

# Peripheral and spinal TRPA1 channels contribute to formalin-induced long-lasting mechanical hypersensitivity

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**Background:** Transient receptor potential ankyrin 1 (TRPA1) is a non-selective cation channel expressed by a subset of nociceptive neurons that acts as a multimodal receptor. Its activity contributes to modulate nociceptive transmission in acute inflammatory pain. However, the role of this channel in chronic pain has been less studied. The purpose of this study was to investigate the local peripheral and spinal participation of TRPA1 channels in formalin-induced long-lasting hypersensitivity.

**Materials and methods:** Formalin (1%)-induced chronic hypersensitivity was determined by the application of von Frey filaments to ipsilateral and contralateral paws and through pharmacological testing using a selective TRPA1 blocker (A-967079). TRPA1 expression in the dorsal root ganglion (DRG) and spinal cord was analyzed by Western blotting.

**Results:** Formalin (1%) injection produced acute flinching behavior (1 h) as well as secondary allodynia and hyperalgesia (12 days). Local peripheral pretreatment (10 min before) or posttreatment (6 days later) with A-967079 (1–100  $\mu$ M) partially prevented and reversed, respectively, in a dose-dependent manner, long-lasting secondary mechanical allodynia and hyperalgesia evoked by 1% formalin. Likewise, intrathecal pretreatment or posttreatment with A-967079 partially prevented and reversed, respectively, formalin-induced long-lasting hypersensitivity. A-967079 (100  $\mu$ M) completely abolished the pro-nociceptive effect of formalin (adjusted to pH 7.4). Finally, formalin injection increased TRPA1 protein expression in the DRG and spinal cord.

**Conclusion:** Results indicate that TRPA1 expressed in the DRG and spinal cord plays a relevant role in formalin-induced long-lasting secondary nociceptive hypersensitivity.

**Keywords:** allodynia, chronic pain, formalin, hyperalgesia, TRPA1

## Introduction

The transient receptor potential ankyrin 1 (TRPA1) is a polymodal cation channel that is highly expressed in nociceptors, where it functions to detect several pro-nociceptive molecules involved in pain and pruritus.<sup>1–4</sup> However, TRPA1 is also expressed in non-neuronal cells such as keratinocytes,<sup>5</sup> megakaryocytes,<sup>6</sup> or enterochromaffin cells.<sup>7</sup> With regard to pain, this channel has been found in the dorsal root ganglion (DRG), trigeminal ganglion, and spinal cord.<sup>1,8–13</sup> Activation of TRPA1 leads to activation of sensory neurons, release of inflammatory neuropeptides, and pain hypersensitivity.<sup>4,14</sup>

Several studies have reported that TRPA1 participates in inflammatory<sup>15–23</sup> and neuropathic<sup>16,24–28</sup> pain models. It has been reported that the TRPA1 channel blocker Chembridge-5861526 (CHEM), given intrathecally, diminishes formalin-induced secondary mechanical hypersensitivity<sup>18</sup> suggesting that TRPA1 channels play an important role in the acute and short-lasting effects of formalin. The role of this channel

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in the development of long-lasting secondary mechanical allodynia and hyperalgesia induced by formalin remains unknown. Accordingly, we hypothesized that local peripheral and spinal TRPA1 underlies formalin-induced acute nociception and long-lasting secondary hyperalgesia and allodynia. Therefore, the purpose of this study was to investigate the participation of peripheral and spinal TRPA1 channels in the development and maintenance of formalin-induced acute nociception and long-lasting secondary mechanical allodynia and hyperalgesia in rats. Moreover, we analyzed TRPA1 protein expression in sites relevant to the nociceptive processing (DRG and spinal cord) in naïve and formalin-treated rats.

## Materials and methods

### Subjects

Experiments were carried out in female Wistar rats (180–200 g) of 8 to 10 weeks old at the beginning of experiments. At the time of the experiment, animals were 1 week old or older. Animals were housed in a room on a 12-h light/dark cycle and had free access to food and drinking water before the experiments. Experiments were conducted in accordance with the Guidelines of Ethical Standards for Investigation of Experimental Pain in Animals.<sup>29</sup> In addition, the Institutional Animal Care and Use Committee approved our study (Cinvestav, Protocol 0092-14). All tests were undertaken during the light phase. The number of experimental animals was kept to a minimum.

### Reagents

Formalin (Sigma-Aldrich, St. Louis, MO, USA) was freshly prepared and dissolved in 0.9% NaCl (sterile saline) in most of the experiments. In one experiment, formalin was dissolved in phosphate-buffered saline (pH 7.4). Then, (1E,3E)-1-(4-Fluorophenyl)-2-methyl-1-penten-3-one oxime (A-967079, Tocris Bioscience, Ellisville, MO, USA) was dissolved in dimethyl sulfoxide (DMSO) to make a stock solution and kept at  $-20^{\circ}\text{C}$  until the day of the experiment. The final concentration of DMSO was less than 1% (v/v). For local peripheral administration, drugs were given in a volume of 50  $\mu\text{L}$  into the formalin-treated paw. For intrathecal (i.t.) administration, drugs were administered in a volume of 10  $\mu\text{L}$  (into the subarachnoid space) through lumbar puncture.

### Induction and assessment of acute nociception and long-lasting secondary mechanical allodynia and hyperalgesia

Rats ( $n=6$  per group) were briefly immobilized in a manner that allowed access to the right hind limb. Then, animals

received a subcutaneous injection of 1% formalin (50  $\mu\text{L}$ ) into the dorsal surface on the right hind paw (ipsilateral) with a 30-gauge needle. Acute nociception was assessed using the formalin test.<sup>30</sup> Rats were placed in open acrylic observation chambers for 30 min to allow them to acclimate to their surroundings; then, they were removed for formalin administration. Mirrors were placed in each chamber to enable unhindered observation. Nociceptive behavior was quantified as the number of flinches of the injected paw during 1-min periods every 5 min, for up to 60 min after injection.<sup>30</sup> Flinching was characterized as rapid and brief withdrawal, or as flexing of the injected paw. Formalin-induced flinching behavior was biphasic. The initial acute phase (Phase 1, 0–10 min) was followed by a relatively short quiescent period, which was then followed by a prolonged tonic response (Phase 2, 15–60 min). After acute nociceptive evaluation, animals were maintained for the next 12 days.

The hypersensitivity induced by formalin was tested at baseline and also 1, 3, 6, and 12 days after injection. However, for subsequent experiments, the sixth day was chosen to evaluate nociceptive behaviors as, at this time, nociceptive behaviors are already established.<sup>31,32</sup> To evaluate the evoked nociceptive behaviors, rats were placed into testing cages on a wire-mesh bottom and allowed to acclimate for 30 min. Rats were stimulated 10 times with two von Frey filaments (Stoelting Co, Wood Dale, IL, USA) to the base of the third toe on the plantar surface of both paws. Three trials were carried out to determine the paw response frequency. A force of 10 mN (1 g) does not activate cutaneous nociceptors in naïve rats, as it does not lead to paw withdrawal. Thus, a response to this filament is an indication of allodynia. Likewise, a force of 250 mN (26 g) is considered a noxious stimulus.<sup>33</sup> An increased response to this filament indicates hyperalgesia. Rats were killed in a  $\text{CO}_2$  chamber at the end of the experiment.

### Western blot analysis

Western blot analysis was used to determine the expression of TRPA1 in ipsilateral DRG and the dorsal spinal cord. For this, naïve and formalin-treated rats ( $n=3$  per group) were killed by decapitation. DRGs (L4–L6), corresponding to the afferent pathway from the hind paw, and the dorsal spinal cord (L4–L6 segment) were carefully removed and processed as previously described.<sup>34</sup> Tissues from individual animals were homogenized in ice-cold lysis buffer (in mM: 150 NaCl, 50 Tris-HCl, 5 EDTA), pH 7.4 for 30 min at  $4^{\circ}\text{C}$ . The protease inhibitors phenylmethylsulfonyl fluoride (1 mM), aprotinin (10  $\mu\text{g}/\text{mL}$ ), leupeptin (10  $\mu\text{g}/\text{mL}$ ), pepstatin A (10  $\mu\text{g}/\text{mL}$ ),

and the surfactant 0.1% Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA) were added to the lysis buffer immediately prior to use. The homogenate was then centrifuged (Eppendorf, Hamburg) at 14,000 rpm for 10 min to remove cellular debris. The resultant supernatant was used to measure protein concentration by using Bradford's method (Bio-Rad, Hercules, CA, USA).

Fifty micrograms of total protein were separated by electrophoresis in 10% SDS-polyacrylamide gels and transferred to polyvinylidene fluoride membranes. Membranes were blocked in 5% non-fat milk in PBS at pH 7.4 (in mM: 137 NaCl, 2.7 KCl, 10 Na<sub>2</sub>HPO<sub>4</sub>, and 2 KH<sub>2</sub>PO<sub>4</sub>) with 0.05% Tween-20 for 1 h. After that, they were washed and incubated overnight at 4°C in 5% non-fat dry milk/PBS containing rabbit anti-TRPA1 antibody (1:2,000, Cat. No. 68847, Abcam, Cambridge, MA, USA). Membranes were incubated for 1 h at room temperature in 1% non-fat milk/PBS containing the horseradish peroxidase-conjugated secondary antibody (bovine anti-rabbit 1:3000, Cat. No. sc-2370, Santa Cruz Biotechnology, Dallas, TX, USA or goat anti-mouse 1:10,000, Cat. No. 115-035-003, Jackson Immuno Research, West Grove, PA, USA). Protein signal detection was achieved with the ECL chemiluminescence system (Millipore, Billerica, MA, USA). The next day, blots were incubated with a monoclonal antibody directed against  $\beta$ -actin (1:5,000, Cat. No. MAB1501R, Millipore, Billerica, MA, USA), which was used as internal control to normalize TRPA1 protein expression level. Bands were analyzed with Image Studio Ver. 4.0 (LI-COR, Lincoln, NE, USA).

## Experimental design

To determine whether blockade of TRPA1 channels at the peripheral or spinal level modifies formalin-induced secondary allodynia and hyperalgesia, we used the highly selective TRPA1 blocker A-967079 (IC<sub>50</sub>: 289 nM).<sup>24</sup> Dose selection was based on previous studies<sup>17,24</sup> and pilot experiments in our laboratory. Formalin (1%) was selected because, at this concentration, formalin activates C fibers wherein TRPA1 is mainly expressed.<sup>3,35</sup>

In order to assess the participation of local peripheral and spinal TRPA1 channels in the development of formalin-induced secondary allodynia and hyperalgesia, rats received a subcutaneous (s.c.) injection into the dorsal surface of the right hind paw (50  $\mu$ L) or an i.t. injection (10  $\mu$ L) of vehicle (1% DMSO) or increasing doses of A-967079 (1–100  $\mu$ M) 10 min before 1% formalin injection into the right (ipsilateral) paw. Allodynia and hyperalgesia were evaluated 6 days later in both paws. To determine whether A-967079 acted locally,

the maximal dose of A-967079 used (100  $\mu$ M) was injected into the left (contralateral) paw 10 min before a 1% formalin injection into the right paw, and the effect on nociceptive behavior was assessed in both paws.

In order to determine the participation of local peripheral and spinal TRPA1 channels in the maintenance of formalin-induced secondary allodynia and hyperalgesia, rats received a s.c. injection into the dorsal surface of the right hind paw (50  $\mu$ L) or an i.t. injection (10  $\mu$ L) of vehicle (1% DMSO) or increasing doses of A-967079 (1–100  $\mu$ M) 6 days after 1% formalin injection into the right (ipsilateral) paw. Animals were tested before drug administration to register a baseline of nociceptive behaviors. Allodynia and hyperalgesia were evaluated 1 h after drug administration because, at this time, we observed the maximal antinociceptive response.

In order to discharge the effects of hydrogen ions (H<sup>+</sup>) in the development of formalin-induced secondary allodynia and hyperalgesia, rats received a local peripheral pretreatment with vehicle (1% DMSO) or A-967079 (100  $\mu$ M) 10 min before the administration of 1% formalin pH 7.4, instead of regular formalin (without controlling the pH).

In order to assess the role of TRPA1 channels in sites related to nociceptive processing induced by formalin, we determined the expression of this channel at the ipsilateral DRG and dorsal spinal cord at 1 h as well as on days 1, 6, and 12 after the 1% formalin injection.

## Data and statistical analysis

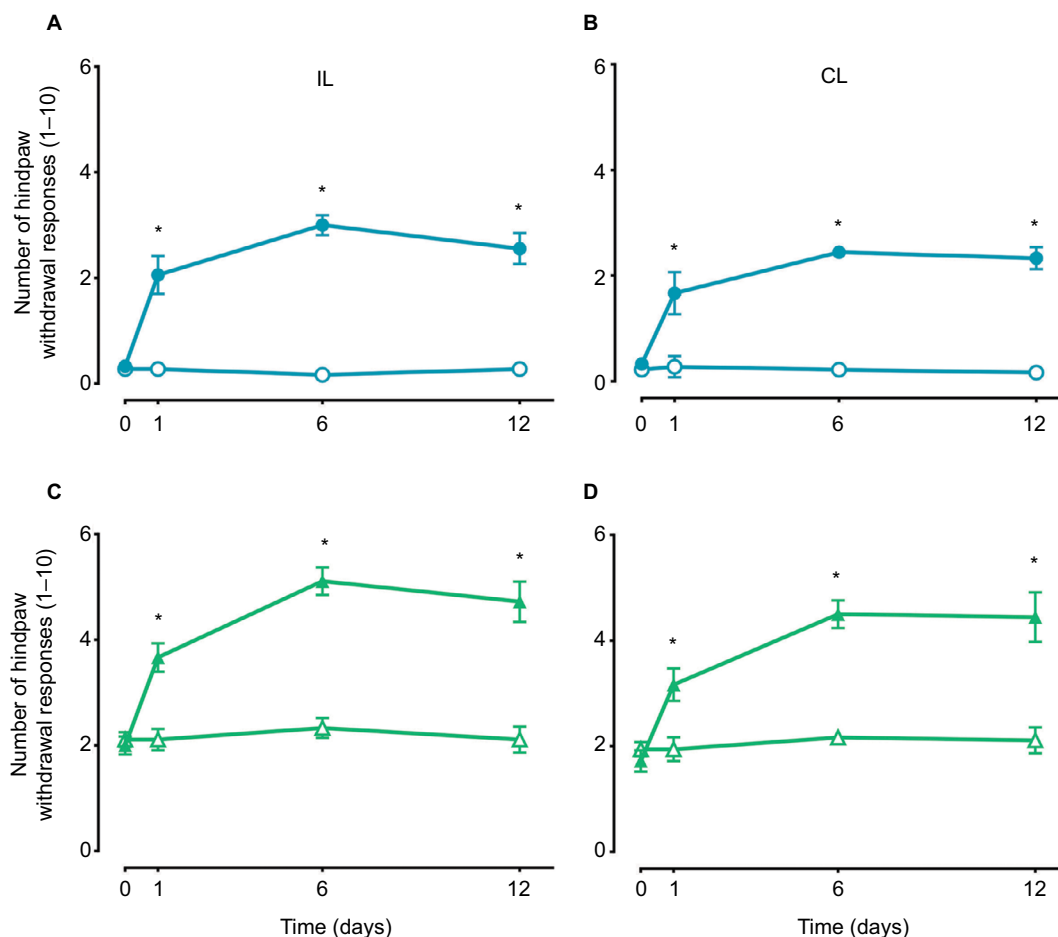
Behavioral data are expressed as mean  $\pm$  SEM of the hind paw withdrawal response of six rats for each group. Protein expression data are the mean  $\pm$  SEM of three independent experiments.

Data were analyzed by one-way analysis of variance (ANOVA) followed by the Student–Newman–Keuls post hoc test; *p*-values less than 0.05 were considered significant. Statistical analyses were conducted using GraphPad Prism 6.0 (GraphPad Software, Inc., La Jolla, CA, USA).

## Results

### Acute and secondary allodynia and hyperalgesia induced by formalin

Formalin administration into the dorsal surface of the right hind paw resulted in acute nociception (spontaneous flinching) lasting approximately 1 h (data not shown) as well as secondary mechanical allodynia and hyperalgesia lasting from 1 to 12 days in the injected (ipsilateral; Figure 1A and C) and in the uninjured (contralateral; Figure 1B and D) hind paw. This was observed as a bilateral increase in the paw withdrawal responses to application of von Frey filaments, which was



**Figure 1** The time course of the mean number of paw withdrawal responses ( $n=6 \pm \text{SEM}$ ) to 10 applications of von Frey filaments of two different bending forces 10 (circles, **A** and **B**) and 250 mN (triangles, **C** and **D**) following subcutaneous administration of 1% formalin (1% F).

**Note:**  $p < 0.05$  by two-way ANOVA followed by the Student–Newman–Keuls test.

**Abbreviations:** IL, ipsilateral; CL, contralateral; ANOVA, analysis of variance.

significant 1 day after formalin administration and lasted for at least 12 days ( $p < 0.05$ ). For experiments with the TRPA1 channel blocker, the sixth day was chosen to evaluate nociceptive behaviors as previously reported.<sup>36</sup> In addition, effects in the contralateral paw were omitted for the sake of clarity.

### Effects of A-967079 on formalin-induced acute and long-lasting hypersensitivity

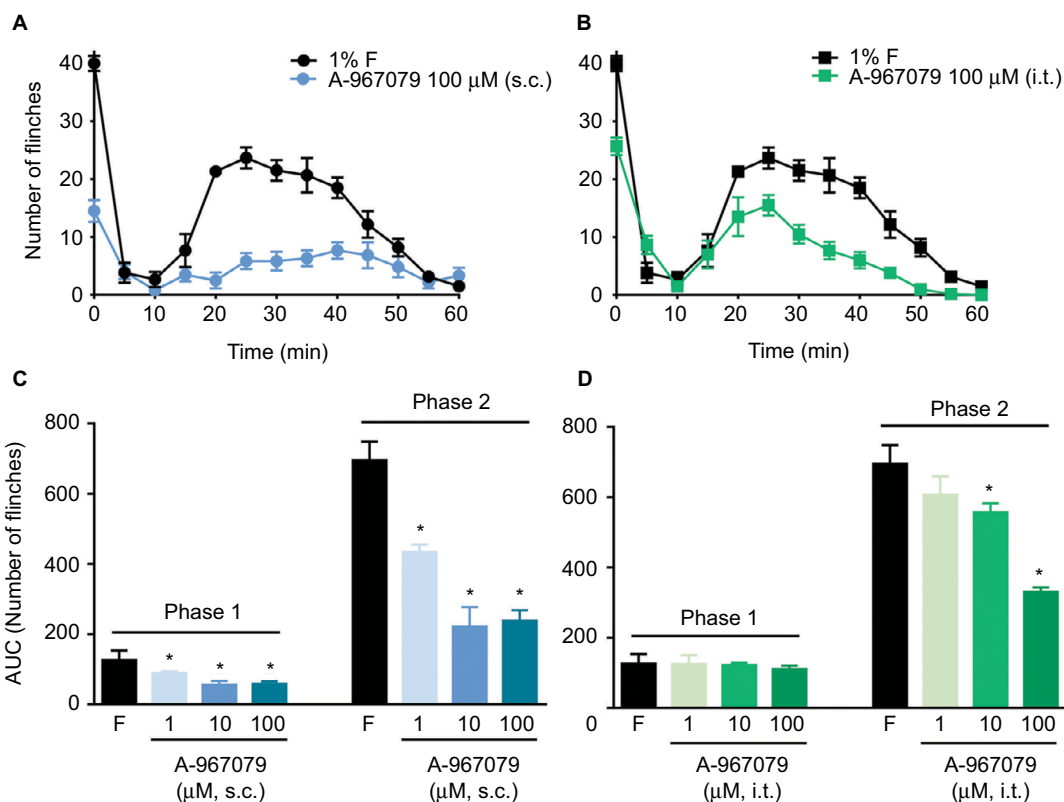
Local peripheral and i.t. pretreatment (10 min before) with A-967079 (1–100  $\mu\text{M}$ ) partially prevented formalin-induced acute (1 h) nociceptive flinching during phases 1 and 2 ( $p < 0.05$ , Figure 2). Moreover, local peripheral ( $p < 0.05$ , Figure 3) or spinal ( $p < 0.05$ , Figure 4) pre- or posttreatment (6 days later) with A-967079 (1–100  $\mu\text{M}$ ) partially prevented and reversed, respectively, 1% formalin-induced secondary mechanical allodynia and hyperalgesia in ipsilateral and contralateral paws. Of note, local peripheral administration of A-967079 (100  $\mu\text{M}$ ) into the contralateral paw did not affect formalin-induced hypersensitivity, indicating that the

antinociceptive effect of A-967079 is mediated by the blockade of TRPA1 located in the periphery (Figure 3).

Formalin (adjusted to pH 7.4) injection produced lesser nociceptive behaviors than formalin dissolved in saline. Interestingly, local peripheral pretreatment with A-967079 (100  $\mu\text{M}$ ) completely prevented formalin (pH 7.4)-induced long-lasting allodynia and hyperalgesia ( $p < 0.05$ , Figure 5).

### Effect of formalin on TRPA1 expression in DRG and spinal cord

Western blot analysis identified a 123-kDa band corresponding to the molecular weight expected for TRPA1 in the ipsilateral DRG and dorsal spinal cord of naïve and formalin-treated rats (Figure 6). Moreover, formalin (1%) injection increased TRPA1 protein expression on days 6 and 1 after injection in the ipsilateral DRG ( $p < 0.05$ , Figure 6A) and dorsal spinal cord ( $p < 0.05$ , Figure 6B). No change in TRPA1 protein expression was detected in the contralateral DRG or dorsal horn (data not shown).



**Figure 2** Antinociceptive effect of the local peripheral (s.c.; **A** and **C**) and intrathecal (i.t.; **B** and **D**) pretreatment with A-967079 (1–100 μM) in rats subjected to the 1% formalin test.

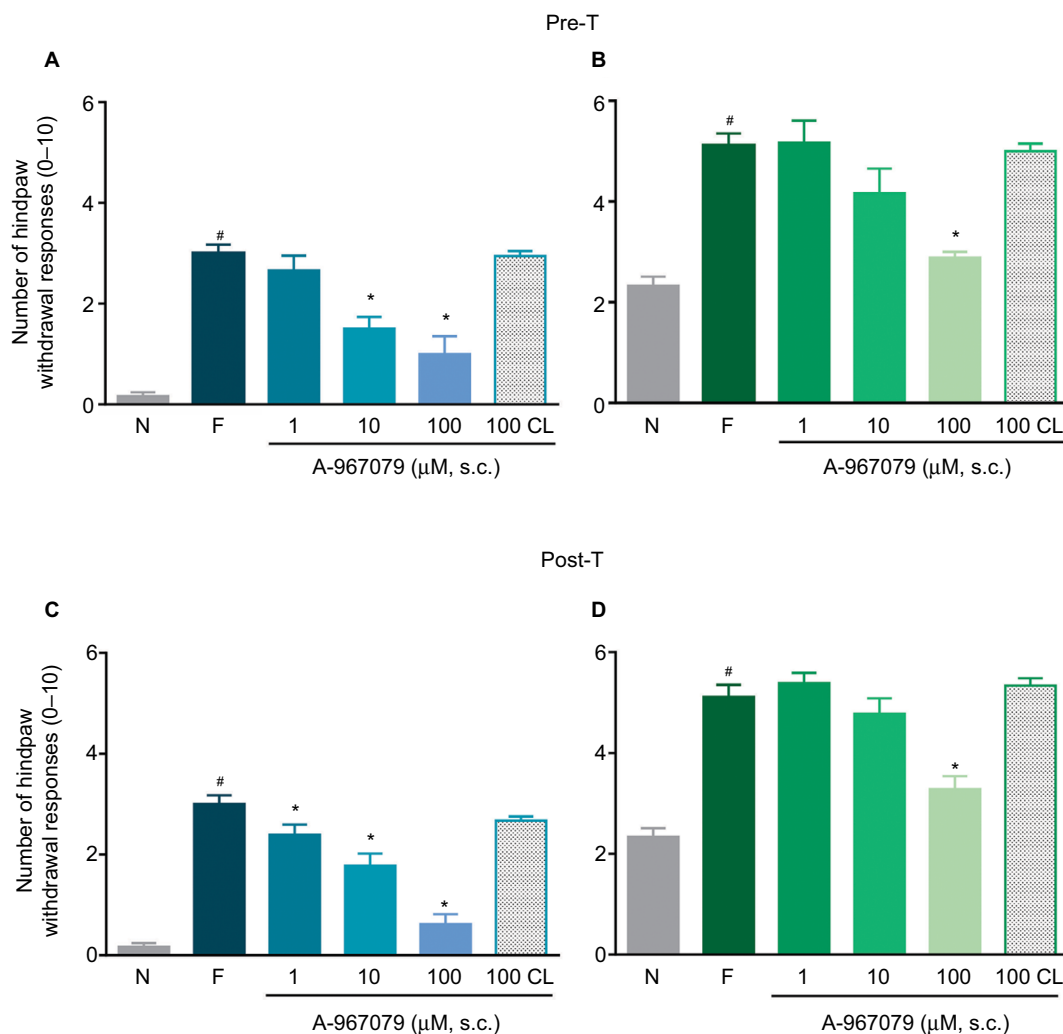
**Notes:** (**A** and **B**) The time course observed during phases 1 and 2 of the formalin test after administration of 100 μM of A-967079 into the dorsal part of the paw (**A**) or i.t. (**B**) 10 min before formalin injection. **C** and **D** show the data on the area under the curve for the phases 1 and 2 for local peripheral and intrathecal administration, respectively. Data are mean ( $n=6 \pm \text{SEM}$ ). In plots **C** and **D**, \* $p < 0.05$ , significantly different from the formalin (F) group, by one-way ANOVA followed by the Student–Newman–Keuls test. **Abbreviation:** ANOVA, analysis of variance.

## Discussion

In this study, we found that either local peripheral or i.t. administration of the TRPA1 channel blocker A-967079 prevented acute nociception in the formalin test. Furthermore, this drug prevented and reversed formalin-induced long-lasting hypersensitivity in rats. As formalin is a TRPA1 channel agonist<sup>35</sup> and A-967079 is a highly selective TRPA1 channel blocker,<sup>24</sup> our data suggest that peripheral and spinal TRPA1 channels participate in acute nociception as well as in the development and maintenance of long-lasting secondary allodynia and hyperalgesia induced by formalin. These data suggest that acute nociception and secondary allodynia and hyperalgesia depend on TRPA1 channel-induced persistent small afferent input as well as a sensitized dorsal horn. Our results agree with previous observations demonstrating that TRPA1, indeed, participate in formalin-induced acute nociception.<sup>18,35</sup> Moreover, the fact that acute nociception induced by the selective TRPA1-channel agonist allyl isothiocyanate (AITC) is prevented by A-967079 further supports that TRPA1 plays an important role in formalin-induced acute nociception.<sup>24</sup> However, to our knowledge, this is the first

report about the anti-allodynic and anti-hyperalgesic effect of A-967079 in formalin-induced long-lasting nociceptive hypersensitivity. Our study agrees with a previous report showing that the TRPA1-channel blocker CHEM diminishes formalin-induced secondary mechanical hypersensitivity (24 h),<sup>18</sup> suggesting that TRPA1 channels play an important role in the short- (1 day) and long-lasting (6–12 days) effects of formalin. Moreover, our data concord with those showing that i.t. injection of the TRPA1-channel blocker HC-030031 reduces long-lasting (28 days) mechanical and cold allodynia induced by complete Freund's adjuvant (CFA) in mice.<sup>11</sup> The fact that A-967079 blocks AITC-induced acute nociceptive behaviors in vivo,<sup>24</sup> further suggest that TRPA1 channels are important for the development and maintenance of nociceptive hypersensitivity induced by formalin. Moreover, our data agree with several studies reporting that other TRPA1 antagonists (HC030031, CHEM) or genetic deletion of *Trpa1* reduces nociceptive hypersensitivity induced by colorectal distension, pancreatitis, colitis, osteoarthritis, REM-sleep deprivation, skin incision, intraplantar capsaicin, carrageenan, and peripheral nerve injury.<sup>15–24,28,37,38</sup> Thus, our





**Figure 3** Antinociceptive effect of the local peripheral (s.c.) pretreatment (**A** and **B**) or posttreatment (**C** and **D**) with A-967079 (1–100  $\mu$ M) on formalin-induced secondary mechanical allodynia (**A** and **C**) and hyperalgesia (**B** and **D**) in rats.

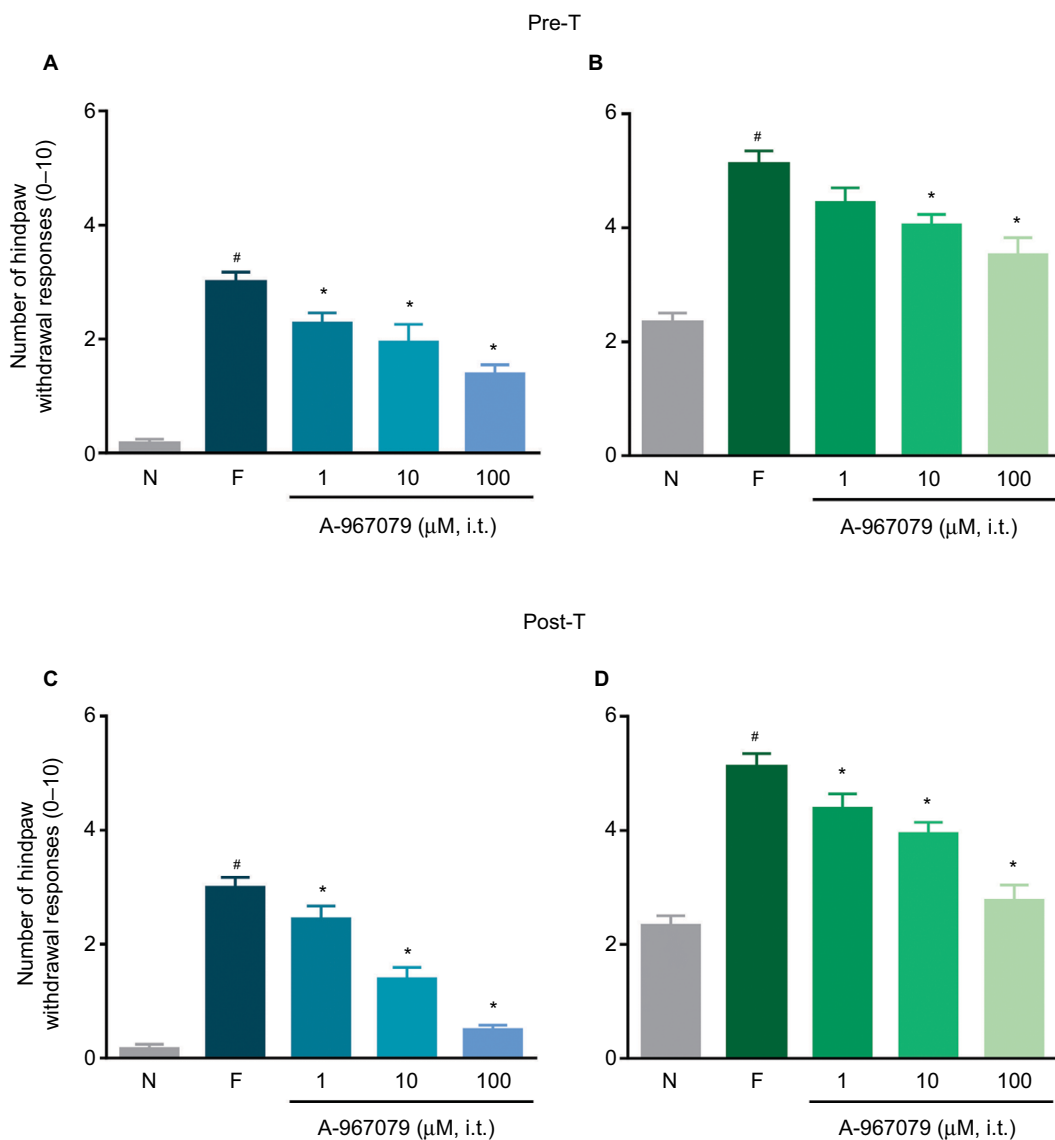
**Notes:** Data are expressed as the mean ( $n=6 \pm$  SEM) paw withdrawal responses to the application of von Frey filaments (10 and 250 mN) to the plantar surface of rat paws before (naive, N) and after 1% formalin (F). In all cases, <sup>#</sup> $p < 0.05$  versus N group; <sup>\*</sup> $p < 0.05$  versus F group, by one-way ANOVA followed by the Student–Newman–Keuls test.

**Abbreviations:** pre-T; pretreatment; post-T, posttreatment; ANOVA, analysis of variance; CL, contralateral.

results extend these observations by showing that blockade of TRPA1 channels has important consequences on the development and maintenance of formalin-induced nociceptive hypersensitivity. Some studies have reported that oral administration of A-967079 does not diminish mechanical allodynia induced by CFA, chronic constriction injury, or spinal nerve ligation models.<sup>24</sup> It is likely that this discrepancy is due to the low amount of compound that reaches local peripheral or spinal sites after oral administration. In support of this, i.e. administration of CHEM or HC-030031 reduces tactile allodynia in neuropathic rats.<sup>26,27</sup>

It has been established that activation of TRPA1 channels by its agonists (cinnamaldehyde, AITC, and formalin) in the paw produces mechanical sensitization in rats.<sup>15,16,35,39</sup> Forma-

lin injection induces peripheral and central sensitization. In the first case, there is evidence that formalin injection leads to serotonin (5-HT), histamine, IL-1 $\beta$ , and TNF $\alpha$  release at the periphery, which in turn activate their receptors to produce direct nociception or sensitization.<sup>40–42</sup> With regard to central effects, formalin injection starts a cascade of events including release of glutamate, prostaglandins, and 5-HT at the dorsal horn spinal cord<sup>43,44</sup> and activation of microglia which, in turn, releases nitric oxide, nerve growth factor, cytokines (IL-1 $\beta$  and TNF $\alpha$ ), and brain-derived neurotrophic factor (BDNF).<sup>31,45,46</sup> Acting at their receptors, these molecules promote central sensitization and nociception. Thus, the local peripheral anti-allodynic and anti-hyperalgesic effects of A-967079 could be due, in the first instance, to the



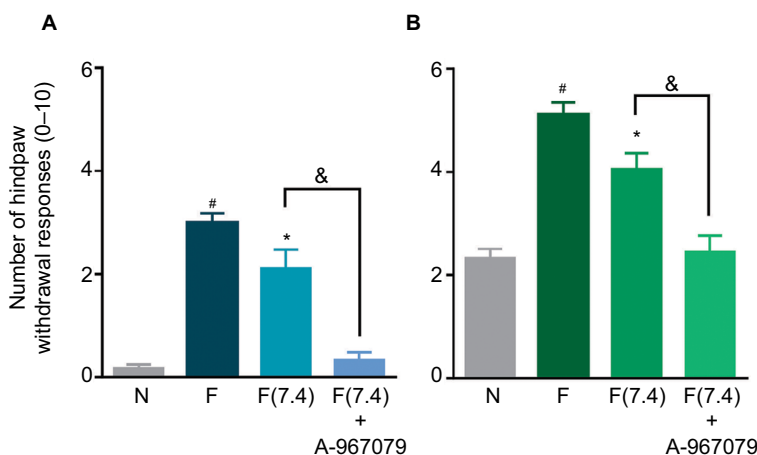
**Figure 4** Antinociceptive effect of the intrathecal (i.t.) pretreatment (**A** and **B**) or posttreatment (**C** and **D**) with A-967079 (1–100 μM) on formalin-induced secondary mechanical allodynia (**A** and **C**) and hyperalgesia (**B** and **D**) in rats.

**Notes:** Data are expressed as the mean ( $n=6 \pm \text{SEM}$ ) paw withdrawal responses to the application of von Frey filaments (10 and 250 mN) to the plantar surface of rat paws before (naïve, N) and after 1% formalin (F). In all cases  $\#p < 0.05$  versus N group;  $*p < 0.05$  versus F group, by one-way ANOVA followed by the Student–Newman–Keuls test. **Abbreviations:** pre-T, pretreatment; post-T, posttreatment; ANOVA, analysis of variance.

blockade of TRPA1 channels located on peripheral endings of primary afferent neurons. This will reduce the release of several pro-nociceptive substances, avoiding peripheral sensitization. On the other hand, A-967079 – given into the spinal cord – may reduce formalin-induced secondary allodynia and hyperalgesia by diminishing release of pro-nociceptive molecules as well as activation of microglia. In addition, i.t. A-967079 could reduce dorsal root reflexes or direct antidromic activation and cutaneous blood flow leading to a reduction in substance P and calcitonin gene-related peptide release in peripheral and central terminals of primary afferent neurons.<sup>24,25,47,48</sup> Moreover, activation of

spinal TRPA1 channels leads to an increase of the excitatory synaptic transmission in substantia gelatinosa neurons,<sup>49,50</sup> which in turn reduces activation thresholds and enhances afferent activity.<sup>39</sup> Thus, i.t. A-967079 could block this excitatory synaptic transmission and some consequences of the descending pain facilitation.<sup>18</sup> All of these actions would reduce the pre- and postsynaptic excitability of afferent and convergent pain-relay neurons.

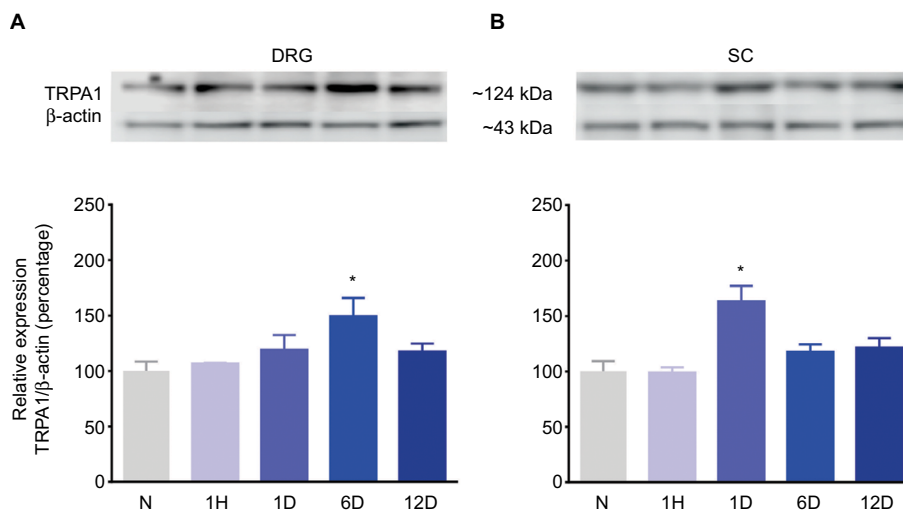
Local peripheral or i.t. administration of A-967079 produced a partial reversal of secondary allodynia and hyperalgesia in the formalin model. This result may suggest that other mechanisms, besides activation of TRPA1 chan-



**Figure 5** The antinociceptive effect of the local peripheral pretreatment with A-967079 (100  $\mu$ M) on 1% formalin (pH 7.4)-induced secondary mechanical allodynia (A) and hyperalgesia (B) in rats.

**Notes:** Data are expressed as the mean ( $n=6 \pm$  SEM) paw withdrawal responses to the application of von Frey filaments (10 and 250 mN) to the plantar surface of rat paws before (naive, N) and after 1% formalin without control of pH [F] or 1% formalin at pH 7.4 [F(7.4)]. In all cases,  $^{\#}p < 0.05$  versus N group;  $^*p < 0.05$  versus F group;  $^{\&}p < 0.05$  versus F(7.4) group, by one-way ANOVA followed by the Student–Newman–Keuls test.

**Abbreviation:** ANOVA, analysis of variance.



**Figure 6** Effect of 1% formalin on the TRPA1 protein expression levels in the ipsilateral DRGs (A) and dorsal SC (L4–L6) (B) from rats.

**Notes:** Insets show representative blots obtained with TRPA1 and  $\beta$ -actin primary antibodies. The specific signal for TRPA1 was quantified and plotted as the percentage of expression against  $\beta$ -actin ( $n=3 \pm$  SEM).  $^*p < 0.05$  compared to the naive group, by one-way ANOVA followed by the Student–Newman–Keuls test.

**Abbreviations:** N, naive; 1H, 1 hour; 1D, 1 day; 6D, 6 days; 12D, 12 days; ANOVA, analysis of variance; DRG, dorsal root ganglion; SC, spinal cord.

nels, are involved in the development and maintenance of secondary allodynia and hyperalgesia. Former studies have established that activation of acid-sensing ion channels (ASICs) and TRPV1 is a common feature in inflammatory pain in which pH is not controlled.<sup>51,52</sup> Furthermore, we have demonstrated that hydrogen ions ( $H^+$ ) indeed contribute to the development of secondary allodynia and hyperalgesia induced by formalin.<sup>53</sup> Thus, injection of formalin without pH control would stimulate several targets, including ASICs, TRPV1,<sup>54</sup> and TRPA1<sup>35</sup> channels. In order to discharge the participation of  $H^+$  and activation of TRPV1 and ASICs, we designed an experiment controlling the pH of formalin to 7.4. We observed that 1% formalin at pH 7.4 produced

lower levels of secondary allodynia and hyperalgesia than 1% formalin (without pH control). This result agrees with a previous observation showing that 0.5% formalin at pH 7.4 induced lower nociceptive behaviors than either 0.5% formalin at pH 5.8 or non-pH-adjusted formalin.<sup>53</sup> In this condition (1% formalin at pH 7.4), local peripheral administration of A-967079 completely abolished development of formalin-induced secondary allodynia and hyperalgesia in rats. Thus, it seems that under controlled conditions of pH, the only target responsible of the nociceptive hypersensitivity is TRPA1 channel.

In line with the behavioral results, Western blot data revealed that formalin injection increases TRPA1 protein



expression in the ipsilateral DRG and dorsal region of the spinal cord. Thus, our data indicate that this channel is located in sites related to the peripheral and spinal processing of pain. Our data are the first to demonstrate that formalin enhances TRPA1 protein expression in the DRG and spinal cord. These results concord with those from other studies demonstrating that CFA, colitis, and nerve injury enhances TRPA1 protein and mRNA expression in the DRG of rats and mice.<sup>1,8,11,39,55–58</sup>

## Conclusion

Formalin produces long-lasting nociceptive hypersensitivity, which is sensitive to TRPA1 blockade. Data suggest that the peripheral and spinal TRPA1 channel located in the DRG and spinal cord is important for the development and maintenance of formalin-induced secondary allodynia and hyperalgesia. This channel could be a target to develop useful drugs to treat long-lasting nociceptive hypersensitivity.

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## Author contributions

JM and VAM-R conceived, designed, and conducted the experiments. VAM-R, RN-N, CGG-P, and GG conducted the experiments. JM, JET-L, and VG-S supervised the experiments and analyzed data. All authors contributed toward data analysis, drafting, and critically revising the paper, gave final approval of the version to be published, and agreed to be accountable for all aspects of the work.

## Disclosure

The authors report no conflicts of interest in this work.

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