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The involvement of the TRPA1 receptor in a mouse model of sympathetically maintained neuropathic pain

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ABSTRACT

Sympathetic fibres maintain some forms of neuropathic pain, but the underlying mechanisms are poorly understood. Therefore, this study investigated the possible involvement of transient receptor potential ankyrin 1 (TRPA1) and the role of the sympathetic nervous system (involved in sympathetically maintained neuropathic pain) in a model of neuropathic pain induced by sciatic nerve chronic constriction injury (CCI) in mice. Systemic injection of the selective TRPA1 antagonist HC-030031 reversed the mechanical and cold allodynia that was induced by sciatic nerve chronic constriction injury (CCI). Nerve injury also sensitised mice to nociception, which was induced by the intraplantar injection of a low dose of the TRPA1 agonist allyl isothiocyanate without changing TRPA1 immunoreactivity in the injected paw. Furthermore, chemical sympathectomy produced by guanethidine largely prevented CCI-induced mechanical and cold allodynia. CCI also induced a norepinephrine-triggered nociception that was inhibited by an α-adrenoceptor antagonist, norepinephrine transporter block and monoamine oxidase inhibition. Finally, the peripheral injection of HC-030031 also largely reduced CCI-induced norepinephrine nociception and mechanical or cold allodynia. Taken together, the present findings reveal a critical role of TRPA1 in mechanical and cold hypersensitivity and norepinephrine hypersensitivity following nerve injury. Finally, our results suggest that TRPA1 antagonism may be useful to treat patients who present sympathetically maintained neuropathic pain.

1. Introduction

Neuropathic pain is a debilitating condition that is poorly understood and often untreatable. It is caused by a lesion or disease of the somatosensory nervous system (Treede et al., 2008). Pain after neuropathy is the consequence of a complex interplay of mechanisms in the peripheral and central nervous systems that are among the most intractable of pain syndromes (Woolf and Mannion, 1999). Neuropathic pain is often resistant to common therapeutic interventions. Common complaints of neuropathic pain patients include spontaneous pain and painful hypersensitivity to mechanical, thermal and chemical stimuli (Baron, 2006). The sympathetic nervous system may mediate some types of neuropathic pain, as demonstrated by pain relief that is achieved by sympathetic blockade or antagonism of α-adrenoceptors (Baron, 2006). Moreover, some neuropathies cause hypersensitivity to norepinephrine, which may stimulate nociceptors and induce spontaneous pain (Ali et al., 2000; Drummond, 2010; Torebjork et al., 1995; Xanthos et al., 2008). Previous data suggest a pathological interaction between sympathetic and afferent neurons in the skin because the intensity of spontaneous pain and the spatial distribution of mechanical dynamic and punctate hyperalgesia are greater during high sympathetic activity than during

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low activity (Baron, 2006; Baron et al., 2002). Furthermore, the interaction between sympathetic and afferent neurons in deep somatic tissues may also play an important role in the pathophysiology of sympathetically maintained pain (Drummond et al., 1991). However, the mechanisms involved in sympathetically maintained neuropathic pain are poorly understood.

Transient receptor potential ankyrin 1 (TRPA1) is a non-selective, calcium-permeable cation channel that is usually expressed in a subset of small diameter primary afferent nerve fibres jointly with the TRP vanilloid 1 receptor (TRPV1) (Pertovaara and Koivisto, 2011; Story et al., 2003). This channel is relevant for the detection of nociceptive stimuli, and it may be activated by various exogenous irritants, such as allyl isothiocyanate from mustard oil, and several endogenous reactive species, such as hydrogen peroxide and unsaturated aldehydes, that are produced during tissue damage and neuropathy (Andersson et al., 2008; Andre et al., 2008; Macpherson et al., 2007; Trevisani et al., 2007). Moreover, the TRPA1 channel is activated and sensitised by different compounds that are produced during tissue damage (e.g., inflammation and neuropathic pain), such as adenosine triphosphate (ATP, via the activation of P_2X_3 receptor) (Krimon et al., 2013) and nerve growth factor (NGF) (Obata et al., 2005). Furthermore, TRPA1 may be important to the development of cold allodynia and mechanical hyperalgesia caused by trauma-, diabetes- or chemotherapy-induced neuropathy (Caspani et al., 2009; Eid et al., 2008; Nassini et al., 2011; Obata et al., 2005; Wei et al., 2009). Notably, it has been suggested that reactive oxygen species and reactive aldehydes generated during norepinephrine uptake and metabolism could be involved in neuropathic pain maintenance (Dina et al., 2008).

However, the influence of TRPA1 on neuropathic pain mediated by the sympathetic nervous system is currently unknown. Therefore, we hypothesised that TRPA1 activation by reactive species that are generated during sympathetic activation could be relevant for the maintenance of sympathetically mediated neuropathic pain. This study investigated the possible involvement of the TRPA1 receptor in a mouse model of sympathetically mediated neuropathic pain.

2. Materials and methods

2.1. Animals

Experiments were conducted using male adult Swiss mice weighing 30–35 g that were bred in the Federal University of Santa Maria animal house. Mice were maintained in home cages under a 12:12 h light–dark cycle (lights on 06:00 h) at a constant room temperature (22 ± 2 °C). The animals were acclimated in the laboratory for at least 2 h before testing. The experiments were performed with the approval of the Ethics Committee of the Federal University of Santa Maria (Process number: 164/2011) and performed in accordance with the current guidelines for the care of laboratory animals and the ethical guidelines for investigations involving experiments on conscious animals (Zimmermann, 1983). The total number of animals (316 animals) and the nociceptive stimuli used in this study were the minimum necessary to demonstrate consistent effects of drug treatments. The specific number of animals used for each experiment is indicated in Section 3 and figure legends. Animal allocation to treatment groups was randomised by a computer using Research Randomizer (www.randomizer.org). Blinded investigators performed the behavioural observations.

2.2. Drugs and reagents

All reagents were obtained from Sigma Chemical Company (St. Louis, MO, USA) unless indicated. HC-030031 (2-(1,3-dimethyl-2,6-dioxo-1,2,3,6-tetrahydro-7H-purin-7-yl)-N-(4-isopropylphenyl) acetamide) was synthesised as previously described (Andre et al., 2008). Acetone was purchased from Vetec (Rio de Janeiro, Brazil). Allyl isothiocyanate, HC-030031 and propranolol stock solutions were prepared in dimethyl sulfoxide (DMSO, 5%), Tween-80 (10%) and phosphate-buffered saline (PBS, 137 mM NaCl and 10 mM phosphate buffer, pH 7.4). The stock solutions of the other drugs were prepared in PBS. All stock solutions were diluted in PBS to the desired concentration just before use. For the actual drug administration, the final concentration of DMSO and Tween-80 did not exceed 0.5%, which did not cause any detectable effects per se.

2.3. Neuropathic pain model

For the induction of chronic mononeuropathy, mice were first anesthetised by an intraperitoneal (i.p.) injection of 90 mg/kg of ketamine plus 3 mg/kg of xylazine hydrochloride. Neuropathy was induced by chronic constriction injury (CCI) of the sciatic nerve using a similar procedure previously described for rats (Bennett and Xie, 1988) and adapted for mice (Sommer et al., 1998). Three loosely constrictive ligatures were placed around the right sciatic nerve under anaesthesia. In sham surgery, animals were anesthetised, and the sciatic nerve was exposed without performing constriction. Sham-operated animals were used as neuropathy controls. Naïve animals were neither anesthetised nor operated, and they were used as surgery controls. Nociceptive tests were performed seven days after the procedures.

2.4. Measurement of mechanical hyperalgesia

We utilised mechanical hyperalgesia as a parameter of nociception, which was characterised by a significant decrease in the mechanical paw withdrawal threshold (PWT) compared with the baseline values. The measurement of mechanical PWT was performed using an up-and-down protocol as described previously (Chaplan et al., 1994). Briefly, mice were first acclimated in individual clear plexiglas boxes ($9 \times 7 \times 11$ cm^3) on an elevated wire mesh platform to allow access to the plantar surface of the right hind paw. Next, von Frey filaments of increasing stiffness (0.02, 0.07, 0.16, 0.4, 1.4, and 4 g) were applied to the hind paw plantar surfaces with enough pressure to bend the filament, starting with the 0.4 g filament. The absence of paw lifting after 5 s led to the use of the next stiffer filament, whereas paw lifting indicated a positive response and led to the use of the next weaker filament. This protocol continued until a total of six measurements were taken or four consecutive positive or negative responses occurred. All measurements were performed on the paw ipsilateral to the surgical or sham procedure. The 50% mechanical PWT response was calculated from the resulting scores as described previously (Dixon, 1980). The 50% PWT was expressed in grams (g), and it was evaluated before (baseline) and after treatment or surgical procedures at different time points (0.5–4 h), as indicated in the protocols.

2.5. Measurement of cold allodynia

We utilised cold allodynia as another nociception parameter, which was characterised by a nocifensive reaction of animals after evaporative cooling of topically applied acetone (Caspani et al., 2009; Nassini et al., 2011). After the measurement of PWT, 20 μl of acetone was applied to the dorsal hind paw ipsilateral to the injury, and the resulting behaviour was assigned an arbitrary score. A score of 0 indicated no response, 0.5 a licking response, 1 flinching and brushing of the paw, 2 strong flinching, and 3 strong flinching and licking. Mouse behaviour was observed during the first 30 s before (baseline) acetone application, after

acetone application, and at different time points (0.5–4 h) as indicated in the protocols after different treatments or surgical procedure.

2.6. Role of the TRPA1 receptor on neuropathic nociception induced by CCI

We verified the role of the TRPA1 receptor on CCI-induced neuropathic nociception by investigating the possible antinociceptive or nociceptive effects of a TRPA1 receptor antagonist and agonist. In addition, we investigated alterations in TRPA1 immunoreactivity in control or nerve-injured mice.

2.6.1. Effect of selective TRPA1 antagonist treatment on CCI-induced mechanical and cold allodynia

The involvement of TRPA1 in CCI-induced allodynia was evaluated using the selective TRPA1 antagonist HC-030031. Seven days after surgery, mice were treated with HC-030031 (10, 30 or 100 mg/kg, i.p.) (da Costa et al., 2010) or vehicle (0.25% DMSO, 0.5% Tween-80 in PBS, i.p.). The effect of the drug on mechanical hyperalgesia or cold allodynia was examined 1 h before treatment and at different time points (0.5–4 h) after i.p. HC-030031 treatment. Separate groups of animals were treated with HC-030031 (100 μg/paw) (da Costa et al., 2010) or vehicle (20 μl/paw) via subcutaneous injection under the dorsal surface of the right hind paw (intraplantar, i.pl.), and the effect of the drug on mechanical hyperalgesia or cold allodynia was examined before and 1 h after treatment.

2.6.2. TRPA1 agonist-induced hypersensitivity

We next investigated whether a low dose of a TRPA1 agonist could induce spontaneous nociception in nerve-injured mice. Spontaneous nociception induced by the TRPA1 receptor agonist allyl isothiocyanate was performed as previously described (Andrade et al., 2008). The animals were placed individually in chambers (transparent glass cylinders 20 cm in diameter) and were allowed to adapt to the chambers for 10 min before treatment. After the adaptation period, 20 μl of a sub-effective dose of allyl isothiocyanate (100 ng/paw) (Andrade et al., 2008) was injected i.pl. into the right hind paw. Separate groups of animals received an i.pl. injection of the appropriate vehicle and were used as controls. After this drug challenge, mice were observed individually for 5 min, and the amount of time spent licking, flinching or lifting the injected paw was measured with a chronometer. These behaviours were considered indicative of nociception. A separate group of animals was also treated with HC-030031 (100 mg/kg, i.p.) or vehicle (0.25% DMSO, 0.5% Tween-80 in PBS, i.p.) 1 h before AITC i.pl. injection.

2.6.3. TRPA1 immunoreactivity analysis

Immunoreactivity to the TRPA1 receptor was assessed using western blot analysis as previously described (Trevisan et al., 2014). The right hind paw skin (the site of nociceptive measures) and the sciatic nerve (the site of nerve injury) were quickly isolated and homogenised (tissue homogeniser for 0.1–50 ml; type EL-770; Insight Equipamentos, Pesquisa e Ensino; Ribeirão Preto; Brazil) in 400 μl or 200 μl, respectively, of ice-cold in a lysis buffer containing 10 mM HEPES, pH 7.9, 10 mM KCl, 2 mM $MgCl_2$, 1 mM ethylenediamine tetraacetic acid (EDTA), 1 mM NaF, 10 μg/ml aprotinin, 10 mM β-glycerophosphate, 1 mM phenylmethanesulphonyl fluoride, 1 mM DL-dithiothreitol (DTT) and 2 mM of sodium orthovanadate and incubated for 15 min on ice. After centrifugation (3000g for 30 min at 4 °C), the supernatant was collected. The protein content was determined using the method of Coomassie Dye (Bradford, 1976) with bovine serum albumin as a standard. Next, 50 and 30 μg of protein from the sciatic nerve or hind paw homogenised samples, respectively, were mixed with loading buffer (200 mM Tris, 10% glycerol, 2% SDS, 2.75 mM β-mercaptoethanol and 0.04% bromophenol blue) and boiled for 10 min. The proteins were separated in 10% sodium dodecyl sulphate-polyacrylamide gels (SDS-PAGE) and transferred to polyvinylidene difluoride membranes according to the manufacturer's instructions (PerkinElmer, Waltham, MA, USA). The membranes were processed using a SNAP I.D. system (Millipore, Temecula, CA, USA), blocked with 1% BSA in TBS-T (0.05% Tween 20 in Tris-borate saline) and incubated for 10 min with a specific rabbit polyclonal IgG antibody to anti-TRPA1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1:150 in TBS-T. The blots were washed thrice with TBS-T followed by incubation with the secondary antibody (1:3000) for 10 min. For measurement, the chemiluminescence system (ECL Western Blotting Systems, GE Healthcare, Little Chalfont, BKM, UK) was used and visualised using the ChemiDoc™ XRS+ System (BioRad, Life Technologies, Carlsbad, CA, USA). To assess the quality of the separation, the same membranes were stained with mouse actin antibody (Sigma, St. Louis, MO, USA) 1:3000. Then, the membranes were incubated with respective secondary antibody conjugated with peroxidase following the same protocol described above. The bands shown are representative of the groups. The quantification was performed by normalisation with control group (medium). The TRPA1 western blots presented a faint background that was corrected during image analysis.

2.7. Role of sympathetic nervous system on CCI-induced neuropathic nociception

We investigated the role of the sympathetic nervous system in CCI-induced neuropathic nociception by investigating the effect of chemical sympathectomy on the cold allodynia or mechanical hyperalgesia caused by CCI and the possible nociceptive effect of norepinephrine in control and nerve-injured mice.

2.7.1. The role of sympathetic fibres in CCI-induced nociception

Chemical sympathectomy was produced by the treatment of mice with guanethidine (30 mg/kg, i.p.) as described previously (Ferreira et al., 2005) to investigate the role of sympathetic fibres in CCI-induced nociception. Treatment was administered on days 4, 5 and 6 after CCI induction. Seven days after CCI induction (1 day after the last guanethidine injection), mechanical hyperalgesia and cold allodynia were measured as described above.

2.7.2. Norepinephrine-induced spontaneous nociception

Spontaneous nociception induced by norepinephrine was performed as previously described (Xanthos et al., 2008) with minor modifications. Seven days after sham or nerve-injury surgery, the animals were placed individually in chambers (transparent glass cylinders 20 cm in diameter) and were allowed 10 min for adaptation before treatment. After the adaptation period, 20 μl of the adrenergic receptor agonist norepinephrine (30 ng/paw) was injected i.pl. under the dorsal surface of the right hind paw. Separate groups of animals received an i.pl. injection of the appropriate vehicle and were used as controls. After challenge, the mice were observed individually for 5 min, and the amount of time spent licking, flinching or lifting the injected paw was measured with a chronometer. These behaviours were considered indicative of nociception.

2.8. Investigation of some mechanisms involved in norepinephrine-induced nociception in nerve-injured mice

Distinct groups of animals were treated with different classes of drugs that were locally co-administered with norepinephrine (30 ng/paw, i.pl.) to investigate some of the possible mechanisms through which the peripheral injection of norepinephrine causes nociception in nerve-injured mice. The following drugs were co-administered with norepinephrine: the α-adrenoceptor antagonist phentolamine (100 ng/paw), the β-adrenoceptor antagonist propranolol (300 pg/paw), the norepinephrine transporter (NET) inhibitor desipramine (100 ng/paw), the monoamine oxidase-A inhibitor (MAO-A) clorgyline (100 ng/paw) and the TRPA1 receptor antagonist HC-030031 (100 μg/paw). A separate group of animals was also treated with HC-030031 (100 mg/kg, i.p.) or its vehicle 1 h before norepinephrine injection. The dosage choice for each drug was based on previous data described in the literature (Xanthos et al., 2008; Ferreira et al., 2005; da Costa et al., 2010) or on preliminary experiments performed in our laboratory.

2.9. Statistical analyses

The results are presented as the means ± S.E.M., except for the cold allodynia scores, which are reported as medians followed by their 25th and 75th percentiles. The inhibition percentages are reported as the means ± S.E.M., which were calculated based on the responses of the control group. Statistical analyses were performed using the GraphPad Prism 4.0 software. The significance of differences between groups was evaluated with Mann–Whitney's test or a Kruskal–Wallis test followed by Dunn's test (for

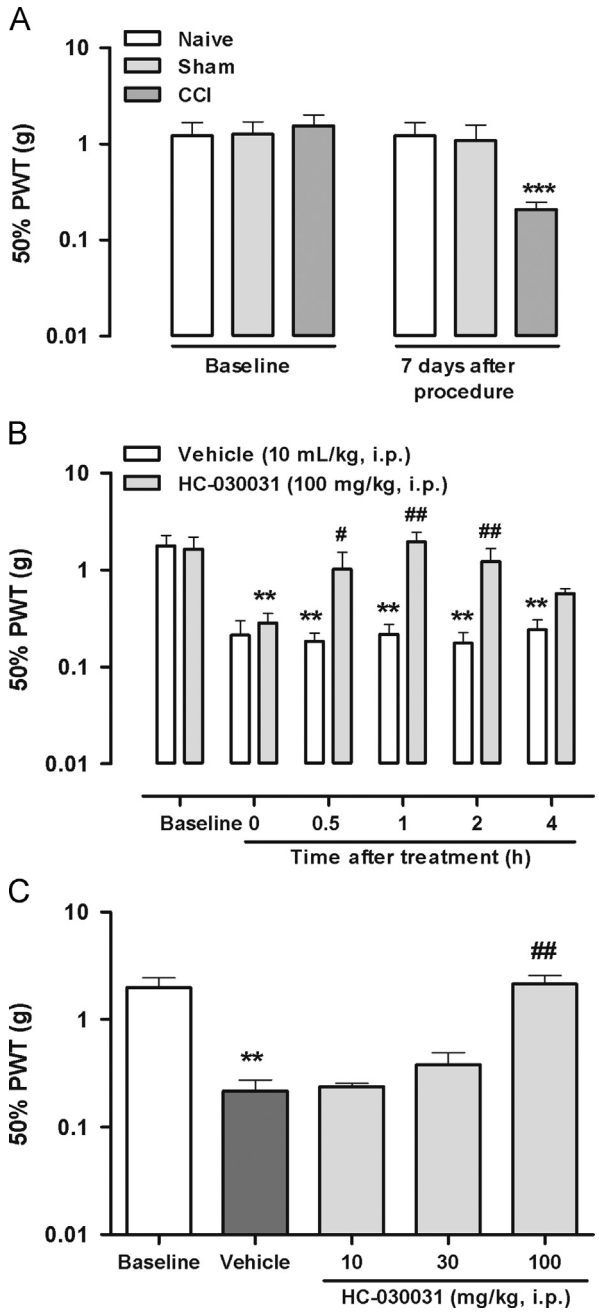

Fig. 1. TRPA1 antagonist reduced neuropathy-induced mechanical allodynia in mice. (A) 50% paw withdrawal thresholds (PWT) before (baseline) and 7 days after procedures in naïve, sham and chronic constriction-injured (CCI) mice ($n=7$ each group). $^{***}P<0.001$, compared with the sham group (one-way ANOVA followed by Student Newman–Keuls' test). (B) Time-course of PWT in neuropathic mice treated with HC-030031 (100 mg/kg, i.p.) or vehicle (10 ml/kg, i.p.) ($n=7$ each group). $^{**}P<0.01$, compared to baseline values; $^{\#}P<0.05$ and $^{\#\#}P<0.01$, compared with vehicle-treated animals (two-way ANOVA followed by Bonferroni's test). (C) Dose–response curve for the anti-allodynic effect of HC-030031 (10–100 mg/kg, i.p.) ($n=7$ each group). $^{**}P<0.01$, compared to baseline, $^{\#\#}P<0.01$, compared with vehicle-treated animals (one-way ANOVA followed by Student Newman–Keuls' test). Data are expressed as the means + S.E.M.

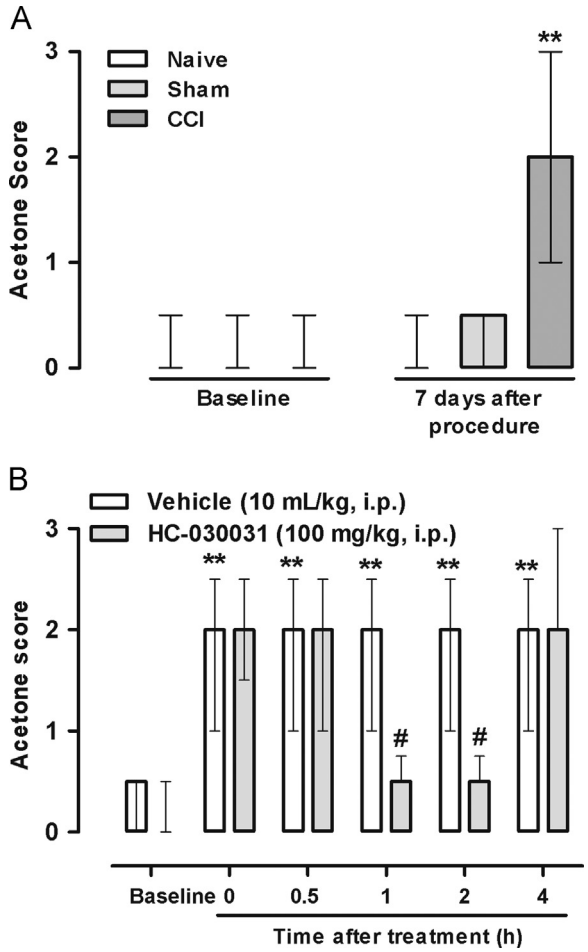

Fig. 2. TRPA1 antagonist reduced neuropathy-induced cold allodynia in mice. (A) Nocifensive reaction scores for the right hind paw of animals after evaporative cooling of acetone before (baseline) and 7 days after the procedure in naïve, sham and chronic constriction-injured (CCI) mice ($n=10$ each group). $^{**}P<0.001$, compared with sham group (Kruskal–Wallis followed by Dunn's test). (B) Time-course of HC-030031 (100 mg/kg, i.p.) and vehicle (10 ml/kg) effects on cold allodynia in neuropathic mice ($n=10$ each group). $^{**}P<0.01$, compared with baseline values; $^{\#}P<0.05$, compared with respective vehicle-treated animals (Mann–Whitney's test). Data are expressed as the medians ± interquartile ranges.

the cold allodynia results) along with an unpaired Student's *t*-test and one-way analysis of variance (ANOVA). ANOVA was followed by a Student–Newman–Keuls' (SNK) test or two-way ANOVA followed by Bonferroni's test (for mechanical allodynia and spontaneous nociception results). $P < 0.05$ was considered significant.

3. Results

3.1. Antinociceptive effect of the selective TRPA1 antagonist HC-030031 on CCI-induced mechanical hyperalgesia

Fig. 1A depicts the 50% paw withdrawal thresholds (PWT) of naïve, sham, and nerve-injured mice. There was no difference in the PWT values between groups before the procedure or between naïve and sham-operated mice 7 days after the procedure. However, CCI of the sciatic nerve induced a marked mechanical allodynia compared with sham surgery mice 7 days after lesion creation (Fig. 1A).

The mechanical allodynia produced by nerve injury was maintained throughout the experimental period in vehicle-treated mice (Fig. 1B). However, the systemic injection of HC-030031 (100 mg/kg, i.p.) reversed CCI-induced mechanical allodynia from 0.5 to 2 h after injection, with a complete inhibition 1 h after treatment (Fig. 1B). The higher dose (100 mg/kg), but not the lower doses (10 or 30 mg/kg, i.p.), of injected HC-030031 reduced mechanical allodynia in nerve-injured mice (Fig. 1C). Notably, HC-030031 treatment (100 mg/kg, i.p.) did not alter the mechanical thresholds of sham-operated mice (results not shown). Therefore, the 100 mg/kg (i.p.) HC-030031 dose was used in subsequent tests. Seven animals were used per group in Fig. 1, with a total of 70 animals.

3.2. Antinociceptive effect of the selective TRPA1 antagonist HC-030031 on CCI-induced cold allodynia

Fig. 2A depicts the nocifensive reactions of the right hind paw of animals after evaporative cooling of acetone. The topical application of acetone did not induce nociception in naïve, sham, and nerve-injured mice before the procedure (baseline) or in naïve or sham animals 7 days after the surgical procedure. In contrast, CCI of the sciatic nerve induced cold allodynia compared to sham surgery in mice after 7 days (Fig. 2A).

Systemic treatment with HC-030031 (100 mg/kg, i.p.) almost completely reversed the cold allodynia induced by CCI compared with the vehicle group at 1 and 2 h after administration (Fig. 2B). HC-030031 treatment did not alter the acetone scores in sham-operated animals (results not shown). Ten animals were used per group in Fig. 2, with a total of 50 animals.

3.3. TRPA1 agonist-induced nociception in neuropathic mice

The sensitivity of peripheral TRPA1 receptors in neuropathic mice was investigated by examining the spontaneous nociception induced by a receptor agonist (Fig. 3). The intraplantar injection of a low dose of the TRPA1 agonist AITC (100 ng/paw) did not induce nociception in sham-operated mice, but it did cause an intense nociceptive response in mice seven days after CCI (Fig. 3A).

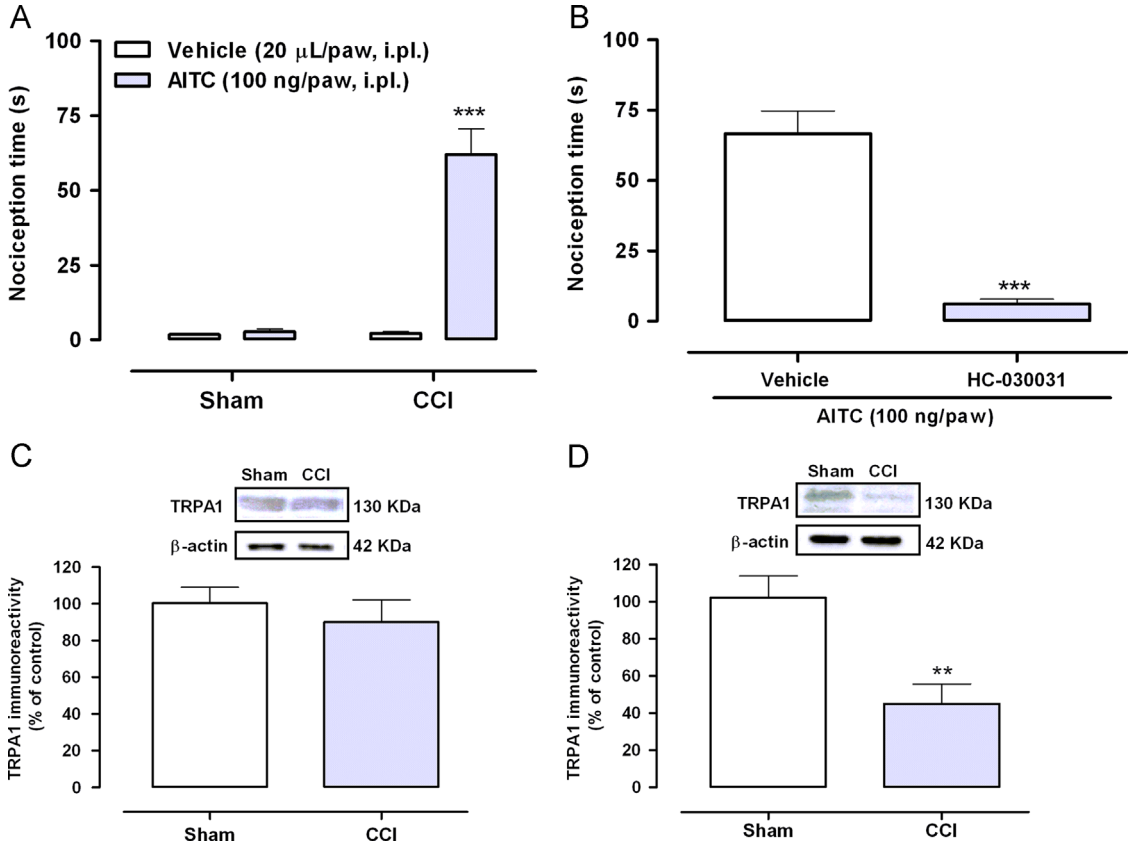

Fig. 3. TRPA1 agonist-induced nociception in neuropathic mice. (A) Effect of intraplantar AITC (100 ng/paw) and vehicle injection on nociception time in chronic constriction-injured (CCI) and sham-operated mice ($n=7$). $^{***}P < 0.01$ compared with respective vehicle-treated group (unpaired *t* test). (B) Antinociceptive effect of HC-030031 (100 mg/kg, i.p.) pre-treatment on the nociception caused by i.pl. injection of AITC (100 ng/paw) in neuropathic mice ($n=7$ each group). $^{***}P < 0.001$ compared with vehicle-treated group (unpaired *t* test). TRPA1 immunoreactivity in the right hind paw (C) and the injured sciatic nerve (D) seven days after surgery ($n=4$ each group). Lane 1, control (sham mice). Lane 2, CCI mice. Western blot results were expressed as % of control. The representative β-actin protein bands demonstrates equal loading. $^{**}P < 0.01$ compared with sham group (unpaired *t* test). Data are expressed as the means + S.E.M.

Furthermore, pre-treatment with HC-030031 (100 mg/kg, i.p.) largely prevented (91 ± 2% inhibition) the spontaneous nociception induced by AITC in nerve-injured mice (Fig. 3B). Seven animals were used per group in Fig. 3A and B, with a total of 28 animals.

3.4. Detection of TRPA1 immunoreactivity in neuropathic mice

We next investigated whether the nociceptive response to the TRPA1 agonist could be due to an increase in TRPA1 expression in the right hind paw (local nociception measurement) or sciatic nerve (location of the nerve injury). We observed that TRPA1 immunoreactivity in the hind paw ipsilateral to the nerve lesion was not different between the sham and nerve-injured mice (Fig. 3C). However, CCI significantly reduced (44 ± 10%) TRPA1 immunoreactivity in the sciatic nerve seven days after lesion creation compared to the sham-operated group (Fig. 3D). Four animals were used per group in Fig. 3C and D, with a total of 8animals.

3.5. CCI-induced nociception is sympathetically maintained

The chemical sympathectomy produced by guanethidine (30 mg/kg, i.p., 3 days before CCI) largely prevented CCI-induced mechanical hyperalgesia (Fig. 4A) and cold allodynia (Fig. 4B); the mechanical threshold and acetone scores similar to baseline values (before the surgical procedure). The same guanethidine treatment did not alter either the mechanical thresholds or acetone scores in sham-operated mice (results not shown).

Next, we investigated whether nerve-injured animals had a nociceptive response to norepinephrine. An intraplantar injection of norepinephrine (30 ng/paw) induced only a few spontaneous nociceptive behaviours in sham-operated animals (8.0 ± 3.2 s of response), which were not different from the responses to vehicle (PBS) in sham and nerve-injured mice (5.1 ± 2.0 and 4.4 ± 2.3 s of response, respectively) (Fig. 4C). In contrast, an intraplantar injection of norepinephrine (30 ng/paw) produced a marked nociceptive response in animals seven days after CCI compared to the PBS group (39.3 ± 7.1 and 8.0 ± 3.2 s of response, respectively) (Fig. 4C). Eight animals were used per group in Fig. 4, with a total of 48 animals.

3.6. Study of the peripheral mechanisms involved in NE-induced nociception

First, we examined the role of adrenoceptors and norepinephrine uptake and degradation on norepinephrine-induced nociception using receptor antagonists, transporter and enzyme inhibitors. The co-administration of the α-adrenoceptor antagonist phentolamine (100 ng/paw), but not the β-adrenoceptor antagonist propranolol (300 pg/paw), only somewhat, but significantly, inhibited (24 ± 7%) norepinephrine-induced nociception (30 ng/paw) (Fig. 5A and B). Furthermore, co-administration with the selective norepinephrine transporter inhibitor desipramine (100 ng/paw) or the selective monoamine oxidase inhibitor clorgyline (100 ng/paw) largely reduced (inhibition of 88 ± 3% and 90 ± 7%, respectively) the nociception caused by norepinephrine (30 ng/paw) in neuropathic mice (Fig. 5C and D). Eight animals were used per group in Fig. 5, with a total of 64 animals.

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3.7. Effect of TRPA1 antagonist in NE-induced nociception, cold and mechanical allodynia caused by nerve injury

The systemic (100 mg/kg, i.p.) or local (100 μg/paw, i.pl.) injection of HC-030031 largely reduced (inhibition of 81 ± 9 and 63 ± 8%, respectively) norepinephrine-induced nociception (30 ng/paw) in nerve-injured animals (Fig. 6A and B). Peripheral injection of HC-030031 (100 μg/paw, intraplantar) almost completely abolished the mechanical hyperalgesia and cold allodynia induced by nerve injury (Fig. 6C and D). Eight animals were used per group in Fig. 6A–D with a total of 48 animals.

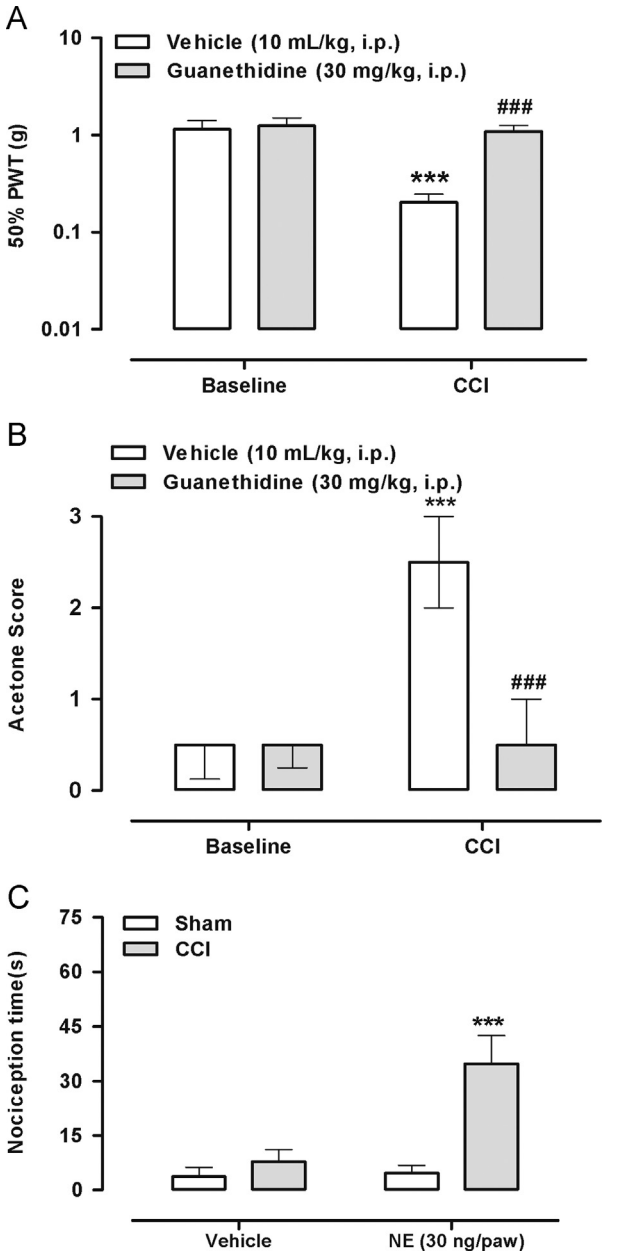

Fig. 4. CCI-induced nociception is sympathetically maintained. (A, B) Effect of the chemical sympathectomy produced by guanethidine (30 mg/kg, i.p.) in mechanical (A) and cold (B) allodynia-induced by CCI in mice ($n=8$ each group). $^{***}P<0.001$ compared with baseline; $^{\#\#\#}P<0.001$ compared with vehicle-treated group (unpaired t test for A and Mann–Whitney's test for B). Data are expressed as the means+S.E.M. (A) or median ± interquartile ranges (B). (C) Effect of intraplantar norepinephrine (NE, 30 ng/paw) and vehicle injection on nociception in chronic constriction-injured (CCI) and sham-operated mice ($n=8$ each group). $^{***}P<0.01$ compared with the respective sham group (unpaired t test).

4. Discussion

Neuropathic pain is among the most intractable of pain syndromes (Treede et al., 2008; Woolf and Mannion, 1999). Therefore, the study of its mechanisms is relevant to the development of more

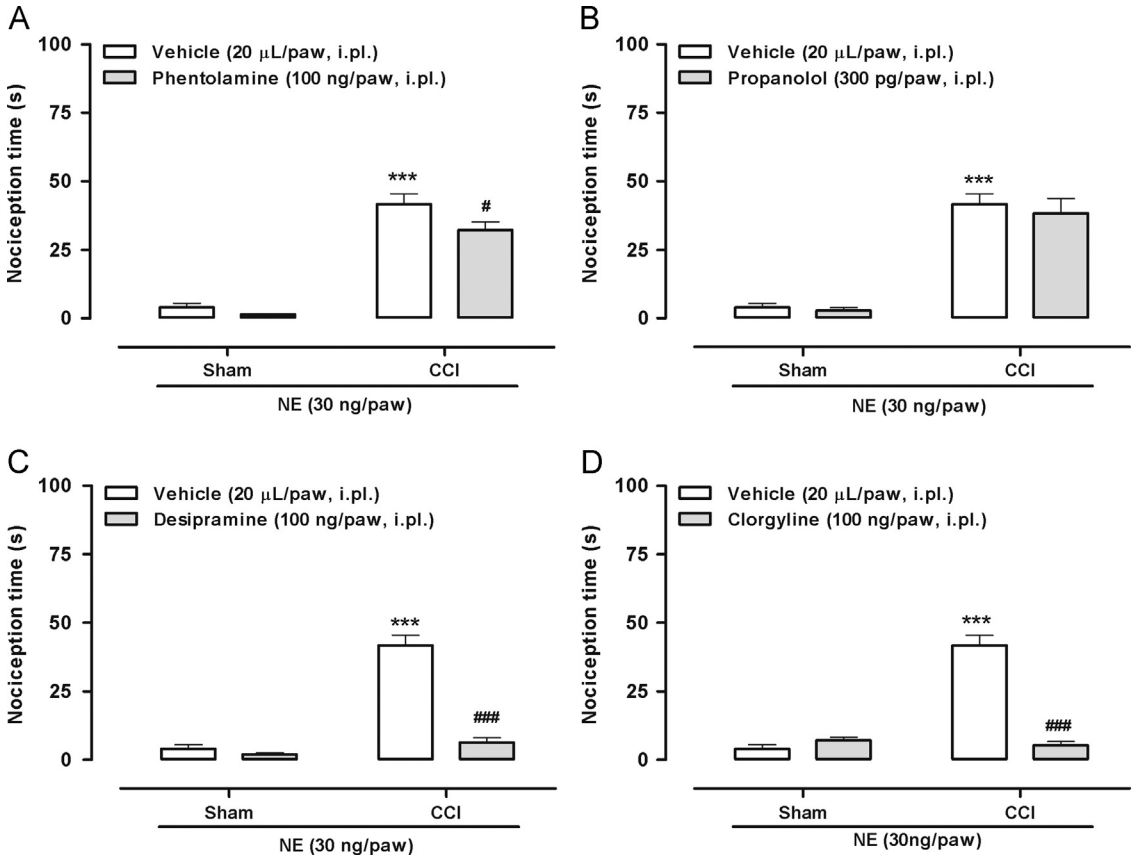

Fig. 5. Peripheral mechanisms involved in norepinephrine-induced nociception in neuropathic mice. Effect of intraplantar co-administration of phentolamine (100 ng/paw, A), propranolol (300 pg/paw, B), desipramine (100 ng/paw, C) and clorgyline (100 ng/paw, D) with NE (norepinephrine, 30 ng/paw) in sham-operated and chronic constriction-injured (CCI) animals ($n=8$ each group). $^{***}P<0.001$, compared with vehicle-treated sham-operated animals, $^{\#}P<0.05$, $^{\#\#\#}P<0.001$, compared with vehicle-treated CCI animals (two-way ANOVA). Data are expressed as the means+S.E.M.

effective treatments for neuropathic pain. Sympathetic nervous system activation and TRPA1 stimulation seem to be important underlying mechanisms of some neuropathic pain syndromes, but the relationship between them is unknown. The present study observed TRPA1 receptor-mediated nociception in a model of sympathetically maintained pain in mice.

We observed that a selective TRPA1 antagonist largely reversed both mechanical and cold allodynia induced by chronic sciatic nerve constriction injury in mice. Our results are consistent with the literature, which indicates that TRPA1 antagonists and antisense oligonucleotides may reduce mechanical and cold allodynia produced in rodents by spinal nerve ligation, nerve injury, streptozotocin and oxaliplatin (del Camino et al., 2010; Eid et al., 2008; Nassini et al., 2011; Obata et al., 2005; Wei et al., 2009). Taken together, these findings demonstrate the critical role of the TRPA1 receptor in pain symptoms related to neuropathies of different aetiologies.

We also investigated the sensitivity of peripheral TRPA1 receptors in neuropathic mice. We observed that the intraplantar injection of the TRPA1 agonist allyl isothiocyanate (the main component of mustard oil) at a dose that did not produce spontaneous nociception in sham-operated mice caused an intense nociceptive response in mice with chronic sciatic nerve constriction injury. Moreover, we observed that HC-030031 treatment abolished AITC-induced nociception, which indicated that this response was mediated by the TRPA1 receptor. Although CCI induced a peripheral sensitisation to TRPA1 agonists, we were unable to detect any differences in the TRPA1 immunoreactivity in the stimulated hind paw between sham and operated mice. Therefore, the nociceptive response to a TRPA1 agonist was not due to an increase in tissue TRPA1 content, but it could be related to an increase in TRPA1 function. However, additional studies must be performed to clarify how this phenomenon occurs. In addition, we observed a significant reduction in TRPA1 immunoreactivity in the sciatic nerve of constriction-injured mice. Similarly, a decrease in TRPA1 mRNA and protein was reported previously in the dorsal root ganglion ipsilateral to a chronic constriction injury in mice (Caspani et al., 2009). These results suggest that cells other than sensory neurons are also important for TRPA1 expression in paw skin tissue. In fact, it has been demonstrated that dermal fibroblasts, keratinocytes and even sympathetic neurons express functional TRPA1 receptors that could be involved in pain sensitisation (Atoyan et al., 2009; Jain et al., 2011; Smith et al., 2004).

Some forms of painful peripheral neuropathy are mediated by the sympathetic nervous system, where pain relief is achieved by sympatholytic procedures (Baron, 2006). Similar to what has been observed in rats (Neil et al., 1991), we detected that chemical sympathectomy produced by guanethidine largely inhibited mechanical hyperalgesia and cold allodynia observed seven days after CCI in mice, which suggests that CCI is a model for sympathetically maintained pain. Moreover, CCI may induce sympathetic sprouting into the ipsilateral dorsal root ganglion and increase plasma norepinephrine levels in rodents one week after injury (Jin et al., 2008; Ramer et al., 1997). In addition to the analgesic effect of sympatholytic procedures, hypersensitivity to norepinephrine has been taken as evidence for sympathetically maintained pain (Xanthos et al., 2008). Consistent with the findings demonstrating that norepinephrine may stimulate nociceptors and induce spontaneous pain after nerve injury (Ali et al., 2000; Drummond, 2010; Torebjork et al., 1995; Xanthos et al., 2008), we found that CCI caused norepinephrine hypersensitivity. Therefore, CCI in rodents may represent a model of

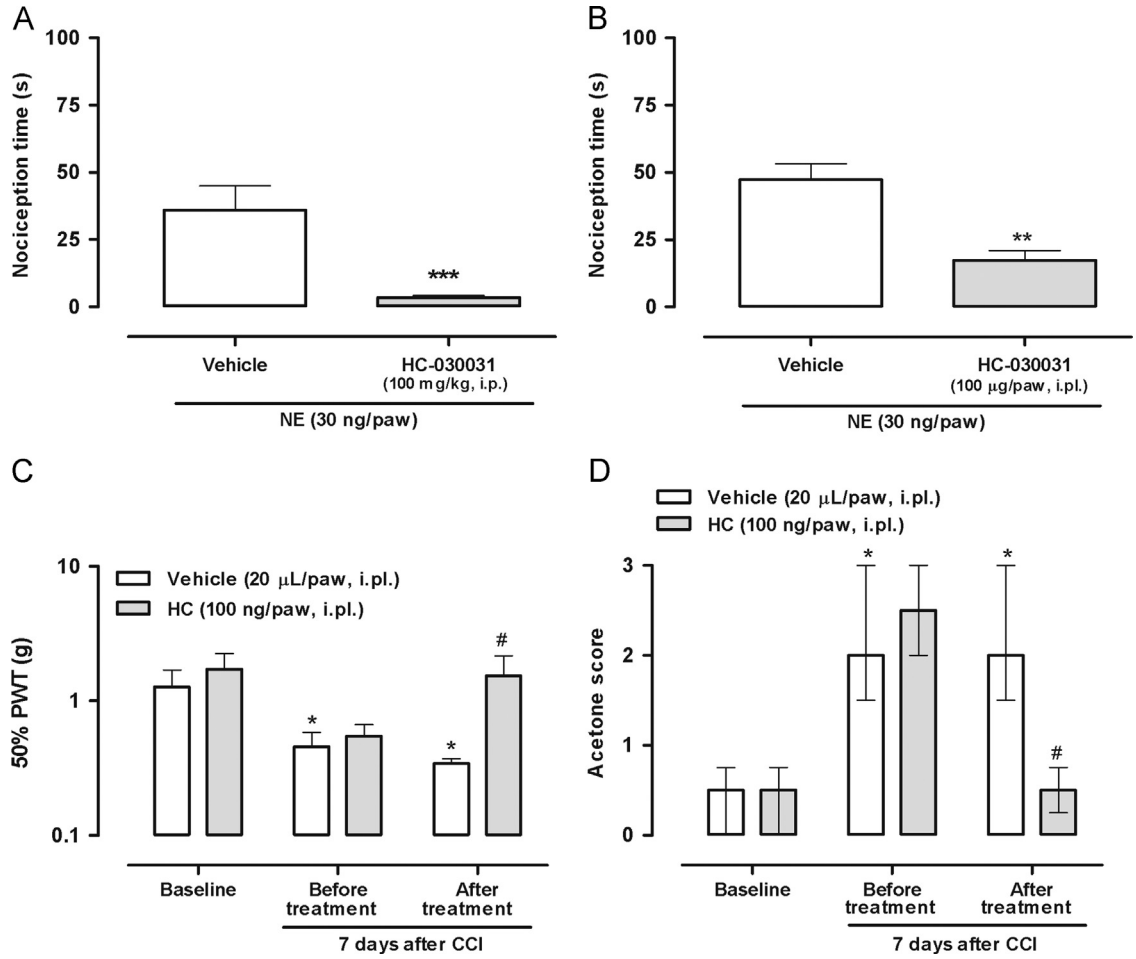

Fig. 6. Peripheral TRPA1 receptor is involved in norepinephrine-induced nociception and cold and mechanical allodynia caused by nerve injury. (A, B) Effect of systemic (A, 100 mg/kg, i.p.) and peripheral (B, 100 µg/paw, i.pl.) HC-030031 treatment on the nociception caused by NE (norepinephrine, 30 ng/paw, i.pl.) in neuropathic mice ($n=8$ each group). $^{***}P<0.001$, $^{**}P<0.01$, compared with vehicle-treated group (unpaired t test). (C, D) Mechanical (C) and cold (D) allodynia induced by CCI in mice before and 1 h after the peripheral treatment with HC-030031 (100 µg/paw, i.pl.) ($n=8$ each group). $^{*}P<0.05$, compared with baseline (two-way ANOVA followed by Bonferroni's test for C and Kruskal–Wallis followed by Dunn's test for D); $^{\#}P<0.05$, compared with vehicle-treated animals (two-way ANOVA followed by Bonferroni's test for C and Mann–Whitney's test for D). Data are expressed as the means+S.E.M. in A–C and as the medians ± interquartile ranges in D.

sympathetically maintained pain, at least one week after initial induction.

Next, we investigated the mechanisms involved in the hypersensitivity to norepinephrine after nerve injury because they are poorly understood. Both α_1 and α_2-adrenoceptors are implicated in norepinephrine-induced pain in humans and nociception in rats with neuropathy (Drummond, 2010; Xanthos et al., 2008). Accordingly, we verified that the α-adrenoceptor antagonist phentolamine, but not the β-adrenoceptor antagonist propranolol, reduced spontaneous nociception to norepinephrine in constriction-injured mice. The α-adrenoceptor antagonism produced a partial effect, but other mechanisms are likely involved in this response. It was recently demonstrated that norepinephrine uptake and metabolism are involved in alcohol-induced painful peripheral neuropathy in rats (Dina et al., 2008). Similarly, treatment with selective inhibitors of the norepinephrine transporter (desipramine) and monoamine oxidase (clorgyline) largely inhibits the nociception caused by norepinephrine in nerve-injured mice. Therefore, norepinephrine metabolism seems to be an important mechanism underlying the hypersensitivity to norepinephrine after nerve injury.

Norepinephrine metabolism via monoamine oxidase generates some reactive oxygen species and reactive aldehydes, such as hydrogen peroxide and 3,4-dihydroxyphenylglycolaldehyde (DOPEGAL), that are neurotoxic (Burke et al., 2004). Dina et al. (2008) indicated that catecholamine metabolites contribute to enhanced mechanical sensitivity of primary sensory afferents, and this possibility was not excluded although they were not able to clearly prove DOPEGAL involvement (Dina et al., 2008). In addition, it is important to note that TRPA1 is implicated in mechanical hyperalgesia and cold allodynia induced by sciatic nerve injury and that it is activated by several endogenous reactive species, such as hydrogen peroxide and unsaturated aldehydes from oxidative stress (Andersson et al., 2008; Trevisani et al., 2007). Therefore, our study indicates that systemic and peripheral administration of a TRPA1 receptor antagonist almost abolished norepinephrine-induced nociception in neuropathic mice. Finally, we demonstrated that the peripheral injection of a TRPA1 receptor antagonist also inhibited CCI-induced mechanical hyperalgesia and cold allodynia in mice. Therefore, these data demonstrate the critical role of peripheral TRPA1 in the induction of painful hypersensitivity in a model of sympathetically maintained pain. Because primary afferent nociceptors possess the necessary machinery for uptake and metabolism of catecholamines, and TRPA1 receptors are largely expressed in peptidergic sensory neurons (Baraldi et al., 2010), these areas could be the sites where these metabolites bind to cause pain.

Taken together, the present findings reveal the critical role of TRPA1 in mechanical and cold hypersensitivity and the hypersensitivity to norepinephrine after nerve injury. Our results suggest that TRPA1 antagonists may be useful to neuropathic patients who present sympathetically maintained pain.

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References

Ali, Z., Raja, S.N., Wesselmann, U., Fuchs, P.N., Meyer, R.A., Campbell, J.N., 2000. Intradermal injection of norepinephrine evokes pain in patients with sympathetically maintained pain. Pain 88, 161–168.

Andersson, D.A., Gentry, C., Moss, S., Bevan, S., 2008. Transient receptor potential A1 is a sensory receptor for multiple products of oxidative stress. J. Neurosci. 28, 2485–2494.

Andrade, E.L., Luiz, A.P., Ferreira, J., Calixto, J.B., 2008. Pronociceptive response elicited by TRPA1 receptor activation in mice. Neuroscience 152, 511–520.

Andre, E., Campi, B., Materazzi, S., Trevisani, M., Amadesi, S., Massi, D., Creminon, C., Vaksman, N., Nassini, R., Civelli, M., Baraldi, P.G., Poole, D.P., Bunnett, N.W., Geppetti, P., Patacchini, R., 2008. Cigarette smoke-induced neurogenic inflammation is mediated by alpha,beta-unsaturated aldehydes and the TRPA1 receptor in rodents. J. Clin. Investig. 118, 2574–2582.

Atoyan, R., Shander, D., Botchkareva, N.V., 2009. Non-neuronal expression of transient receptor potential type A1 (TRPA1) in human skin. J. Investig. Dermatol. 129, 2312–2315.

Baraldi, P.G., Preti, D., Materazzi, S., Geppetti, P., 2010. Transient receptor potential ankyrin 1 (TRPA1) channel as emerging target for novel analgesics and anti-inflammatory agents. J. Med. Chem. 53, 5085–5107.

Baron, R., 2006. Mechanisms of disease: neuropathic pain – a clinical perspective. Nat. Clin. Pract. Neurol. 2, 95–106.

Baron, R., Fields, H.L., Janig, W., Kitt, C., Levine, J.D., 2002. National Institutes of Health Workshop: reflex sympathetic dystrophy/complex regional pain syndromes—state-of-the-science. Anesth. Analg. 95, 1812–1816.

Bennett, G.J., Xie, Y.K., 1988. A peripheral mononeuropathy in rat that produces disorders of pain sensation like those seen in man. Pain 33, 87–107.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Burke, W.J., Li, S.W., Chung, H.D., Ruggiero, D.A., Kristal, B.S., Johnson, E.M., Lampe, P., Kumar, V.B., Franko, M., Williams, E.A., Zahm, D.S., 2004. Neurotoxicity of MAO metabolites of catecholamine neurotransmitters: role in neurodegenerative diseases. Neurotoxicology 25, 101–115.

Caspani, O., Zurborg, S., Labuz, D., Heppenstall, P.A., 2009. The contribution of TRPM8 and TRPA1 channels to cold allodynia and neuropathic pain. PLoS One 4, e7383.

Chaplan, S.R., Bach, F.W., Pogrel, J.W., Chung, J.M., Yaksh, T.L., 1994. Quantitative assessment of tactile allodynia in the rat paw. J. Neurosci. Methods 53, 55–63.

da Costa, D.S., Meotti, F.C., Andrade, E.L., Leal, P.C., Motta, E.M., Calixto, J.B., 2010. The involvement of the transient receptor potential A1 (TRPA1) in the maintenance of mechanical and cold hyperalgesia in persistent inflammation. Pain 148, 431–437.

del Camino, D., Murphy, S., Heiry, M., Barrett, L.B., Earley, T.J., Cook, C.A., Petrus, M.J., Zhao, M., D'Amours, M., Deering, N., Brenner, G.J., Costigan, M., Hayward, N.J., Chong, J.A., Fanger, C.M., Woolf, C.J., Patapoutian, A., Moran, M.M., 2010. TRPA1 contributes to cold hypersensitivity. J. Neurosci. 30, 15165–15174.

Dina, O.A., Khasar, S.G., Alessandri-Haber, N., Bogen, O., Chen, X., Green, P.G., Reichling, D.B., Messing, R.O., Levine, J.D., 2008. Neurotoxic catecholamine metabolite in nociceptors contributes to painful peripheral neuropathy. Eur. J. Neurosci. 28, 1180–1190.

Dixon, W.J., 1980. Efficient analysis of experimental observations. Annu. Rev. Pharmacol. Toxicol. 20, 441–462.

Drummond, P.D., 2010. Sensory disturbances in complex regional pain syndrome: clinical observations, autonomic interactions, and possible mechanisms. Pain Med. 11, 1257–1266.

Drummond, P.D., Finch, P.M., Smythe, G.A., 1991. Reflex sympathetic dystrophy: the significance of differing plasma catecholamine concentrations in affected and unaffected limbs. Brain 114, 2025–2036.

Eid, S.R., Crown, E.D., Moore, E.L., Liang, H.A., Choong, K.C., Dima, S., Henze, D.A., Kane, S.A., Urban, M.O., 2008. HC-030031, a TRPA1 selective antagonist, attenuates inflammatory- and neuropathy-induced mechanical hypersensitivity. Mol. Pain 4, 48.

Ferreira, J., Triches, K.M., Medeiros, R., Calixto, J.B., 2005. Mechanisms involved in the nociception produced by peripheral protein kinase c activation in mice. Pain 117, 171–181.

Jain, A., Bronneke, S., Kolbe, L., Stab, F., Wenck, H., Neufang, G., 2011. TRP-channel-specific cutaneous eicosanoid release patterns. Pain 152, 2765–2772.

Jin, Y., Sato, J., Yamazaki, M., Omura, S., Funakubo, M., Senoo, S., Aoyama, M., Mizumura, K., 2008. Changes in cardiovascular parameters and plasma norepinephrine level in rats after chronic constriction injury on the sciatic nerve. Pain 135, 221–231.

Krimon, S., Araldi, D., do Prado, F.C., Tambeli, C.H., Oliveira-Fusaro, M.C., Parada, C.A., 2013. P2X3 receptors induced inflammatory nociception modulated by TRPA1, 5-HT3 and 5-HT1A receptors. Pharmacol. Biochem. Behav. 112, 49–55.

Macpherson, L.J., Dubin, A.E., Evans, M.J., Marr, F., Schultz, P.G., Cravatt, B.F., Patapoutian, A., 2007. Noxious compounds activate TRPA1 ion channels through covalent modification of cysteines. Nature 445, 541–545.

Nassini, R., Gees, M., Harrison, S., De Siena, G., Materazzi, S., Moretto, N., Failli, P., Preti, D., Marchetti, N., Cavazzini, A., Mancini, F., Pedretti, P., Nilius, B., Patacchini, R., Geppetti, P., 2011. Oxaliplatin elicits mechanical and cold allodynia in rodents via TRPA1 receptor stimulation. Pain 152, 1621–1631.

Neil, A., Attal, N., Guilbaud, G., 1991. Effects of guanethidine on sensitization to natural stimuli and self-mutilating behaviour in rats with a peripheral neuropathy. Brain Res. 565, 237–246.

Obata, K., Katsura, H., Mizushima, T., Yamanaka, H., Kobayashi, K., Dai, Y., Fukuoka, T., Tokunaga, A., Tominaga, M., Noguchi, K., 2005. TRPA1 induced in sensory neurons contributes to cold hyperalgesia after inflammation and nerve injury. J. Clin. Investig. 115, 2393–2401.

Pertovaara, A., Koivisto, A., 2011. TRPA1 ion channel in the spinal dorsal horn as a therapeutic target in central pain hypersensitivity and cutaneous neurogenic inflammation. Eur. J. Pharmacol. 666, 1–4.

Ramer, M.S., French, G.D., Bisby, M.A., 1997. Wallerian degeneration is required for both neuropathic pain and sympathetic sprouting into the DRG. Pain 72, 71–78.

Smith, M.P., Beacham, D., Ensor, E., Koltzenburg, M., 2004. Cold-sensitive, menthol-insensitive neurons in the murine sympathetic nervous system. Neuroreport 15, 1399–1403.

Sommer, C., Schmidt, C., George, A., 1998. Hyperalgesia in experimental neuropathy is dependent on the TNF receptor 1. Exp. Neurol. 151, 138–142.

Story, G.M., Peier, A.M., Reeve, A.J., Eid, S.R., Mosbacher, J., Hricik, T.R., Earley, T.J., Hergarden, A.C., Andersson, D.A., Hwang, S.W., McIntyre, P., Jegla, T., Bevan, S., Patapoutian, A., 2003. ANKTM1, a TRP-like channel expressed in nociceptive neurons, is activated by cold temperatures. Cell 112, 819–829.

Torebjork, E., Wahren, L., Wallin, G., Hallin, R., Koltzenburg, M., 1995. Noradrenaline-evoked pain in neuralgia. Pain 63, 11–20.

Treede, R.D., Jensen, T.S., Campbell, J.N., Cruccu, G., Dostrovsky, J.O., Griffin, J.W., Hansson, P., Hughes, R., Nurmikko, T., Serra, J., 2008. Neuropathic pain: redefinition and a grading system for clinical and research purposes. Neurology 70, 1630–1635.

Trevisan, G., Hoffmeister, C., Rossato, M.F., Oliveira, S.M., Silva, M.A., Silva, C.R., Fusi, C., Tonello, R., Minocci, D., Guerra, G.P., Materazzi, S., Nassini, R., Geppetti, P., Ferreira, J., 2014. TRPA1 receptor stimulation by hydrogen peroxide is critical to trigger hyperalgesia and inflammation in a model of acute gout. Free Radic. Biol. Med. 72, 200–209.

Trevisani, M., Siemens, J., Materazzi, S., Bautista, D.M., Nassini, R., Campi, B., Imamachi, N., Andre, E., Patacchini, R., Cottrell, G.S., Gatti, R., Basbaum, A.I., Bunnett, N.W., Julius, D., Geppetti, P., 2007. 4-Hydroxynonenal, an endogenous aldehyde, causes pain and neurogenic inflammation through activation of the irritant receptor TRPA1. Proc. Natl. Acad. Sci. USA 104, 13519–13524.

Wei, H., Hamalainen, M.M., Saarnilehto, M., Koivisto, A., Pertovaara, A., 2009. Attenuation of mechanical hypersensitivity by an antagonist of the TRPA1 ion channel in diabetic animals. Anesthesiology 111, 147–154.

Woolf, C.J., Mannion, R.J., 1999. Neuropathic pain: aetiology, symptoms, mechanisms, and management. Lancet 353, 1959–1964.

Xanthos, D.N., Bennett, G.J., Coderre, T.J., 2008. Norepinephrine-induced nociception and vasoconstrictor hypersensitivity in rats with chronic post-ischemia pain. Pain 137, 640–651.

Zimmermann, M., 1983. Ethical guidelines for investigations of experimental pain in conscious animals. Pain 16, 109–110.