphin. Eur. J. Pharmacol. 277, 71–76.

Ueda, H., Inoue, M., Yoshida, A., Mizuno, K., Yamamoto, H., Maruo, J., Matsuno, K., Mita, S., 2001. Metabotropic neurosteroid/σ-receptor involved in stimulation of nociceptor endings of mice. J. Pharmacol. Exp. Ther. 298, 703–710.

Wegert, S., Ossipov, M.H., Nichols, M.L., Bian, D., Vanderah, T.W., Malan Jr., T.P., Porreca, F., 1997. Differential activities of intrathecal MK-801 or morphine to alter responses to thermal and mechanical stimuli in normal or nerve-injured rats. Pain 71, 57–64.

Waldhoer, M., Bartlett, S.E., Whistler, J.L., 2004. Opioid receptors. Annu. Rev. Biochem. 73, 953–990.