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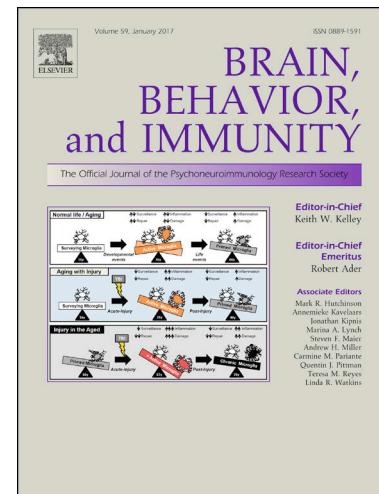
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Macrophages and Schwann cell TRPA1 mediate chronic allodynia in a mouse model of complex regional pain syndrome type I

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ABSTRACT

Complex regional pain syndrome type I (CRPS-I) is characterized by intractable chronic pain. Poor understanding of the underlying mechanisms of CRPS-I accounts for the current unsatisfactory treatment. Antioxidants and antagonists of the oxidative stress-sensitive channel, the transient receptor potential ankyrin 1 (TRPA1), have been found to attenuate acute nociception and delayed allodynia in models of CRPS-I, evoked by ischemia and reperfusion (I/R) of rodent hind limb (chronic post ischemia pain, CPIP). However, it is unknown how I/R may lead to chronic pain mediated by TRPA1. Here, we report that the prolonged (day 1-15) mechanical and cold allodynia in the hind limb of CPIP mice was attenuated permanently in *Trpa1*^{-/-} mice and transiently after administration of TRPA1 antagonists (A-967079 and HC-030031) or an antioxidant (α -lipoic acid). Indomethacin treatment was, however, ineffective. We also found that I/R increased macrophage (F4/80⁺ cell) number and oxidative stress markers, including 4-hydroxynonenal (4-HNE), in the injured tibial nerve. Macrophage-deleted MaFIA (Macrophage Fas-Induced Apoptosis) mice did not show I/R-evoked endoneurial cell infiltration, increased 4-HNE and mechanical and cold allodynia. Furthermore, *Trpa1*^{-/-} mice did not show any increase in macrophage number and 4-HNE in the injured nerve trunk. Notably, in mice with selective deletion of Schwann cell TRPA1 (*Plp1-Cre;Trpa1*^{fl/fl} mice), increases in macrophage infiltration, 4-HNE and mechanical and cold allodynia were attenuated. In the present mouse model of CRPS-I, we propose that the initial oxidative stress burst that follows reperfusion activates a feed forward mechanism that entails resident macrophages and Schwann cell TRPA1 of the injured tibial nerve to sustain chronic neuroinflammation and allodynia. Repeated treatment one hour before and for 3 days after I/R with a TRPA1 antagonist permanently protected CPIP mice against neuroinflammation and allodynia, indicating possible novel therapeutic strategies for CRPS-I.

Keywords: macrophage, nociception, Schwann cells, HC-030031, A-967079, 4-HNE, allodynia.

INTRODUCTION

Complex regional pain syndromes (CRPSs), previously known as reflex sympathetic dystrophy or causalgia (Norman and Bruehl, 2006), comprise two painful conditions (Gierthmühlen et al., 2014; Jänig and Baron, 2003) that develop following minor or major injuries with either little or no obvious nerve damage (CRPS type I, CRPS-I), or clear evidence of nerve damage (CRPS type II, CRPS-II) (Birklein and Dimova, 2017; Gierthmühlen et al., 2014). CRPS-I is more prevalent than CRPS-II (Gierthmühlen et al., 2012; Jänig and Baron, 2003), usually involves a single limb, is associated with pain, sensory, motor, or autonomic impairment, and afflicts patient's quality of life with a huge emotional and psychological burden. Usually, CRPS-I develops from an exaggerated inflammatory response to tissue trauma that may be treated using anti-inflammatory drugs, but can evolve towards a neuropathic pain condition which may become intractable (Birklein and Dimova, 2017; Gierthmühlen et al., 2014; Goh et al., 2017). The mechanisms implicated in the chronification of pain in CRPS-I are poorly understood, thus limiting the possibility to provide satisfactory treatments to affected patients (Goh et al., 2017). Therefore, the identification of the underlying mechanisms of CRPS-I is necessary in order to offer novel and effective pain relief strategies to patients.

The transient receptor potential ankyrin 1 (TRPA1) channel, expressed in peptidergic nociceptors and glial cells (De Logu et al., 2017; Moran and Szallasi, 2017), is exquisitely sensitive to oxidants generated at sites of tissue damage or inflammation, such as hydrogen peroxide (H₂O₂) and 4-hydroxynonenal (4-HNE) (Taylor-Clark et al., 2009; Trevisani et al., 2007). TRPA1 is implicated in mechanical and cold hypersensitivity observed in a variety of models of inflammatory, neuropathic and cancer pain (Antoniazzi et al. 2018; Bonet et al. 2013; Eid et al. 2008; De Logu et al. 2016; McGaraughty et al. 2010; Petrus et al. 2007; Trevisan et al. 2013). We recently showed that a single systemic administration of a TRPA1 antagonist (HC-030031) produced a transient attenuation of mechanical and cold allodynia in a rat (Klafke et al., 2016) and mouse (De Prá et al. 2019) model of CRPS-I, evoked by ischemia and reperfusion (I/R) of the rodent hind limb (chronic post-ischemia pain, CPIP) (Coderre et al., 2004). Oxidative stress increase has been documented in models of CRPS-I (Coderre et al., 2004; Klafke et al., 2016), and some clinical reports showed that antioxidant treatment reduces symptoms of CRPS-I caused by trauma and surgery (Birklein and Dimova, 2017; Lin et al., 2018; Perez et al., 2003). Oxidants produced by I/R have the potential to activate TRPA1-dependent pain-like behaviors (Sasaki et al., 2014; So et al., 2016). However, the mechanism responsible for the activation of oxidative stress and the consequent implication of TRPA1 to sustain chronic allodynia in CPIP mice is unknown.

In the present study, we have investigated the cellular and biochemical mediators that contribute to the TRPA1-dependent mechanisms that, evoked by I/R, initiate and sustain mechanical and cold allodynia. Results showing that TRPA1 genetic deletion provided permanent protection against allodynia in CPIP mice support previous evidence obtained with pharmacological antagonism (De Prá et al., 2019; Klafke et al., 2016) and strengthen the hypothesis that TRPA1 plays a major role in CPIP. Recently, we reported in a mouse model of neuropathic pain (partial sciatic nerve ligation) that nerve injury promotes the recruitment of macrophages, which, *via* their own oxidative burst, activate

TRPA1 in Schwann cells. Such activation results in a NADPH oxidase 1 (NOX1)-dependent amplification of the oxidative burden of the injured tissue that eventually signal pain (De Logu et al., 2017). Thus, we wondered whether macrophages could contribute to CPIP in mice and whether Schwann cell TRPA1 was implicated in the neuroinflammatory pathway that underlies allodynia. By using mice in which the monocyte/macrophage population is depleted and mice harboring a selective deletion of TRPA1 in Schwann cells, we found that both macrophages invading the endoneural space and Schwann cell TRPA1 are essential for promoting and maintaining allodynia. Finally, permanent attenuation of allodynia by a brief (before and after tourniquet application) treatment with a TRPA1 antagonist indicates possible novel therapeutic strategies for CRPS-I.

RESULTS

Mechanical and cold allodynia induced by prolonged IR are mediated by TRPA1 in mice

Ischemia was produced by placing a tourniquet on one hind limb of anesthetized mice just proximal to the ankle joint for 2 hours, and then removing it to allow reperfusion. C57BL/6J mice undergoing I/R developed a prolonged mechanical and cold allodynia that was already maximal at day 1 and was maintained until the last day of observation (day 15) (Figure 1A). As previously reported (De Prá et al., 2019) no differences were observed between female and male mice in I/R-induced mechanical and cold allodynia (Figure 1A). C57BL/6J mice undergoing I/R developed a mild but already significant mechanical and cold allodynia 6, 12 and 24 hours after I/R (Suppl Figure 1A). The essential role of the TRPA1 channel was underlined by the observation that mechanical and cold allodynia were similar in both *Trpa1*^{+/+} and C57BL/6J mice, while no allodynia was present in *Trpa1*^{-/-} mice throughout the entire period of observation from day 1 to 15 (Figure 1B). On day 15 after I/R, a single systemic (intragastric, i.g.) administration of two chemically unrelated TRPA1 antagonists, A-967079 (100 mg/kg, i.g.) (Figure 1C) and HC-030031 (300 mg/kg, i.g.) (Figure 1D) attenuated mechanical and cold allodynia in C57BL/6J mice. Complete inhibition was observed at 1 and 2 hours, while allodynia completely recovered 3 hours after drug administration (Figure 1C, D). Expression of TRPA1 mRNA measured by RT-qPCR was similar in homogenates of dorsal root ganglia (DRGs) obtained from C57BL/6J mice at day 15 from I/R or sham procedure. In fact, TRPA1 mRNA expression relative to β -actin was $4.78 \cdot 10^{-7} \pm 7.40 \cdot 10^{-8}$ (n=6 mice) and $3.26 \cdot 10^{-7} \pm 3.58 \cdot 10^{-8}$ (n=6 mice), respectively, indicating that allodynia is not associated with changes in channel expression in nociceptors.

I/R-evoked increase in oxidative stress is TRPA1-dependent

To explore the role of oxidative stress in the pain-like responses evoked by I/R, the effect of the antioxidant, α -lipoic acid, was investigated. On day 15 after the tourniquet application, systemic administration of α -lipoic acid (100 mg/kg, i.g.) attenuated both mechanical and cold allodynia evoked in C57BL/6J mice. Mechanical and cold allodynia

were markedly attenuated 1 hour after and returned to baseline values 3 hours after α -lipoic acid administration (Figure 2A).

To support these pharmacological results, some oxidative stress biomarkers, such as H_2O_2 levels, 4-HNE staining and NOX and superoxide dismutase (SOD) activities, were measured in segments of the tibial nerve from the ankle of C57BL/6J after I/R. At day 15 after I/R, H_2O_2 level, and NOX and SOD activities were markedly increased in CPIP as compared to sham C57BL/6J mice (Figure 2B, C and D). In particular, 4-HNE, an end-product of oxidative stress (Dalle-Donne et al., 2006), was used to further explore the mechanisms responsible for the generation of oxidative stress. At day 15 a robust increase in 4-HNE staining was detected in sections of nerve of C57BL/6J CPIP mice (Figure 2E). Notably, the marked increase in 4-HNE staining in sections of nerve present in *Trpa1*^{+/+} was completely absent in *Trpa1*^{-/-} CPIP mice, indicating an essential role of the channel in generating oxidative stress (Figure 2F).

Macrophage accumulation is essential to evoke I/R-evoked mechanical and cold allodynia

Next, we explored the molecular and biochemical events responsible for the pain-producing oxidative burden. The observation that, at day 15 after I/R, indomethacin administration (30 mg/kg, i.g.) did not affect mechanical or cold allodynia in C57BL/6J CPIP mice, indicates that inhibition of prostaglandin synthesis cannot reverse these pain-like responses (Figure 3A). In another model of CRPS induced by tibial fracture, the activation and recruitment of innate immune cells, including myeloid cells, such as macrophages, have been reported in the spinal cord and brain (Cropper et al., 2019). In our model, we observed that at day 15 after I/R the number of infiltrating F4/80⁺ cells in the injured tibial nerve trunk of C57BL/6J CPIP mice was significantly higher than that observed in sham mice (Figure 3B). As reported for behavioral responses, no difference between males and females was observed in the number of macrophages infiltrating the tibial nerve (Figure 3B). The F4/80⁺ cell infiltration in the tibial nerve showed a time-dependent increase (6, 12 and 24 hours after I/R) that was temporally associated with the increase in mechanical and cold allodynia (Suppl Figure 1B).

Next, we investigated the mechanism underlying the recruitment of macrophages and whether they sustain mechanical and cold allodynia. The increase in F4/80⁺ cell numbers, which was documented in *Trpa1*^{+/+}, was reduced in *Trpa1*^{-/-} CPIP mice (Figure 3C). This finding underlines the key role of TRPA1 in promoting the macrophage accumulation into the endoneurial space of the nerve trunk at the site of the I/R-evoked injury. To better understand the role of macrophages in pain and neuroinflammation, we used Macrophage Fas-Induced Apoptosis (MaFIA) mice, expressing a green fluorescent protein (GFP) reporter and, a drug inducible Fas suicide gene under control of the macrophage colony stimulating factor (M-CSF) receptor promoter (Burnett et al., 2004). MaFIA mice were initially treated for 5 consecutive days (day 10-14) after tourniquet application with the B/B homodimerizer (B/B-HmD) agent, AP20187 (2 mg/kg, i.p.), to induce apoptosis in the entire monocyte/macrophage population (Burnett et al., 2004). At day 4 after initiation of B/B-HmD treatment, mechanical and cold allodynia (Figure 3D), the number of infiltrating macrophages/GFP⁺ cells (Figure 3E) and the staining of 4-HNE (Figure 3F) in the injured tibial nerve trunk were markedly attenuated, thus underlining

the crucial role of macrophage accumulation in the injured nerve trunk to sustain pain-like responses. To understand whether macrophage expansion was closely associated with the initiation of mechanical and cold allodynia, MaFIA mice were treated with AP20187 for 5 consecutive days before the tourniquet application. This procedure attenuated mechanical and cold allodynia throughout the 15 days of observation (Figure 3G), as well as the number of infiltrating macrophages/GFP⁺ cells (Figure 3H) and the 4-HNE staining (Figure 3I) in the tibial nerve at day 15 after I/R. These findings indicate that recruitment of macrophages is the early and crucial factor in developing mechanical and cold allodynia.

Schwann cell TRPA1 mediates I/R-induced allodynia and neuroinflammation

In a previous mouse model of neuropathic pain caused by partial sciatic nerve ligation, we showed that TRPA1 expressed in Schwann cells, *via* calcium-dependent NOX1 activation, is essential to amplify the oxidative stress signal that sustains neuropathic pain and neuroinflammation (De Logu et al., 2017). To explore whether Schwann cell-expressing TRPA1 could be involved in I/R-evoked responses, we used *Plp1-Cre;Trpa1^{f/f}* mice, which harbor a selective deletion of TRPA1 in the Schwann cell/oligodendrocyte lineage (De Logu et al., 2017). Mechanical and cold allodynia, (Figure 4A), F4/80⁺ cell infiltration and increase in 4-HNE staining (Figure 4B and 4C) in *Plp1-Cre;Trpa1^{f/f}* CPIP mice were attenuated as compared to sham mice. This finding implicates Schwann cell TRPA1 in the control of pain-like responses and neuroinflammation evoked by I/R of the mouse hind limb.

To specifically investigate the contribution of neuronal TRPA1, we studied mice with selective deletion of TRPA1 in primary sensory neurons (*Adv-Cre;Trpa1^{f/f}*) (Marone et al., 2018). In these mice, while I/R-evoked mechanical and cold allodynia were attenuated (Figure 5A), the F4/80⁺ cell infiltration and the increase in 4-HNE staining were unaffected (Figure 5B and 5C). Thus, in contrast with the Schwann cell channel, TRPA1 expressed by nociceptors signals pain, but does not contribute to macrophage expansion and oxidative stress increase.

TRPA1 antagonism during and after I/R application attenuates pain and neuroinflammation

Finally, we explored whether a brief treatment with a TRPA1 antagonist before and shortly after I/R may prevent the sustained mechanical and cold hypersensitivity. HC-030031 (300 mg/kg i.p.) was given 3 times a day, with the first administration 30 minutes before and 30 and 90 minutes after tourniquet application. Further administrations were given at 8:00 am, 2:00 pm and 8:00 pm on the 3 subsequent days. Under these circumstances, mechanical and cold allodynia were significantly attenuated throughout the 15 days of observation (Figure 6A). In addition, at day 15 after tourniquet application, a marked reduction in F4/80⁺ cell infiltration and 4-HNE staining was assessed in the injured tibial nerve trunk (Figure 6B and 6C). To better understand the role of TRPA1 activation in maintaining mechanical and cold allodynia and neuroinflammation, a prolonged (daily 3 times a day from day 12 to day 15) treatment with the TRPA1 antagonist HC-030031 was performed. This treatment schedule significantly reduced mechanical and cold allodynia (Figure 6D), and, at day 15, F4/80⁺ cell (Figure 6E) and 4-HNE (Figure 6F) increase. These findings imply that prolonged TRPA1 blockade, either

before or after (when the allodynia was already established) I/R, can prevent allodynia and neuroinflammation.

DISCUSSION

Our data support previous reports that TRPA1 antagonists were able to attenuate mechanical and cold allodynia evoked by I/R in the rat (Klafke et al., 2016) or mouse (De Prá et al., 2019) hind limb, as they show that *Trpa1*^{+/+} mice exhibited allodynia similar to that observed in C57BL/6J mice, but *Trpa1*^{-/-} mice were completely protected from the painful responses caused by application and removal of the tourniquet. In addition, mechanical and cold allodynia was reversed by the intragastric administration of two different TRPA1 antagonists (A-967079 and HC-030031), thus strengthening the key role of the channel. TRPA1 implication in painful dysesthesia induced by I/R has been previously reported (Sasaki et al., 2014; So et al., 2016). However, the cellular and biochemical mechanisms that result in the TRPA1 activation and the consequent generation of mechanical and cold allodynia are still poorly understood.

The proalgesic role of oxidative stress in rodent models of pain evoked by I/R has been supported by the ability of antioxidants to attenuate mechanical allodynia in CPIP rats (Coderre et al., 2004). This early observation in experimental animals has been corroborated by reports of increased levels of oxidative stress in CRPS-I patients and by the beneficial effect of antioxidants in CRPS-I (Aïm et al., 2017; Birklein and Dimova, 2017; Perez et al., 2003). Oral treatment with the antioxidant, vitamin C, starting on the day of a wrist fracture halved the risk of CRPS-I within the first year after injury (Ekrol et al., 2014; Zollinger et al., 2007, 1999). Patients treated with the free radical scavenger, dimethyl sulfoxide (Zuurmond et al., 1996), and oral treatment with the free radical scavenger, N-acetyl-L-cysteine (Perez et al., 2003), improved the CRPS-I impairment level sum score, upper and lower extremity skills and functions, and general health. Altogether, these data are in line with our findings, showing that the antioxidant, α -lipoic acid, reduced mechanical and cold allodynia in the present mouse model of CPRS-1.

Moreover, CPIP mice showed increased NOX and SOD activities and increased levels of H₂O₂ and 4-HNE in the injured tibial nerve trunk. It has been known for a long time that the neuropathic pain caused by nerve injury (Wallerian degeneration) is associated with a robust infiltration of macrophages within the damaged nerve trunk (Gaudet, Popovich, and Ramer 2011; De Logu et al. 2017; Ramer, French, and Bisby 1997; Trevisan et al. 2016). However, the mechanism by which invading macrophages generate pain has been addressed only recently. Findings obtained in a model of trigeminal neuralgia (Trevisan et al. 2016) proposed a key role of nociceptor TRPA1, which, activated by the oxidative burst of invading macrophages, conveys the pain signal to the brain. However, a subsequent study in a different model of neuropathic pain (partial sciatic nerve ligation) provided evidence that the macrophage-dependent oxidative stress does not directly target TRPA1 in nociceptors, but activates the channel in Schwann cells that ensheath the injured nerve trunk (De Logu et al., 2017). Then, *via* a NOX1-dependent pathway, Schwann cell TRPA1 amplifies and sustains the oxidative stress signal which exerts a dual function. An outwardly directed release of oxidants maintains macrophage

recruitment inside the perineurial space, and an inwardly directed oxidative stress targets nociceptor TRPA1 to signal pain (De Logu et al., 2017). We reasoned that a similar mechanism could be implicated in the prolonged pain and neuroinflammation of CPIP mice. However, additional mechanisms may contribute, as recent findings proposed a role of angiotensin II type 2 receptor (AT2-R) expressed on macrophages infiltrating the injured nerve, by showing that local AT2-R activation in peripheral macrophages is a critical trigger mechanism of chronic pain hypersensitivity associated with nerve injury/neuropathy (Shepherd et al., 2018a, 2018b).

Although a previous study detected a moderate difference in the development of mechanical allodynia in male and female mice (Tang et al., 2017), our research group previously reported (De Prá et al., 2019) and confirm in the current study, that there is no difference in non-evoked and evoked pain responses evoked by I/R in female vs. male mice. The absence of sex-related differences in the severity of pain in CRPS-I patients (Velzen et al., 2019) is in agreement with our mouse findings.

The use of macrophage-depleted MaFIA mice clearly showed that mechanical allodynia evoked by I/R was dependent on the inflammatory cell expansion in the nerve trunk. In fact, sustained attenuation of mechanical and cold allodynia was obtained by treatment with the macrophage depleting agent, AP20187, given either before or after I/R. These two findings imply that macrophage expansion is necessary and sufficient to initiate and sustain the pain-like responses. Increased levels of 4-HNE staining were also reduced in macrophage-depleted MaFIA mice after both AP20187 treatment regimens, supporting the hypothesis that macrophages are implicated in the oxidative burden of the injured nerve trunk. Thus, it is possible that invading macrophages increase the endoneurial oxidative burden that targets nociceptor TRPA1 to signal pain.

In addition to exhibiting a markedly attenuated allodynia, *Trpa1*^{-/-} CPIP mice showed reduced macrophage infiltration and 4-HNE staining. Thus, TRPA1 is not only implicated in sensing the oxidative stress that evokes allodynia, but also contributes to sustain neuroinflammation. There is no evidence that nociceptor TRPA1 favors macrophage recruitment at sites of inflammation or tissue injury, and macrophages do not express TRPA1 (De Logu et al., 2017). Therefore, the channel promoting neuroinflammation in the injured tibial nerve trunk should be expressed in cells other than macrophages or primary sensory neurons. These considerations suggested that an analogy exists between the CPIP model and the partial sciatic nerve ligation model (De Logu et al., (De Logu et al., 2017), where TRPA1 expressed in Schwann cells ensheathing the injured nerve is implicated. To test this hypothesis we used mice with deletion of TRPA1 in the Schwann cell/oligodendrocyte lineage (*Plp1-Cre;Trpa1*^{fl/fl}) (De Logu et al., 2017). The finding that mechanical and cold allodynia, macrophage infiltration and 4-HNE staining were attenuated in *Plp1-Cre;Trpa1*^{fl/fl} CPIP mice indicates that Schwann cell-expressing TRPA1 is essential to attract macrophages into the endoneurial space and to generate the oxidative burden that eventually sustains the persistent pain signal. However, as the myelin proteolipid protein-1 (PLP1) is not exclusively expressed in the Schwann cell/oligodendrocyte lineage, we investigated the contribution of neuronal TRPA1 by using *Adv-Cre;Trpa1*^{fl/fl} mice, which harbor a selective deletion of TRPA1 in primary sensory neurons. In these mice we revealed that TRPA1 expressed in Schwann cells plays

a major role in the neuroinflammation and allodynia evoked by I/R, while the nociceptor TRPA1 is solely implicated in pain signaling.

The failure of indomethacin to reduce mechanical and cold allodynia in CPIP mice indicates that cyclooxygenase metabolites are not involved, and supports the clinical observation of the limited effectiveness of non-steroidal anti-inflammatory drugs in CRPS-I patients (Birklein and Dimova, 2017; Gierthmühlen et al., 2014; Goh et al., 2017). Improvement of the pain condition by limiting the oxidative stress burden has been proposed in CRPS-I patients (Aïm et al., 2017; Birklein and Dimova, 2017; Perez et al., 2003). The limited results obtained with this therapeutic approach may be due to the poor pharmacokinetic of antioxidants, or other unknown reasons (Aïm et al., 2017; Birklein and Dimova, 2017; Perez et al., 2003). The present findings propose TRPA1 as a novel therapeutic target for the treatment of CRPS-I. While *Trpa1*^{-/-} mice were permanently protected from the development of CPIP, the administration of a single dose of TRPA1 antagonists provided a robust attenuation of allodynia that was, however, transient, as allodynia returned to baseline values 3 hours after drug administration.

In a model of chemotherapeutic-evoked neuropathic pain induced by a single administration of the proteasome inhibitor, bortezomib, we reported that the administration of a TRPA1 antagonist shortly before and after the chemotherapeutic drug administration produced a permanent inhibition of the prolonged (10 days) mechanical and cold allodynia (Trevisan et al. 2013). This finding suggested that TRPA1-dependent events rapidly ensuing bortezomib administration, including an increase in oxidative stress, are critical to maintain the prolonged allodynia. We hypothesized a similar pathway operating in CPIP mice. Thus, we devised a therapeutic schedule based on the administration of a TRPA1 antagonist just before and for 3 consecutive days after the application of the tourniquet. This therapeutic protocol resulted particularly efficient in preventing both mechanical and cold allodynia, thus underlining the possibility that a similar early and timely treatment may provide some benefit in patients affected by CRPS-I. The sometimes marked temporal delay between the causal injury (Dommerholt, 2004) and the development of CRPS-I may, however, prevent similar therapeutic strategies in CRPS-I patients. For this reason, the effect of a prolonged treatment schedule with a TRPA1 antagonist was tested 12 days after I/R, when allodynia was maximal. This therapeutic protocol efficiently tempered mechanical and cold allodynia and neuroinflammation, thus proposing a rationale for the prolonged use of TRPA1 antagonists for the treatment of CRPS-I.

Reperfusion of an ischemic tissue with oxygenated blood has long been known to produce an early and massive ROS release (So et al., 2016), due to the activation of several mechanisms, including mitochondrial dysfunction (Zweier and Talukder, 2006). Furthermore, reperfusion of the mouse hind paw after a 10-minute ischemia caused a marked release of H₂O₂ that resulted in TRPA1 activation and sensitization (Sasaki et al., 2014; Takahashi et al., 2011). Finally, H₂O₂ may serve as a chemoattractant to monocytes, as proven in zebrafish larvae, whose wounds produce H₂O₂ to facilitate rapid macrophage recruitment (Niethammer et al., 2009), or in mice at sites of nerve injury (De Logu et al., 2017). These previous findings and our current results suggest that the initial oxidative stress burst that follows reperfusion activates a feed forward mechanism that entails resident macrophages and Schwann cell TRPA1 of the injured tibial nerve to sustain

chronic neuroinflammation and pain. All the same, it is possible that additional mechanisms contribute, including the recently identified ability of macrophage AT2-R to generate a proalgesic release of oxidative stress (Shepherd et al., 2018b, 2018a).

MATERIALS AND METHODS

Animals

Adult C57BL/6J (male and female 25-30g, 5-6 weeks), littermate wild type (*Trpa1*^{+/+}) and TRPA1-deficient (*Trpa1*^{-/-}) mice (male, 25-30g, 5-8 weeks), generated by heterozygotes on a C57BL/6J background (B6.129P-*Trpa1*^{tm1Kykw/J}; Jackson Laboratories, Bar Harbor, ME, USA) (Kwan et al., 2006), were used. To generate mice in which the *Trpa1* gene was conditionally silenced in Schwann cells/oligodendrocytes, homozygous 129S-*Trpa1*^{tm2Kykw/J} (*floxed TRPA1*, *Trpa1*^{fl/fl}, Stock No: 008649, Jackson Laboratories, Bar Harbor, ME, USA), were crossed with hemizygous B6.Cg-Tg(*Plp1-Cre*^{ERT})3Pop/J mice (*Plp1-Cre*^{ERT}, Stock No: 005975, Jackson Laboratories), expressing a tamoxifen-inducible Cre in myelinating cells (*Plp1*, proteolipid protein myelin 1) (De Logu et al., 2017; Doerflinger et al., 2003). The progeny (*Plp1-Cre*; *Trpa1*^{fl/fl}) was genotyped by standard PCR for *Trpa1* and *Plp1-Cre*^{ERT} (De Logu et al., 2017). Mice negative for *Plp1-Cre*^{ERT} (*Plp1-Cre*⁻; *Trpa1*^{fl/fl}) were used as control. Both positive and negative mice to *Cre*^{ERT} and homozygous for floxed *Trpa1* (*Plp1-Cre*^{ERT}; *Trpa1*^{fl/fl} and *Plp1-Cre*^{ERT-/-}; *Trpa1*^{fl/fl}, respectively) were treated with intraperitoneal (i.p.) tamoxifen (1 mg/100 µl in corn oil, once a day, for 5 consecutive days) (De Logu et al., 2017; Doerflinger et al., 2003), resulting in Cre-mediated ablation of *Trpa1* in PLP-expressing Schwann cells/oligodendrocytes. Successful Cre-driven deletion of TRPA1 mRNA was confirmed by RT-qPCR (De Logu et al., 2017).

To selectively delete *Trpa1* gene in primary sensory neurons, 129S-*Trpa1*^{tm2Kykw/J} mice (*floxed Trpa1*, *Trpa1*^{fl/fl}, Stock No: 008649; Jackson Laboratories), which possess loxP sites on either side of the S5/S6 transmembrane domains of the *Trpa1* gene, were crossed with hemizygous *Advillin-Cre* male mice (Guan et al., 2016; Zurborg et al., 2011). The progeny (*Adv-Cre*; *Trpa1*^{fl/fl}) were genotyped by standard PCR for *Trpa1* and *Advillin-Cre* (Guan et al., 2016). Mice negative for *Advillin-Cre* (*Adv-Cre*⁻; *Trpa1*^{fl/fl}) were used as control. Successful *Advillin-Cre* driven deletion of TRPA1 mRNA was confirmed by RT-qPCR (Zappia et al., 2017). To evaluate the involvement of macrophages, Macrophage Fas-Induced Apoptosis (MaFIA; stock No: 005070, Jackson Laboratories, Bar Harbor, ME, USA) mice were used. MaFIA mice were treated with the B/B-HmD (AP20187; 2 mg/kg, i.p.) or vehicle (0.9% NaCl plus 10% v/v PEG-400 plus 1.7% v/v Tween 80) once a day for 5 consecutive days, either before or 10 days after I/R or sham procedure to induce selective depletion of macrophages (Burnett et al., 2006; Shepherd et al., 2018b, 2018a).

Study design

The group size of n=6-8 animals for behavioral experiments was determined by sample size estimation using G*Power (v3.1) (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. Allocation

concealment of mice to vehicle(s) or treatment(s) group was performed using a randomization procedure (<http://www.randomizer.org/>). The assessors were blinded to the identity (genetic background or allocation to treatment group) of the animals. Identity of the animals was unmasked to assessors only after data collection. Mice were housed in a temperature- and humidity-controlled *vivarium* (12 hours dark/light cycle, free access to food and water, 5 animals per cage).

At least 1 hour before behavioral experiments, mice were acclimatized to the experimental room and the evaluations were performed 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 (Zimmermann, 1983). In addition, all experiments and sample collections were carried out according to the European Union (EU) guidelines for animal care procedures and the Italian legislation (DLgs 26/2014) application of the EU Directive 2010/63/EU. Studies were conducted under University of Florence research permits (#194/2015-PR and #844/2019-PR), University of the Extreme South of Santa Catarina (Unesc; Brazil, protocol #009/2015-1) and Federal University of Santa Maria (UFSM, Brazil, protocol #5371190617/2017) approved all procedures. The behavioral studies followed the animal research reporting *in vivo* experiment (ARRIVE) guidelines (McGrath and Lilley, 2015). Animals were anaesthetized with a mixture of ketamine and xylazine (90 mg/kg and 3 mg/kg, respectively, i.p.) and euthanized with inhaled CO₂ plus 10-50% O₂.

Chronic Post-ischemia Pain (CPIP)

CPIP was used as an animal model of CRPS-I and was performed by hind paw transient I/R (Coderre et al., 2004; De Prá et al., 2019; Millecamps et al., 2010). Briefly, in anesthetized mice, a nitrile 70 durometer O-ring (O-Rings West, Seattle, WA, USA) with 7/3200 internal diameter was positioned around the mouse right hind limb just proximal to the ankle joint, to act as a tourniquet for 2 hours. Then, after the O-ring removal, mice were fully recovered within 30-60 minutes of reperfusion. Control (sham) mice received the same anesthetic treatment, but the O-ring was cut so that it only loosely surrounded the ankle without occluding the blood flow to the right hind paw.

Treatment protocols

Unless otherwise indicated, reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA or Milan, Italy). TRPA1 selective antagonists, HC-030031 (300 mg/kg, i.g.) and A-967079 (100 mg/kg, i.g.), the antioxidant α -lipoic acid (100 mg/kg, i.g.), the nonsteroidal anti-inflammatory drug, indomethacin (30 mg/kg, i.p.) or their vehicle (dimethyl sulfoxide, DMSO 1% in NaCl 0.9%) were given at day 15 after I/R or sham procedure. In another experimental setting, HC-030031 (300 mg/kg, i.g.) was given 3 times a day the same day of I/R (30 minutes before tourniquet application and 30 and 90 minutes after) and for the 3 subsequent days (8:00 AM, 2:00 PM and 8:00 PM) and 3 times a day (8:00 AM, 2:00 PM and 8:00 PM) starting from day 12 until day 15.

Assessment of mechanical allodynia and cold hypersensitivity

Mechanical allodynia and cold hypersensitivity were assessed before (baseline) and 6 and 12 hours and 1, 5, 10, and 15 days after I/R or sham procedure in C57BL/6J; and 1, 5, 10, and 15 days after I/R or sham procedure in *Trpa1*^{+/+}, *Trpa1*^{-/-}, *Plp1-Cre*^{ERT}, *Trpa1*^{fl/fl} and *Adv-Cre;Trpa1*^{fl/fl}. The effect of the various pharmacological treatments was assessed at day 15, before and 1, 2 and 3 hours after drug administration. MaFIA mice were tested before (baseline) and 1, 5, 10, 15 days after I/R or sham procedure and from day 10 to 15 after AP20187 or vehicle administration.

The measurement of mechanical paw-withdrawal threshold was carried out using von Frey filaments of increasing stiffness (0.02-2 g) applied to the plantar surface of the mouse hind paw, according to the up-and-down paradigm (Chaplan et al., 1994; De Prá et al., 2019). The 50% mechanical paw-withdrawal threshold (in g) response was then calculated from the resulting scores (Dixon, 1980).

Cold allodynia of the hind paw was assessed by measuring the acute nociceptive response to acetone-evoked evaporative cooling (Trevisan et al. 2013). Briefly, a drop (20 µl) of acetone was gently applied to the plantar surface of the mouse hind paw, and the time spent in elevation and licking of the plantar region was measured for 60 seconds. Acetone was applied three times at 10-15-minute intervals, and the average elevation/licking time was calculated. Cold allodynia was considered as an increase in the nociceptive time observed after exposure to acetone when compared to basal values.

RT-qPCR

RNA was extracted from L4-L6 dorsal root ganglia obtained from euthanized C57BL/6J mice after IR or sham procedure, throughout the standard Trizol® extraction method. RNA levels and purity were assessed spectrophotometrically by determining the absorbance at 260 nm and 280 nm. The RNA (100 ng) was reverse transcribed using the iScript™ cDNA Synthesis kit (Bio-Rad, Hercules, USA), according to the manufacturer's protocol. Real time PCR was performed on Rotor Gene® Q (Qiagen, Hilden, GE) for the relative quantification of mRNA. The sets of probes for mouse tissue were as follows: ACTB, primer1 GACTCATCGTACTCCTGCTTG, primer2 GATTACTGCTCTGGCTCCTAG Probe/56-FAM/CTGGCCTCA/ZEN/CTGTCCACCTTCC/3IABkFQ/ (NCBI Ref Seq: NM_001101); TRPA1: primer1 5'GTACTTCTTGTCTGTTTCTTGC-3', primer2 5'-ACCATCGTGTATCCAAATAGACC-3', probe:/56-FAM/AAAACCGTA/ZEN/GCATCCTGCCGTG/3IABkFQ/(NCBI Ref Seq: NM_177781). The chosen reference gene was ACTB. The QuantiTect Probe PCR Kit (Qiagen, Hilden, GE) was used for amplification, and the cycling conditions used were: samples were heated to 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds, and 65°C for 20 seconds. PCR reaction was carried out in triplicate. Relative expression of mRNA was calculated using the 2^{-Δ(ΔCT)} comparative method, with each gene normalized against the internal endogenous reference ACTB gene for the same sample.

Biochemical assays

All assays were performed in segments of the tibial nerve taken just proximal to the ankle joint from euthanized mice at day 15 after I/R or sham procedure.

H₂O₂ production. H₂O₂ levels were assessed by the phenol red-horseradish peroxidase (HRP) method (Antoniazzi et al., 2018). Briefly, samples were homogenized in 50 mM phosphate buffer (pH 7.4) containing 5 mM sodium azide at 4°C for 60 seconds, and the homogenate was centrifuged (12.000xg for 20 minutes at 4°C). The supernatant obtained was used to determine H₂O₂ levels (Nakamura et al., 1998). The H₂O₂ levels were expressed as μmol of H₂O₂ based on a standard curve of HRP-mediated oxidation of phenol red by H₂O₂, corrected by protein content (in milligrams) (Bradford, 1976).

NADPH oxidase activity. NOX activity was assessed using a commercially available assay kit (CY0100, cytochrome C reductase, NADPH assay kit,). Briefly, samples were homogenized in 50 mM phosphate buffer (pH 7.4) and centrifuged at 3.000x g for 10 minutes at 4°C. The supernatant obtained was centrifuged for 40 minutes at 10.000x g at 4°C and NADPH activity was assayed. The NADPH oxidase activity was expressed as U/mL/mg of protein (Bradford, 1976).

Superoxide dismutase (SOD) activity. The SOD activity was assayed using a nitro blue tetrazolium (NBT)-based assay (Oberley and Spitz, 1984). Briefly, samples were homogenized in a Tris-HCL buffer (100 mM, pH 7.4) containing 0.5% Triton™ X-100, 5 mM beta-mercaptoethanol, 0.1 g/ml phenylmethanesulfonyl fluoride, centrifuged at 14.000x g at 4°C for 5 minutes and assayed according to the manufacturer's instructions. Results were expressed as the percent inhibition of the rate of NBT-diformazan formation.

Immunofluorescence

The tibial nerve was dissected from C57BL/6J, *Trpa1*^{+/+}, *Trpa1*^{-/-}, *Plp1-Cre*^{ERT}, *Trpa1*^{fl/fl}, *Adv-Cre*^{ERT}, *Trpa1*^{fl/fl} and MaFIA mice anesthetized and transcardially perfused with phosphate buffered saline (PBS), followed by 4% paraformaldehyde. The tibial nerve was postfixed for 24 hours, and paraffin embedded. Immunofluorescence staining was performed according to standard procedures. Briefly, after antigen retrieval (Ethylenediaminetetraacetic acid, EDTA, solution pH 9.0, Dako) for 20 minutes at 98°C, sections (5 μm) were incubated with the following primary antibodies: F4/80 [1:50, MA516624, rat monoclonal (Cl:A3-1), Thermo Fisher Scientific, Rockford, USA] or 4-HNE [1:40, ab216020, mouse monoclonal (HNEJ-2), Abcam, Cambridge, UK) diluted in antibody diluent (Roche Diagnostics, Mannheim, Germany) 1 hour at room temperature. Sections were then incubated for 2 hours in the dark with fluorescent secondary antibodies: polyclonal Alexa Fluor® 488, polyclonal Alexa Fluor 594, polyclonal Alexa Fluor 546, and polyclonal Alexa Fluor 647 (1:600, Invitrogen, Milan, Italy). Sections were coverslipped using a water-based mounting medium with 4'6'-diamidino-2-phenylindole (DAPI, Abcam, Cambridge, UK). The analysis of negative controls (non-immune serum) was simultaneously performed to exclude the presence of non-specific immunofluorescent staining, cross-immunostaining, or fluorescence bleed-through. For histological evaluation, sections were stained with hematoxylin/eosin and, based on the morphology, the boundaries of the nerve trunk corresponding to the epineurium were identified and reported in adjacent immunofluorescence images with dashed lines. The number of F4/80⁺ cells was counted in 10⁴ μm^2 boxes in the tibial nerve (inside the nerve trunk). The 4-HNE staining was evaluated as the fluorescence

intensity measured by an image processing program (ImageJ 1.32J, National Institutes of Health, Bethesda, USA).

Statistical analysis

Results are shown as the mean and standard error of the mean (SEM). The statistical significance of differences between groups was assessed using Student's t-test, one-way or two-way analysis of variance (ANOVA) followed by Bonferroni post hoc where appropriate. Statistical analyses were performed on raw data using Prism 5 GraphPad software (GraphPad Software Inc.). P values less than 0.05 ($P < 0.05$) were considered significant.

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Declaration of interest

The authors declare no conflict of interest.

Author contributions

All the authors discussed the results, commented on the manuscript, and approved this final version.

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Figure legends

Figure 1. *TRPA1 mediates mechanical and cold allodynia in chronic post-ischemia pain (CPIP) mice.*

Mechanical and cold allodynia assessed at different time points before (baseline, BL) and after (day 1, 5, 10 and 15) ischemia and reperfusion (I/R) (CPIP) or sham procedure in C57BL/6J male and female mice (A) and *Trpa1*^{+/+} or *Trpa1*^{-/-} mice (B). (C, D) Mechanical and cold allodynia in C57BL/6J mice was assessed at BL and at day 15 after I/R or sham procedure, 1 hour before (time 0) and after (1, 2 and 3 hours) administration of A-967079 (100 mg/kg, intragastric), HC-030031 (300 mg/kg, intragastric) or their vehicles. Data are mean \pm SEM (n = 8 mice). **P*<0.05, ***P*<0.01, ****P*<0.001 when compared to sham, sham/*Trpa1*^{+/+} or sham/vehicle; ##*P*<0.01, ###*P*<0.001 when compared to CPIP/*Trpa1*^{+/+} or CPIP/vehicle (Two-way ANOVA followed by Bonferroni's post hoc test in A-D).

Figure 2. *Increase in oxidative stress in chronic post-ischemia pain (CPIP) mice is dependent on TRPA1.*

(A) Mechanical and cold allodynia in C57BL/6J mice was assessed at baseline (BL) and at day 15 after I/R or sham procedure, 30 minutes before (time 0) and after (1, 2 and 3 hours) administration of α -lipoic acid (100 mg/kg, intragastric) or vehicle. H₂O₂ level (B) and NADPH oxidase (NOX) (C) and superoxide dismutase (SOD) activities (D) in tibial nerve of C57BL/6J after ischemia and reperfusion (I/R) (CPIP) or sham procedure. Representative photomicrographs and mean fluorescence intensity of 4-HNE staining in the tibial nerve of C57BL/6J (E) and *Trpa1*^{+/+} or *Trpa1*^{-/-} (F) after I/R or sham procedure. Data are mean \pm SEM (n = 6-8 mice). **P*<0.05, ***P*<0.01, ****P*<0.001, when compared to sham/vehicle or sham; ####*P*<0.001, when compared to the CPIP/vehicle or CPIP/*Trpa1*^{+/+} (A, Two-way ANOVA followed by Bonferroni's post hoc test; B-E, Student's t-test; F, One-way ANOVA followed by Bonferroni's post hoc test). Where not indicated, the measurements are on day 15 after I/R or sham procedure.

Figure 3. *Endoneurial macrophage infiltration that sustains mechanical and cold allodynia in chronic post-ischemia pain (CPIP) mice is TRPA1-dependent.*

(A) Mechanical and cold allodynia in C57BL/6J mice was assessed at baseline (BL) and at day 15 after ischemia and reperfusion (I/R) (CPIP) or sham procedure, 30 minutes before (time 0) and after (1, 2 and 3 hours) administration of indomethacin (30 mg/kg, intragastric) or vehicle. Representative photomicrographs and number of F4/80⁺ cells in the tibial nerve in male and female C57BL/6J (B) and in *Trpa1*^{+/+} or *Trpa1*^{-/-} (C) mice on day 15 after I/R or sham procedure. (D) Mechanical and cold allodynia assessed at different time points (1 to 15 days) in MaFIA mice after I/R or sham procedure and treated for 5 consecutive days (day 10-14) with the dimerizing agent, B/B-HmD (2 mg/kg/day, intraperitoneal) or vehicle. Representative photomicrographs and number of F4/80⁺ cells (E) and mean fluorescence intensity of 4-HNE staining (F) in the tibial nerve of MaFIA mice on day 15 after I/R or sham procedure. (G) Mechanical and cold allodynia assessed at different time points (1 to 15 days) in MaFIA mice after

I/R or sham procedure, pretreated for 5 consecutive days before I/R with the dimerizing agent, B/B-HmD (2 mg/kg/day, intraperitoneal) or vehicle. Representative photomicrographs and number of F4/80⁺ cells (H) and mean fluorescence intensity of 4-HNE staining (I) in the tibial nerve of MaFIA mice on day 15 after I/R or sham procedure. Red arrows show the days of B/B-HmD administration. Data are mean \pm SEM (n = 6-8). *** P <0.001, when compared to sham/vehicle, sham or sham/ *Trpa1*^{+/+}; ### P <0.001 when compared to CPIP/*Trpa1*^{+/+} or CPIP/veh (A, D, G, Two-way ANOVA followed by Bonferroni's post hoc test; B, Student's t-test; C, E, F, H, I, one-way ANOVA followed by Bonferroni's post hoc test).

Figure 4. *Allodynia, macrophage accumulation, and 4-HNE production in Chronic post-ischemia pain (CPIP) mice is mediated by Schwann cell TRPA1.*

(A) Mechanical and cold allodynia assessed at different time points before (baseline, BL) and after (day 1, 5, 10 and 15) ischemia and reperfusion (I/R) (CPIP) or sham procedure in *Plp1-Cre*^{ERT};*Trpa1*^{fl/fl} or *control* mice. Representative photomicrographs and number of F4/80⁺ cells (B) and mean fluorescence intensity of 4-HNE staining (C) in the tibial nerve of *Plp1-Cre*^{ERT};*Trpa1*^{fl/fl} or *control* mice on day 15 after I/R or sham procedure. Data are mean \pm SEM (n=7). *** P <0.001, when compared to sham/control; ## P <0.01, ### P <0.001, when compared to CPIP/control (A, Two-way ANOVA followed by Bonferroni's post hoc test; B, C, One-way ANOVA followed by Bonferroni's post hoc test).

Figure 5. *Allodynia, but not macrophage accumulation, and 4-HNE production in Chronic post-ischemia pain (CPIP) mice are mediated by neuronal TRPA1.*

(A) Mechanical and cold allodynia assessed at different time points before (baseline, BL) and after (day 1, 5, 10 and 15) ischemia and reperfusion (I/R) (CPIP) or sham procedure in *Adv-Cre*;*Trpa1*^{fl/fl} or *control* mice. Representative photomicrographs and number of F4/80⁺ cells (B) and mean fluorescence intensity of 4-HNE staining (C) in the tibial nerve of *Adv-Cre*;*Trpa1*^{fl/fl} or *control* mice on day 15 after I/R or sham procedure. Data are mean \pm SEM (n = 6). *** P <0.001, when compared to sham/control, and ### P <0.001, when compared to CPIP/control (A, Two-way ANOVA followed by Bonferroni's post hoc test; B, C, One-way ANOVA followed by Bonferroni's post hoc test).

Figure 6. *A repeated pre- and post-treatment with a TRPA1 antagonist prevents the mechanical and cold allodynia in chronic post-ischemia pain (CPIP) in mice.*

(A, D) Drawing presenting the experimental protocol and mechanical and cold allodynia assessed at different time points before (baseline, BL) and after ischemia and reperfusion (I/R) (CPIP) or sham procedure in C57BL/6J mice after HC-030031 (300 mg/kg, intragastric, i.g.) administered 3 times a day, 1 day before and 3 days after I/R or sham procedure (A) and after HC-030031 (300 mg/kg, intragastric, i.g.) administered 3 times a day, daily from day 12 to day 15 after I/R or sham procedure (D). Representative photomicrographs and number of F4/80⁺ cells (B, E) and mean fluorescence intensity of 4-HNE staining (C, F) in the tibial nerve of C57BL/6J mice on day 15 after I/R or sham procedure and repeated HC-030031 administration. Dash (-)

represents the vehicle of HC-030031 (HC03). Data are mean \pm SEM (n = 6-8). *** P <0.001, when compared to sham/vehicle or sham, and # P <0.005, ## P <0.01, ### P <0.001, when compared to CPIP/vehicle or -/CP/IP (A, D, Two-way ANOVA followed by Bonferroni's post hoc test; B, C, E, F, One-way ANOVA followed by Bonferroni's post hoc test).

Supplementary Figure 1. *Time course of mechanical and cold allodynia and macrophage recruitment in chronic post-ischemia pain (CPIP) mice.* (A) Mechanical and cold allodynia assessed at different time points before (baseline, BL) and after (6, 12 and 24 hours) ischemia and reperfusion (I/R) (CPIP) or sham procedure in C57BL/6J mice. (B) Representative photomicrographs and number of F4/80⁺ cells in the tibial nerve of mice 6, 12 and 24 hours after I/R or sham procedure. Data are mean \pm SEM (n = 6). ** P <0.01, *** P <0.001, when compared to sham or BL (A, Two-way ANOVA followed by Bonferroni's post hoc test; B, One-way ANOVA followed by Bonferroni's post hoc test).

Highlights

- TRPA1 mediates allodynia in a mouse model of complex regional pain syndrome type I.
- Allodynia is associated with macrophage accumulation in the injured tibial nerve.
- Allodynia is dependent of TRPA1 agonists production by macrophage accumulation.
- Schwann cell TRPA1 is essential to sustain neuroinflammation and allodynia.

