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Sigma-1 receptors regulate activity-induced spinal sensitization and neuropathic pain after peripheral nerve injury

Beatriz de la Puente [a], Xavier Nadal [b], Enrique Portillo-Salido [a], Ricard Sánchez-Arroyos [a], Sergio Ovalle [a], Gabriel Palacios [a], Asunción Muro [a], Luz Romero [a], José Manuel Entrena [c], José Manuel Baeyens [c], José Antonio López-García [d], Rafael Maldonado [b], Daniel Zamanillo [a], José Miguel Vela [a,*]

[a] Department of Pharmacology, Laboratorios Esteve. Av. Mare de Déu de Montserrat, 221. 08041 Barcelona, Spain
[b] Laboratory of Neuropharmacology, Facultat de Ciències de la Salut i de la Vida, Universitat Pompeu Fabra. Doctor Aguader, 88. 08003 Barcelona, Spain
[c] Department of Pharmacology and Institute of Neuroscience, Faculty of Medicine, Universidad de Granada, Av. de Madrid 11. 18012 Granada, Spain
[d] Department of Physiology, Faculty of Medicine, Universidad de Alcalá de Henares. Alcalá de Henares, 28871 Madrid, Spain

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ABSTRACT

Sigma-1 receptor (σ_1R) is expressed in key CNS areas involved in nociceptive processing but only limited information is available about its functional role. In the present study we investigated the relevance of σ_1R in modulating nerve injury-evoked pain. For this purpose, wild-type mice and mice lacking the σ_1R gene were exposed to partial sciatic nerve ligation and neuropathic pain-related behaviors were investigated. To explore underlying mechanisms, spinal processing of repetitive nociceptive stimulation and expression of extracellular signal-regulated kinase (ERK) were also investigated. Sensitivity to noxious heat of homozygous σ_1R knockout mice did not differ from wild-type mice. Baseline values obtained in σ_1R knockout mice before nerve injury in the plantar, cold-plate and von Frey tests were also indistinguishable from those obtained in wild-type mice. However, cold and mechanical allodynia did not develop in σ_1R null mice exposed to partial sciatic nerve injury. Using isolated spinal cords we found that mice lacking σ_1R showed reduced wind-up responses respect to wild-type mice, as evidenced by a reduced number of action potentials induced by trains of C-fiber intensity stimuli. In addition, in contrast to wild-type mice, σ_1R knockout mice did not show increased phosphorylation of ERK in the spinal cord after sciatic nerve injury. Both wind-up and ERK activation have been related to mechanisms of spinal cord sensitization. Our findings identify σ_1R as a constituent of the mechanisms modulating activity-induced sensitization in pain pathways and point to σ_1R as a new potential target for drugs designed to alleviate neuropathic pain.

1. Introduction

Sigma receptors have been classified into two subtypes (σ_1R and σ_2R) [15,22]. Sigma-1 receptor (σ_1R) is the only subtype that has been cloned so far, and its sequence has no known homology with other mammalian protein. The gene encodes a protein of 223 amino acids with at least one putative transmembrane domain anchored to endoplasmic reticulum and plasma membranes [23,60,62].

From a functional point of view, the σ_1R has been proposed to be a modulator of a variety of receptors and ion channels, acting as amplifiers in signal transduction cascades [67]. At the endoplasmic reticulum the σ_1R acts as a chaperone regulating the flow of Ca^{2+} via inositol 1,4,5-trisphosphate (IP_3) receptors [26,27]. In addition, σ_1R regulates interorganellar Ca^{2+} signaling [28] and lipid transport from the endoplasmic reticulum to plasma membrane lipid rafts [29,30]. Other relevant functions are to regulate components of plasma membrane-bound signal transduction such as phospholipase C and protein kinase C activities [55] and, importantly, modulation of activity of neurotransmitter receptors and ion channels, including K^+ channels, Ca^{2+} channels, *N*-methyl-D-aspartate (NMDA), dopamine and γ-aminobutyric acid (GABA) receptors [2,44,51,56,61,66].

σ_1R has been proposed as a putative target in several therapeutic fields such as cognition and neurodegeneration, depression and anxiety, schizophrenia, and drug addiction [15,22,46,47,52]. Existing evidence also supports a role in pain. σ_1Rs are widely expressed in the nervous system [1,40,58,59,72] including areas important for pain control. During long time, data supporting a role for σ_1Rs in modulating pain were restricted to studies describing a tonic inhibitory control of σ_1R on opioid receptor-mediated antinociception: σ_1R agonists inhibit antinociception induced by morphine whereas σ_1R antagonists and σ_1R antisense

* Corresponding author. Tel.: +34 93 4466244; fax: +34 93 4466220.
E-mail address: jvela@esteve.es (J.M. Vela).

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oligodeoxynucleotides (ODNs) enhance the antinociceptive effect of morphine and other μ-, δ- and κ-opioid receptor agonists in acute thermal nociceptive tests [10,11,39,48,60]. The notion that σ_1R may also play a role in nociception in the absence of opioids came from recent studies showing that formalin-induced nociception was attenuated in σ_1R knockout mice [8] and by systemic administration of haloperidol and haloperidol metabolites I and II (with an order of potency which correlated with their affinity for σ_1Rs) [7]. Furthermore, intrathecal administration of σ_1R antagonists dose-dependently reduced formalin- and nerve injury-induced pain behaviors [37,63].

The selectivity and pharmacology of σ_1R ligands are unclear. Certain neurosteroids interact with σ_1Rs and have been proposed to be their endogenous ligands [47,52], but the precise nature of endogenous high-affinity σ_1R ligands is still under debate [20]. In this study, we took advantage of the genetic approach using σ_1R knockout mice to examine the role of σ_1Rs in mechanisms underlying neuropathic pain and spinal sensitization. We previously showed that deletion of this gene results in viable and fertile σ_1R-deficient mice with no detectable abnormalities [41]. Here we show that σ_1R modulates behavioral hypersensitivity and spinal hyperactivity secondary to nerve injury. Our findings identify σ_1R as a key constituent of the mechanisms modulating activity-induced sensitization in nociceptive pathways and point to σ_1R as a new potential target for the treatment of neuropathic pain.

2. Methods

2.1. Animals

Wild-type, heterozygous ($\sigma_1R^{+/-}$) and homozygous ($\sigma_1R^{-/-}$) male sigma receptor knockout mice backcrossed (N10 generation) to a CD1 albino genetic background (Harlan Ibérica, Barcelona, Spain) from 6 to 8 weeks old at the beginning of the experiments were used. Null mutant mice were generated by targeted removal of most of the coding region of the mSR1 gene, as described previously [41]. Mice 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 A.M.). Behavioral testing was conducted in a sound attenuated room and was done in blind respect to genotype and surgical procedure. Experimental procedures and animal husbandry were conducted according to ethical principles for the evaluation of pain in conscious animals [74] and to 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.

2.2. Neuropathic pain model: partial sciatic nerve ligation

The partial sciatic nerve ligation model was used to induce neuropathic pain, according to the protocol previously described [43]. Briefly, mice were anesthetized with isoflurane (induction: 5%; surgery: 2%) and the common sciatic nerve was exposed at the level of the mid-thigh of the right hindpaw. At about 1 cm proximally to the nerve trifurcation, a tight ligation using 9–0 non-absorbable virgin silk suture (Alcon surgical, Texas, 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.

2.3. Nociceptive behavioral tests

2.3.1. Evaluation of thermal nociception (tail-flick and hot plate tests)

Acute heat nociceptive responses were assessed by the tail-flick and hot plate tests in uninjured independent groups of σ_1R knockout and wild-type mice (n = 12 per group). For the tail-flick test, animals were placed in a loose Plexiglas restrainer on a tail-flick apparatus (Panlab SL, Barcelona, Spain). A photobeam was focused on the tail about 4 cm from the tip, and the latency to tail-flick response was recorded automatically to the nearest 0.1 s. The intensity of the radiant heat source was adjusted to yield baseline latencies between 3 and 5 s in wild-type mice. A cut-off latency time of 10 s was imposed to avoid damage to tail tissues. Thermal nociceptive responses were also assessed by the hot plate test at 50 ± 0.5 °C. Briefly, mice were placed on the surface of the hot plate (Panlab SL, Barcelona, Spain) and the latency to the beginning of forepaw licking and jumping was recorded. Animals were removed from the hot plate after the jumping response was recorded. In order to avoid burns, the maximal time (cut-off) in the hot plate did not exceed 160 s.

2.3.2. Evaluation of neuropathic pain-related behaviors

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 (n = 12 per group) by using the plantar, cold-plate and von Frey test, respectively. Animals were first habituated for one hour to each different experimental test once daily for 4 days. After the habituation period, baseline responses were established during 2 consecutive days for each paradigm in the following sequence: von Frey, plantar (30 min later) and cold-plate test (15 min later). One day after baseline measurements, sciatic nerve injury was induced. σ_1R knockout and wild-type mice were tested in each paradigm on days 3, 6 and 10 after the surgical procedure using the same experimental sequence as for baseline responses.

Mechanical allodynia was quantified by measuring the hindpaw withdrawal response to von Frey filament stimulation [9]. 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., San Jose 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 responding. The threshold of response was calculated by using the up–down Excel program generously provided by Basbaum's laboratory (UCSF, San Francisco, USA). Clear paw withdrawal, shaking or licking was considered as a nociceptive-like response. Both ipsilateral and contralateral hindpaws were tested.

Thermal hyperalgesia was assessed with a plantar test apparatus (Ugo Basile, Varese, Italy), as previously reported [24], by measuring hindpaw 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 hindpaw and activated with a light beam intensity chosen in preliminary studies to give baseline latencies from 8 to 9 s in control wild-type 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 the absence of response. The mean withdrawal latencies for the ipsilateral and contralateral hindpaws were determined from the average of three separate trials, taken at 5-min intervals to prevent thermal sensitization and behavioral disturbances.

Cold allodynia was assessed by using a hot/cold-plate analgesia meter (Columbus, OH, USA) as previously described [4]. Briefly, mice were placed into compartment enclosures on the cold surface of the plate which is maintained at a temperature of 5 ± 0.5 °C. The number of elevations of each hindpaw was then recorded for 5 min. A score was calculated by subtracting the number of elevations of the right hindpaw (ipsilateral) from left hindpaw (contralateral). A positive difference score indicates development of cold allodynia.

2.4. ERK immunoblotting and immunohistochemistry

2.4.1. Western blotting

Immediately after deep anesthesia with sodium pentobarbital (100 mg/kg, i.p.), spinal cords were carefully removed from wild-type and $\sigma_1R^{-/-}$ mice 14 days ($n = 6$ per group) after surgery and ipsilateral spinal hemicord segments L4–S1 were dissected, frozen immediately in dry ice and stored at −80 °C. Spinal cord tissue was homogenized by sonication in 1% sodium dodecyl sulfate (SDS) containing a protease and phosphatase inhibitor cocktail purchased from Calbiochem (San Diego, CA, USA). The homogenate was centrifuged at 10,000*g* for 10 min at 4 °C. The supernatant was decanted from the pellet and used for Western blot analyses. The concentration of protein in the homogenate was measured using the DC Protein Assay from Bio-Rad Laboratories Inc. (Hercules, CA, USA). Equal amounts of protein (50 μg) were fractionated by 8% (w/v) SDS–PAGE and transferred onto a polyvinylidene difluoride membrane, blocked with 5% non-fat dry milk in Tris–Tween 20-buffered Saline (T–TBS) overnight at 4 °C. Membranes were then incubated with rabbit primary polyclonal antibodies recognizing the mitogen-activated protein kinase (MAPK, total ERK 1/2) or mouse monoclonal antibodies recognizing the activated MAPK (diphosphorylated MAPK, p-ERK 1/2) obtained from Sigma–Aldrich Co. (Madrid, Spain) at a 1:20,000 and a 1:5000 dilution, respectively, in 5% non-fat dry milk in T–TBS. The blots were washed three times for 10 min with T–TBS and then incubated with horseradish peroxidase–conjugated goat anti-rabbit IgG or goat anti-mouse IgG, purchased from Pierce Biotechnology Inc. (Rockford, IL, USA), diluted 1:5000 in 5% non-fat dry milk in T–TBS. The membranes were then washed with T–TBS three times for 10 min, one time with TBS for 10 min and the peroxidase reaction revealed by chemiluminescence (Immun-Star HRP Chemiluminescent Kit) from Bio-Rad. Chemiluminescence was detected with the ChemiDoc XRS System from Bio-Rad. The blots were then incubated for 15 min at room temperature in stripping buffer (Re-Blot Plus Strong Antibody Stripping Solution) from Chemicon International Inc. (Temecula, CA, USA) and re-probed for β-tubulin, using rabbit polyclonal antibodies diluted 1:1000 obtained from Abcam Inc. (Cambridge, MA, USA), as a loading control. The densitometric analysis of immunoreactive bands was done using the Quantity One software (Bio-Rad) and normalized respect to the intensity of the corresponding β-tubulin immunoreactive bands to obtain relative optical density ERK and p-ERK values. p-ERK was finally normalized respect to total ERK protein.

2.4.2. Immunohistochemistry

Wild-type and $\sigma_1R^{-/-}$ mice 14 days after surgery ($n = 12$ per group) were deeply anesthetized with sodium pentobarbital (100 mg/kg, i.p.) and perfused intracardially with 200 ml of cold saline solution followed by 500 ml of a cold 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 (PB). After perfusion, spinal cord segments L4–S1 were dissected out and postfixed for 4 h in 4% paraformaldehyde in PB at 4 °C. Then, spinal cord segments were washed in PB and serial coronal sections (40 μm-thick) were obtained with the aid of a vibratome (vibrocut FTB, Frankfurt, Germany) and collected in phosphate-buffered saline (PBS) to be processed immunohistochemically as free-floating sections. Sections were pre-incubated with 0.3% H_2O_2 in PBS for 30 min to block endogenous peroxidase activity and, after washing three times with PBS, with normal goat serum diluted 1:100 in PBS for 1 h at room temperature (RT) to prevent unspecific staining. Sections were then incubated for 48 h at 4 °C with the primary antibody (anti-p-ERK 1/2) diluted 1:500 in PBS with 1% bovine serum albumin and 0.4% Triton X-100. After washing three times for 10 min in PBS sections were incubated with goat anti-mouse biotinylated antibodies from Vector Laboratories Inc. (Burlingame, CA, USA) diluted 1:200 in PBS for 1 h at RT. After washing the sections three times in PBS, an avidin–biotin–peroxidase complex (Vector) was applied diluted 1:100 in PBS for 1 h at RT. The sections were washed again in PBS and placed in a chromogen solution containing 0.05% 3,3′-diaminobenzidine and 0.01% H_2O_2 in PBS for 5 min. The immunostained sections were placed on slides and coverslipped with glycergel mounting medium (DakoCytomation, Inc., Barcelona, Spain) for microscopic observation and photography.

Possible changes in p-ERK immunoreactivity in the dorsal horn were assessed by quantifying the density of immunostaining. Sections were simultaneously processed for immunohistochemistry in order to avoid methodological changes affecting the intensity of staining. Three appropriate fields per mice containing the dorsal horn were digitized by a video camera connected to a microscope and interfaced to a computer. The boundary of the dorsal horn (laminae I + II) was traced and the mean density of immunostaining was measured based on the inverse computer grayscale (from 0 = white to 255 = black) by using the National Institutes of Health (NIH) Image J software. Individual immunodensity values were corrected by subtracting the background (labeling in the white matter) for each section.

2.5. Electrophysiology

Spinal cords were obtained from newborn 5- to 10-day-old $\sigma_1R^{-/-}$ and wild-type mice after urethane (2 mg/kg, i.p.) anesthesia followed by dorsal laminectomy. In vitro AC recordings were performed as previously described [45] using a total of 13 spinal cords from wild-type and 10 cords from homozygous $\sigma_1R^{-/-}$ knockout mice. Briefly, the spinal cord was fixed to the Sylgard base of a recording chamber and continuously superfused (6–9 ml/min) with oxygenated (95% O_2; 5% CO_2) artificial cerebrospinal fluid (ACSF) at pH 7.4 and RT. 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. After setting up, the spinal cord was left untouched for one hour. Stimuli were then applied to the dorsal root in trains of shocks (20 stimuli at 1 Hz) and responses were recorded from the corresponding ventral root. Stimuli were adjusted to activate only thick and myelinated fibers (50 μs and 50 μA) or increased up to activate all fibers in the root including nociceptive C fibers (300 μs and 300 μA). 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 from Cambridge Electronic Design Ltd. (Cambridge, UK). AC recordings from ventral roots were analyzed based on threshold criteria to count spikes to each stimulus of the train. Spike counts were performed for each stimulus of the train in a window between 20 and 950 ms from stimulus artifact.

2.6. Statistical analysis

Data obtained from thermal nociceptive tests were compared by one-way ANOVA and neuropathic pain-related behaviors were compared on each experimental day by using a two-way ANOVA repeated measures (paw and genotype as between factor of variation; day and surgery as within group levels) followed by corresponding one-way ANOVA and post hoc comparisons (Fisher's LSD test) when appropriate. Similarly, the difference between the groups for ERK immunoreaction in Western blots and immunohistochemistry was compared using ANOVA followed by post hoc comparison.

In electrophysiological studies, response profiles from the different groups of animals were compared by means of one-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.

In all cases, the criterion for statistical significance was established when p value was below 0.05.

3. Results

3.1. Nociceptive behavior of σ_1 receptor deficient mice: baseline control and neuropathic pain-related behaviors

3.1.1. Response of non-injured mice to thermal and mechanical stimuli

Sensitivity to noxious heat of homozygous σ_1R knockout mice ($\sigma_1R^{-/-}$), measured as the latency time of response to thermal stimulation in the hot plate (50 °C) and tail-flick tests, did not differ from wild-type mice (Fig. 1). Similarly, baseline values obtained in σ_1R knockout mice before nerve injury in the plantar, cold-plate and von Frey tests were indistinguishable from those obtained in wild-type mice (Fig. 2–4). Therefore, homozygous ($\sigma_1R^{-/-}$) knockout mice perceive and respond normally to acute thermal and mechanical nociceptive stimuli.

3.1.2. Thermal (heat) hyperalgesia (plantar test) secondary to nerve injury

No differences in baseline paw withdrawal latencies before surgery were found in the plantar test when compared to wild-type, σ_1R heterozygous ($\sigma_1R^{+/-}$) and homozygous ($\sigma_1R^{-/-}$) knockout mice (Fig. 2). Sham-operated wild-type, $\sigma_1R^{+/-}$ and $\sigma_1R^{-/-}$ mice also showed similar paw withdrawal latencies 3, 6 and 10 days after surgery in both the ipsilateral and the contralateral paws.

As expected, a significant decrease in paw withdrawal latencies (thermal hyperalgesia) was observed in the ipsilateral, nerve-injured paw of wild-type mice 3, 6 and 10 days after surgery (Fig. 2). Thermal (heat) hyperalgesia also developed in heterozygous and homozygous σ_1R knockout mice exposed to sciatic nerve injury, as significantly reduced withdrawal latencies of their ipsilateral paws, indistinguishable from those in wild-type mice, were found 3, 6 and 10 days after surgery (Fig. 2). In the same way, no differences were found in the contralateral paw when compared paw withdrawal latencies of wild-type, $\sigma_1R^{+/-}$ and $\sigma_1R^{-/-}$ mice exposed to sciatic nerve injury 3, 6 and 10 days after surgery (Fig. 2).

3.1.3. Cold allodynia (cold-plate test) secondary to nerve injury

Baseline values before surgery did not reveal significant differences between wild-type, $\sigma_1R^{+/-}$ and $\sigma_1R^{-/-}$ mice. Likewise, sham-operated wild-type, $\sigma_1R^{+/-}$ and $\sigma_1R^{-/-}$ mice showed similar cold-plate scores 3, 6 and 10 days after surgery (Fig. 3). The responses of the contralateral paw were similar in all the experimental groups at the different times.

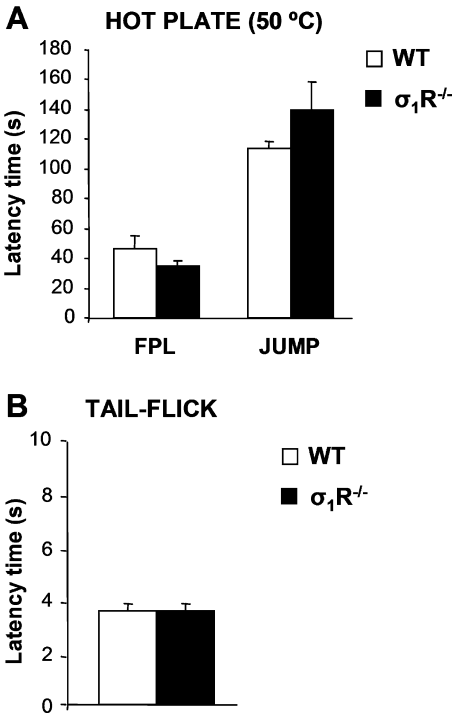

Fig. 1. Nociceptive response to noxious heat (hot plate and tail-flick tests) in control wild-type and homozygous $\sigma_1R^{-/-}$ mice. (A) The latency time for fore paw licking (FPL) and jumping (JUMP) responses on exposure to a hot plate kept at 50 °C did not differ in $\sigma_1R^{-/-}$ knockout mice respect to wild-type (WT) mice. (B) Similarly, the latency of the tail-flick response evoked by noxious heat stimulation using a light beam was in homozygous σ_1R knockout mice indistinguishable from wild-type mice. Each bar and vertical line represent the mean ± SEM (n = 12 per group). No statistically significant differences were found between wild-type and $\sigma_1R^{-/-}$ mice either in the hot plate or in the tail-flick test.

As expected, a significant increase of the cold-plate score (cold allodynia) was found in wild-type mice exposed to the sciatic nerve injury 3, 6 and 10 days after surgery when compared to sham-operated mice. Heterozygous $\sigma_1R^{+/-}$ mice exposed to the sciatic nerve injury also developed cold allodynia as significant increases of the cold-plate score 3, 6 and 10 days after surgery were found when compared to sham-operated mice (Fig. 3). However, cold-plate scores in homozygous $\sigma_1R^{-/-}$ exposed to nerve injury were not significantly different from values obtained in sham-operated mice at 3, 6 and 10 days after surgery (Fig. 3).

3.1.4. Mechanical allodynia (von Frey test) secondary to nerve injury

The pressure threshold eliciting paw withdrawal responses at baseline, before surgery, did not differ when wild-type, $\sigma_1R^{+/-}$ and $\sigma_1R^{-/-}$ mice were compared (Fig. 4). In the same way, sham-operated wild-type, $\sigma_1R^{+/-}$ and $\sigma_1R^{-/-}$ mice required similar pressure to elicit withdrawal of both the ipsilateral and the contralateral paw 3, 6 and 10 days after surgery. Wild-type, $\sigma_1R^{+/-}$ and $\sigma_1R^{-/-}$ mice exposed to sciatic nerve injury also required similar pressure to elicit withdrawal of the contralateral paw 3, 6 and 10 days after surgery.

Partial sciatic nerve ligation induced mechanical allodynia in wild-type mice, as evidenced by a significant reduction from day 3 post-injury of the pressure required to evoke withdrawal of the ipsilateral, nerve-injured paw compared to baseline pre-injury and contralateral paw values (Fig. 4). Heterozygous $\sigma_1R^{+/-}$ mice exposed to sciatic nerve injury also showed decreases in the pressure threshold eliciting withdrawal of the ipsilateral paw 3, 6 and 10 days after surgery. However, mechanical allodynia was significantly attenuated in $\sigma_1R^{+/-}$ mice, pressure thresholds being

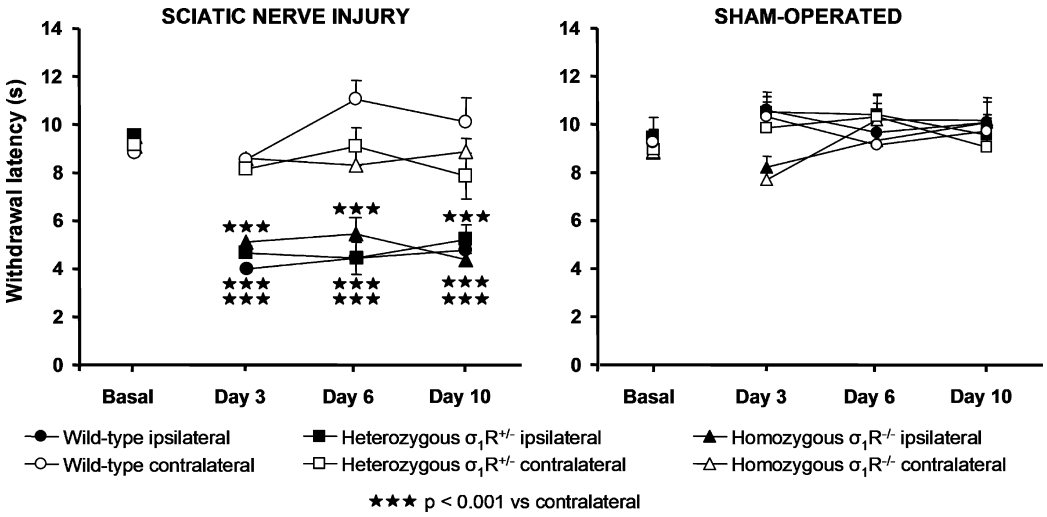

Fig. 2. Development of thermal (heat) hyperalgesia (plantar test) following nerve injury in control wild-type and σ_1R-deficient mice. Thermal hyperalgesia, evidenced as a significant reduction in the latency time for withdrawal of the ipsilateral, respect to the contralateral, hindpaw in response to radiant heat was found 3, 6 and 10 days after sciatic nerve injury in wild-type, σ_1R heterozygous and σ_1R homozygous knockout mice. The development of thermal hyperalgesia did not differ when the different genotypes were compared. Similarly, no effect of genotype was found when compared to responses in sham-operated mice. Each point and vertical line represent the mean ± SEM (n = 12 per group).

significantly modified respect to wild-type on days 6 and 10 after nerve injury. Interestingly, in the homozygous $\sigma_1R^{-/-}$, mechanical allodynia was strongly reduced 3 days after nerve injury and was not observed on days 6 and 10 post-injury (pressure thresholds eliciting withdrawal of the ipsilateral and contralateral paws were not significantly different). In fact, significant differences between $\sigma_1R^{-/-}$ knockout and wild-type mice were found in the withdrawal thresholds of the ipsilateral, nerve-injured paw on all evaluated days (3, 6 and 10 post-injury) (Fig. 4).

3.2. Activation of ERK in the spinal cord secondary to sciatic nerve injury

Activation (phosphorylation) of mitogen-activated protein kinases (MAPKs), particularly extracellular signal-regulated kinase (ERK 1/2; p44/42 MAPK), occurs in dorsal horn neurons and glial cells following nerve injury and contributes to the induction and maintenance of neuropathic pain [34,42,73]. We explored the possibility that differential activation of ERK in the spinal cord could underlie the differences in neuropathic pain-related behaviors (i.e., allodynia) between wild-type and $\sigma_1R^{-/-}$ knockout mice. ERK activation in spinal cords from sham-operated and nerve-injured mice was analyzed by Western immunoblotting and immunohistochemistry. Immunoblots using proteins from ipsilateral spinal hemicord segments L4–S1 14 days after surgery were done for total ERK and its phosphorylated form (p-ERK) (Fig. 5A). No significant changes in total ERK protein were found either in wild-type or in $\sigma_1R^{-/-}$ knockout mice when nerve-injured and sham-operated mice were compared. As expected, the phosphorylation of ERK was increased (7.8-fold; $p < 0.01$) in the ipsilateral spinal hemicord of wild-type mice exposed to sciatic nerve injury with respect to sham-operated wild-type mice. However, the significant

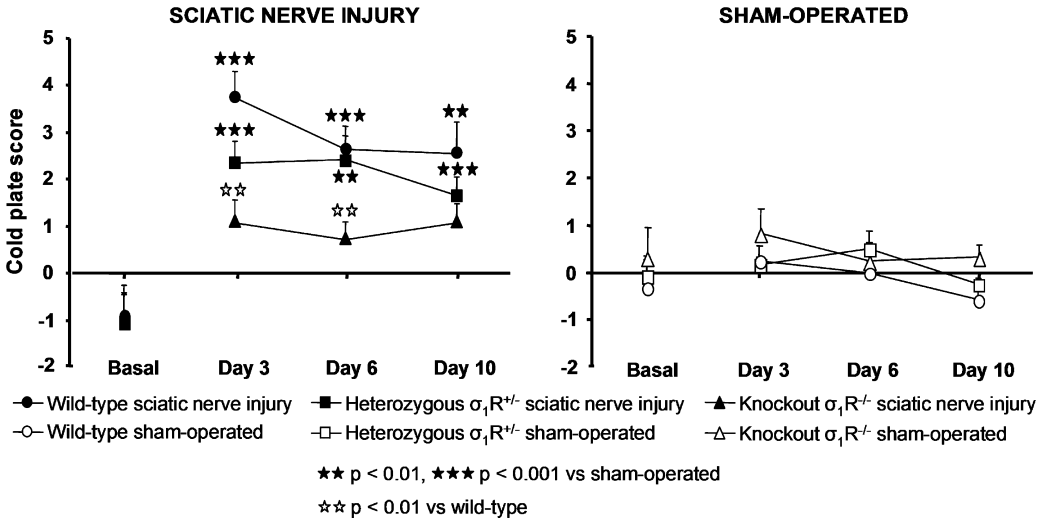

Fig. 3. Development of cold allodynia (cold-plate test) following nerve injury in control wild-type and σ_1R-deficient mice. Cold allodynia, evidenced as a significantly increased number of elevations of the right hindpaw (ipsilateral) respect to left hindpaw (contralateral) was evidenced in nerve-injured, respect to sham-operated mice, wild-type and σ_1R heterozygous mice, but not in σ_1R homozygous knockout mice. No effect of genotype was found when compared to responses in sham-operated mice. Each point and vertical line represent the mean ± SEM (n = 12 per group).

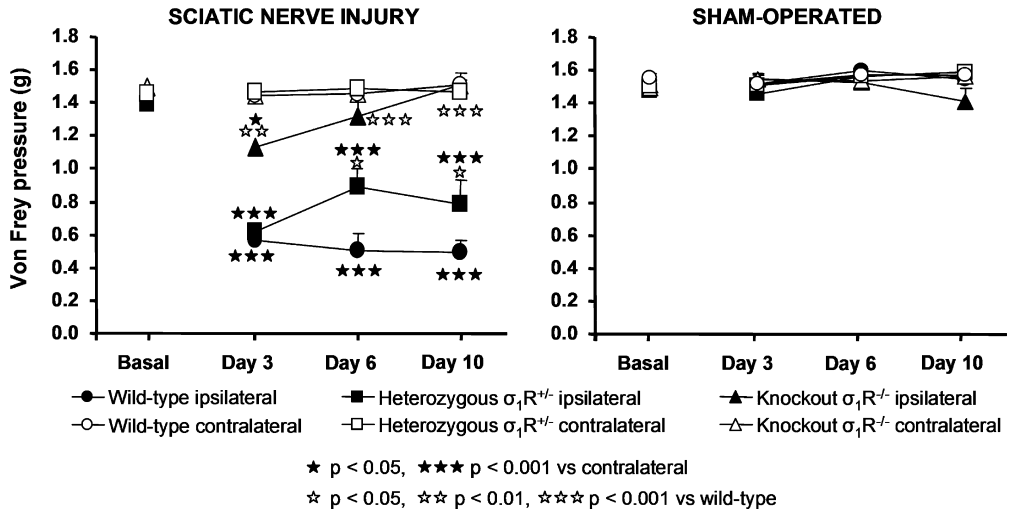

Fig. 4. Development of mechanical allodynia (von Frey test) following nerve injury in control wild-type and σ_1R-deficient mice. Mechanical allodynia, evidenced as a significant reduction in the pressure evoking withdrawal of the nerve-injured hindpaw was clearly evidenced in wild-type mice on all days of measurement. It was attenuated in heterozygous σ_1R mice on days 6 and 10 after nerve injury, and it was strongly reduced 3 days after nerve injury and was not observed on days 6 and 10 post-injury in homozygous σ_1R knockout mice. No effect of genotype was found when responses in sham-operated mice were compared. Each point and vertical line represent the mean ± SEM (n = 12 per group).

upregulation subsequent to nerve injury found in the wild-type did not occur in the knockout mice, as no significant change in the intensity of p-ERK immunoreactive bands was found when compared ipsilateral cords from $\sigma_1R^{-/-}$ mice exposed to nerve injury and sham operation (1.3-fold increase in nerve-injured mice with respect to sham-operated mice).

By immunohistochemistry, increased activation of ERK in the spinal cord (L4–S1 segments) was apparent in the ipsilateral dorsal horn of nerve-injured respect to sham-operated wild-type mice 14 days after surgery. This was evidenced by both the presence of increased numbers of p-ERK immunoreactive cells and the increased p-ERK immunostaining intensity showed by cells (Fig. 5B–H). Immunostaining for p-ERK was present in numerous cells in superficial laminae I and II, but also with smaller numbers in more ventral laminae, and was located in the soma as well as in the nucleus. In the $\sigma_1R^{-/-}$ knockout mice no differences in p-ERK immunostaining in the spinal cord were found when nerve-injured and sham-operated mice were compared. When p-ERK immunoreaction in the ipsilateral dorsal horn (laminae I–II) was quantified in the sections, the density was significantly increased after nerve injury (respect to sham operation) in wild-type but not in $\sigma_1R^{-/-}$ knockout mice (Fig. 5B).

3.3. Spinal processing of repetitive nociceptive stimulation

In order to provide an electrophysiological correlate to the nociceptive behavioral findings described above, in vitro AC recordings were performed in spinal cords from wild-type and homozygous $\sigma_1R^{-/-}$ knockout mice. Repetitive stimulation of the dorsal root at stimulus intensities sufficient to activate C-fibers produced a typical wind-up response in ventral root recordings, which manifested as a progressive increase in action potential firing (Fig. 6). Stimulus intensities insufficient to activate C-fibers (i.e., 50 μs and 50 μA) did not produce wind-up upon the arrival of the afferent volleys. Interestingly, spinal cords from wild-type animals showed stronger wind-up responses than those from homozygous $\sigma_1R^{-/-}$ animals (Fig. 6). This was evidenced as a reduction in the total number of action potentials induced by trains of C-fiber intensity stimuli in spinal cords from $\sigma_1R^{-/-}$ mice respect to wild-type mice. For 200 μs and 200 μA stimuli, wild-type cords produced the smaller mean spike count in response to the second stimulus of the train (22 ± 5 counts) and the greater in response to the last stimulus (223 ± 36 counts). The rise rate of the response was 8.7 ± 1.6 spikes per second in wild-type cords. For the same stimulus intensity, spinal cords from $\sigma_1R^{-/-}$ animals showed slightly larger spike counts than wild-types at the beginning of the train of stimuli (71 ± 26 counts) and much smaller spike counts towards the end of the train (141 ± 37 counts). This resulted in a significantly smaller rise rate for knockout animals (3.2 ± 0.9 spikes per second) compared to controls ($p < 0.05$; two-tailed unpaired t-test). Responses to repetitive A-fiber intensity stimulation did not produce spike wind-up and were similar in wild-type and $\sigma_1R^{-/-}$ knockout mice.

4. Discussion

In the present study we took advantage of a genetic approach using $\sigma_1R^{-/-}$ knockout mice to examine the role of σ_1R in nociception secondary to nerve injury. Our results indicated that increased sensitivity (allodynia) to mechanical and thermal (cold) stimuli found after nerve injury is markedly attenuated to absent in mice lacking σ_1Rs. Differential modulation of spinal excitability and ERK signaling could contribute to the reduced behavioral hypersensitivity seen in σ_1R knockout mice after nerve injury.

The sensitivity to noxious heat stimulation in the $\sigma_1R^{-/-}$ knockout mice did not differ from wild-type mice, as evidenced in the hot plate and tail-flick tests. In the same way, baseline (before surgery) responses of σ_1R-deficient mice to thermal (heat and cold) and mechanical stimuli were undistinguishable from wild-type ones. Responses of sham-operated mice also remained unchanged (respect to wild-type) in σ_1R-deficient mice. Thus, in the absence of nerve injury, no differences in the performance of the sensory/nociceptive pathway were seen when compared σ_1R-deficient mice to wild-type mice, suggesting that basic mechanisms for transduction, transmission and perception of sensory and nociceptive inputs are intact in mice lacking σ_1Rs. This is in agreement with previous pharmacological studies showing that σ_1 ligands did not influence nociceptive behaviors by themselves in normal conditions [7,11,18,38].

However, after sciatic nerve injury, when hypersensitivity in the ipsilateral paw would result in perception of pain from non-noxious stimuli (allodynia), the scenario is different. We found that

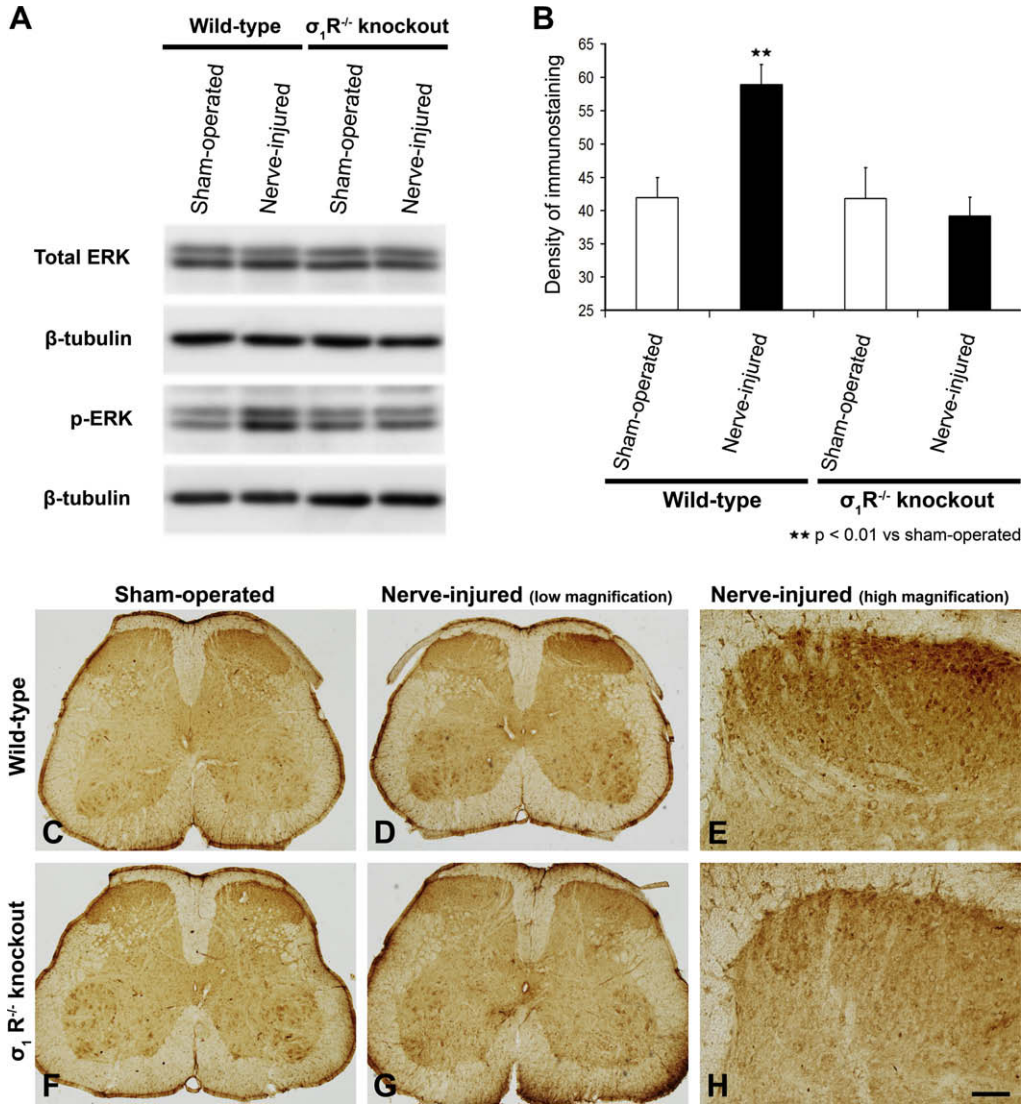

Fig. 5. Activation of ERK (immunoblotting and immunohistochemistry) following nerve injury in control wild-type and homozygous $\sigma_1R^{-/-}$ mice. (A) Western immunoblotting for total ERK and p-ERK in ipsilateral spinal hemicord segments L4–S1 14 days after surgery. β-tubulin was used as a loading control. (B) Quantification of immunostaining for p-ERK in the ipsilateral dorsal horn (laminae I and II) of spinal cord sections (L4–S1 segments) from sham-operated and nerve-injured wild-type and homozygous σ_1R knockout mice 14 days after surgery. Each bar and vertical line represent the mean ± SEM. (A and B) Note that p-ERK immunoreactivity was increased after nerve injury in wild-type but not in σ_1R null mice. (C–H) p-ERK immunostaining in spinal cord sections from sham-operated and nerve-injured wild-type and homozygous σ_1R knockout mice. The right side of photomicrographs C, D, F and G corresponds to the side of injury (ipsilateral side). Note that p-ERK immunoreactivity in superficial laminae of the ipsilateral dorsal horn after nerve injury was remarkably higher in wild-type (E) than in σ_1R null mice (H). Scale bar = 500 μm in C, D, F and G; and 100 μm in E and H.

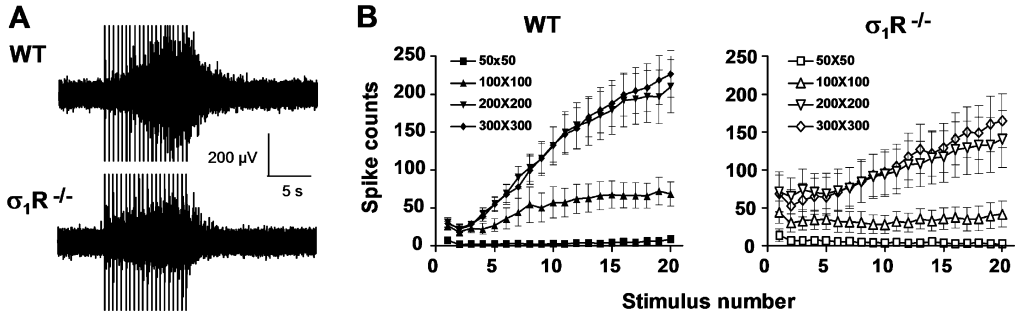

Fig. 6. Electrophysiological recordings of spinal cord responses upon application of repetitive electrical stimuli of constant intensity in control wild-type and homozygous $\sigma_1R^{-/-}$ mice. (A) Original AC recordings of responses to a train of 200 μs and 200 μA obtained from cords of different genotypes. Vertical lines at regular intervals correspond to stimuli artifact. Spikes are viewed as a progressive thickening of the baseline noise. (B) Spike counts obtained following stimulation at different intensities as stated (in μs × μA) obtained from cords of different genotypes. Note the large differences between phenotypes for high intensity (C-fiber intensity) stimuli (e.g., 200 μs × 200 μA). Significant differences ($p < 0.001$) were found between curves for high intensity stimuli (200 μs × 200 μA and 300 μs × 300 μA) in control and homozygous σ_1R knockout animals using two-way ANOVA.

mechanical allodynia was attenuated in heterozygous $\sigma_1R^{+/-}$ mice and was not observed in homozygous $\sigma_1R^{-/-}$ mice 6 and 10 days after nerve injury. Cold allodynia in homozygous (but not in heterozygous) σ_1R knockout mice exposed to nerve injury was also attenuated. In contrast, nerve injury-induced heat hyperalgesia was not modified in σ_1R knockout mice respect to wild-type mice. A differential modulation by σ_1Rs of sensory/nociceptive pathways depending on the stimulus quality and modality is thus suggested. This adds to previous findings that different receptor systems and mechanisms are involved in the diverse sensory abnormalities (i.e., mechanical versus thermal) associated with neuropathic pain [3,49,57,68].

The conception that σ_1Rs plays a role in modulating pain behaviors in sensitizing conditions is also supported in previous studies. Formalin-induced pain was reduced in σ_1R knockout mice [8] and haloperidol and its metabolites I and II, which behave as antagonists at this receptor [13,14], inhibited formalin-induced pain [7]. Similarly, intraplantar injection of capsaicin induced mechanical allodynia in wild-type but not in σ_1R knockout mice and σ_1R antagonists were able to inhibit this pain behavior [18]. Regarding the site of action, intrathecal pretreatment with the σ_1R antagonist BD-1047 has been shown to dose-dependently reduce pain behaviors in second phase of the formalin test [37]. The analgesic effect occurred concomitantly with a reduction in formalin-evoked Fos expression and phosphorylation of the NMDA receptor NR1 subunit (pNR1) at protein kinase C (PKC) and protein kinase A (PKA) dependent sites in spinal dorsal horn [37]. In separate recent studies, activation of spinal σ_1Rs has been found to have opposite effects: intrathecal administration of σ_1R agonists facilitated nociception involving Ca^{2+}-dependent second messenger cascades [64], and enhanced NMDA-induced pain in parallel to an increased pNR1 expression [38]. Interestingly, the effect of pharmacological blockage of spinal σ_1Rs in a neuropathic pain model, the chronic constriction injury of the sciatic nerve in rats, has recently been investigated [63]. The σ_1R antagonist BD1047 intrathecally administered on postoperative days 0–5 significantly attenuated the development of mechanical allodynia, but not thermal hyperalgesia, concurrent with an inhibition of nerve injury-induced NMDA receptor NR1 subunit expression and phosphorylation [63]. These results fully agree with the observation reported here that mechanical allodynia, but not thermal hyperalgesia, is suppressed in nerve-injured σ_1R knockout mice.

Collectively, results described above support a modulatory role of σ_1Rs in spinal sensitization and point to NMDA receptors and Ca^{2+}-dependent intracellular cascades as underlying mechanisms. This is not surprising as σ_1Rs are functionally coupled to NMDA receptors [5,25,44,51,53] and regulate intracellular Ca^{2+} concentration via phospholipase C (PLC) and IP_3 receptors [26,27,54,55,67]. The overall effect of activating σ_1Rs is to increase intracellular Ca^{2+} concentration by potentiating Ca^{2+} entry at the plasma membrane level (NMDA-induced Ca^{2+} influx) and Ca^{2+} mobilization from endoplasmic stores (IP_3-induced Ca^{2+} mobilization). Indeed, NMDA and IP_3 receptors, as well as downstream Ca^{2+}-dependent second messengers including PKC and other calcium/calmodulin-dependent kinases (e.g., CaMKII) are involved in central (spinal) sensitization and pain hypersensitivity [6,16,19,32,33,36,70]. Hence, according to our results, blocking/absence of σ_1Rs might be associated with reduced nerve injury-evoked activity of Ca^{2+}-permeable NMDA and IP_3 receptors (Fig. 7), thus causing reduction in central sensitization-related pain hypersensitivity.

The σ_1R is expressed abundantly in the spinal cord, mainly in the two superficial laminae, in dendritic processes and neuronal perikarya, both on the plasma and endoplasmic reticulum membranes [1]. Its expression in the spinal cord is upregulated early and transiently during the induction phase of neuropathic pain [63]. We focused on functional changes in excitability and synaptic plasticity in the spinal cord as an integrative correlate of the role played by σ_1Rs. Central sensitization is a complex synaptic plasticity phenomenon characterized by the rapid-onset, activity-dependent increase in the excitability of nociceptive spinal dorsal horn neurons. Several types of synaptic plasticity arise in response to noxious stimuli that modify nociceptive transmission in the spinal cord. These include immediate-onset phenomena such as wind-up and long-term potentiation (LTP). Wind-up is interpreted as an amplification in the spinal cord of the nociceptive message that arrives from peripheral nociceptors, whereby repetitive stimulation of dorsal horn neurons induces an increase in their evoked responses with each stimulus [17,31,50]. In our study, in spinal cords from wild-type mice, repetitive stimulation of primary afferent fibers in the dorsal root produced a typical wind-up response manifested as a progressive increase in action potential firing. Interestingly, spinal cords from mice lacking σ_1Rs showed reduced wind-up responses respect to wild-type ones, as evidenced by a smaller rise rate of the response and a subsequent reduction in the total number of action potentials induced by trains of stimuli. These data point to a modulatory role of σ_1R on spinal excitability after repetitive nociceptor stimulation, such as that expected to occur as a consequence of nerve injury. Similar to our findings in the spinal cord, σ_1Rs are known to modulate LTP in the hippocampus [44]. In this way, glutamatergic NMDA and substance P tachykinin receptors (i.e., NK1) have been identified as key mediators of wind-up and central sensitization [31,69], and σ_1Rs modulate elevations of cytosolic Ca^{2+} incurred not only upon activation of NMDA but also of NK1 receptors through the PLC-IP_3 pathway (Fig. 7).

Ca^{2+} entry into neurons via NMDA receptors or mobilized via IP_3 receptors can initiate the intracellular ERK signaling cascade. In fact, neurotransmitters released by primary afferent fibers, acting through a variety of receptors and ion channels, activate Ca^{2+}-dependent enzymes such as PKC, CaMKs, adenylate cyclase-PKA and tyrosine kinases, which lead to ERK activation (phosphorylation) in dorsal horn neurons [34–36] (Fig. 7). As a common effector of multiple signals, ERK activation is activity-dependent in dorsal horn neurons. Indeed, activation of ERK in dorsal horn neurons is induced by repetitive C-fiber electrical stimulation and blockage of spinal ERK suppresses wind-up [21]. Similarly, LTP of C-fiber-evoked field potentials induces ERK activation in spinal dorsal horn and inhibition of ERK blocks the induction and maintenance of LTP [71]. In this way, in addition to its kinase-dependent posttranslational regulation, the ERK pathway also regulates long-term pain hypersensitivity via transcriptional regulation of key gene products [36,65,71]. After nerve injury, ERK activation occurs early and it is long lasting. It is immediately induced in neurons of the superficial dorsal horn and this is followed by a widespread sequential induction in spinal microglia and astrocytes [42,73]. Activated glia produce inflammatory mediators that sensitize dorsal horn neurons. Activity of dorsal horn neurons, in turn, promotes activation of spinal glia. This neuron–glia interaction involves ERK signaling in the positive feedback enhancing and prolonging pain sensitization. Indeed, in several nerve injury models, intrathecal ERK inhibitors (i.e., MEK inhibitors or ERK antisense ODNs) reduce pain hypersensitivity, particularly mechanical allodynia, when administered in both the induction and maintenance phases of neuropathic pain [12,42,65,73]. In the present study, ERK activation was increased in spinal cords of wild-type mice exposed to sciatic nerve injury 14 days after surgery, but no significant changes respect to sham-operated mice were found in the spinal cords from nerve-injured $\sigma_1R^{-/-}$ knockout mice. This finding supports that σ_1R is involved in the regulation of intracellular cascades, probably Ca^{2+} dependent, that lead to phosphorylation of ERK (Fig. 7). The inability to upregulate ERK activation subsequent to nerve injury found in the spinal cords of σ_1R knockout mice could be interpreted along the lines of reduced sensitization and pain hypersensitivity

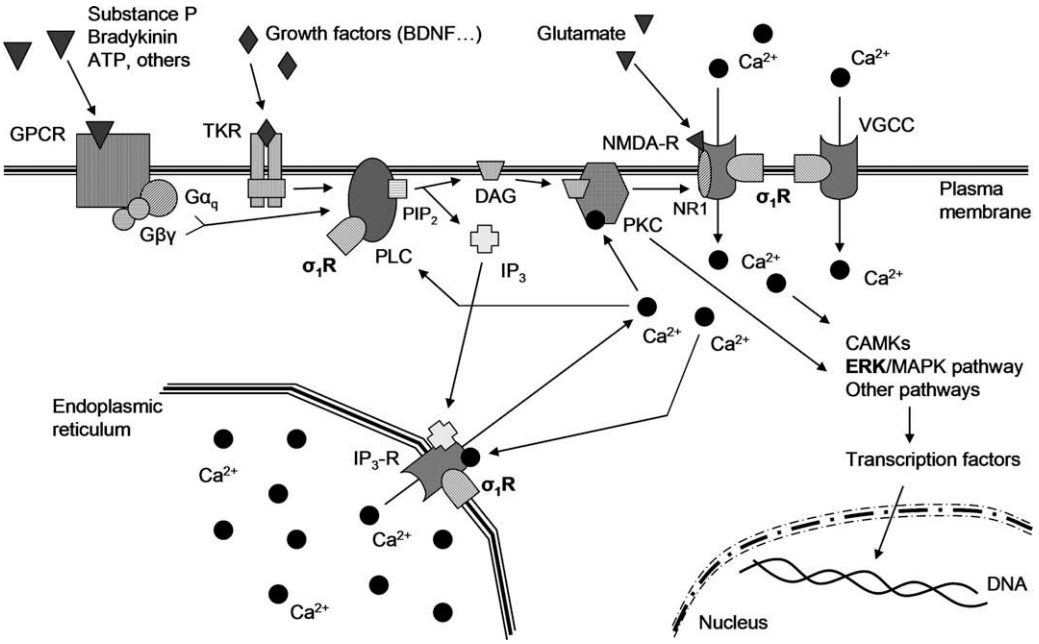

Fig. 7. Involvement of σ_1R in signal transduction pathways. Activation by nociceptive mediators released into the dorsal horn of G-protein-coupled receptors (GPCRs) involving $G\alpha_q$-coupling and other phospholipase C (PLC)-activating G proteins, and/or tyrosine kinase receptors (TKRs), stimulates PLC enzymes to hydrolyze phosphorylated phosphatidylinositols (e.g., PIP_2) to produce diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3). IP_3 binds then to IP_3 receptors (IP_3-R) in the endoplasmic reticulum to promote the efflux of Ca^{2+} to the cytoplasm. Raises of cytosolic Ca^{2+} are also produced by Ca^{2+} influx through ionotropic *N*-methyl-D-aspartate receptors (NMDA-Rs) and voltage-gated Ca^{2+} channels (VGCCs). In turn, some PLC isoforms are activated by Ca^{2+} and low increases in intracellular Ca^{2+} concentrations activate IP_3-R (Ca^{2+}-induced Ca^{2+} release) to promote more Ca^{2+} to be released from the endoplasmic reticulum. Interestingly, protein kinase C (PKC) as well as calcium/calmodulin-dependent kinases (CAMKs) is activated by Ca^{2+}. These enzymes phosphorylate diverse plasma membrane receptors and ion channels (e.g., NR1 subunit of NMDA-R), being responsible for their rapid sensitization, and activate different signaling pathways, including the ERK pathway, leading to both rapid kinase-dependent posttranslational regulation and long-term changes via transcriptional regulation in dorsal horn neurons. σ_1R plays a key role in the control of intracellular Ca^{2+} levels. Activation of σ_1Rs increases intracellular Ca^{2+} concentrations by potentiating both Ca^{2+} entry at the plasma membrane level (via NMDA-R and VGCC) and Ca^{2+} mobilization from endoplasmic stores (via PLC and IP_3-R), which is followed by increased kinase sensitization (rapid) and transcriptional activation (long-term) of key gene products underlying pain hypersensitivity. Absence/blocking of σ_1Rs would avoid upregulation of Ca^{2+}-dependent sensitizing intracellular cascades.

(allodynia) and it is consonant with the reduced wind-up responses found in spinal cords from mice lacking σ_1Rs.

Taken together, our findings propose the σ_1R as a new constituent of the mechanisms modulating activity-induced sensitization in nociceptive pathways and thus as a new potential target of action for drugs designed to alleviate neuropathic pain.

Conflict of interest

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

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