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RESEARCH PAPER

Pharmacological properties of S1RA, a new sigma-1 receptor antagonist that inhibits neuropathic pain and activity-induced spinal sensitization

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BACKGROUND AND PURPOSE

The sigma-1 (σ_1) receptor is a ligand-regulated molecular chaperone that has been involved in pain, but there is limited understanding of the actions associated with its pharmacological modulation. Indeed, the selectivity and pharmacological properties of σ_1 receptor ligands used as pharmacological tools are unclear and the demonstration that σ_1 receptor antagonists have efficacy in reversing central sensitization-related pain sensitivity is still missing.

EXPERIMENTAL APPROACH

The pharmacological properties of a novel σ_1 receptor antagonist (S1RA) were first characterized. S1RA was then used to investigate the effect of pharmacological antagonism of σ_1 receptors on *in vivo* nociception in sensitizing conditions and on *in vitro* spinal cord sensitization in mice. Drug levels and autoradiographic, *ex vivo* binding for σ_1 receptor occupancy were measured to substantiate behavioural data.

KEY RESULTS

Formalin-induced nociception (both phases), capsaicin-induced mechanical hypersensitivity and sciatic nerve injury-induced mechanical and thermal hypersensitivity were dose-dependently inhibited by systemic administration of S1RA. Occupancy of σ_1 receptors in the CNS was significantly correlated with the antinociceptive effects. No pharmacodynamic tolerance to the antiallodynic and antihyperalgesic effect developed following repeated administration of S1RA to nerve-injured mice. As a mechanistic correlate, electrophysiological recordings demonstrated that pharmacological antagonism of σ_1 receptors attenuated the wind-up responses in spinal cords sensitized by repetitive nociceptive stimulation.

CONCLUSIONS AND IMPLICATIONS

These findings contribute to evidence identifying the σ_1 receptor as a modulator of activity-induced spinal sensitization and pain hypersensitivity, and suggest σ_1 receptor antagonists as potential novel treatments for neuropathic pain.

Abbreviations

ACSF, artificial cerebrospinal fluid; S1RA, 4-[2-[[5-methyl-1-(2-naphthalenyl)-1H-pyrazol-3-yl]oxy]ethyl] morpholine (E-52862); UPLC-MS/MS, ultra performance liquid chromatography with tandem mass spectrometric detection

Introduction

Sigma (σ) receptors have been classified into two subtypes: σ_1 and σ_2. The σ_1, but not the σ_2, receptor has been cloned and the gene encodes a protein of 223 amino acids anchored to the endoplasmic reticulum and plasma membranes (Su and Hayashi, 2003; Cobos *et al.*, 2008; Maurice and Su, 2009; Tsai *et al.*, 2009). Certain neurosteroids interact with σ_1 receptors, but the precise nature of endogenous high-affinity σ_1 receptor ligands is still unclear (Maurice and Su, 2009).

Confused with opioid receptors for a while due to the cross-reactivity of some ligands, the σ_1 receptor has been recently identified as a unique ligand-regulated molecular chaperone. The most prominent action of σ_1 receptors is the modulation of intracellular signalling cascades incurred when the target protein they are interacting with becomes activated (Su and Hayashi, 2003; Su *et al.*, 2010). Notably, while having no effects by themselves under normal physiological conditions, σ_1 receptor ligands exert their modulatory activity under conditions involving a disturbance, when target proteins become conformationally unstable and prone to the assistance of σ_1 receptor chaperones (Hayashi *et al.*, 2000; Su and Hayashi, 2003; Su *et al.*, 2010).

The best characterized chaperoning by σ_1 receptors occurs at the endoplasmic reticulum, where inositol-1,4,5-trisphosphate (IP_3) receptors mediate the efflux of Ca^{2+} from the endoplasmic reticulum into the mitochondria. Under normal, resting conditions, the σ_1 receptor is in a dormant state with regard to chaperone activity, but under pathological/stressful conditions, in the presence of high concentrations of cytosolic IP_3, the Ca^{2+} concentration at the endoplasmic reticulum dramatically falls and σ_1 receptors bind to unstable IP_3 receptors, preventing their degradation and ensuring the proper Ca^{2+} influx into the mitochondria (Tsai *et al.*, 2009). At the plasma membrane, σ_1 receptors modulate NMDA receptors (Monnet *et al.*, 1990; Bergeron *et al.*, 1996), probably by regulating the opening of a small conductance Ca^{2+}-activated K^+ current (SK channels) that shunts NMDA receptor-mediated responses (Martina *et al.*, 2007), as well as a number of other ion channels (Zhang and Cuevas, 2002; Cheng *et al.*, 2008; Herrera *et al.*, 2008) and G protein-coupled receptors (Kim *et al.*, 2010).

The therapeutic potential of σ_1 receptor ligands has been recognized for many years (Maurice and Su, 2009; Tsai *et al.*, 2009). In terms of pain, σ_1 receptors modulate opioid receptor-mediated antinociception (σ_1 receptor agonists inhibit whereas antagonists enhance the antinociceptive effect of opioids) (Chien and Pasternak, 1994; Mei and Pasternak, 2002), and a direct physical interaction between the μ-opioid receptor and the σ_1 receptor has been recently described (Kim *et al.*, 2010). However, evidence that σ_1 receptors may also play a role in nociception in the absence of opioids came from more recent studies using mice lacking σ_1 receptors and behavioural models involving sensitization of pain pathways. Mice with σ_1 receptors deleted showed attenuated pain responses in the formalin test (Cendán *et al.*, 2005b) and mechanical hypersensitivity did not develop following capsaicin sensitization (Entrena *et al.*, 2009b) or sciatic nerve injury (de la Puente *et al.*, 2009). Treatment with haloperidol and its metabolites I and II, which act as antagonists at σ_1 receptors (Cobos *et al.*, 2007), produced similar results – inhibition of formalin-induced pain (Cendán *et al.*, 2005a) and capsaicin-induced mechanical hypersensitivity (Entrena *et al.*, 2009a). Furthermore, activation of spinal σ_1 receptors evoked mechanical and thermal hypersensitivity (Roh *et al.*, 2010) and enhanced NMDA-induced pain, concomitant with increased phosphorylation of the NR1 subunit of NMDA receptors (Kim *et al.*, 2008). Both nociceptive behaviours and phosphorylation of NR1 subunits in the spinal cord were inhibited by spinal antagonism of σ_1 receptors (Kim *et al.*, 2006; 2008; Roh *et al.*, 2008; 2010). Nevertheless, the selectivity and pharmacology of σ_1 receptor ligands used as pharmacological tools are unclear and the demonstration that σ_1 receptor antagonists have any efficacy in reversing central sensitization and increased pain sensitivity is still missing (Drews and Zimmer, 2009).

In the present study, we have addressed this issue by assessing the effects of S1RA (E-52862; Laggner *et al.*, 2006), a new selective σ_1 receptor antagonist, on increased pain sensitivity. As mechanistic correlates, the effects on electrophysiological recordings from spinal cords sensitized by nociceptive dorsal root stimulation as well the relationship between the occupancy of σ_1 receptors and the antinociceptive activity were analysed. Overall, this study found that selective antagonism of the σ_1 receptor with S1RA inhibits behavioural nociception in sensitizing conditions, including neuropathic pain in mice. Electrophysiological and σ_1 receptor occupancy data point to a modulatory effect on central (spinal) hyperexcitability arising from repetitive nociceptive stimulation as the underlying mechanism.

Methods

Animals

All animal husbandry and experimental procedures complied with European guidelines regarding protection of animals used for experimental and other scientific purposes (Council Directive of 24 November 1986, 86/609/ECC) and received approval by the local Ethical Committee. The results of all studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (McGrath *et al.*, 2010). Male CD1 mice (6 to 8 weeks old; 234 in total; Charles River, France) were used in the

behavioural, pharmacokinetic and *ex vivo* binding experiments, whereas newborn 7–10-day-old mice (total 24) were used for electrophysiological studies. Some *in vitro* binding studies were performed in male Dunkin Hartley guinea pigs (200–250 g; total 3; Harlan, Barcelona). Animals had access to food and water *ad libitum* and were kept in controlled laboratory conditions with temperature at 21 ± 1°C and a light–dark cycle of 12 h (lights on at 7:00 h). Experimental behavioural testing was carried out in a soundproof and air-regulated experimental room and was carried out without knowledge of treatments and surgical procedures.

Surgery

The partial sciatic nerve ligation model was used to induce neuropathic pain. Briefly, mice were anaesthetized with isoflurane (Abbott–Esteve, Spain) (induction: 3%; surgery: 1%) and the common sciatic nerve was exposed at the level of the mid-thigh of the right hind paw. At about 1 cm proximally to the nerve trifurcation, a tight ligation using 9–0 non-absorbable virgin silk suture (Alcon Laboratories Inc., Fort Worth, TX, USA) was created enclosing the outer 33–50% of the diameter of the sciatic nerve, leaving the rest of the nerve 'uninjured'. Care was taken to ensure that the ligation was not too tight so as to occlude the perineural blood flow. The muscle was then stitched with 6–0 silk suture and the skin incision closed with wound clips. Control, sham-operated mice underwent the same surgical procedure and the sciatic nerve was exposed, but not ligated.

Drugs and drug administration

Capsaicin (8-methyl-*N*-vanillyl 6-nonamide) was purchased from Sigma–Aldrich (Madrid, Spain), and dissolved in 1% DMSO (vehicle) in physiological saline. Capsaicin (1 µg in 20 µL of 1% DMSO) was intraplantarly (i.pl.) injected into the mid-plantar surface of the right hind paw using a 1710 TLL Hamilton microsyringe (Teknokroma, Barcelona, Spain) with a 30.5-gauge needle. Formalin at 2.5% was prepared from a solution of 37% formaldehyde (Sigma–Aldrich) and was i.pl. injected into the right hind paw as in the case of capsaicin.

The new selective σ_1 receptor antagonist (S1RA; E-52862; 4-[2-[[5-methyl-1-(2-naphthalenyl)-1H-pyrazol-3-yl]oxy] ethyl] morpholine), developed and supplied by Laboratories Esteve (Barcelona, Spain), was used as the hydrochloride and doses are expressed as weights of this form. For *in vivo* administration the compound was dissolved in aqueous solutions: 0.9% physiological saline (partial sciatic nerve ligation model) or 0.5% hydroxypropyl methyl cellulose (HPMC) (Sigma–Aldrich) (rest of the *in vivo* models). The compound and the vehicle were administered in a volume of 10 mL·kg^{-1} through the i.p. or p.o. route, as shown in the text. Pregabalin (from Laboratories Esteve) was used as a control in electrophysiological studies.

In vitro *pharmacological profile of S1RA*

Binding to σ_1 receptor and σ_2 receptors. Guinea pig brain membrane preparation and binding assays for σ_1 receptors were performed as previously described (DeHaven-Hudkins *et al.*, 1992) with some modifications. The radioligand used was [^{3}H](+)-pentazocine (PerkinElmer, Boston, MA, USA). Non-specific binding was determined using 1 µM haloperidol (Sigma–Aldrich). Microplates were quantified by liquid scintillation spectrophotometry. The human σ_1 receptor radioligand binding assay was performed in the CEREP (France), according to its specifications (http://www.cerep.com).

Guinea pig membrane preparation and binding assays for the σ_2 receptor were performed as described (Ronsisvalle *et al.*, 2001) with some modifications. The radioligand used was [^{3}H]di-*o*-tolylguanidine (DTG) (Perkin Elmer). Non-specific binding was determined with 5 µM DTG and σ_1 receptors were blocked with (+)SKF-10047 (Tocris Bioscience, Bristol, UK) at 400 nM. Microplates were also quantified by liquid scintillation spectrophotometry. Rat σ_2 receptor binding assays were performed according to CEREP's specifications (http://www.cerep.com). Assays were done at least twice and concentrations were tested in triplicate.

Selectivity profile. Binding affinities of S1RA for proteins other than σ receptors were determined by commercial radioligand binding assays by CEREP and MDS Pharma (now Ricerca Biosciences, Lyon, France). A selectivity profile including a panel of 170 radioligand binding assays for different receptors, ion channels and enzymes was performed according to their standard assay protocols (http://www.cerep.com; http://www.ricerca.com). When significant affinity (K_i < 1 µM or % inhibition at 1 µM > 50%) for a given target included in the panel was detected, the binding assay was followed by a cell-based functional assay to assess the functional profile (agonist/activator or antagonist/inhibitor) of the compound. On this basis, the functional activity of the compound was tested in CHO cells stably transfected with the human 5-HT_{2B} receptor according to CEREP's specifications (http://www.cerep.com). Assays were done with concentrations tested in duplicate.

Functional profile on σ_1 receptors. Guinea pig brain membrane binding assays for the σ_1 receptor were conducted either in the absence or presence of 1 mM phenytoin, as previously reported (Cobos *et al.*, 2005), to identify the functional (agonistic or antagonistic) nature of S1RA. Assays were conducted as described earlier using [^{3}H](+)-pentazocine. In parallel, several drugs were evaluated as controls: dextromethorphan (Sigma–Aldrich) (+)SKF-10047 and PRE-084 (Tocris) as σ_1 receptor agonists; and NE-100 (Tocris), BD-1063 (Tocris) and haloperidol as σ_1 receptor antagonists. Assays were done at least twice and concentrations were tested in triplicate.

Electrophysiology

Spinal cords were obtained from newborn 7–10-day-old mice after urethane (2 mg·kg^{-1}, i.p.) anaesthesia followed by dorsal laminectomy and *in vitro* AC recordings were performed. Briefly, spinal cords were fixed to the Sylgard base of a recording chamber and continuously superfused (6–9 mL·min^{-1}) with oxygenated (95% O_2; 5% CO_2) artificial cerebrospinal fluid (ACSF) at pH 7.4 and room temperature. The composition of the ACSF was (in mM): NaCl (128), KCl (1.9), KH_2PO_4 (1.2), $MgSO_4$ (1.3), $CaCl_2$ (2.4), $NaHCO_3$ (26) and glucose (10). A period of 60 min was allowed for the preparation to stabilize before testing spinal reflexes. The lumbar dorsal root (L4 or L5) and the corresponding ventral root were placed in tight-fitting glass suction electrodes. Electrical stimulation

was then applied to the dorsal root and responses were recorded from the corresponding ventral root. The signal coming from the ventral root suction electrode was amplified to record fast compound spikes produced by the firing of action potentials by motor neurons using a Neurolog AC amplifier. Signals were digitized at 5 kHz and stored for offline computer-aided analysis using a CED 1401 interface and Spike 2 software (Cambridge Electronic Design Ltd, Cambridge, UK). AC recordings were analysed based on threshold criteria to count spikes to each stimulus when a train of stimuli was applied. Spike counts were performed for each stimulus of the train in a window between 20 and 950 ms from stimulus artefact.

The electrical stimulation test consisted of one A-fibre intensity stimulus adjusted to activate only thick and myelinated fibres (20 μA, 200 μs), followed by one C-fibre intensity stimulus (200 μA, 200 μs) and then followed by a train of 15 consecutive C-fibre intensity stimuli applied at 1 Hz (200 μA, 200 μs, 1 Hz) to produce wind-up responses. Stimuli were applied at 45 s intervals and the electrical stimulation tests were done at 30 min intervals. This protocol allowed us to evaluate drug effects on both non-nociceptive somato-motor circuits and nociceptive circuits (number of spikes produced by single C-fibre stimuli as well as spike wind-up to trains of stimuli).

After two to four repetitions of the stimulation test to obtain stable baseline responses, S1RA was superfused (dissolved in ACSF at 3, 10 or 30 μM) for a 15 min period. Pregabalin at 300 μM was used as a control. Timing was arranged so as to perform the electrical stimulation test at the end of the superfusion of the compound. After testing the effect of the compound, electrical stimulation tests continued at 30 minute intervals for a prolonged washout period (four successive washing periods). Only one concentration was tested per preparation and five to seven preparations were used to test the effect of each concentration.

Motor coordination: rotarod test

The motor performance of mice treated with S1RA or vehicle (n = 7–10 per group) was assessed by means of an automated rotarod (Panlab SL, Barcelona, Spain). Before drug treatments, mice were trained and animals that were unable to stay moving on the rod for 240 s at 10 r.p.m. were discarded for the study. In single-dose studies, mice were required to walk against the motion of an elevated rotating drum at 10 r.p.m and the latency to fall-down was recorded automatically. With the selected animals rotarod latencies were measured 30, 60, 120 and 180 min after administration of vehicle or S1RA either i.p. (32, 64 and 128 mg·kg^{-1}) or p.o. (40, 80 and 160 mg·kg^{-1}). In repeated treatment studies mice were required to walk against the motion of an elevated rotating drum under an incremental procedure following the schedule: 4, 5, 6, 7, 8, 9 and 10 r.p.m. during 30, 20, 20, 20, 10, 10 and 10 s, respectively. This procedure allowed a better response from control animals that were heavier, than in the single-dose study at the time of evaluation. The latency to fall-down was recorded. Animals were treated with either vehicle or 25 mg·kg^{-1} S1RA i.p. twice daily for 21 days. On the test day, rotarod performance was evaluated at 30, 60 and 120 min after last administration of vehicle or S1RA.

Mechanical hypersensitivity induced by capsaicin

Mice were habituated for 2 h in individual test compartments placed on an elevated mesh-bottomed platform with a 0.5 cm^2 grid to provide access to the ventral side of the paws. Animals were then given an i.pl. injection of capsaicin (1 μg in 20 μL of 1% DMSO) or its solvent into the mid-plantar surface of the right hind paw. Fifteen minutes after i.pl. capsaicin or vehicle injection, mechanical stimulation was applied onto the plantar surface of the hind paw using an automated testing device (dynamic plantar aesthesiometer; Ugo Basile, Italy). The device lifts a straight monofilament (0.5 mm in diameter) exerting a constant upward pressure of 0.5 g (4.90 mN) onto the plantar surface and when the animal withdraws its hind paw, the mechanical stimulus automatically stops and the latency time is recorded. Latency was defined as the time from the onset of exposure to the filament to the cessation of the pressure when the sensor detected the paw withdrawal. Paw-withdrawal latencies were measured in triplicate for each animal at 30 s intervals. A cut-off latency of 60 s was used in each trial. Mice (n = 8–12 per group) received vehicle or S1RA either i.p. (16, 32 and 64 mg·kg^{-1}) or p.o. (32, 64 and 128 mg·kg^{-1}) 15 min before capsaicin injection and withdrawal latencies to mechanical stimulation were determined 15 min after c treatments).

The effect of treatments on mechanical hypersensitivity induced by capsaicin was calculated with equation 1

$$\%\ \text{reduction of mechanical hypersensitivity} = \left(\frac{LTD - LTV}{CT - LTV}\right) \times 100; \quad (1)$$

Where, LTD and LTV are the latency time in drug- and vehicle-treated animals, respectively, and CT is the cut-off time (60 s).

Formalin-induced nociceptive behaviour

A diluted formalin solution (20 μL of a 2.5% formalin solution; 0.92% of formaldehyde) was injected into the mid-plantar surface of the right hind paw of the mouse. Formalin-induced nociceptive behaviour was quantified as the time spent licking or biting the injected paw during 45 min (divided into nine periods of 5 min each) after the injection of formalin. The initial acute phase (0–5 min; phase I) was followed by a relatively short quiescent period, which was then followed by a prolonged response (15–45 min; phase II). Mice (n = 8–12 per group) received i.p. administration of vehicle or S1RA (20, 40 and 80 mg·kg^{-1}) 15 min before i.pl. formalin injection.

The antinociception induced by the treatments in the formalin test was calculated with equation 2

$$\%\ \text{antinociception} = \left(\frac{LTV - LTD}{LTV}\right) \times 100; \quad (2)$$

where, LTV and LTD represent the licking-biting time in vehicle- and drug-treated animals, respectively.

Neuropathic pain-related behaviours

Hyperalgesia to noxious thermal stimulus and allodynia to cold and mechanical stimuli were used as outcome measures

of neuropathic pain in sham and nerve-injured mice by using the plantar, cold plate and von Frey test, respectively. Animals were first habituated for 1 h to each different experimental test once daily for 4 days. After the habituation period, baseline responses were established during two consecutive days for each paradigm in the following sequence: von Frey, plantar (15 min later) and cold-plate (15 min later) test. One day after baseline measurements, sciatic nerve injury or sham operation was induced according to the protocol described above. Mice were tested in each paradigm on corresponding days (as described below), using the same experimental sequence as for baseline responses.

Mechanical allodynia was quantified by measuring the hind paw withdrawal response to von Frey filament stimulation. Briefly, animals were placed into compartment enclosures in a test chamber with a framed metal mesh floor through which the von Frey monofilaments (bending force range from 0.008 to 2 g) (North Coast Medical, Inc., Gilroy, CA, USA) were applied and thresholds were measured using the up–down paradigm. The filament of 0.4 g was used at first. Then, the strength of the next filament was decreased when the animal responded or increased when the animal did not respond. This up-down procedure was stopped four measures after the first change in animal response. The threshold of response was calculated by using the up–down Excel program generously provided by Basbaum's laboratory (UCSF, San Francisco, CA, USA). Clear paw withdrawal, shaking or licking was considered as a nociceptive-like response. Both ipsilateral and contralateral hind paws were tested.

Thermal (heat) hyperalgesia was assessed with a plantar test apparatus (Ugo Basile), by measuring hind paw withdrawal latency in response to radiant heat. Briefly, mice were placed into compartment enclosures on a glass surface. The heat source was then positioned under the plantar surface of the hind paw and activated with a light beam intensity chosen based on preliminary studies to give baseline latencies from 8 to 9 s in control mice. The digital timer connected to the heat source automatically recorded the response latency for paw withdrawal to the nearest 0.1 s. A cut-off time of 20 s was imposed to prevent tissue damage in absence of response. The mean withdrawal latencies for the ipsilateral and contralateral hind paws were determined from the average of three separate trials, done at 5 min intervals.

Cold allodynia was assessed using a hot-/cold-plate analgesia meter (Columbus, OH, USA). Briefly, mice were placed into compartment enclosures on the cold surface of the plate, which was maintained at a temperature of 5 ± 0.5°C. The number of elevations of each hind paw was then recorded for 5 min. A score was calculated by subtracting the number of elevations of the right hind paw (ipsilateral) from left hind paw (contralateral). A positive difference score indicated cold allodynia.

Acute treatment protocol design (effect on the expression of neuropathic pain). Two tests were performed: von Frey test first (30 min post-treatment) and plantar test second (45 min post-treatment). One day after baseline measurements, sciatic nerve injury or sham operation was induced and mice were tested on days 5 and 10 after the surgical procedure to monitor the development of neuropathic pain-related behaviours. On day 10, neuropathic pain-related behaviours were already apparent and mice received a vehicle injection. On days 11, 12 and 13, mice received i.p. administration of three different doses of S1RA (16, 32 and 64 mg·kg^{-1}) following a Latin square design (n = 10–12 per group). Finally, on day 14 after surgery, mice were administered with vehicle and responses were evaluated as an internal control to know if mechanical and thermal hypersensitivity were influenced by previous treatments.

Repeated (21 days) treatment protocol design (effect on the development of neuropathic pain). Three tests were sequentially performed: von Frey test first (30 min post-treatment), plantar test second (45 min post-treatment) and cold-plate test third (60 min post-treatment). One day after baseline measurements, sciatic nerve injury or sham operation was induced. Treatment twice a day (b.i.d.) (morning and afternoon) by i.p. route with S1RA at 25 mg·kg^{-1} or vehicle (n = 10–12 per group) started the day of surgery and was maintained up to day 20. The effect of treatments was evaluated on days 3, 6, 9, 12, 15 and 20 after nerve injury, starting the tests 30 min after the first daily (morning) administration. On day 22 and 25 after surgery (treatment washout), mice received vehicle and were tested again in the absence of active compound.

Ex vivo *binding*

To determine brain penetration and σ_1 receptor occupancy by S1RA, *ex vivo* binding experiments using [^{3}H](+)-pentazocine were performed in brain sections 30 min after single *in vivo* i.p. administration of vehicle or three different doses of S1RA (16, 32 and 64 mg·kg^{-1}). Briefly, mice (n = 3 per group) were given either vehicle or S1RA i.p. and 30 min later brains were quickly removed under isoflurane (3%) anaesthesia, frozen on dry ice and stored at –80°C prior sectioning. Mid-forebrain coronal sections (20 µm thick) containing the dorsal hippocampus were obtained with a cryostat (Leica Microsystems Nussloch GmbH, Nussloch, Germany), mounted onto gelatin-coated glass slices and stored at –20°C until incubation. Brain sections were incubated with 14 nM [^{3}H](+)-pentazocine (in presence or absence of 10 µM haloperidol) for 90 min at room temperature in 50 mM Tris-HCl buffer (pH = 7.4). Incubation was stopped in ice-cold Tris-HCl buffer and slides were then washed three times (10 min each) in the same buffer followed by a final quick wash in distilled water. Slides were then dried completely using a stream of fresh air. Experiments varying the assay conditions (incubation time, radioligand concentration) and emission image acquisition time (not shown) were done for setting up the method as described earlier.

Slides were made conductive by disposing a copper foil tape (3 M, Belgium) on the free side and radioactivity emission was measured using a β-imager (Biospace Lab, Paris, France). Data from brain sections were collected during 16 h using the β-Acquisition software (Biospace). The levels of bound radioactivity were directly determined by counting the number of β-particles emerging from the delineated areas by using the β-Vision+ software (Biospace). The radioligand binding signal was expressed in counts per minute per square millimetre. Brain sections incubated in absence of haloperidol were used to calculate the total binding and brain sections incubated in the presence of 10 µM haloperidol to determine the non-specific binding. Specific σ_1 receptor binding was

obtained by subtracting the corresponding non-specific binding from the total binding. Three adjacent brain sections per animal were collected per slide. Two brain slices were used to measure the total binding, and the third one for non-specific binding. *Ex vivo* receptor labelling by [^{3}H](+)-pentazocine in whole brain section autoradiograms of S1RA-treated mice was expressed as the percentage of receptor labelling respect to vehicle-treated animals. Radioligand *ex vivo*-receptor labelling is inversely proportional to the receptor occupancy by the *in vivo*-administered drug. Therefore, *in vivo*-receptor occupancy of central σ_1 receptors after peripheral administration of S1RA was determined as the percentage of inhibition of specific [^{3}H](+)-pentazocine *ex vivo* binding using equation 3

$$\%\ \text{receptor occupancy} = 100\left(\frac{SBi - Bi}{SBv - Bv}\right)\times 100 \qquad (3)$$

where SBi is the specific binding at the dose i, SBv is the specific binding from vehicle, Bi is the background binding outside section boundaries at the dose i, and Bv the background binding outside section boundaries from vehicle. Both the percentage of receptor occupancy and the percentage of effect were plotted against log dose and the sigmoid curves of best fit were calculated by nonlinear regression analysis. The ED_{50} value was then estimated from the dose–response curve.

Correlations between σ_1 receptor occupancy and antinociceptive efficacy produced by treatment with S1RA in different pain models were also made. To analyse the relationship between the binding of the compound to the receptor and the observed behavioural response, data from the dose–receptor occupancy and dose–antinociceptive effects curves were fitted by an operational model. Equation 4, derived from the operational model of pharmacological agonism (Black and Leff, 1983), was used

$$\%\ \text{antinociceptive effect} = \frac{100}{1+\left(\frac{100}{\tau\times\%\ \text{receptor occupancy}}\right)^n} \qquad (4)$$

where tau (τ), operational efficacy, is a measure of the efficiency of transduction of receptor occupancy into pharmacological effect and n provides the necessary flexibility to account for steeper or flatter curves than the rectangular hyperbola ($n = 1$).

Determination of the concentration of S1RA in plasma, forebrain and spinal cord following single or repeated administrations

Two groups of five nerve-injured mice each were treated with a single (25 mg·kg^{-1}, i.p.) or repeated dose twice daily for 12 days (25 mg·kg^{-1}, i.p., b.i.d). Acute single treatment was done on day 12 post-surgery whereas repeated treatment with S1RA started the day of surgery. Two control, non-operated groups of five mice each receiving identical treatments (single dose or repeated dose for 12 days) were also included in the study. Samples from plasma, forebrain and spinal cord were obtained 30 min following single (acute treatment) or last (repeated treatment) dose, time point at which pharmacological activity with manual von Frey filaments was assessed in the behavioural studies. Mice were anaesthetized with isoflurane (3%), exsanguinated by cardiac puncture and plasma, forebrain and spinal cord were collected.

Blood samples (c. 1 mL) were delivered into heparinized tubes, centrifuged at 2280× *g* for 10 min at 4°C and the supernatant transferred to a clean vial. Forebrain and spinal cord samples from each individual were macerated with scissors. A subsample was taken, weighed and homogenized with four volumes of methanol using an Ultra Turrax mixer (IKA-Werke GmbH & Co. KG, Staufen, Germany). Samples were then centrifuged (3000× *g* for 5 min) and the resulting supernatant transferred to a clean vial. Plasma samples and forebrain and spinal cord extracts (25 µL aliquots) were submitted to automated solvent extraction.

Concentrations of S1RA in mouse plasma, forebrain and spinal cord samples were determined by ultra performance liquid chromatography with tandem mass spectrometric detection (UPLC-MS/MS) using an Acquity UPLC (Waters Corporation, Mildford, MA, USA) coupled to an API4000 detector (Sciex, Foster City, CA, USA) with a BEH C18 2.1 × 50 mm 1.7 µm analytical column (Waters). Mobile phase consisted of a gradient of 10 mM ammonium formate + 0.2% formic acid and methanol + 0.2% formic acid and the flow rate was 0.6 mL·min^{-1}. The transitions m/z 338.2 → 114.1 and 346.3 → 122.1 were used to monitor S1RA and the corresponding internal standard ($^2H_8$)S1RA. The calibration range was 0.5–2000 ng·mL^{-1}.

Statistical analysis

Data obtained for the capsaicin, formalin and rotarod models were subjected to a one-way ANOVA followed, when significant differences were found, by *post hoc* Bonferroni's test. The neuropathic pain-related behaviours were compared on each experimental day by using a two-way ANOVA repeated measures (paw and treatment as between-factor of variation; day and surgery as within group levels) followed by corresponding one-way ANOVA and *post hoc* comparisons (Bonferroni's test) when appropriate. In electrophysiological studies, response profiles from the different treatment groups were compared by means of two-way ANOVA. Rise rate of spike counts was calculated as the maximal number of spikes minus the minimum number divided by the interval between them in seconds and non-paired Student's *t*-test was used for comparison. Two-way ANOVA followed by Bonferroni's test was used to analyse differences in the concentration of S1RA in plasma and CNS samples from control and nerve-injured mice following single and repeated administrations. Density in forebrain and spinal cord was assumed to be 1 g·mL^{-1} in order to compare ng·mL^{-1} (plasma) and ng·g^{-1} (forebrain and spinal cord). Non-parametric correlation analysis (Spearman) was applied to analyse the relation between the extent of CNS receptor occupancy and the antinociceptive effect elicited by S1RA. GraphPad Prism software (GraphPad Software Inc., La Jolla, CA, USA) was used. In all cases, the criterion for statistical significance was established when the *P* value was below 0.05.

Results

In vitro *pharmacological profile of S1RA*

S1RA behaved as a highly selective σ_1 receptor antagonist. It showed high affinity for human (K_i = 17 nM) and guinea pig

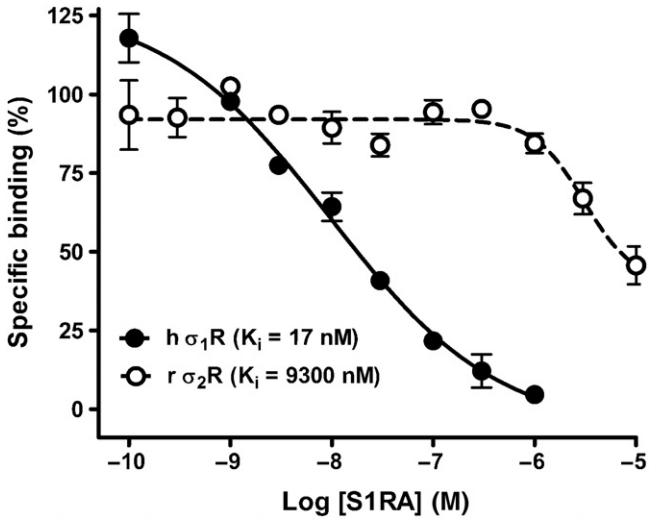


Figure 1

Binding of S1RA to σ_1 receptors (σ_1R) and σ_2 receptors (σ_2R). Equilibrium-competition binding curves of S1RA vs. [^{3}H](+)-pentazocine using Jurkat human T lymphocyte cell line (h σ_1R) and vs. [^{3}H]DTG using rat cerebral cortex (r σ_2R). Concentrations were tested in triplicate.

(K_i = 23.5 nM) σ_1 receptors but no significant affinity for the σ_2 receptors (K_i > 1000 nM for guinea pig and rat σ_2 receptors) (Figure 1). Moderate affinity (K_i = 328 nM) and antagonistic activity, with very low potency (IC_{50} = 4700 nM) was found at the human 5-HT_{2B} receptor. S1RA showed no significant affinity (K_i > 1 μM or % inhibition at 1 μM < 50%) for other additional 170 targets (receptors, transporters, ion channels and enzymes) (Table 1).

Phenytoin, a low-potency allosteric modulator of σ_1 receptors, has been shown to differentially modulate affinities of σ_1 receptor ligands depending on their agonistic or antagonistic nature (Cobos *et al.*, 2005). It shifts known σ_1 receptor agonists to significantly higher affinities (K_i ratios without phenytoin vs. with phenytoin > 1) while σ_1 receptor antagonists show no shift or a very little shift to lower affinity values (K_i ratios without phenytoin vs. with phenytoin ≤ 1). S1RA produced a small shift to lower-affinity values when incubated in the presence of phenytoin (K_i without phenytoin/K_i with phenytoin = 0.8), which indicated antagonist properties at the σ_1 receptor (Table 2). In the same conditions, the σ_1 receptor antagonists haloperidol, BD-1063 and NE-100 showed ratios ≤ 1, whereas the σ_1 receptor agonists dextromethorphan (+)SKF-10047 and PRE-084 showed shifts to higher affinity in the absence of phenytoin (ratios > 1) (Table 2).

Effects of S1RA on spinal processing of repetitive nociceptive stimulation: wind-up phenomenon

S1RA applied up to the higher concentration (30 μM) did not exert any significant effect on spikes evoked by the activation of non-nociceptive Aβ fibres (32.2 ± 12.2 mV in control vs. 30.2 ± 11.2 mV after S1RA). Similarly, the response to single stimuli at C-fibre intensity (200 μA, 200 μs) was not significantly affected by S1RA at any concentration (3, 10 and 30 μM) as spike counts were unchanged with respect to vehicle (Figure 2A). Repetitive stimulation of the dorsal root at stimulus intensities activating C-fibres (200 μA, 200 μs, 1 Hz) in vehicle-treated cords produced a typical wind-up response in ventral root recordings, which manifested as a progressive increase in action potential firing. S1RA inhibited the spinal nociceptive C-fibre–dependent wind-up phenomenon found when trains of stimuli were applied (Figure 2B, C). The compound reduced in a concentration-dependent manner the number of spikes produced in response to repetitive stimulation of nociceptive afferent fibres. The reduction by S1RA of the total spike counts and the rise rate of the response to repetitive stimulation of C-fibres was significant for the 10 and 30 μM applications. The effect was related to the presence of compound in the media as it disappeared following successive washing. As observed with S1RA, pregabalin (300 μM) did not significantly affect the response evoked by the activation of non-nociceptive Aβ fibres or by single stimuli at C-fibre intensity, but it inhibited the spinal C-fibre dependent wind-up phenomenon when trains of stimuli were applied (Figure 2C).

Effect of S1RA on rotarod

To investigate the possibility that S1RA could interfere with motor coordination and thus with the response of mice in the nociceptive and neuropathic pain-related behavioural tests, the motor performance was assessed in the rotarod test after single and repeated-dose treatment with S1RA. S1RA did not induce any significant effect on motor coordination in mice after single p.o. administration, even at the highest dose tested (160 mg·kg^{-1}). After single i.p. administration, no significant effects were found at 32 and 64 mg·kg^{-1}, but a significant reduction in the permanence time on the rotating rod was observed at 128 mg·kg^{-1} at 30 (maximum effect) and 60 min reading times (Figure 3A). In the repeated treatment, using the same administration protocol and dose used in the partial sciatic nerve ligation study (25 mg·kg^{-1} i.p. twice daily for 21 days), no significant effect of S1RA was observed (data not shown). Moreover, no other treatment-related adverse effects were recorded. Altogether, the antinociceptive activity and the effects of S1RA on neuropathic pain-related behaviours (see later) cannot be attributed to effects on motor coordination (no effects were found in the rotarod test at the same doses and routes of administration).

Effect of S1RA on nociceptive models involving chemical (capsaicin and formalin) sensitization

No effects were elicited by S1RA in mice injected i.pl. with vehicle. However, S1RA, given either i.p or p.o. at doses devoid of effects in the rotarod test, dose-dependently reversed capsaicin-induced mechanical hypersensitivity in mice. The efficacy was similar when comparing both routes of administration, but the potency was higher by the i.p. route (Figure 3B). Similarly, S1RA given i.p. exerted a clear dose-dependent analgesic effect on both phase I and phase II of formalin-evoked nociceptive behaviours, the efficacy and potency being similar in both phases (Figure 3C).

Table 1

Binding profile of S1RA

Receptor	Affinity [K_i (nM)]
σ_1(h)	17
σ_1 (gp)	23.5
σ_2 (r)	9300
σ_2 (gp)	>1000
5-HT_{2B} (h)	328
	Antagonist (IC_{50} = 4700 nM)
Other receptors, ion channels and enzymes[a]	n.s.

h, human; gp, guinea pig; r, rat; n.s., not significant.
K_i > 1 μM or % inhibition at 1 μM < 50%.
[a]Commercial *in vitro* pharmacology screening package including the following assays for 170 additional targets:
CEREP: 5-HT receptors, 5-HT_{1D}(r), 5-HT_{2A}(h), 5-HT_{4e}(h), 5-HT_7(h).
MDS Pharma Services (Ricerca Biosciences): adenosine receptors, A_1(h), A_{2A}(h), A_3(h); adrenoceptors α_{1A}(r), α_{1B}(r), α_{1D}(h), α_{2A}(h), α_{2C}(h), β_1(h), β_2(h), β_3(h); adrenomedullin receptors, AM_1(h), AM_2(h); aldosterone(r); anaphylatoxin C5a(h); androgen (testosterone) AR(r); angiotensin AT_1(h), AT_2(h); apelin APJ(h); atrial natriuretic factor (ANF)(gp); bombesin BB_1(h), BB_2(h), BB_3(h); bradykinin B_1(h), B_2(h); calcitonin(h); calcitonin gene-related peptide CGRP(h); calcium channel L-type, benzothiazepine(r), L-type, dihydropyridine(r), L-type, phenylalkylamine(r), N-type(r); cannabinoid CB_1(h), CB_2(h); chemokine CCR1(h), CCR2B(h), CCR4(h), CCR5(h), CX3CR1(h), CXCR2 (IL-8RB)(h); cholecystokinin CCK_1 (CCKA)(h), CCK_2 (CCKB)(h); acetylcholinesterase (ACES)(h); butyrylcholinesterase (CHLE)(h); colchicine(r); corticotropin releasing factor (CRF_1) (h); dopamine D_1(h), D_{2S}(h), D_3(h), $D_{4.2}$(h), D_5(h); endothelin ET_A(h), ET_B(h); epidermal growth factor (EGF)(h); erythropoietin EPOR(h); estrogen ERα(h), ERβ(h); G protein-coupled receptor GPR103(h), GPR8(h); $GABA_A$, chloride channel TBOB(r), flunitrazepam central(r), muscimol central(r), $GABA_{B1(a)}$(h), $GABA_{B1(b)}$(h); gabapentin(r); galanin GAL_1(h), GAL_2(h); glucocorticoid(h); glutamate AMPA(r), kainate(r), NMDA, agonism(r), NMDA, glycine(r), NMDA, phencyclidine(r), NMDA, polyamine(r), glycine, strychnine-sensitive(r); growth hormone secretagogue (GHS, ghrelin)(h); histamine H_1(h), H_2(h), H_3(h), H_4(h); imidazoline I2, central(r); inositol trisphosphate IP_3(r); insulin(r); IL-1(m), IL-2(m), IL-6(h); leptin(m); leukotriene BLT_1 (LTB_4)(h), cysteinyl $CysLT_1$(h), $CysLT_2$(h); melanocortin MC_1(h), MC_3(h), MC_4(h), MC5(h); melatonin MT_1(h), MT_2(h); motilin(h); muscarinic M_1(h), M_2(h), M_3(h), M_4(h), M_5(h); neuromedin U, NMU1(h), NMU2(h); neuropeptide Y, Y_1(h), Y_2(h); neurotensin NTS_1(h); N-formyl peptide receptor FPR1(h), peptide receptor-like FPRL1(h); nicotinic acetylcholine(h), acetylcholine α1, bungarotoxin(h), acetylcholine α7, bungarotoxin(r); opiate δ (OP_1, DOP)(h), κ (OP_2, KOP)(h), μ (OP_3, MOP)(h), orphanin ORL1(h); peroxisome proliferator-activated receptor PPARα(h), PPARγ(h); phorbol ester(m); platelet-activating factor (PAF)(h); platelet-derived growth factor (PDGF)(m); potassium channel [KA](r), [K_{ATP}](ham), [SKCA](r), hERG(h); progesterone PR-B(h); prostanoid CRTh2 (h), DP(h), EP_2(h), EP4(h), thromboxane A_2 (TP)(h); purinergic P2X(rb), P2Y(r); retinoid X-receptor RXRα(h); rolipram(r); ryanodine RyR3(r); 5-HT receptors, 5-HT_{1A}(h), 5-HT_{1B}(r), 5-HT_{2C}(h), 5-HT_3(h), 5-HT_4(gp), 5-HT_{5A}(h), 5-HT_6(h); sodium channel, site 2(r); somatostatin sst_1(h), sst_2(h), sst_3(h), sst_4(h), sst_5(h); tachykinin NK_1(h), NK_2(h), NK_3(h); thyroid hormone(r); thyrotropin-releasing hormone (TRH)(r); TGF-β(m); transporter, adenosine(gp), choline(r), dopamine (DAT)(h), GABA(r), monoamine(rb), noradrenaline (NET)(h), 5-HT (SERT)(h); TNF, non-selective(h); urotensin II(h); vanilloid(r); VEGF(h); vasoactive intestinal peptide VIP_1(h); vasopressin V_{1A}(h), V_{1B}(h), V_2(h); vitamin D3(h).
(h), human; (ham), hamster; (r), rat; (m), mouse; (rb), rabbit.

Table 2

Ratio of K_i values with or without phenytoin

	Ratio K_i Without/with phenytoin
Dextromethorphan	20
(+)SKF-10047	3.6
PRE-084	3.6
NE-100	1.0
BD-1063	0.8
Haloperidol	0.6
S1RA	0.8

Effect of S1RA on the expression of neuropathic pain-related behaviours (acute treatment)

Partial sciatic nerve ligation induced mechanical allodynia and thermal (heat) hyperalgesia. Mechanical allodynia was shown by a reduced pressure threshold evoking withdrawal of the ipsilateral hind paw on day 5 and 10 post-surgery compared with baseline pre-surgery values (Figure 4A). In turn, thermal hyperalgesia was shown by a decreased withdrawal latency of the ipsilateral hind paw in response to a thermal stimulus on day 5 and 10 post-surgery compared with baseline pre-surgery values (Figure 4A). Neither mechanical allodynia nor thermal hyperalgesia developed in the contralateral paw (data not shown). Similarly, sham operation did not induce mechanical hypersensitivity or thermal hyperalgesia (Figure 4B) as no significant changes of

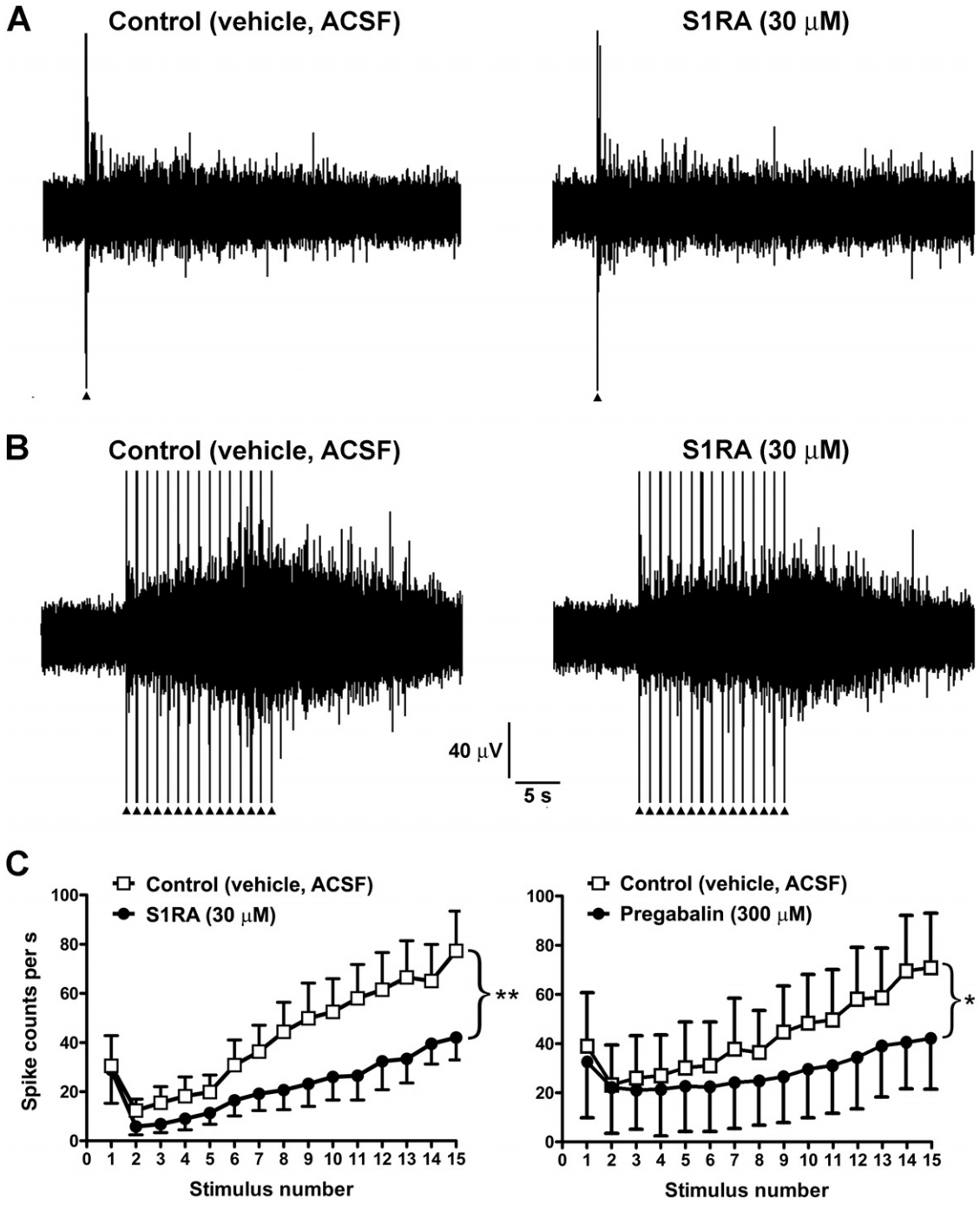


Figure 2

Effect of S1RA on electrophysiological recordings of spinal cord responses upon application of electrical C-fibre intensity stimuli. Original AC recordings of responses to a single C-fibre intensity stimulus (200 μA, 200 μs) (A) and to a train of C-fibre intensity stimuli applied at 1 Hz (B) obtained from spinal cords superfused with vehicle (left) or S1RA at 30 μM (right). Stimulation was applied to the dorsal root of the lumbar spinal cord (L4 or L5) and responses were recorded at the ventral root. Vertical lines at regular intervals correspond to stimuli artefacts whereas response action potentials (spikes) are viewed as a thickening of the baseline noise. Note that the response to single C-fibre intensity stimulus (A) was not modified whereas the wind-up response to repetitive C-fibre stimulation (B), which manifested as a progressive increase in action potential firing, was attenuated in cords superfused with S1RA respect to vehicle (control). Graphs show the number of spikes evoked by each of the 15 consecutive C-fibre intensity stimuli of the train applied at 1 Hz (200 μA, 200 μs, 1 Hz) in control (vehicle) and after superfusion of S1RA at 30 μM or pregabalin at 300 μM (C). Note that the wind-up response (progressive increase in spike counts evoked by repetitive stimulation of C-fibres) was attenuated in drug-treated compared with vehicle-treated cords. Data obtained from the average of five to seven spinal cord preparations per treatment group. *$P < 0.05$; **$P < 0.01$, significantly different from vehicle treatment; ANOVA.

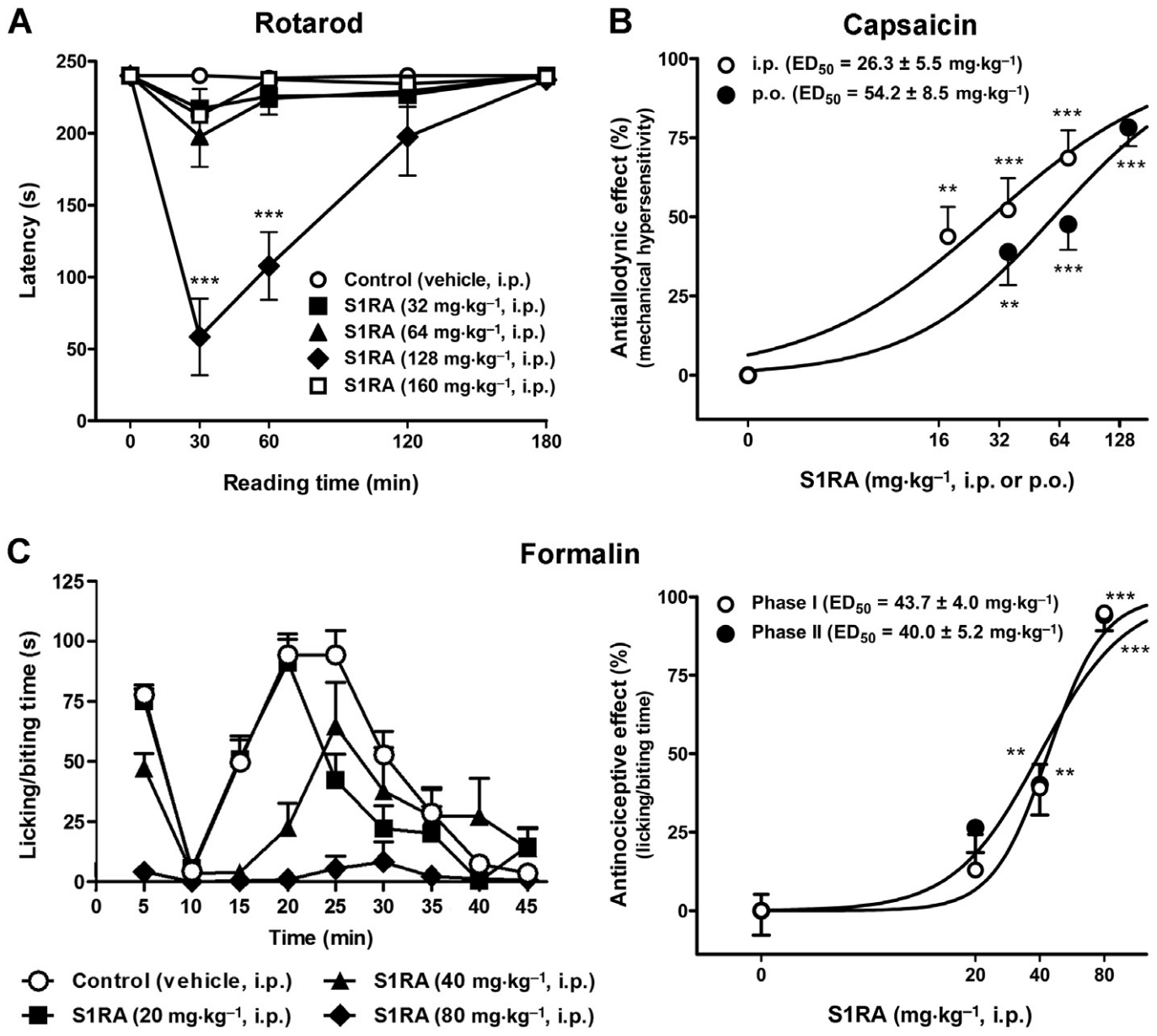


Figure 3

Dose–response effect of S1RA on the rotarod, capsaicin-induced mechanical hypersensitivity and formalin (phase I and II) tests. In the rotarod test (A), the permanence time on the rotating rod (latency) was measured up to 180 min after i.p. and p.o. treatment. Note that S1RA had no effect after p.o. administration at 160 mg·kg^{-1}, and that a significant reduction in latency was only observed when administered at 128 mg·kg^{-1} by the i.p. route. In the capsaicin model (B) treatments were administered i.p. or p.o. 30 min before the test (i.e. 15 min before i.pl. capsaicin injection). Note that S1RA dose-dependently inhibited capsaicin-induced mechanical hypersensitivity, the potency being higher by the i.p. than by the p.o. route. To study the effect on formalin-evoked nociception (C), treatments were administered i.p.15 min before i.pl. formalin injection and the time spent licking or biting the injected paw was recorded. Both the time-course (left panel) and the percent antinociceptive effect (right panel) at 0–5 min (phase I) and 15–30 min (phase II) after the injection of formalin were shown. Note that S1RA dose-dependently inhibited the behavioural response to formalin, efficacy and potency being similar in both phases. Data, obtained from seven to 10 (rotarod) and eight to 12 (capsaicin and formalin) mice per group, are presented as the mean ± SEM latency (A) or percentage of maximum possible effect (B and C). $^{**}P < 0.01$, $^{***}P < 0.001$, significantly different from corresponding vehicle group (dose 0 in B and C); ANOVA followed by Bonferroni's test.

the response to mechanical and thermal stimulation were observed in sham-operated mice after surgery compared with baseline pre-surgery values.

Systemic treatment with S1RA (16, 32 and 64 mg·kg^{-1}, i.p.) on days 11–13 post-surgery dose-dependently inhibited both mechanical allodynia and thermal (heat) hyperalgesia after partial sciatic nerve ligation in the ipsilateral paw (Figure 4A). Post-treatment values on day 14 (1 day after treatments) were not significantly different from the pre-treatment post-surgery values on day 10, indicating that the

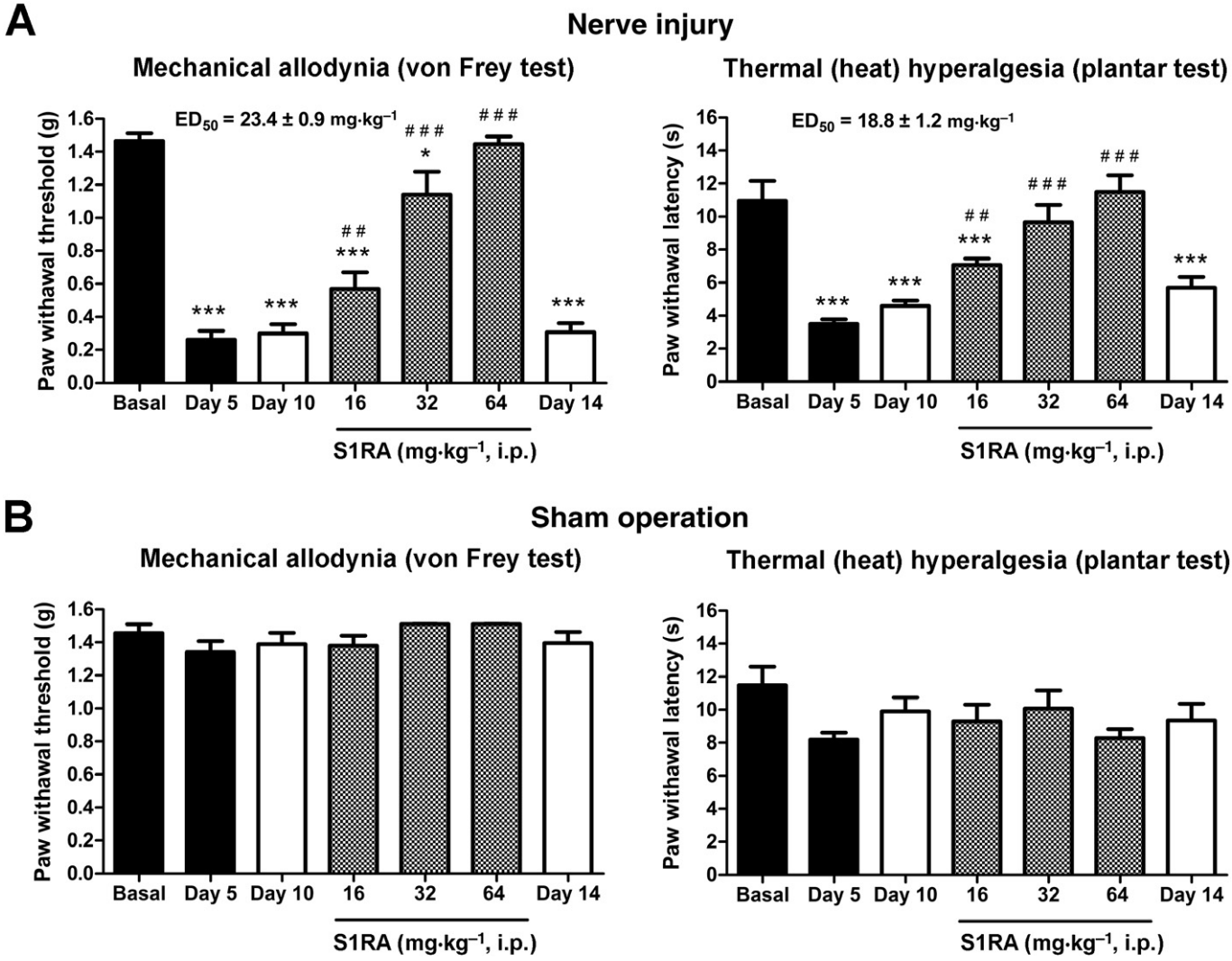


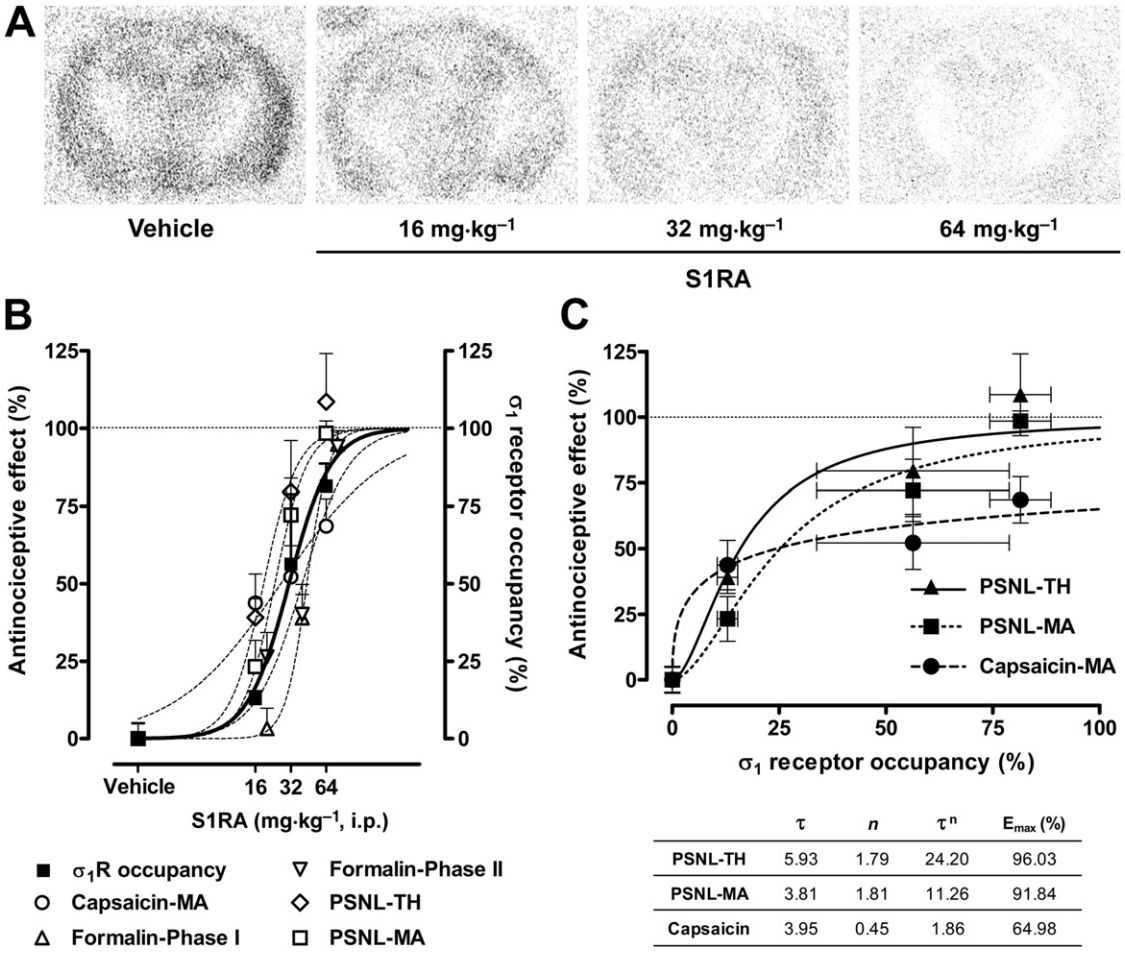
Figure 4

Dose–response effect of acute treatment with S1RA on the expression of neuropathic pain in nerve-injured and sham-operated mice. Pressure thresholds evoking withdrawal (left panel) and latency to withdrawal (right panel) of the ipsilateral paw in response to mechanical von Frey filament and thermal (heat) stimulation, respectively, were evaluated in nerve-injured (A) and sham-operated (B) mice before surgery (Basal), on day 5 after surgery, on day 10 post-surgery following vehicle treatment, on days 11–13 post-surgery 30 min (von Frey test) and 45 min (plantar test) after treatment with three different doses of S1RA, and finally on day 14 post-surgery after treatment with vehicle. Note that acute, single treatment with S1RA dose-dependently reversed both mechanical allodynia and thermal (heat) hyperalgesia after partial sciatic nerve ligation in the ipsilateral paw. Note also that post-treatment values on day 14 (one day after treatments) were not significantly different from pre-treatment post-surgery values on day 10. Sham operation did not induce significant changes in mechanical and thermal sensitivity respect to basal pre-surgery values. S1RA did not significantly modified mechanical and thermal sensitivity in sham-operated mice. Data obtained from 10 to 12 mice per group and expressed as mean ± SEM pressure threshold (g) evoking paw withdrawal or latency (s) to paw withdrawal. $^{*}P < 0.05$; $^{***}P < 0.001$, significantly different from corresponding basal pre-surgery values. $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, significantly different from vehicle treatment on day 10; ANOVA with Bonferroni's test.

effect of the drug treatment was reversible. S1RA did not produce significant effects on mechanical and thermal sensitivity in the contralateral paw of nerve-injured mice (data not shown) or in sham-operated mice (Figure 4B).

Brain penetration and σ_1 receptor occupancy: correlation with antinociceptive activity

To investigate if S1RA actually penetrates into the brain and binds to σ_1 receptors when administered systemically, and to study the relationship of its antinociceptive activity with σ_1 receptor occupancy in the CNS, *ex vivo* binding experiments were performed. In these experiments, σ_1 receptor occupancy in the CNS, measured as inhibition of [^{3}H](+)-pentazocine *ex vivo* binding on brain sections, was determined 30 min after the single *in vivo* i.p. administration of vehicle or S1RA at 16, 32 and 64 mg·kg^{-1} (the same time point, route of administration and doses used to determine pharmacodynamic efficacy in behavioural studies).



	τ	*n*	τ^n	E_{max} (%)
PSNL-TH	5.93	1.79	24.20	96.03
PSNL-MA	3.81	1.81	11.26	91.84
Capsaicin	3.95	0.45	1.86	64.98

Figure 5

Dose-dependent σ_1 receptor occupancy and correlation with antinociceptive activity. Receptor occupancy, measured as inhibition of [^{3}H](+)-pentazocine *ex vivo* binding on brain section autoradiograms, was determined 30 min after single *in vivo* i.p. administration of vehicle or S1RA at 16, 32 and 64 mg·kg^{-1} (A). Note that receptor occupancy was visually apparent in sections from mice treated with S1RA. When quantified, a typical dose-dependent occupancy of σ_1 receptors by S1RA was obtained (ED_{50} = 31.7 ± 4.4 mg·kg^{-1}) (B). Similar sigmoid log dose–effect curves with significant correlation (Spearman, $P < 0.001$) between the extent of CNS receptor occupancy and the antinociceptive effect elicited by S1RA were found: partial sciatic nerve ligation-induced thermal hyperalgesia (PSNL-TH; $r = 0.899$) and mechanical allodynia (PSNL-MA; $r = 0.888$) as well as capsaicin-induced mechanical allodynia (capsaicin-MA; $r = 0.739$). An operational model for the transduction of receptor occupancy into antinociceptive effect in the nerve-injury and capsaicin models was generated (C). Experimental mean values and theoretical curves were represented. Tau (τ; a measure of the transduction of occupancy into antinociceptive effect) and *n* (a measure of the shape of the curve) parameter values from the curve fittings and the calculated asymptotic maximum % antinociceptive effect ($E_{max} = \frac{100}{1+\frac{1}{\tau^n}}$) when the % of receptor occupancy = 100 are shown.

Specific [^{3}H](+)-pentazocine *ex vivo* radiolabelling was found in various brain areas, including the hippocampus, cerebral cortex, amygdala and hypothalamus of vehicle-treated mice (Figure 6A). This regional brain distribution matches that previously reported by immunohistochemical localization of σ_1 receptors in brain (Alonso *et al.*, 2000).

Receptor occupancy (i.e. inhibition of (^{3}H)(+)-pentazocine *ex vivo* binding) was apparent in sections from mice treated *in vivo* with S1RA (Figure 5A), and increased with increased dose as expected from a competitive binding to the same receptor. This supports that S1RA administered systemically has access and binds to brain σ_1 receptors.

A typical dose-dependent occupancy of σ_1 receptors by S1RA was obtained (ED_{50} = 31.7 ± 4.4 mg·kg^{-1}). Plotting the percent σ_1 receptor occupancy and the percent antinociceptive effect in the capsaicin, formalin and nerve-injury models against dose revealed similar sigmoid log dose-effect curves (Figure 5B). Indeed, a significant correlation between the extent of CNS receptor occupancy and the antinociceptive effect elicited by S1RA in the different pain models was found when non-parametric correlation tests (Spearman) were applied.

An operational model for the transduction of receptor occupancy into antinociceptive effect in the capsaicin and

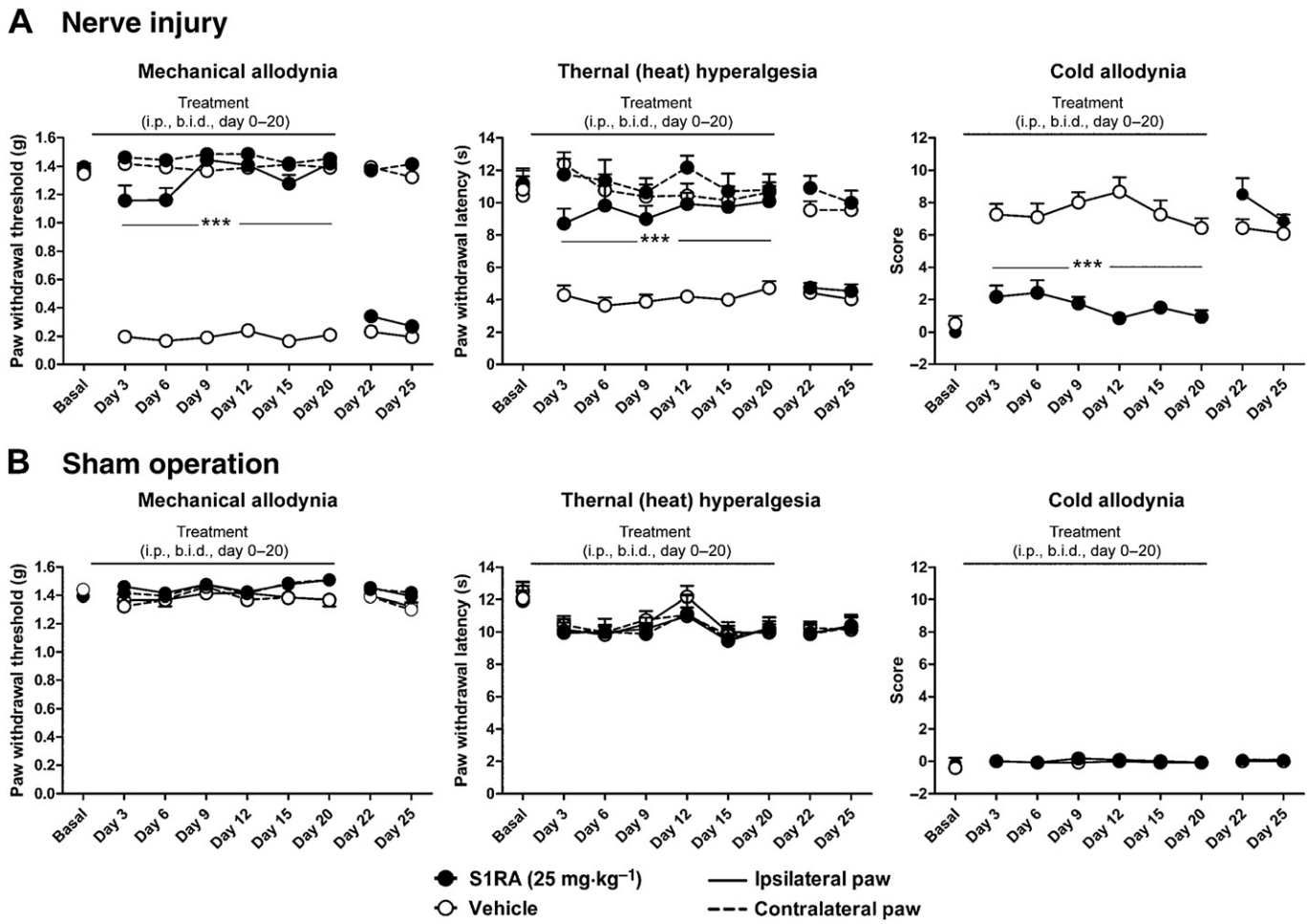


Figure 6

Effect of repeated treatment with S1RA on the development of neuropathic pain. The response to mechanical and thermal (heat and cold) stimulation of the ipsilateral and contralateral paws of nerve-injured (A) and sham-operated (B) mice was evaluated before surgery (Basal), on days 3, 6, 9, 12, 15 and 20 post-surgery following repeated treatment (21 days i.p. twice daily with either vehicle or S1RA at 25 mg·kg^{-1}) and after treatment discontinuation on days 22 and 25 post-surgery. Note that mechanical and thermal hypersensitivity developed in the ipsilateral (but not in the contralateral) paw of nerve-injured (but not in sham-operated) mice as compared with basal pre-surgery values. Nerve injury-induced mechanical and thermal hypersensitivity of the ipsilateral paw were inhibited by repeated administration of S1RA, but no effects were exerted by the compound on the mechanical and thermal sensitivity of the contralateral paw and in both paws of sham-operated mice. Note that no tolerance to its antiallodynic and antihyperalgesic effects was seen as its efficacy increased overtime with the succession of administrations, maximum activity being attained from day 9 to 12 of treatment in all the tests. Note also that the antiallodynic and antihyperalgesic effects exerted by S1RA during the treatment period (up to day 20) disappeared after treatment discontinuation (day 22 and 25). Data obtained from 10 to 12 mice per group and expressed as mean ± SEM. ***$P < 0.001$, significantly different from corresponding vehicle treatment group; ANOVA followed by Bonferroni's test.

nerve-injury models was generated (Eq. 4; Figure 5C). Experimental mean values and theoretical curves did not exactly match, as expected by the required transduction steps between the occupancy of the receptor and the physiological effects. Thus, although a simple logistic equation such as equation 4 did not yield a perfect fit to the data, the model resembles the data closely enough to provide a description of the relationship between binding and functional processes in qualitative and quantitative terms. Theoretical curves distinguish between low- and high-receptor occupancy. At very low-receptor occupancy, the highest effects corresponded to capsaicin; however, because of the very low *n* value, the opposite occurs at higher concentrations in which capsaicin yielded the lowest maximum response.

Effect of S1RA on the development of neuropathic pain-related behaviours (repeated 21 day treatment)

Partial sciatic nerve ligation resulted in mechanical and thermal (heat and cold) hypersensitivity in the ipsilateral, nerve-injured hind paw from day 3 to 25 post-surgery compared with baseline pre-surgery values in vehicle-treated mice (Figure 6A). Mechanical allodynia was demonstrated by a

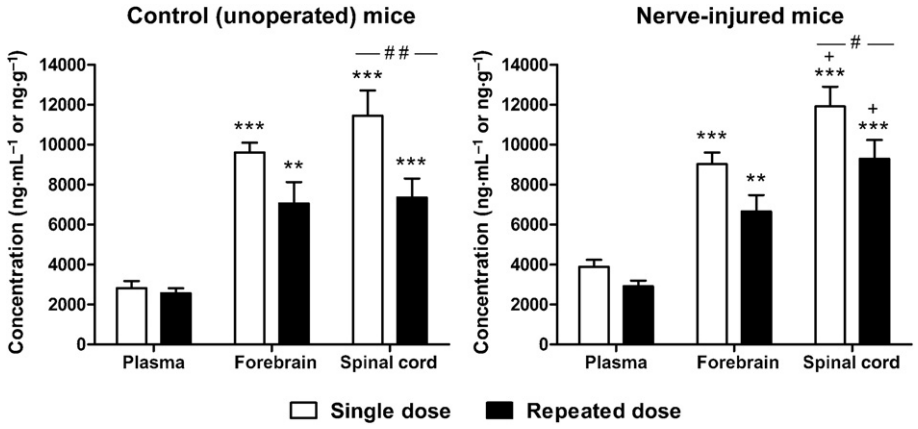


Figure 7

Concentrations of S1RA in plasma, forebrain and spinal cord following a single or repeated administration. Concentrations of S1RA in plasma, forebrain and spinal cord was measured in control non-operated and nerve-injured mice receiving a single or repeated (twice daily for 12 days) i.p. administration of S1RA at 25 mg·kg^{-1}. Single (acute) treatment was done on day 12 post-surgery whereas repeated treatment with S1RA started the day of surgery. Tissue/organ samples for analysis were obtained 30 min after single (acute) or last (repeated) dosage, time point at which pharmacodynamic efficacy was assessed in the behavioural studies. No significant differences in S1RA concentration were found in plasma and CNS samples between nerve-injured and non-operated control mice, but S1RA concentrations were higher in brain and spinal cord than in plasma, both in the single- and repeated-dose regimens. Note that S1RA concentrations in these tissues/organs were similar or even higher (spinal cord) in mice exposed to single than to 12 days repeated, twice daily dosing. Data obtained from five mice per group and expressed as mean ± SEM ng·mL^{-1} (plasma) or ng·g^{-1} (forebrain and spinal cord). $^{**}P < 0.01$, $^{***}P < 0.001$, significantly different from corresponding plasma values. $^{+}P < 0.05$, spinal cord significantly different from corresponding forebrain values. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ single significantly different from corresponding repeated-dose values; ANOVA followed by Bonferroni's test.

reduced pressure threshold evoking withdrawal of the right, ipsilateral hind paw; thermal hyperalgesia by a decreased withdrawal latency of the ipsilateral hind paw in response to heat; and cold allodynia by an increased number of elevations of the ipsilateral hind paw with respect to the contralateral hind paw in response to cold. Neuropathic pain-related behaviours did not develop in the left hind paw (contralateral) of mice exposed to the sciatic nerve injury (Figure 7A) and, similarly, no significant changes of the response to mechanical and thermal stimulation were found in sham-operated mice up to 25 days after surgery compared with baseline pre-surgery values (Figure 6B).

Mechanical allodynia, thermal (heat) hyperalgesia and thermal (cold) allodynia induced by the sciatic nerve ligation in the ipsilateral paw were suppressed by the repeated (21 days) administration of S1RA at 25 mg·kg^{-1}. The antiallodynic and antihyperalgesic effects were already significant (compared with vehicle treatment) from day 3 of administration (first day evaluated) and were maintained throughout the treatment period (Figure 6A). No pharmacodynamic tolerance to the analgesic (i.e. antiallodynic and antihyperalgesic) effect was observed following repeated systemic administration of the selective σ_1 receptor antagonist S1RA twice daily for 21 days. On the contrary, efficacy increased overtime with the succession of repeated administrations and maximum activity in all tests, indistinguishable from baseline pre-surgery values, was attained from day 9 to 12 of treatment (Figure 6A). Note that the dose of 25 mg·kg^{-1} chosen for the repeated-dose study was just about the ED_{50} obtained for mechanical allodynia in the acute, single-dose study (it corresponded to 56% antiallodynic effect based on the dose–response curve).

The antiallodynic and antihyperalgesic effects exerted by S1RA during the treatment period (up to day 20) disappeared after treatment discontinuation: allodynia and hyperalgesia values on day 22 and 25 in the ipsilateral paw were undistinguishable from those in the vehicle-treated group (Figure 6A). No significant effects on mechanical and thermal sensitivity were elicited by repeated treatment with S1RA either in the contralateral paw of nerve-injured mice or in sham-operated mice (Figure 6B).

Concentration of S1RA in plasma, brain and spinal cord after the single and repeated administration

To investigate the possibility that the increased efficacy seen over time following repeated dosing could be caused by an increase of concentration of S1RA at the time of the test in plasma, forebrain and spinal cord, samples from mice, single and multiple dosed, were analysed by UPLC-MS/MS. Control (non-operated) and nerve-injured mice received a single or repeated (twice daily for 12 days) i.p. administration of S1RA at 25 mg·kg^{-1}, the same dose used for the assessment of behavioural hypersensitivity in the chronic treatment study. Acute treatment was given on day 12 post-surgery and repeated treatment with S1RA started the day of surgery, as in the behavioural studies. Samples from plasma, forebrain and spinal cord were obtained 30 min after the single (acute) or last (repeated) dosage, time point at which pharmacodynamic efficacy was assessed in the behavioural studies.

No significant differences in S1RA concentration were found in plasma and CNS samples when comparing nerve-injured and non-operated control mice after the single- and repeated-dose regimens (Figure 7), suggesting that neither

surgery for partial ligation of the sciatic nerve nor subsequent pain have an effect on the levels of S1RA at 30 min post-dose.

S1RA concentrations were higher in brain and spinal cord ($ng \cdot g^{-1}$) than in plasma ($ng \cdot mL^{-1}$), both in the single- and repeated-dose regimens, as expected from a rapid CNS distribution following the i.p. administration but a higher drug metabolism/clearance in plasma than in CNS. Interestingly, S1RA concentrations in plasma, forebrain and spinal cord were similar or slightly higher following the single than the repeated regimen (Figure 7), probably because of variability secondary to the fast absorption and disposition observed in mice in previous pharmacokinetic studies (t_{max} = 0.25 h and $t_{1/2}$ = 1.4 h following single i.p. administration to male mice; data not shown). Slight changes in the absorption, disposition or clearance between single and multiple administrations around the maximum plasma concentration could also contribute to the differences. In any case, the increased efficacy over time observed with the succession of repeated administrations was not due to an increase in the S1RA concentration in plasma, forebrain or spinal cord but rather reflected a sustained pharmacodynamic effect.

Discussion

In the present study, a novel CNS penetrant selective σ_1 receptor antagonist, S1RA, was used to examine the effect of pharmacological antagonism on σ_1 receptors on behavioural nociception in sensitizing conditions. Formalin-induced nociception, capsaicin-induced mechanical hypersensitivity and nerve injury-induced mechanical and thermal hypersensitivity were dose-dependently inhibited by acute systemic administration of S1RA. Electrophysiological data point to a modulatory effect of S1RA on spinal hyperexcitability arising from repetitive nociceptive stimulation, such as that expected to follow nerve injury, capsaicin or formalin sensitization.

Data from radioligand binding assays showed that S1RA binds to σ_1 receptors with high affinity (K_i = 17 nM) and behaves as an antagonist at these receptors. It was selective for σ_1 receptors compared with σ_2 receptors and a panel of other 170 receptors, enzymes, transporters and ion channels except for the 5-HT_{2B} receptor, where it acts as a low-potency antagonist (IC_{50} = 4700 nM). It crosses the blood–brain barrier and binds to σ_1 receptors in the CNS. Altogether, this makes S1RA suitable to selectively antagonize the σ_1 receptors and to investigate the role of these receptors in nociception.

Responses to mechanical and thermal stimuli in sham-operated mice remained unchanged after surgery. S1RA had no effect on the responses in sham-operated mice after either acute or chronic treatment. Similarly, no effects were elicited by S1RA on mechanical sensitivity in mice i.pl. injected with vehicle (in the absence of capsaicin), suggesting that normal transduction, transmission and perception of sensory and nociceptive inputs remain intact following pharmacological antagonism of σ_1 receptors. Indeed, responses of σ_1 receptor-deficient mice to mechanical and thermal stimuli were found to be undistinguishable from those of wild-type mice (Entrena *et al.*, 2009b; de la Puente *et al.*, 2009). This is in agreement with the observation that σ_1 receptor ligands do not normally exert any effect by themselves in physiological conditions (Su and Hayashi, 2003; Su *et al.*, 2010). However, following sensitization with capsaicin or formalin, and after sciatic nerve injury, when hypersensitivity is induced, the outcome was clearly different. S1RA dose-dependently inhibited capsaicin-induced mechanical hypersensitivity, phase I and II of formalin-evoked nociceptive behaviours, and both mechanical and thermal hypersensitivity secondary to partial sciatic nerve ligation. These effects of S1RA are consistent with previous findings showing reduced nociceptive behaviours in mice lacking σ_1 receptors when tested in the same animal models (Cendán *et al.*, 2005b; Entrena *et al.*, 2009b; de la Puente *et al.*, 2009). Overall, we concluded that antagonism of σ_1 receptors did not result in a pure analgesic effect as normal mechanical and thermal sensitivity thresholds were not modified; and that, under sensitizing pain conditions (i.e. nerve injury), the effect of antagonizing σ_1 receptors was antiallodynic and antihyperalgesic, enabling the reversal of diminished nociceptive thresholds back to normal levels.

No pharmacodynamic tolerance was observed following repeated systemic administration of the selective σ_1 receptor antagonist S1RA twice daily for 21 days to nerve-injured mice. On the contrary, efficacy slightly increased over time with the succession of repeated administrations. Maximum activity (100%) in all tests (mechanical and cold allodynia and heat hyperalgesia), indistinguishable from baseline pre-surgery values, was attained from day 12 of treatment using a dose (25 $mg \cdot kg^{-1}$) that was just about the ED_{50} (i.e. 56% efficacy) for mechanical allodynia in the acute dose study. An increase of the S1RA concentration was not responsible for this increased efficacy, as concentrations of S1RA in plasma, forebrain and spinal cord were similar or slightly greater following single than repeated 12 day administrations. In contrast, upregulation of σ_1 receptors following nerve injury could increase the chance of the drug to exert its effect and thus the increased drug effectiveness over time. The two available studies at this regard support this possibility: up-regulation of σ_1 receptor expression in the dorsal horn of the spinal cord has been reported in neuropathic pain conditions subsequent to chronic constriction of the sciatic nerve (Roh *et al.*, 2008) and chronic compression of the dorsal root ganglion (Son and Kwon, 2010) in rats. An alternative explanation could be a 'disease modification' effect associated with an attenuation of the plastic changes (e.g. central sensitization) following nerve injury. However, if it does occur, it is short-lasting or requires continuous σ_1 receptor antagonism as neuropathic pain-related behaviours returned to baseline nerve injury values found in vehicle-treated mice, 2 days after treatment discontinuation. This suggests instead an improvement of 'disease symptoms' related to the presence and influence of the drug at the time of the test.

Based on UPLC-MS/MS analysis and autoradiographic *ex vivo* binding assays we here have shown that, when administered systemically, S1RA had access and bound to CNS σ_1 receptors. Interestingly, a close correspondence (significant correlation) was found between the extent of CNS receptor occupancy and the antinociceptive efficacy exerted by S1RA on the different pain models, as revealed also when an operational model was applied to transduce receptor occupancy into antinociceptive effect. Regarding the site of action, intrathecal (i.t.) administration of σ_1 receptor agonists has

been shown to enhance NMDA-induced (Kim *et al.*, 2008), formalin-induced (Kim *et al.*, 2006), and nerve injury-induced (Roh *et al.*, 2008) nociceptive behaviours. Actually, activation of spinal σ_1 receptors by single i.t. administration of a σ_1 receptor agonist to normal, naïve mice is enough to induce increased paw withdrawal responses to mechanical and thermal stimuli (Roh *et al.*, 2010). Thus, it is clear that the σ_1 receptors modulated nociceptive signalling at the spinal cord level, where they are expressed abundantly in the two superficial laminae, in dendritic processes and neuronal perikarya (Alonso *et al.*, 2000).

To investigate if pharmacological antagonism of σ_1 receptors actually modulates spinal excitability, isolated mice spinal cords were superfused with S1RA and responses to electrical stimulation of the lumbar dorsal root were recorded. S1RA did not modify the Aβ-fibre-mediated non-nociceptive signalling and the response to single stimuli at C-fibre intensity, which is consistent with the behavioural observation that S1RA did not alter the normal response to sensory and nociceptive inputs in non-sensitizing conditions. However, S1RA inhibited the wind-up phenomenon found when trains of stimuli at intensities activating C-fibres were applied. The observation that the wind-up response is attenuated in mice lacking σ_1 receptors (de la Puente *et al.*, 2009) supports the pharmacological finding herein. Wind-up can be defined as an increase in the excitability of spinal cord neurons evoked by repetitive stimulation of afferent C-fibres, and stands for a spinal amplification of the message coming from peripheral nociceptors (Dickenson and Sullivan, 1987; Herrero *et al.*, 2000). Hence, electrophysiological data point to a modulatory role of σ_1 receptors on spinal excitability, whereby pharmacological antagonism inhibits the amplified spinal response that would normally arise from repetitive nociceptor stimulation. As sustained, repetitive afferent drive comes to the spinal cord following nerve injury, capsaicin or formalin injection, inhibition of spinal hyperexcitability could underlie the effects exerted by S1RA on the earlier-mentioned pain models.

Increased signalling through the glutamatergic NMDA receptor on dorsal horn neurons is a key mediator of spinal wind-up (Woolf and Thompson, 1991; Herrero *et al.*, 2000), and account for sensitization and pain hypersensitivity (Latremoliere and Woolf, 2009). Activation of the NMDA receptor produces influx of Ca^{2+} and thus increased cytosolic concentration of Ca^{2+} in dorsal horn neurons. In turn, intracellular Ca^{2+} activates Ca^{2+}-dependent second messengers including protein kinase C and other calcium/calmodulin-dependent kinases that are ultimately responsible for the plastic changes underlying central (spinal) sensitization and pain hypersensitivity (Kawasaki *et al.*, 2004; Latremoliere and Woolf, 2009). Ligands of σ_1 receptors are postsynaptic regulators of NMDA receptor-mediated synaptic transmission. Activation of σ_1 receptors enhanced the NMDA receptor-mediated rise in cytosolic Ca^{2+} concentration and currents (Monnet *et al.*, 1990; Bergeron *et al.*, 1996), as well as NMDA-dependent central sensitization/synaptic plasticity phenomena (i.e. long-term potentiation) (Martina *et al.*, 2007). In contrast, the NMDA receptor-mediated responses are inhibited and the enhancing effects of σ_1 receptor agonists on NMDA receptors blocked by antagonizing σ_1 receptors (Monnet *et al.*, 1990; Hayashi *et al.*, 1995; Martina *et al.*, 2007). Moreover, the NMDA receptor NR1 subunit is phosphorylated in dorsal horn neurons following noxious stimulation or nerve injury, and this facilitates NMDA responses and contributes to central sensitization and pain hypersensitivity (Ultenius *et al.*, 2006). Again, activation of spinal σ_1 receptors has been found to evoke (Roh *et al.*, 2010) or enhance (Kim *et al.*, 2008) pain concomitant with increased phosphorylation of the NMDA receptor NR1 subunit, and both increased phosphorylation of NR1 in the spinal cord and nociceptive behaviours were inhibited by antagonizing spinal σ_1 receptors (Kim *et al.*, 2006; 2008; Roh *et al.*, 2008; 2010).

Overall, our evidence supports a role for σ_1 receptors in modulating nociception following prolonged noxious stimulation (i.e. capsaicin or formalin injection) and persistent abnormal afferent input (i.e. nerve injury) at the spinal cord level, and points to inhibition of augmented spinal excitability secondary to sustained afferent drive as a mechanism underlying its modulatory effect. The effects reported by pharmacologically antagonizing σ_1 receptors are consistent with a role for σ_1 receptors in central (spinal) sensitization and pain hypersensitivity and suggest a potential therapeutic use of σ_1 receptor antagonists for the management of neuropathic pain. In this context, S1RA has recently completed single- and multiple-dose phase I clinical studies demonstrating good safety, tolerability and pharmacokinetic profiles in humans (EudraCT numbers 2008-000751-94 and 2009-009424-37, respectively), which support proceeding to phase II clinical trials in pain.

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Conflicts of interest

The authors state that there were no conflicts of interest in respect to the work reported in this paper.

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