Non-neuronal TRPA1 encodes mechanical allodynia evoked by neurogenic inflammation and partial nerve injury in rats

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Abstract

Background and Purpose. The proalgesic transient receptor potential (TRP) ankyrin 1 (TRPA1) channel, expressed by a subpopulation of primary sensory neurons, has been implicated in various pain models in mice. However, evidence in rats indicates that TRPA1 conveys nociceptive signals elicited by channel agonists but not those associated with tissue inflammation or nerve injury. Here, in rats, we explored the TRPA1 role in mechanical allodynia associated with neurogenic inflammation and moderate (partial sciatic nerve ligation, pSNL) or severe (chronic constriction injury, CCI) sciatic nerve injury. Experimental Approach. Acute nociception and mechanical hypersensitivity associated with neurogenic inflammation and sciatic nerve injury (pSNL and CCI) were investigated in rats with TRPA1 pharmacological antagonism or genetic silencing. TRPA1 presence and function was analyzed in cultured rat Schwann cells. Key Results. Hind paw mechanical allodynia (HPMA), but not acute nociception, evoked by local injection of the TRP vanilloid 1 (TRPV1) agonist, capsaicin, or the TRPA1 agonist, allyl isothiocyanate, was mediated by calcitonin gene related peptide (CGRP) released from peripheral nerve terminals. CGRP-evoked HPMA was sustained by a reactive oxygen species (ROS)-dependent TRPA1 activation, probably in Schwann cells. HPMA evoked by pSNL, but not that evoked by CCI, was mediated by ROS and TRPA1 without the involvement of CGRP. Conclusions and Implications. As found in mice, TRPA1 mediates mechanical allodynia associated with neurogenic inflammation and moderate nerve injury in rats. The channel implication in mechanical hypersensitivity following inflammation and partial nerve damage is a common rodent feature and might be explored in humans.

Non-neuronal TRPA1 encodes mechanical allodynia evoked by neurogenic inflammation and partial nerve injury in rats Francesco De Logu[1§] Gaetano De Siena[1§], Lorenzo Landini[1], Matilde Marini[1], Daniel Souza Monteiro de Araujo[1], Antonia Romitelli[1], Luigi F. Iannone[1], Pierangelo Geppetti[1], Romina Nassini[1]1Department of Health Sciences, Clinical Pharmacology and Oncology Section, University of Florence, Florence, 50139, Italy Corresponding author * Pierangelo Geppetti,*geppetti@unifi.it*§ These authors contributed equally to this work

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AbstractBackground and Purpose. The proalgesic transient receptor potential (TRP) ankyrin 1 (TRPA1) channel, expressed by a subpopulation of primary sensory neurons, has been implicated in various pain models in mice. However, evidence in rats indicates that TRPA1 conveys nociceptive signals elicited by channel agonists but not those associated with tissue inflammation or nerve injury. Here, in rats, we explored the TRPA1 role in mechanical allodynia associated with neurogenic inflammation and moderate (partial sciatic nerve ligation, pSNL) or severe (chronic constriction injury, CCI) sciatic nerve injury. Experimental Approach. Acute nociception and mechanical hypersensitivity associated with neurogenic inflammation and sciatic nerve injury (pSNL and CCI) were investigated in rats with TRPA1 pharmacological antagonism or genetic silencing. TRPA1 presence and function was analyzed in cultured rat Schwann cells. Key Results. Hind paw mechanical allodynia (HPMA), but not acute nociception, evoked by local injection of the TRP vanilloid 1 (TRPV1) agonist, capsaicin, or the TRPA1 agonist, allyl isothiocyanate, was mediated by calcitonin gene related peptide (CGRP) released from peripheral nerve terminals. CGRP-evoked HPMA was sustained by a reactive oxygen species (ROS)-dependent TRPA1 activation, probably in Schwann cells. HPMA evoked by pSNL, but not that evoked by CCI, was mediated by ROS and TRPA1 without the involvement of CGRP. Conclusions and Implications. As found in mice, TRPA1 mediates mechanical allodynia associated with neurogenic inflammation and moderate nerve injury in rats. The channel implication in mechanical hypersensitivity following inflammation and partial nerve damage is a common rodent feature and might be explored in humans. Keywords: TRPA1, neurogenic inflammation, nerve injury, oxidative stress, Schwann cells.**1 Introduction**The transient receptor potential (TRP) family of channels encompasses several nonselective cation channels expressed in a variety of cells, including a subpopulation of primary sensory neurons, where they encode sensory modalities that span from thermosensation to mechanical and chemical stimuli (Story et al., 2003) (Talavera et al., 2020). Major attention has been paid to the TRP vanilloid 1 (TRPV1), also known as the capsaicin (hot pepper) receptor, and the TRP ankyrin 1 (TRPA1), also known as the allyl isothiocyanate (AITC, wasabi) receptor (Talavera et al., 2020) (Szallasi et al., 1999). TRPV1 and TRPA1 are abundantly expressed in a heterogeneous subpopulation of primary sensory neurons that consists of peptidergic and non-peptidergic C-fibre and Aδ-fibre nociceptors (Bhattacharya et al., 2008). TRPV1 and TRPA1 stimulation results in the release of the neuropeptides, substance P (SP), and calcitonin gene-related peptide (CGRP) (Nassini et al., 2014), which mediate neurogenic inflammatory responses (Geppetti et al., 1996). In recent years, the role of TRPA1 in sustaining mechanical allodynia has been identified in mouse models of inflammatory, neuropathic, cancer, and migraine pain. These include intraarticular injection of monosodium urate (Trevisan et al., 2014), hind limb ischemia and reperfusion (De Logu et al., 2020), partial sciatic nerve ligation (pSNL) (De Logu et al., 2017), alcoholic polyneuropathy (De Logu et al., 2019), melanoma cells inoculation (De Logu et al., 2021), and CGRP injection into the periorbital skin (De Logu et al., 2022). In addition to the contribution of neuronal TRPA1, which signals agonist-induced acute nociception, a critical role of Schwann cell TRPA1 has been identified in macrophage-dependent (De Logu et al., 2017) (De Logu et al., 2021) and macrophage-independent (De Logu et al., 2019) (De Logu et al., 2022) sustained mechanical allodynia. Pain-like responses produced in rat models of inflammatory and neuropathic conditions have been reported to be reduced by first generation TRPA1 receptor antagonists (Petrus et al., 2007) (Eid et al., 2008) (McNamara et al., 2007) (Wei et al., 2009), which, however, suffered from poor selectivity or suboptimal pharmacokinetics. More recently, TRPA1 deletion in rats by CRISPR technology, while attenuating acute nociception by AITC, failed to reduce mechanical hypersensitivity in models of inflammatory and neuropathic pain, including those evoked by chronic constriction injury (CCI), the chemotherapeutic agent bortezomib, and complete Freund adjuvant (Reese et al., 2020). These findings led to the concluion that TRPA1 implication in pathophysiological models of pain diseases is confined to mice and cannot be replicated in other rodent species (Reese et al., 2020). Here, we examined in rats the role of TRPA1 in mechanical allodynia in a model of neurogenic inflammation and in two models of neuropathic pain. We found that while acute nociceptive responses elicited by AITC and capsaicin injection in the rat hind paw were dependent on their respective selective targets (TRPA1 and TRPV1, respectively), mechanical allodynia was exclusively due to CGRP release and the activation of non-neuronal TRPA1, most likely in Schwann cells, that senses, amplifies, and sustains the pro-allodynic oxidative stress signal. We also confirmed in rats the failure of TRPA1 antagonism to attenuate allodynia in a severe model of neuropathic pain (CCI), while

TRPA1 and oxidative stress were markedly implicated in allodynia in the less severe pSNL model. Thus, TRPA1 seems to have a conserved ability to encode various pain modalities across different mammal species, including rats, in pathophysiological pain models.**2 Methods 2.1 Animals** Sprague-Dawley rats (male, 150 g, Charles River, Milan, Italy, RRID:RGD_734476) were use throughout. The group size of n = 6 animals for behavioral experiments was determined by sample size estimation using G*Power (Version 3.1.9.6-available from*https://gpower.software.informer.com/3.1/*)(Faul et al., 2007) to detect size effect in a post-hoc test with type 1 and 2 error rates of 5 and 20%, respectively. Rats were allocated to vehicle or treatment groups using a randomization procedure (*http://www.randomizer.org/*). Investigators were blinded to treatments, which were revealed only after data collection. No animals were excluded from experiments. All behavioral experiments were in accordance with European Union (EU) guidelines for animal care procedures and the Italian legislation (DLgs 26/2014) application of EU Directive 2010/63/EU. Study was approved by the Italian Ministry of Health (research permit 360/2022-PR). The behavioral studies followed the animal research reporting *in vivo*experiment (ARRIVE) guidelines (Kilkenny et al., 2010) and the recommendations made by the British Journal of Pharmacology (Lilley et al., 2020). Rats were housed in a temperature- and humidity-controlled*vivarium* (12 hr dark/light cycle, free access to food and water, 5 animals per cage). At least 1 hr before behavioral experiments, rats were acclimatized to the testing room and behavior was evaluated between 9:00 am and 5:00 pm. All the procedures were conducted following the current guidelines for laboratory animal care and the ethical guidelines for investigations of experimental pain in conscious animals set by the International Association for the Study of Pain(Kilkenny et al., 2010). Animals were euthanized with inhaled CO_2 plus 10-50% O_2.**2.2 Partial ligation of the sciatic nerve** Partial ligation of the sciatic nerve (pSNL) was performed as previously described (Seltzer et al., 1990). Briefly, rats were anesthetized with a mixture of ketamine (100 mg kg^{-1}) and xylazine (10 mg kg) and the right sciatic nerve was exposed at high-thigh level. Under a magnification of 25x, the dorsum of the nerve was carefully freed from surrounding connective tissues at a site near the trochanter just distal to the point at which the posterior bicep semitendinosus nerve branches off the common sciatic nerve. The nerve was fixed in its place by pinching the epineurium on its dorsal aspect, taking care not to press the nerve against underlying structures. A silicon treated silk suture was inserted into the nerve and tightly ligated so that the dorsal l/3-1/2 of the nerve thickness was trapped in the ligature. The wound was then closed. In sham-operated mice, used as controls, the right sciatic nerve was exposed, but not ligated. Rats were monitored, adequately rehydrated, and maintained in a controlled temperature (37 °C) until fully recovered from anesthesia.**2.3 Chronic constriction injury to sciatic nerve**Chronic constriction injury (CCI) to sciatic nerve was performed as previously described (Bennett et al., 1988). Briefly, rats were anesthetized with a mixture of ketamine (100 mg kg^{-1}) and xylazine (10 mg kg^{-1}) and the common sciatic nerve was exposed at the level of the middle of the thigh by blunt dissection through the bicep femoris. Proximal to the sciatic trifurcation, about 7 mm of nerve was freed of adhering tissue, and four ligatures (5.0 Ethicon chromic catgut) were tied loosely around it with about 1-mm spacing. Great care was taken to tie the ligatures, such that the diameter of the nerve was seen to be just barely constricted. In sham-operated mice, used as controls, the right sciatic nerve was exposed, but not ligated. Rats were monitored, adequately rehydrated, and maintained in a controlled temperature (37 °C) until fully recovered from anesthesia.**2.4 Treatment protocols**Rats received unilateral (right hindpaw) intraplantar (i.pl.) injection (20 µl/site) of allyl isothiocyanate (AITC, 200 nmol solution diluted in mineral oil) or vehicle (mineral oil), capsaicin (CPS, 10 nmol) or vehicle (0.5% DMSO), CGRP (1.5 nmol), SP (3.5 nmol) or vehicle (0.9%NaCl). Some rats were treated (0.5 h before or 0.5 h after the stimulus) with i.pl. (20 µl/site) A967079 (300 nmol), L733,060 (20 nmol), olcegepant (1 nmol), SQ22536 (25 nmol), L-NAME (1 µmol), N-tert-butyl-α-phenylnitrone (PBN, 670 nmol) or vehicle (4% dimethylsulfoxide, DMSO 4% Tween 80 in 0.9% NaCl). Other rats received intraperitoneal (i.p., 10 ml kg^{-1}) A967079 (10, 30 and 100 mg kg^{-1}), AMG0902 (AMG, 10, 30 and 100 mg kg^{-1}) capsazepine (CPZ, 4 mg kg^{-1}) or vehicle (4% DMSO 4% Tween 80 in 0.9% NaCl) before the stimulus or at day 15 after pSNL, CCI or sham surgery. In different experiments, rats were randomly allocated to the groups receiving perineural (p.n., 10 µl) or intrathecal (i.th., 10 µl) treatment with TRPA1 antisense (AS) or mismatch (MM) oligonucleotide (ODN) (10 nmol), once a day for 4 consecutive days, or once a day for 4 consecutive days, starting from day 10 to day 14 after pSNL, CCI, or sham surgery. TRPA1 AS-ODN sequence was 5'-TATCGCTCCACATTGCTAC-3', TRPA1 MM-ODN

sequence was 5'-ATTCGCCTCACATTGTCAC-3'. Perineural injections were performed by injecting the compound into the region surrounding the sciatic nerve at high thigh level of right hind limbs without skin incision using a microsyringe fitted with a 30-gauge needle.**2.5 Behavioral assay**Acute nociception Each rat was lightly restrained in a towel, and an intraplantar injection of 20 µl was made to the right hindpaw using a 30-gauge disposable needle attached to a luer-tipped Hamilton syringe. Immediately after the i.pl. injection, rats were placed inside a plexiglass chamber and spontaneous nociception was assessed for 10 min by measuring the time (seconds) that the animal spent licking/lifting the injected paw. Paw mechanical allodynia. Paw mechanical allodynia was evaluated by measuring the paw withdrawal threshold using the up-down paradigm (Chaplan et al., 1994; Dixon, 1980). Rats were acclimatized (1 hr) in individual clear plexiglass boxes on an elevated wire mesh platform, to allow for access to the plantar surfaces of the hind paws. Von Frey filaments of increasing stiffness (0.4, 0.6, 1.0, 1.4, 2, 4,8,10, and 15 g) were applied to the hind paw plantar surfaces of rats with enough pressure to bend the filament. The absence of a paw being lifted after 5 s led to the use of the next filament with an increased force, whereas a lifted paw indicated a positive response, leading to the use of a subsequently weaker filament. Six measurements were collected for each rat or until four consecutive positive or negative responses occurred. The 50% mechanical withdrawal threshold (expressed in g) was then calculated.**2.6 RNAscope**Frozen tissue sections of mouse sciatic nerve (10 µm) were baked for 30 min at 60 °C and washed with 1X PBS. Sciatic nerve tissues were treated with hydrogen peroxide (#322335, ACD HybEZ) for 10 min at room temperature (RT). Target retrieval was performed for 5 min at 99-100degC, followed by Protease Plus (#322331, ACD HybEZ) pre-treatment for 30 min at 40*C. Samples were subsequently hybridized with a probe specific to rat TRPA1 (#312511, ACD HybEZ) and negative (#310043, ACD HybEZ) control probe for 2h at 40*C. Sequential signal amplification and red chromogenic detection was performed. Sciatic nerve slides were subjected to an immunofluorescent labeling using Alexa Fluor(r) 488 anti-S100 beta antibody [EP1576Y] (#ab196442, monoclonal rabbit, 1:100, Abcam) overnight at 4degC. Both DRG and sciatic nerve sections were coverslipped using mounting medium with DAPI - Aqueous, Fluoroshield (#ab104139, Abcam). Fluorescent images were acquired using a Zeiss Axio lmager 2, Zeiss ZEN imaging 2020.**2.7 Primary culture of rat Schwann cells**Rat Schwann cells were isolated from sciatic nerve of Sprague-Dawley rats (Tao, 2013). Briefly, sciatic nerve was dissected, the epineurium was removed, and nerve explants were divided into 1 mm segments and dissociated enzymatically using collagenase (0.05%) and hyaluronidase (0.1%) in HBSS (2 h, 37 degC). Cells were collected by centrifugation (150 x g, 10 min, room temperature) and the pellet was resuspended and cultured in DMEM containing fetal calf serum (10%), L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 mg/ml), neuregulin (10 nM), and forskolin (2 µM). Three days later, cytosine arabinoside (Ara-C, 10 mM) was added to remove fibroblasts. Cells were cultured at 37 °C in 5% CO_2 and 95% O_2. Purity of primary Schwann cells cultured according to the present protocol reaches almost 100%. The culture medium was replaced every 3 days and cells were used after 15 days of culture.**2.8 Ca^{2+} imaging**Cells were plated on poly- L-lysine-coated (8.3 µM) 35 mm glass coverslips and maintained at 37 °C in 5% CO_2 and 95% O_2 for 24 h. Cells were loaded for 40 min with Fura-2 AM-ester (5 µM) added to the buffer solution (37 °C) containing (in mM) 2 $CaCl_2$; 5.4 KCl; 0.4 MgSO4; 135 NaCl; 10 D-glucose; 10 HEPES and bovine serum albumin (BSA, 0.1%). Cells were washed and transferred to a chamber on the stage of a fluorescent microscope for recording (Axio Observer 7 with fast filterwheel and Digi-4 for the excitation, Zeiss). Cells were exposed to AITC (1 mM) or vehicle (0.5% DMSO) or CGRP (10 µM) or vehicle (0.9% NaCl), and the Ca^{2+} response was monitored for 6 min or 0.5 h after stimulus, respectively. The Ca^{2+} response to AITC and CGRP and was also monitored in the presence of A967079 (50 µM), olcegepant (100 nM), or vehicle (0.1% DMSO). Results were expressed as percent increase in $Ratio_{340/380}$ over baseline normalized to the maximum effect induced by ionomycin (5 µM) added at the end of each experiment.**2.9 Data and Statistical Analysis**Results are expressed as mean ± standard error of the mean (SEM). For multiple comparisons, a one-way analysis of variance (ANOVA) followed by the post-hoc Bonferroni's test was used. For behavioral experiments with repeated measures, the two-way mixed model ANOVA followed by the post-hoc Bonferroni's test was used. Statistical analyses were performed on raw data using Graph Pad Prism 8 (GraphPad Software Inc.). P values less than 0.05 ($P<0.05$) were considered significant. The data and statistical analysis comply with the recommendations of the British Journal of Pharmacology on experimental design and analysis in pharmacology (Curtis et

al., 2018).**2.10 Drugs and chemicals**If not otherwise indicated, reagents were obtained from Merck Life Science SRL (Milan, Italy).**3 Results3.1 AITC and capsaicin-evoked nocifensive behaviour and mechanical allodynia**If not otherwise specified, all compounds were given by the intraplantar (i.pl., 20 ml) route of administration. Injection of AITC (200 nmol) in the hind paw of Sprague-Dawley rats caused a spontaneous, acute, and transient (10 min) local nocifensive behaviour that was followed by a delayed and prolonged (2 hours) hind paw mechanical allodynia (HPMA) (Figure 1A). Systemic (intraperitoneal, i.p. 100 mg kg^{-1}) or local (i.pl., 300 nmol) pretreatment with the selective TRPA1 antagonist, A967079, prevented both nocifensive behaviour and HPMA induced by AITC (Figure 1A). Selective involvement of the TRPA1 channel was strengthened by the failure of the TRPV1 antagonist capsazepine to affect either response (Supplementary Figure S1). Capsaicin (10 nmol) injection elicited a similar biphasic response, consisting of an acute nocifensive behaviour (10 min) and a delayed HPMA (2 hours), which were abated by pretreatment (i.p., 4 mg kg^{-1}) with the selective TRPV1 antagonist, capsazepine (Figure 1B). Notably, pretreatment with A967079 (i.p. or i.pl.) did not affect the nocifensive behaviour but did prevent HPMA induced by capsaicin (Figure 1C and 1D). To understand the mechanism underlying TRPA1-dependent HPMA, antagonists were given after the administration of the stimulus. Posttreatment with capsazepine did not affect HPMA elicited by both capsaicin and AITC (Figure 1E), whereas posttreatment with A967079 markedly attenuated both responses (Figure 1F). These data suggest that spontaneous nocifensor behaviour elicited by TRPV1 and TRPA1 agonists are mediated by the activation of the respective channel in the sensory nerve terminal. However, whatever the initial stimulus targeting the peptidergic nerve terminal, the prolonged HPMA is due to a common pathway implicating the TRPA1 channel.**3.2 SP and CGRP-evoked mechanical allodynia**Based on the notion that, in rodents, both capsaicin and AITC release the proinflammatory neuropeptides, CGRP and SP, (Geppetti et al., 1996), and on previous findings obtained in mice (De Logu et al., 2022), we hypothesized that a neurogenic inflammatory mechanism is implicated in HPMA evoked by capsaicin or AITC. Pretreatment with an antagonist of the SP NK1 receptor, L733,060 (20 nmol), attenuated HPMA evoked by SP (3.5 nmol) (Figure 2A), and pretreatment with the CGRP receptor antagonist, olcegepant (1 nmol), reduced HPMA elicited by CGRP (1.5 nmol) (Figure 2B). No spontaneous acute nocifensive behaviour was observed following injection of either SP or CGRP (Figure 2A and B). Neither olgecepant nor L733,060 did inhibit the nocifensor behaviour evoked by AITC or capsaicin (Figure 2C-F). However, whereas pretreatment with L733,060 was ineffective (Figure 2E and F), pretreatment with olcegepant prevented HPMA elicited by both AITC and capsaicin (Figure 2C and D). Notably, both pretreatment and posttreatment with A967079 (300 nmol) attenuated HPMA evoked CGRP (Figure 2G and H). In contrast, pretreatment with capsazepine (i.p., 4 mg kg^{-1}) (Figure 2I) or posttreatment with olcegepant (1 nmol) (Figure 2J) failed to reduce HPMA evoked by CGRP. In addition, posttreatment with olcegepant did not affect HPMA elicited by either AITC (Figure 2K) or capsaicin (Figure 2L). These data indicate that, in rats, HPMA associated with neurogenic inflammation is due to CGRP released from TRPV1/TRPA1 expressing nerve terminals and, while TRPV1 activation and CGRP release have an initial role, solely TRPA1 sustains the 2-3 hours of HPMA.**3.3 Cellular and molecular mediators of neurogenic inflammation associated allodynia**Having established the critical role of CGRP in capsaicin- and AITC-induced HPMA and based on mouse findings (De Logu et al., 2022), we explored the signalling pathway underlying CGRP-induced HPMA. Pretreatment, but not posttreatment, with the adenylyl cyclase inhibitor, SQ22536 (25 nmol), or the nitric oxide synthase (NOS) inhibitor, L-NG-nitro arginine methyl ester (L-NAME, 1 μmol), prevented CGRP-evoked HPMA (Figure 3A and B). Both pretreatment and posttreatment with the reactive oxygen species (ROS) scavenger, N-tert-butyl-alpha-phenylnitrone (PBN, 670 nmol), inhibited HPMA evoked by CGRP (Figure 3C), capsaicin (Figure 3D), or AITC (Figure 3E). However, PBN did not affect acute nocifensor behaviour evoked by capsaicin or AITC (Figure 3D and E). Thus, HPMA induced by CGRP capsaicin or AITC in rats shares a final common pathway which encompasses an early and transient activation of adenylyl cyclase and NOS and a sustained generation of ROS. To investigate the role of extraneuronal TRPA1 expressed in local cells of the rat paw in neurogenic inflammation-dependent HPMA, a TRPA1 antisense oligonucleotide (AS-ODN), or the mismatched ODN (MM-ODN), were injected perineurally (p.n., 10 nmol) to silence TRPA1 in local perineural cells, or intrathecally (i.th., 10 nmol) to silence TRPA1 in nociceptors (Bonet et al., 2013). Injection (i.th.) of TRPA1 AS-ODN eliminated both the

acute nocifensor behaviour and HPMA to AITC (Figure 3F). In contrast, (p.n.) TRPA1 AS-ODN, which does not interfere with neuronal RNA, did not affect acute nocifensor behavior, but attenuated HPMA evoked by AITC (Figure 3G). Treatment (p.n.) with TRPA1 AS-ODN did not affect acute nocifensor behaviour evoked by capsaicin, but reduced HPMA elicited by either capsaicin or CGRP (Figure 3H and I). The expression of TRPA1 mRNA in rat Schwann cells was confirmed by RNAscope in rat sciatic nerve tissue by coexpression of TRPA1 mRNA with staining for the Schwann cell marker, S100 (Figure 4A). Primary rat Schwann cells were harvested and grown in culture and their identity was verified by qRTPCR with the S100 primer (Figure 4B). TRPA1 mRNA expression was also confirmed in cultured rat Schwann cells (Figure 4B). Exposure of cultured primary rat Schwann cells to AITC rapidly increased intracellular calcium mobilization (Figure 4C), a response that was inhibited in the presence of A967079. Exposure to CGRP of rat Schwann cells elicited a delayed increase in intracellular calcium mobilization that was attenuated by olcegepant and A967079 (Figure 4D). These data confirm the presence of the functional CGRP receptor and TRPA1 channel in rat Schwann cells.**3.4 TRPA1 role in chronic constriction injury (CCI) and partial sciatic nerve ligation (pSNL)**Rats undergoing CCI or pSNL developed, at day 15 after surgery, a robust HPMA ipsilateral to the lesion that was not observed in sham rats. Systemic (i.p.) administration of two different TRPA1 antagonists, AMG-0902 and A967079, while not affecting HPMA associated with CCI (Figure 4A), dose-dependently attenuated HPMA associated with pSNL (Figure 4B). Capsazepine (i.p.) failed to reduce HPMA in either the CCI or the pSNL model (Figure 4C and D). Repeated (from day 10 to day 14) injection (p.n.) of the TRPA1 AS-ODN did not affect HPMA in the CCI model, whereas it reduced HPMA in the pSNL model (Figure 4E and F). The role of CGRP in both models was also explored. At day 15 after surgery, olcegepant (1 nmol) failed to reduce HPMA in either the pSNL or the CCI model (Figure 4G and H). Finally, the role of oxidative stress was tested in both models. Inhibition of HPMA by PBN was superior in pSNL (area under curve, AUC, 42.56 ± 1.73 n=6) than in the CCI (AUC, 33.20 ± 3.03 n=6, $P<0.05$) model (Figure 4 I and J).**4 Discussion**A large series of evidence supports the role of TRPA1 in models of inflammatory, neuropathic, cancer, and migraine pain in mice (De Logu et al., 2021; De Logu et al., 2022; De Logu et al., 2017; Trevisan et al., 2014). However, recent findings obtained in rats with genetic deletion of the TRPA1 channel by the CRISPR technology showed that, while the agonist-induced TRPA1-mediated acute nocifensor behaviour was attenuated, mechanical allodynia produced by neuropathic and inflammatory pain models was unaffected (Reese et al., 2020). These findings led us to conclude that the proalgesic role of TRPA1 is confined to mice, and it cannot be replicated in another rodent species and possibly in other species (Reese et al., 2020). Here, based on recent findings obtained in mice (De Logu et al., 2022), we explored in rats the role of TRPA1 in mechanical allodynia associated with neurogenic inflammation. We report that selective activation of TRPV1 and TRPA1 in the rat hind paw elicited acute and transient nocifensor behaviour that were most likely due to the direct TRPV1 or TRPA1 gating by the respective agonists and the ensuing depolarization that conveys pain signals to the central nervous system. The transient nature of the nocifensor responses could be associated to the limited time of channel occupancy by the agonists. About 30 min after the agonist injection, rats developed a delayed and sustained mechanical allodynia that lasted 2-3 hours. A similar acute and transient nocifensor behaviour followed by a delayed and sustained periorbital mechanical allodynia (PMA) has been observed in mice after the periorbital injection of capsaicin (De Logu et al., 2022). The underlying mechanism of the capsaicin-evoked HPMA in rats and PMA in mice is apparently identical. In rats and mice, pretreatment with a CGRP receptor antagonist, but not with a SP receptor antagonist, prevented capsaicin-evoked HPMA and PMA, respectively. These findings indicate the CGRP-mediated component of neurogenic inflammation as the sole proalgesic mechanism observed across different rodent species. The sequence of intracellular mediators that in mice (De Logu et al., 2022) mediate the proalgesic action of CGRP was replicated in rats, as adenylyl cyclase and NOS inhibitors, a ROS scavenger and a TRPA1 antagonist, attenuated CGRP- and capsaicin-evoked HPMA. However, whereas CGRP receptor antagonist and adenylyl cyclase and NOS inhibitors prevented HPMA if drugs were given before, but not after, the stimulus, both pretreatment and posttreatment with a ROS scavenger or a TRPA1 antagonist were equally effective in reducing HPMA. The interpretation of these findings suggests that CGRP, cyclic AMP, and nitric oxide exert an early and transient role in HPMA associated with neurogenic inflammation, whereas ROS and TRPA1 activate a feed-forward pathway which sustains mechanical

allodynia over 2-3 hours. As AITC-evoked HPMA was attenuated by the same pharmacological interventions and with the same timing that were shown to inhibit capsaicin-evoked HPMA, an identical common pathway is proposed to sustain mechanical allodynia encoded by neurogenic inflammation, independently from the stimulus that triggers the activation of peptidergic nociceptors and the ensuing neuropeptide release. In mice, we were able to identify the Schwann cells surrounding peptidergic nerve terminals as the cell type that expresses the TRPA1 that sustains the CGRP-mediated and capsaicin-evoked PMA (De Logu et al., 2022). The aim of the present study did not have this purpose, and, accordingly, specific tools to selectively silence Schwann cell TRPA1 in rats were not developed. Notwithstanding, a series of findings suggests the implication of peripheral glial cells. First, in rats, RNAscope showed the colocalization of TRPA1 mRNA with immunofluorescence for the Schwann cell specific protein, S100. Second, AITC evoked a calcium response in primary cultures of rat Schwann cells that was attenuated by a TRPA1 antagonist. Third, and importantly, rat Schwann cells, like mouse and human Schwann cells (De Logu et al., 2022), responded to CGRP with a delayed and sustained calcium response that was reduced by a CGRP receptor antagonist and a TRPA1 antagonist. These *in vitro*findings were recapitulated by *in vivo* results, as HPMA was attenuated by inhibitors of the CGRP receptor and TRPA1. Regarding the two neuropathic pain models investigated in the present study, we confirmed (Reese et al., 2020) that, in the more severe model (CCI), HPMA was not reduced by two different TRPA1 antagonists. However, in the less severe model (pSNL), HPMA was attenuated in a dose-dependent manner by the two TRPA1 antagonists. However, in the two neuropathic pain models, CGRP release does not apparently play any role as HPMA was unaffected by olcegepant. It is known that neuropathic pain caused by peripheral nerve lesion (Wallerian degeneration) is due to hematogenic macrophage accumulation at the site of the injury (De Logu et al., 2017; Van Steenwinckel et al., 2015). Oxidative stress generated by invading macrophages mediates mechanical allodynia in both models, although its contribution seems higher in the pSNL model, as the ROS scavenger, PBN, appeared superior in reducing HPMA in the pSNL model than in the CCI model. The unique redox-sensitivity of TRPA1 (Hinman et al., 2006; Macpherson et al., 2007) and the higher oxidative burden in the pSNL might be the reason for the channel involvement in this model. Additional explanations may be proposed. There is evidence of remarkable differences in the nerve lesions in the footpad skin produced in rats by CCI and pSNL (Ma et al., 2000). Whereas in CCI PGP-9.5+, nerve fibers dramatically decreased within two weeks, in the pSNL model the decrease was partial and underwent a time-dependent recovery (Ma et al., 2000). It is possible to hypothesize that the less severe pSNL lesion preserves the integrity of the unit composed by the nerve fiber and the surrounding Schwann cells that contribute to mechanical allodynia. In the more severe CCI lesion, the structural loss of peripheral sensory axons excludes their contribution to generating pain signals. A TRPA1 antagonist failed to reduce pain symptoms in patients with chronic painful diabetic neuropathy (Jain et al., 2022). However, it did produce a statistically significant attenuation in a subgroup of patients with preserved sensory nerve function, and therefore with a less severe neuropathy (Jain et al., 2022). These findings show some similarity with the present rat data, where HPMA associated with a less severe nerve lesion was TRPA1-dependent. The lack of effect of a CGRP antagonist in either the CCI or pSNL model underlines the unique role of CGRP in migraine (Edvinsson et al., 2018; Nassini et al., 2014) and not in other pain conditions, as indicated by the failure of an anti-CGRP monoclonal antibody in reducing pain in patients with osteoarthrosis (Jin et al., 2018). It is possible that in Wallerian degeneration the oxidative burden required for targeting Schwann cell TRPA1 is provided by the massive ROS generation produced by invading macrophages, while under these circumstances the contribution of CGRP to the overall oxidative burden is negligible. Primary hyperalgesia, a pain response due to sensitization of peripheral nociceptors, has been reported in the cutaneous area of inflammation following the application of a variety of stimuli, including capsaicin, (LaMotte et al., 1992). The proposal by Sir Thomas Lewis (Lewis, 1936) that a chemical substance, released from collateral branches by the antidromic invasion of propagated action potentials originating from the injured nerve terminal, causes the flare (inflammation) and increases the sensitivity of other fibers responsible for pain can be applied to the present findings in rats. CGRP released from TRPV1+ and TRPA1+ nerve fibers *via* oxidative stress and TRPA1 lowers the threshold to mechanical stimuli. This mechanism, recently reported in mice (De Logu et al., 2022) and confirmed here in rats, might be a common feature that should be explored also in humans. In conclusion, we have reported that the contribution of

TRPA1 to mechanical allodynia appears to be present in models of neuropathic pain characterized by moderate nerve injury, although in this case the contribution of CGRP and neurogenic inflammation is absent. 'What is already known',

The TRPA1 channel has been implicated in various pain models in mice.

'What this study adds'

TRPA1 role in mechanical allodynia in neurogenic inflammation and moderate/severe nerve injury in rats.

'Clinical significance'.

TRPA1 implication in mechanical hypersensitivity is a common feature in rodents and may be explored in humans.

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Figure Legends Figure 1. AITC and capsaicin-evoked nocifensive behaviour and mechanical allodynia(A) Acute nocifensive behaviour and HPMA after intraplantar (i.pl., 20 µl), AITC (200 nmol) or vehicle (veh) in rats pre-treated (0.5 h) with intraperitoneal (i.p.) or local (i.pl.) A967079 (A96, 100 mg kg^{-1} and 300 nmol, respectively) or veh. (B) Acute nocifensive behaviour and HPMA after capsaicin (CPS, 10 nmol, i.pl.) or veh in rats pre-treated (0.5 h) with capsazepine (CPZ, 4 mg kg^{-1}, i.p.) or veh. (C) Acute nocifensive behaviour and HPMA after CPS (10 nmol, i.pl.) or veh in rats pre-treated (0.5 h) with A96 (100 mg kg^{-1}, i.p.) and A96 (300 nmol, i.pl.) or veh. (D) HPMA after CPS (10 nmol, i.pl.), AITC (200 nmol, i.pl.) or veh in rats post-treated (0.5 h) with CPZ (4 mg kg^{-1}, i.p.), A96 (100 mg kg^{-1}, i.p.) or veh. Mean±SEM, n=6 rats per group. Dash (-) is a combination of vehicles. Arrows indicates time of administration. ***P<0.001 vs. Veh;$^{\#\#\#}$P<0.001 vs. AITC/Veh A96, CPS/Veh CPZ, CPS/Veh A96. 1-way and 2-way ANOVA, Bonferroni correction.**Figure 2. SP and CGRP-evoked mechanical allodynia**(A) Acute nocifensive behaviour and HPMA after intraplantar (i.pl., 20 µl), SP (3.5 nmol) or vehicle (veh) in rats pre-treated (0.5 h) with local (i.pl.) L733,060 (20 nmol) or veh. (B) Acute nocifensive behaviour and HPMA after CGRP (1.5 nmol, i.pl.) or veh in rats pre-treated (0.5 h) with olcegepant (1 nmol) or veh. Acute nocifensive behaviour and HPMA after AITC (C) (200 nmol, i.pl.), capsaicin (D) (CPS, 10 noml, i.pl.) or veh in rats pre-treated (0.5 h) with olcegepant (1 nmol) or veh. Acute nocifensive behaviour and HPMA after AITC (E) (200 nmol, i.pl.), capsaicin (F) (CPS, 10 noml, i.pl.) or veh in rats pre-treated (0.5 h) with L733,060 (20 nmol) or veh. HPMA after CGRP (1.5 nmol, i.pl.) or veh in rats pre-treated (G) (0.5 h) or post-treated (H) (0.5 h) with A967079 (A96, 300 nmol, i.pl.) or veh. HPMA after CGRP (1.5 nmol, i.pl.) or veh in rats pre-treated (0.5 h) with capsazepine (I) (CPZ, 4 mg kg^{-1}, intraperitoneal, i.p.) or veh, or (K) in rats post-treated (0.5 h) with olcegepant (1 nmol) or veh. Mean±SEM, n=6 rats per group. Dash (-) is a combination of vehicles. Arrows indicates time of administration. ***P<0.001 vs. Veh;$^{\#}$P<0.05,$^{\#\#\#}$P<0.001 vs. SP/Veh L733,060, CGRP/Veh olcegepant, CPS/Veh olcegepant, CGRP/Veh

A96. 1-way and 2-way ANOVA, Bonferroni correction.**Figure 3. CGRP, AITC and capsaicin induce mechanical allodynia*via* NO production, and ROS production.** HPMA after intraplantar (i.pl., 20 µl) CGRP (1.5 nmol) or vehicle (veh) in rats pre-treated (0.5 h) or post-treated (0.5 h) with local (i.pl.) (A) SQ22536 (25 nmol), (B) L-NAME (1 µmol), (C) PBN (670 nmol) or veh. Acute nocifensive behaviour and HPMA after (D) capsaicin (CPS, 10 nmol, i.pl.), (E) AITC (200 nmol, i.pl.) or veh in rats pre-treated (0.5 h) or post-treated (0.5 h) with local (i.pl.) PBN (670 nmol) or veh. Acute nocifensive behaviour and HPMA after AITC (200 nmol, i.pl.) or veh in rats treated (once a day for 4 consecutive days) with (F) intratechal (i.th, 10 µl) or (G) perineural (p.n., 10 µl) TRPA1 antisense (AS) or mismatch (MM) oligonucleotide (ODN) (10 nmol). Acute nocifensive behaviour and HPMA after (H) CPS (10 nmol, i.pl.), (I) CGRP (1.5 nmol, i.pl.) or veh in rats treated (once a day for 4 consecutive days) TRPA1 AS/MM ODN (10 nmol). Mean±SEM, n=6 rats per group. Arrows indicates time of administration. ***P<0.001 vs. Veh;###P<0.001 vs. CGRP/Veh SQ22536, L-NAME, PBN, CPS-AITC/Veh PBN, AITC-CPS-CGRP/MM. 1-way and 2-way ANOVA, Bonferroni correction.**Figure 4. TRPA1 expression and function in primary rat Schwann cells.** (A) Representative images of TRPA1 mRNA expression in rat dorsal root ganglia (DRG) and sciatic nerve (Scale bar: 20 µm, inset 10 µm) (n=3 subjects). DapB, negative control. (B) Representative real-time PCR plot and cumulative data for S100 and Trpa1 mRNA in rat Schwann cells (n=3 independent experiments). (C and D) Typical traces and cumulative data of Ca^{2+} response (F_{340}/F_{380}) in primary rat Schwann cells stimulated with AITC (1 mM), CGRP (10 µM) or vehicle (veh) in presence of A967079 (A96, 50 µM), olcegepant (100 nM) or veh (n=3 independent experiments). Mean±SEM. Dash (-) is a combination of vehicles. ***P<0.001 vs. Veh; ###P<0.001 vs. AITC CGRP. 1-way ANOVA, Bonferroni correction.**Figure 5. TRPA1 activation mediates HPMA in partial sciatic nerve ligation (pSNL), but not in chronic constriction injury (CCI) model.** HPMA in rats 15 days after (A) CCI, (B) pSNL or sham procedure treated with intraperitoneal (i.p.) AMG0902 (AMG, 30 and 100 mg kg^{-1}), A967079 (A96, 10, 30 and 100 mg kg^{-1}) or vehicle (veh). HPMA in rats 15 days after (C) CCI, (D) pSNL or sham procedure treated with capsazepine (CPZ, 4 mg kg^{-1}, i.p.) or veh. HPMA in rats 15 days after (E) CCI, (E) pSNL or sham procedure treated (once a day for 4 consecutive days starting from day 10 to day14 after surgery) with perineural (p.n., 10 µl) TRPA1 antisense (AS) or mismatch (MM) oligonucleotide (ODN) (10 nmol). HPMA in rats 15 days after (G) CCI, (H) pSNL or sham procedure treated with intraplantar (i.pl.) olcegepant (1 nmol) or veh. HPMA in rats 15 days after (I) CCI, (J) pSNL or sham procedure treated with PBN (670 nmol, i.pl.) or veh. Mean±SEM, n=6 rats per group. Arrows indicates time of administration. ***P<0.001 vs. Sham/Veh;###P<0.001 vs. pSNL-CCI/veh AMG/A96, pSNL/MM, pSNL-CCI/veh PBN. 2-way ANOVA, Bonferroni correction.