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RESEARCH ARTICLE

Schwann cell TRPA1 elicits reserpine-induced fibromyalgia pain in mice

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Background and Purpose: Fibromyalgia is a complex clinical disorder with an unknown aetiology, characterized by generalized pain and co-morbid symptoms such as anxiety and depression. An imbalance of oxidants and antioxidants is proposed to play a pivotal role in the pathogenesis of fibromyalgia symptoms. However, the precise mechanisms by which oxidative stress contributes to fibromyalgia-induced pain remain unclear. The transient receptor potential ankyrin 1 (TRPA1) channel, known as both a pain sensor and an oxidative stress sensor, has been implicated in various painful conditions.

Experimental Approach: The feed-forward mechanism that implicates reactive oxygen species (ROS) driven by TRPA1 was investigated in a reserpine-induced fibromyalgia model in C57BL/6J mice employing pharmacological interventions and genetic approaches.

Key Results: Reserpine-treated mice developed pain-like behaviours (mechanical/cold hypersensitivity) and early anxiety-depressive-like disorders, accompanied by increased levels of oxidative stress markers in the sciatic nerve tissues. These effects were not observed upon pharmacological blockade or global genetic deletion of the TRPA1 channel and macrophage depletion. Furthermore, we demonstrated that selective silencing of TRPA1 in Schwann cells reduced reserpine-induced neuroinflammation (NADPH oxidase 1-dependent ROS generation and macrophage increase in the sciatic nerve) and attenuated fibromyalgia-like behaviours.

Conclusion and Implications: Activated Schwann cells expressing TRPA1 promote an intracellular pathway culminating in the release of ROS and recruitment of macrophages in the mouse sciatic nerve. These cellular and molecular events sustain mechanical and cold hypersensitivity in the reserpine-evoked fibromyalgia model. Targeting TRPA1 channels on Schwann cells could offer a novel therapeutic approach for managing fibromyalgia-related behaviours.

Abbreviations: 4-HNE, 4-hydroxynonenal; Adv, Advillin; AITC, allyl-isothiocyanate; MaFIA, macrophages Fas-induced apoptosis; NOX, NADPH oxidases; PBN, phenyl-α-tert-butyl nitrone; Plp1, proteolipid protein 1; TRPA1, Transient Receptor Potential Ankyrin 1; TRPV1, transient receptor potential vanilloid 1; Veh, vehicle.

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KEYWORDS
depression, macrophages, NADPH oxidase, nociception, nociplastic pain, oxidative stress

1 | INTRODUCTION

Fibromyalgia (FM) is a chronic pain syndrome which affects around 3% of the worldwide population and is characterized by widespread musculoskeletal pain, reduced tolerance to heat and cold (McBeth & Mulvey, 2012; Wolfe & Walitt, 2013) and co-morbidity with anxiety and depression (Clauw, 2015; Häuser et al., 2015; Sarzi-Puttini et al., 2020). Recently, the definition of 'nociplastic pain' was introduced to explain central sensitization in the pathogenesis of fibromyalgia (Kosek et al., 2016; Sarzi-Puttini et al., 2020). Other evidence suggests fibromyalgia as a neuropathic pain syndrome in which the central sensitization is secondary to peripheral mechanisms, including dorsal root ganglia (DRG) activation by infiltrating neutrophils (Caxaria et al., 2023; Martínez-Lavín, 2022). Thus, fibromyalgia aetiology and pathogenesis remain poorly understood. Antidepressants and antiepileptics (**duloxetine**, **milnacipran** and **pregabalin**) are drugs approved by the Food and Drug Administration (FDA) for the treatment of fibromyalgia, but they provide limited pain relief (Häuser et al., 2015; Sarzi-Puttini et al., 2020). Therefore, identifying more specific pharmacological targets implicated in the fibromyalgia mechanism is essential to develop better treatments.

Several mouse models of painful diseases, including chemotherapy-induced peripheral neuropathy, migraine, multiple sclerosis, complex regional pain syndrome type I, alcoholic neuropathy and cancer, have been associated with the overproduction of reactive oxygen species (ROS) (Dalenogare et al., 2020; De Logu et al., 2020, 2021, 2022; De Logu, Landini, et al., 2019; De Logu, Li Puma, et al., 2019; Shim et al., 2019). Protein carbonyls (Cordero et al., 2009; Eisinger et al., 1996), malondialdehyde (Bagis et al., 2005), thiobarbituric acid reactive substances (TBARS) (Akkuş et al., 2009; Cordero et al., 2010, 2011, 2012; Hung et al., 2020; Ozgocmen et al., 2006) and peroxide level (Altindag & Celik, 2006) are increased in skin biopsies, plasma and blood mononuclear cells of fibromyalgia patients (Akkuş et al., 2009; Cordero et al., 2010, 2011, 2012; Hung et al., 2020). In contrast, levels of antioxidants, including vitamins A and E (Akkuş et al., 2009), coenzyme Q10 (Cordero et al., 2009), nitrite (Ozgocmen et al., 2006) and superoxide dismutase, are lower than in controls (Bagis et al., 2005). However, little is known about the consequences and the impact of uncontrolled and imbalanced oxidative stress in fibromyalgia.

The pro-algesic **transient receptor potential ankyrin 1 (TRPA1)** channel is abundantly expressed in a subset of nociceptive sensory neurons and, although with different expression levels, in several non-neuronal cells (De Logu et al., 2017; Patil et al., 2023; Souza Monteiro de Araujo et al., 2020; Story et al., 2003). TRPA1 has been identified as an oxidative stress sensor, as it is activated by a considerable number of reactive oxidants produced at the sites of tissue damage, including hydrogen peroxide (H_2O_2) and the byproduct of lipid peroxidation, 4-**hydroxynonenal (4-HNE)** (Andersson et al., 2008; Taylor-Clark et al., 2009; Trevisani et al., 2007). Here, we evaluated the involvement of oxidative stress and TRPA1 in a mouse model induced by systemic administration of **reserpine** that mimics some clinical features of fibromyalgia, including decreased levels of biogenic amines, induction of pain and a depression-like syndrome (Brum et al., 2022; Nagakura et al., 2009). Secretion of various cytokines and chemokines and recruitment of macrophages constitute potential critical players in fibromyalgia and have been implicated in the pro-algesic ROS/TRPA1-dependent pathway (Kim et al., 2008; Littlejohn & Guymer, 2018; Theoharides et al., 2019).

Recently, the crucial contribution of TRPA1 expressed in Schwann cells (SCs) in sustaining mechanical hypersensitivity has been highlighted in different mouse models of pain induced by peripheral nerve injury, ischemia/reperfusion, alcoholic neuropathy, cancer and migraine (De Logu et al., 2017, 2020, 2021, 2022; De Logu, Li Puma, et al., 2019). ROS generated by different cellular sources, including hematogenic or resident endoneurial macrophages, target Schwann cells TRPA1 to amplify a ROS-dependent feed-forward mechanism that sustains neuroinflammation and chronic pain. Thus, we have explored the involvement of this neuroinflammatory pathway in the reserpine-induced fibromyalgia model.

What is already known

- Fibromyalgia is a poorly understood and treated chronic pain syndrome accompanied by anxiety and depression.
- Oxidative stress in fibromyalgia contributes to the severity of patients' painful symptoms.

What does this study add

- TRPA1 mediates pain-like and early anxiety-depressive-like behaviours in a mouse fibromyalgia model.
- Sciatic nerve oxidative stress and monocytes/macrophage accumulation and fibromyalgia-behaviours are triggered by Schwann cell TRPA1.

What is the clinical significance

- Schwann cell TRPA1 blockade represents a new therapeutic target for the treatment of fibromyalgia-related symptoms.

We report that reserpine-treated mice developed pain-like behaviours (mechanical and cold hypersensitivity) and early anxiety-depressive-like disorders associated with increased levels of oxidative stress markers and macrophages in the sciatic nerve tissues. We also revealed that neuroinflammation and pain-like behaviours induced by reserpine were reduced by pharmacological blockade and global genetic deletion of the TRPA1 channel or by selective silencing of Schwann cell TRPA1. Thus, targeting Schwann cell TRPA1 might represent a novel therapeutic strategy for treating fibromyalgia pain.

2 | METHODS

2.1 | Animals

The experimental protocols using animals were approved by the Institutional Animal Care and Use Committee of the University of Florence and of the Federal University of Santa Maria (UNIFI protocols #1194/2015PR and #50/2024-PR; CEUA-UFSM protocols #3525100119/2019 and #5070100119/2019). C57BL/6J mice (n = 156 male and n = 12 female, 20–25 g, 5 weeks) (Harlan Laboratories), wild-type (n = 12 *Trpa1*$^{+/+}$) and TRPA1-deficient (n = 12 *Trpa1*$^{-/-}$; B6129P-Trpa1$^{tm1Kykw/J}$; Jackson Laboratories) mice (25–30 g, 6–8 weeks) generated by heterozygous mice on a C57BL/6J background were used throughout. The animals were kept in IVC cages (4 mice/cage) with environmental enrichment under constant environmental conditions with a 12:12 h light-dark cycle, ambient temperature (22 ± 1°C) and relative humidity of 55 ± 10%. The animals were fed a standard pellet diet and allowed free access to water. Mice were euthanised with an overdose of sodium thiopental (150 mg/kg, i.p.) followed by cervical dislocation (to ensure the animals' euthanasia process); or they were euthanised with inhaled CO_2 plus 10-50% O_2, in this case, the death confirmation was achieved by a physical method of killing (decapitation) (Leary et al., 2020).

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) was crossed with hemizygous B6.Cg-Tg (Plp1-CreERT)3Pop/J mice (*Plp1-Cre*^{ERT}, Stock No: 005975, Jackson Laboratories), expressing a tamoxifen-inducible Cre in myelinating cells (Plp1, proteolipid protein myelin 1). The progeny (*Plp1-Cre*^{ERT};*Trpa1*$^{fl/fl}$) was genotyped by standard PCR for Trpa1 and Plp1-Cre^{ERT}. Mice negative for Plp1-Cre^{ERT} (*Plp1-Cre*$^{ERT-}$;*Trpa*$^{fl/fl}$) were used as control. Both positive and negative mice to Cre^{ERT} and homozygous for floxed Trpa1 (n = 12 *Plp1-Cre*$^{ERT+}$;*Trpa1*^{fl}/^{fl} and n = 12 *Plp1-Cre*$^{ERT-}$;*Trpa1*^{fl}/^{fl}, respectively) were treated with intraperitoneal (i.p.) **tamoxifen** (1 mg 100 μl^{-1} in corn oil, once a day, for five consecutive days), resulting in Cre-mediated ablation of *Trpa1* in PLP-expressing Schwann cells/oligodendrocytes (De Logu et al., 2017, 2020). Successful Cre-driven deletion of TRPA1 mRNA was confirmed by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). To selectively delete the *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. The progeny (n = 12 *Adv-Cre;Trpa1*$^{fl/fl}$) were genotyped by standard PCR for Trpa1 and Advillin-Cre (Guan et al., 2016). Mice negative for Advillin-Cre (n = 12 *Adv-Cre*$^{-}$;*Trpa1*$^{fl/fl}$) were used as control. Successful Advillin-Cre driven deletion of TRPA1 mRNA was confirmed by reverse transcription-quantitative polymerase chain reaction (De Logu et al., 2017, 2020).

To evaluate the involvement of macrophages, macrophage Fas-induced apoptosis (MaFIA; stock No: 005070, Jackson Laboratories, Bar Harbor, ME, USA) mice were used, which exhibit a marked reduction in the number of F4/80^{+} cells after injection of the apoptosis inducer, AP20187 (Burnett et al., 2004). MaFIA mice (n = 24) were treated with the B/B homodimerizer (B/B-HmD) agent AP20187 (2 mg·kg^{-1}, i.p.) or vehicle (10% polyethylene glycol-400, 1.7% Tween 80 in saline solution [0.9%]) once a day for five consecutive days before the induction of experimental fibromyalgia protocol to selectively deplete macrophages (De Logu et al., 2020; Shepherd et al., 2018).

Animal studies are reported in compliance with the ARRIVE guidelines (Percie du Sert et al., 2020) and with the recommendations made by the British Journal of Pharmacology (Lilley et al., 2020). The behavioural studies followed the national and international legislation (guidelines of Brazilian Council of Animal Experimentation and the U.S. Public Health Service's Policy on Humane Care and Use of Laboratory Animals). The group size of n = 6 animals for behavioural 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) with an effect size of 0.25, terror probability of 0.05 and proof power of 0.8. Mice were allocated to the vehicle or treatment groups using a randomization procedure (http://www.randomizer.org/). Investigators were blinded to treatments, which were revealed only after data collection. Human end-point measures did not need to be carried out before, during or after the experiments, and no animals were excluded from the experiments. 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).

2.2 | Experimental model of fibromyalgia

The fibromyalgia was experimentally induced using reserpine. This *in vivo* experimental method in mice replicates behavioural and pathological alterations of fibromyalgia (Brum et al., 2020, 2022; Brusco et al., 2019; Fischer et al., 2020; Nagakura et al., 2009). The dosing regimen of reserpine consisted of 1 mg·kg^{-1} injected subcutaneously (s.c.) in the dorsum (back) of mice once daily for three consecutive days (Brusco et al., 2019; Nagakura et al., 2009). The dose of 1 mg·kg^{-1} instead of 0.25 mg·kg^{-1} was used to obtain more

reproducible results in terms of mechanical and cold hypersensitivity in C57BL/6J mice. The control group received the vehicle (10 ml·kg^{-1}, s.c.; 0.5% acetic acid with 99.5% saline solution [0.9%]) with the same schedule of administration. The animals were subjected to behaviour assessments 1 day after fibromyalgia induction (i.e. on the fourth day after the first reserpine or vehicle injections). C57BL/6J male and female, *Trpa1*$^{+/+}$ and *Trpa1*$^{-/-}$, MaFIA, *Plp1-Cre*$^{ERT+}$;*Trpa1*$^{fl/fl}$ and *Adv-Cre*$^{+}$;*Trpa1*$^{fl/fl}$ and control mice were submitted to the induction of the experimental fibromyalgia model and were tested 1 day after fibromyalgia induction.

2.3 | Treatment protocols

Mice received oral treatment with **A-967079** (10, 30 and 100 mg·kg^{-1}, orally [p.o.]) (Dalenogare et al., 2020; Trevisan et al., 2016) or vehicle (10 ml·kg^{-1}, p.o.) 1 day after fibromyalgia induction. The A-967079 dose of 100 mg·kg^{-1} (p.o.) was chosen for subsequent experiments and the animals were subjected to behavioural tests at 1 h after the treatments. The effect of the clinically approved drugs on fibromyalgia treatment, pregabalin (30 mg·kg^{-1}, p.o.; positive control for pain-like behaviours) and duloxetine (30 mg·kg^{-1}, p.o.; positive control for depressive-like behaviours) (Brusco et al., 2019; Fischer et al., 2020; Jones et al., 2005; Xu et al., 2018) were also tested. **α-Lipoic acid** (100 mg·kg^{-1}, p.o.) (Dalenogare et al., 2020), phenyl-α-tert-butyl nitrone (PBN; 100 mg·kg^{-1}, i.p.) (De Logu et al., 2020) or vehicle (10 ml·kg^{-1}, p.o. or i.p.) were administered 1 day after fibromyalgia induction. Additionally, other sets of animals received the **NADPH oxidase (NOX)** inhibitors, the unselective NOX inhibitor (apocynin, 100 mg·kg^{-1}, i.p.) (Marone et al., 2018) or selective NOX1 inhibitor (ML171; 60 mg·kg^{-1}, i.p.) (De Logu et al., 2017), following the same cited-above schedule of treatment.

2.4 | Behavioural assessment

2.4.1 | Mechanical threshold

Mechanical threshold was evaluated before (baseline [BL] measurement) and 1 day after fibromyalgia induction. The animals that received the treatment with different compounds were tested again several times (h) after treatments. The measurement of the mechanical threshold was carried out using the up-and-down method (Chaplan et al., 1994; Oliveira et al., 2013). The mechanical paw withdrawal threshold response, expressed in grams (g), was calculated from the resulting scores using von Frey filaments of increasing stiffness (0.02–10 g), as previously described (Dixon, 1980). The 50% mechanical withdrawal threshold (expressed in g) was calculated from these scores by using a δ value of 0.469. The development of mechanical allodynia was defined as a reduction in the 50% threshold compared with the baseline values.

2.4.2 | Acetone cooling test

Cold allodynia was evaluated before (baseline [BL] measurement) and 1 day after fibromyalgia induction. The animals that received the treatment with different compounds were tested again several times (h) after treatments. Cold allodynia was assessed with the acetone drop method as previously described (Brum et al., 2020; Brusco et al., 2019). Mice were placed on a wire mesh floor and a drop of acetone (20 μl) was applied three times to the plantar surface of the right hind paw. The behavioural response was analysed for 30 s and recorded in scores. Score 0 = no response; 1 = quick withdrawal, flick or stamp the paw; 2 = prolonged withdrawal or repeated paw flicking; 3 = repeated paw flicking with licking directed at the ventral side of the paw. The sum of the three scores was used for data analysis.

2.4.3 | Forced swimming test

After the mechanical threshold and acetone cooling test were evaluated, the forced swimming test, an established method to assess depression-like behaviour (Deuis et al., 2017), was performed, placing mice in a cylinder (20 cm diameter and 45 cm height) filled with water (23–25°C) at a height of 30 cm. The test was performed for 6 min and the immobility time of the animals in the water was evaluated during the last 4 min, as previously reported (Bai et al., 2018; Pereira et al., 2020). The immobility time was defined as the absence of all movements except those required to maintain the head above water.

2.4.4 | Locomotor activity tests: open field and rotarod

Spontaneous locomotor activity in mice was evaluated using an open-field box (30 cm^3). Each mouse was transferred to the apparatus and recorded for 10 min. The video files were processed by a MATLAB script (version 2020a), which provided the distance travelled (m) (Zhang et al., 2020). The Rotarod test was used to evaluate the mice's forced locomotor activity. All animals were trained in the rotarod (3.7 cm in diameter, 8 rpm) until they could remain in the apparatus for 60 s without falling. One day after fibromyalgia induction, the number of falls from the apparatus was recorded for up to 240 s (Brum et al., 2016, 2020).

2.5 | Tissue H_2O_2 measurement

The sciatic nerve tissues were collected and homogenized in tris–HCl buffer (50 mM, pH 7.4) containing 5-mM sodium azide and kept at 4°C. The tissue homogenate was centrifuged at 1000 × *g* for 10 min, the supernatant was collected and the assays were immediately

performed. The protein content from each sample was determined using the BCA Protein Assay Kit. H_2O_2 levels were determined using the phenol red-peroxidase from the horseradish method (Brum et al., 2020). First, the supernatant was centrifuged again at 12,000 × *g* for 20 min at 4°C. A mixture containing supernatant (50 µl), phenol red (40 µl; 200 µg·ml^{-1}) and peroxidase from horseradish (HRP) type II (EC: 1.11.1.7) (10 µl; 100 µg·ml^{-1}) was incubated in the dark at 25°C. After 10 min, this reaction was stopped by adding NaOH (20 µl; 1 M) and recorded spectrophotometrically at 610 nm. The absorbance was evaluated using a FlexStation 3 Multi-Mode Microplate Reader®. H_2O_2 levels were calculated as nmol H_2O_2 mg protein^{-1}, based on a standard curve of phenol red oxidation by H_2O_2 (0.001–100 µM; $R^2 = 0.9958$) mediated by HRP.

2.6 | Immunofluorescence

Sciatic nerves were collected from mice and post-fixed for 24 h and cryoprotected overnight at 4°C in 30% sucrose until cryosectioning. Cryosections (10 µm) were washed and incubated with a fresh blocking solution (PBS, pH 7.4, 5% NGS or 5% normal donkey serum) for 1 h at room temperature. Next, sections were incubated with the primary antibody 4-HNE (1:25 in 5% NGS, IgG1 mouse monoclonal [HNEJ-2], Abcam Cat# ab48506, RRID:AB_867452; Landini et al., 2023) or primary antibody F4/80 (1:50 in 5% normal donkey serum, MA516624, IgG2b rat monoclonal [CI:A3-1], Thermo Fisher Scientific Cat# MA5-16624, RRID:AB_2538120, which recognizes the murine F4/80 antigen, approximately 160 kDa cell surface glycoprotein member of the EGF-TM7 family of proteins which shares 68% overall amino acid identity with human EGF module containing mucin-like hormone receptor 1 [EMR1]; De Logu et al., 2017, 2020; de Souza Monteiro Araújo et al., 2020), diluted in blocking solution for 1 h at room temperature. Sections were then incubated for 2 h at room temperature in the dark with the fluorescent secondary antibody polyclonal IgG Alexa Fluor® 594 (Thermo Fisher Scientific Cat# A-11005, RRID:AB_2534073) or IgG Alexa Fluor® 488 (Thermo Fisher Scientific Cat# A-21208, RRID:AB_2535794) (1:600 in 5% NGS or 5% normal donkey serum). Sections were coverslipped using a water-based mounting medium with **4′6′-diamidino-2-phenylindole (DAPI)** (Abcam, Cambridge, UK). Tissues were visualized and digital images were captured using an Olympus BX51 or confocal scan LEICA TCS SP5. The 4-HNE staining was evaluated as the fluorescence intensity measured by a Zeiss Axio Imager 2, Zeiss ZEN imaging 2020, while the number of F4/80$^+$ cells was counted in 10^4 µm^2 boxes in the sciatic nerve (inside the nerve trunk). Investigators were blinded for image analysis. 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 edges of the tissue have been excluded from the fluorescence analysis to avoid the 'edge effect', a non-specific binding of fluorescent markers. The Immuno-related procedures used comply with the recommendations made by the *British Journal of Pharmacology* (Alexander et al., 2018).

2.7 | Cell culture

Primary cultures of human Schwann cells (HSCs, #1700, ScienCell Research Laboratories) were grown and maintained in Schwann cell medium (#1701, ScienCell Research Laboratories) at 37°C in 5% CO_2 and 95% O_2. Cells were passaged at 90% confluency and discarded after 12 passages (De Logu et al., 2022). The human monocytic U937 cell line (#CRL-1593.2™; ATCC; RRID:CVCL_0007) was maintained at 37°C, 5% CO_2 and 95% O_2 in RPMI-1640 medium supplemented with heat-inactivated foetal bovine serum (FBS; 10%), L-glutamine (2 mM), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; 10 mM) and sodium pyruvate (1 mM). The differentiation of U937 cells (1×10^5 cells ml^{-1}) to macrophage-like lineage was promoted by exposure to **phorbol 12-myristate 13-acetate (PMA**, 200 ng·ml^{-1}) for 24 h before experiments (Yang et al., 2017). To mimic the inflammatory response of activated macrophages, the macrophage-like U937 cells were further stimulated with *E. coli* lipopolysaccharide (LPS, 10 µg·ml^{-1}) for 2 h before the migration assay experiments (Baek et al., 2009).

2.7.1 | Cells H_2O_2 measurements

H_2O_2 levels were assessed in human Schwann cells using the Amplex Red® assay. Cells were plated in 96-well clear-bottom black plates (5×10^5 cells per well) and maintained in 5% CO_2 and 95% O_2 (24 h, 37°C). The cultured medium was replaced with Krebs/HEPES supplemented with ML171 (1 µM) or vehicle (0.3% DMSO) for 10 min at room temperature. Human Schwann cells were stimulated with reserpine (30 µM) or vehicle (0.01% DMSO) supplemented with Amplex Red (50 µM) and HRP (1 U·mL^{-1}, 30 min, room temperature, protected from light). A signal was detected 60 min after exposure to stimuli. H_2O_2 content was calculated using the H_2O_2 standard and expressed as µmol H_2O_2 lt^{-1} (De Logu, Li Puma, et al., 2019).

2.7.2 | H_2O_2 *in vitro* imaging

A genetically encoded probe for H_2O_2-HyPer (HyPer7.2, Pak et al., 2020, kindly donated by Dr. Emrah Eroglu, Harvard Medical School, Boston, USA) was used. Briefly, human Schwann cells were plated on poly-L-lysine-coated (8.3 µM) 96-well black wall clear bottom plates (5×10^5 cells/well; Corning Life Sciences, Tewksbury, MA, USA) and transfected with DNA (0.2 µg) of HyPer7.2 using jet-OPTIMUS® DNA transfection reagent (Polyplus, Lexington, MA, USA). After 24 h, the human Schwann cells were washed with the buffer solution (37°C) containing (in mM) 2 $CaCl_2$, 5.4 KCl, 0.4 $MgSO_4$, 135 NaCl, 10 D-glucose and 10 HEPES at pH 7.4 and transferred on the stage of a fluorescent microscope for recording (Axio Observer 7; with a fast filterwheel and Digi-4 lens to record excitations; ZEISS, Stuttgart, Germany) (Pak et al., 2020). Cells were exposed to **allyl isothiocyanate (AITC**; 10 µM), in the presence of A-967079 (30 µM) or its vehicle (0.001% DMSO), and H_2O_2 were

recorded for approximately 6 min. In another set of experiments, the response to AITC was evaluated using a $CaCl_2$ free buffer solution. Responses were measured as a ratio of the two excitation wavelengths ($\lambda_{ex1}408/\lambda_{ex2}455$). The ΔF/F0 ratio was calculated for each experiment and the results were expressed as the area under the curve (AUC).

2.7.3 | Transwell migration assay

A noncontact coculture transwell cell culture system between U937 and human Schwann cells was obtained using the Boyden migration assay as previously reported (Yang et al., 2017). Briefly, cells were grown as follows: U937 cells were seeded at a density of 2.5×10^4/chamber into the upper side of a 24-well transwell cell culture system (6.5 mm in diameter, with 8-μm pores, #CLS3458™; Corning) by using the complete medium containing PMA for 24 h before migration assay as previously described (Guy et al., 2017). Human Schwann cells were plated on a 24-well plate. On the day of the experiment, all the cells were replaced with serum-free media and the PMA-U937 cells cultured on the membrane of a 24-well transwell insert were placed into the 24-well plate cultures with human Schwann cells. Three different conditions were tested: PMA-U937 cells without LPS plus human Schwann cells and PMA-U937 cells with LPS plus human Schwann cells in the presence or the absence of A-967079 (30 μM). After 16 h of incubation at 37°C, 5% CO_2 and 95% O_2, migrated cells on the lower surface of the filter were fixed and stained with Diffquick staining, following the manufacturer's instructions, and nonmigratory cells on the upper surface of the filter were wiped with a cotton swab. Random fields were counted under a light microscope using a 20× objective (1.5×10^5 μm^2). In addition, the H_2O_2 level was assessed in the human Schwann cells culture medium using the Amplex Red® assay following manufacturer instructions.

2.8 | Data and statistical analysis

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., 2022). Statistical analyses were carried out using Graph Pad Prism 8.0 software (Graph Pad, USA). Data were expressed as mean ± standard error mean (SEM). Before statistical significance analysis was performed, data were tested for normality using the Kolmogorov–Smirnov test and homogeneity using the Bartlett test. To meet parametric assumptions, mechanical threshold data were log-transformed before analysis. A one-way analysis of variance (ANOVA) followed by the post hoc Bonferroni's test was used for multiple comparisons. Two groups were compared using Student's *t* test. The two-way mixed model ANOVA followed by the post hoc Bonferroni's test was used for behavioural experiments with repeated measures. Post hoc tests were conducted only if F in ANOVA achieved $P < 0.05$. Sample sizes subjected to statistical analysis were at least 5 mice/group ($n = 5$), where $n =$ the number of independent values. *P* values less than 0.05 ($P < 0.05$) were considered significant. Statistical tests and the sample size used for each analysis are listed in the figure legends.

2.9 | Materials

All reagents were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA) other than where indicated. Primary antibody 4-HNE, normal goat serum (NGS), normal donkey serum (NDS) and 4′6′-diamidino-2-phenylindole (DAPI) were purchased from Abcam (Cambridge, UK), while the secondary antibody polyclonal Alexa Fluor® 594 and polyclonal Alexa Fluor® 488 were acquired from Invitrogen (Milan, Italy). Primary monoclonal antibody F4/80 was acquired from Thermo Fisher Scientific (Rockford, USA), Fura-2AM-ester was acquired from Alexis Biochemicals (Lausen, Switzerland) and Amplex Red® was obtained from Invitrogen (Waltham, Massachusetts, USA). Phenol red and sodium azide were purchased from Vetec (Rio de Janeiro, RJ, Brazil), and hydrogen peroxide (H_2O_2) was acquired from Impex (Brasilia, Distrito Federal, Brazil). Reserpine was dissolved to a final concentration of 0.5% acetic acid with a 99.5% saline solution (0.9%). A-967079 was dissolved in 10% DMSO, 5% tween 80 and 85% saline solution (0.9%). α-Lipoic acid (αLA) was dissolved in 20% polyethylene glycol (PEG) and 80% saline solution (0.9%). Phenyl-α-tert-butyl nitrone (PBN), apocynin and ML171 were dissolved in 4% DMSO, 4% tween 80 and 92% saline solution (0.9%). Pregabalin was dissolved in 0.9% saline solution and duloxetine in 10% DMSO and 90% saline solution (0.9%). The control groups (vehicles) received the vehicles in which the treatments were solubilized. All the oral and subcutaneous treatments were administered in mice at a volume of 10 ml·kg^{-1}. Details of other materials and suppliers are provided in the specific sections.

2.10 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in the IUPHAR/BPS Guide to PHARMACOLOGY http://www.guidetopharmacology.org and are permanently archived in the Concise Guide to PHARMACOLOGY 2023/24 (Alexander et al., 2023a,b).

3 | RESULTS

3.1 | Oxidative stress mediates reserpine-induced experimental fibromyalgia symptoms in mice

Reserpine subcutaneous (s.c.) injection in the dorsum of mice (once daily for three consecutive days) (Figure 1a) elicited hind paw

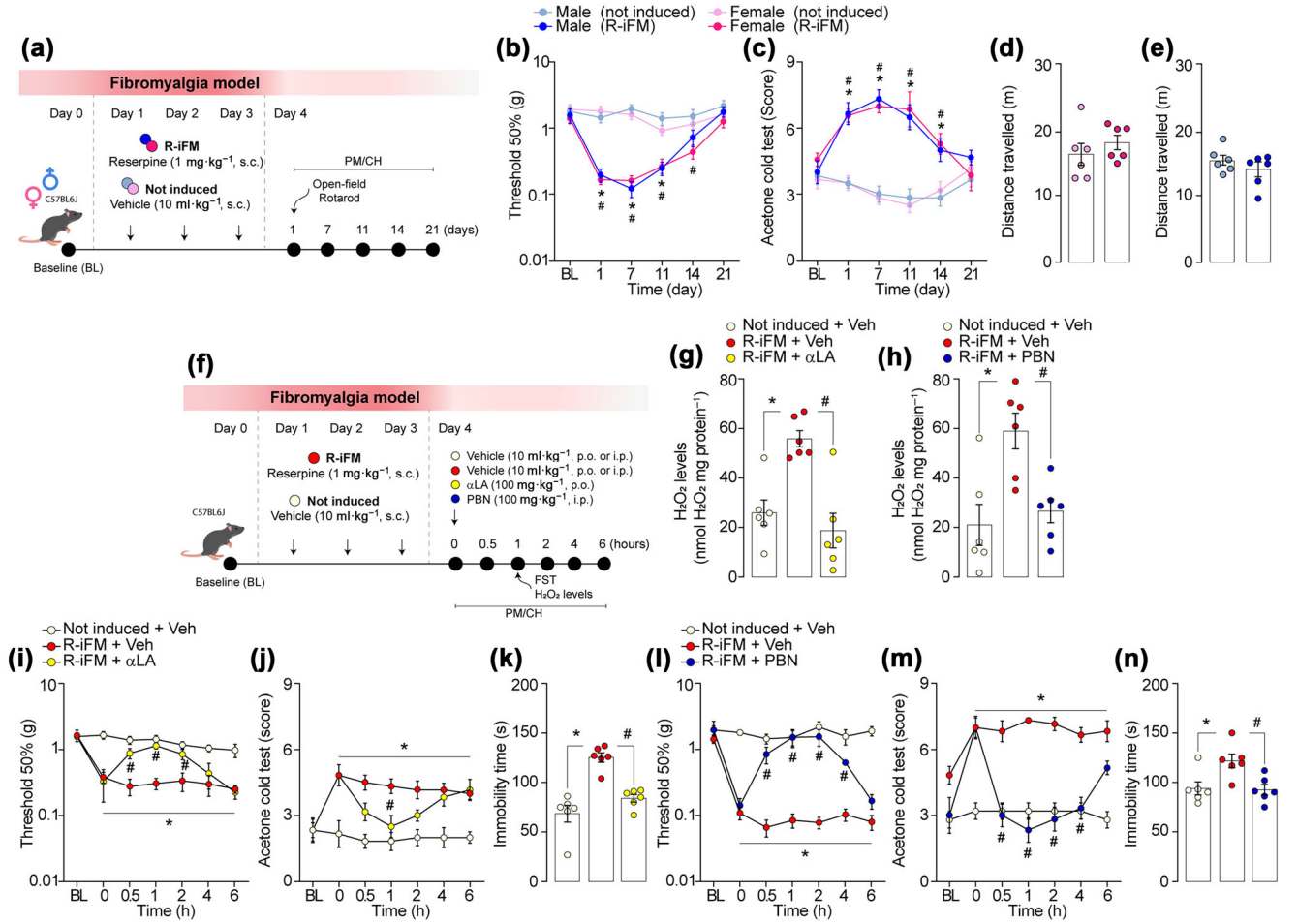

FIGURE 1 Reserpine induces prolonged mechanical allodynia, cold hypersensitivity and depressive-like behaviour in an oxidative stress-dependent way. (a) Scheme of the fibromyalgia (FM) experimental procedure and timeline. (b) Mechanical allodynia, (c) cold hypersensitivity and (d and e) locomotor activity in the open field test evoked by reserpine (1 mg·kg^{-1}, subcutaneously, s.c., reserpine-induced fibromyalgia [R-iFM]) or vehicle (10 ml·kg^{-1}, s.c., Not induced) in C57BL/6J male and female mice. (f) Scheme of the antioxidant treatment in the FM experimental procedure and timeline. (g and h) H_2O_2 levels in the sciatic nerve of R-iFM and not induced mice treated with α-lipoic acid (αLA, 100 mg·kg^{-1}, orally, p.o.), phenyl-α-tert-butyl nitrone (PBN, 100 mg·kg^{-1}, intraperitoneal, i.p.) or vehicle (Veh; 10 ml·kg^{-1}, p.o. or i.p.). (i and l) Mechanical allodynia, (j and m) cold hypersensitivity and (k and n) depressive-like behaviour in R-iFM and not induced mice treated with αLA (100 mg·kg^{-1}, p.o.), PBN (100 mg·kg^{-1}, i.p.) or vehicle (10 ml·kg^{-1}, p.o. or i.p.) (n = 6 mice per group). Mean ± SEM. *$P < 0.05$ versus not induced + Veh. #$P < 0.05$ versus R-iFM + Veh or versus not induced + Veh. Data were analysed by one- or two-way ANOVA followed by the Bonferroni's post hoc test. R-iFM: reserpine-induced fibromyalgia; Veh: vehicle. n = 12 male and n = 12 female C57BL/6J mice were used in (a)–(e); n = 36 C57BL/6J male mice were used in (f)–(n).

mechanical allodynia/cold hypersensitivity (PM/CH) from day 1 to day 14 after the last reserpine administration in C57BL/6J mice (Figure 1b,c), without causing any locomotor alteration in the open-field test (Figure 1d,e) and in the rotarod test (male mice, vehicle-treated 0.3 ± 0.2 falls and reserpine-treated 0.2 ± 0.2 falls, n = 6 per group; female mice, vehicle-treated 0.5 ± 0.2 falls and reserpine-treated 0.0 ± 0.0 falls, n = 6 per group). Despite the higher prevalence of fibromyalgia in women (Sarzi-Puttini et al., 2020), as no gender difference was observed in paw mechanical/cold hypersensitivity, only male mice were used in subsequent experiments to avoid the influence of hormone fluctuation in pain perception and to minimize the number of used animals.

One day after fibromyalgia induction, reserpine-increased H_2O_2 levels were detected in mouse sciatic nerve homogenates, a response that was prevented by the antioxidant α-lipoic acid (αLA; intragastric, p.o., 100 mg·kg^{-1}; Figure 1f) and the ROS spin trap phenyl-α-tert-butyl nitrone (PBN; intraperitoneal, i.p., 100 mg·kg^{-1}; Figure 1f) (Figure 1g,h). Treatment with αLA or PBN reduced paw mechanical/cold hypersensitivity in reserpine-treated mice (Figure 1i,j,l,m). We also observed that reserpine-treated mice developed an increased immobility time in the forced swimming test, a depression-like behaviour, which was reduced by treatment with αLA or PBN (Figure 1k,n). Together, our data indicate that oxidative stress plays a pivotal role in paw mechanical/cold hypersensitivity and associated comorbidity caused by reserpine.

3.2 | TRPA1 mediates fibromyalgia-like behaviours and neuroinflammation induced by reserpine

TRPA1 activation promotes calcium-dependent intracellular ROS generation (De Logu et al., 2017, 2021; Mori et al., 2016). Treatment with the selective TRPA1 antagonist A-967079 (Figure 2a) reduced paw mechanical/cold hypersensitivity induced by reserpine in a dose- (30–100 mg·kg^{-1}, p.o.) and time- (0.5 up to 2 h) dependent manner (Figure 2b,c). Depression-like behaviour and sciatic nerve H_2O_2 levels were also reduced by A-967079 (100 mg·kg^{-1}, p.o.) (Figure 2d,e). In mice with *Trpa1* global genetic deletion (*Trpa1*$^{-/-}$ mice) (Figure 2f), the reserpine-induced increases in paw mechanical/cold hypersensitivity, depression-like behaviour (Figure 2g–i) and H_2O_2 and 4-HNE intrasciatic levels (Figure 2j,k) were attenuated compared to control (*Trpa1*$^{+/+}$) mice.

Previously, we reported that neuroinflammation is pivotal in several neuropathic pain mouse models (De Logu et al., 2017, 2020, 2021; Trevisan et al., 2016). Reserpine increased the number of macrophages (MΦs) (F4/80$^+$ cells) in the sciatic nerve trunk but not in dorsal root ganglia (Figure 3a). To explore their role in paw mechanical/cold hypersensitivity and neuroinflammation, we used macrophages Fas-induced apoptosis (MaFIA) transgenic mice (Figure 3b). Reserpine-induced paw mechanical/cold hypersensitivity, depression-like behaviour and intrasciatic F4/80$^+$ cell accumulation in MaFIA mice were similar to those evoked in C57BL/6J mice and were attenuated by AP20187 treatment (Figure 3c–f). The present observation that reserpine injection in *Trpa1*$^{-/-}$ mice failed to increase intrasciatic F4/80$^+$ cells (Figure 3g) indicates that neuroinflammation like paw mechanical/cold hypersensitivity depends on TRPA1.

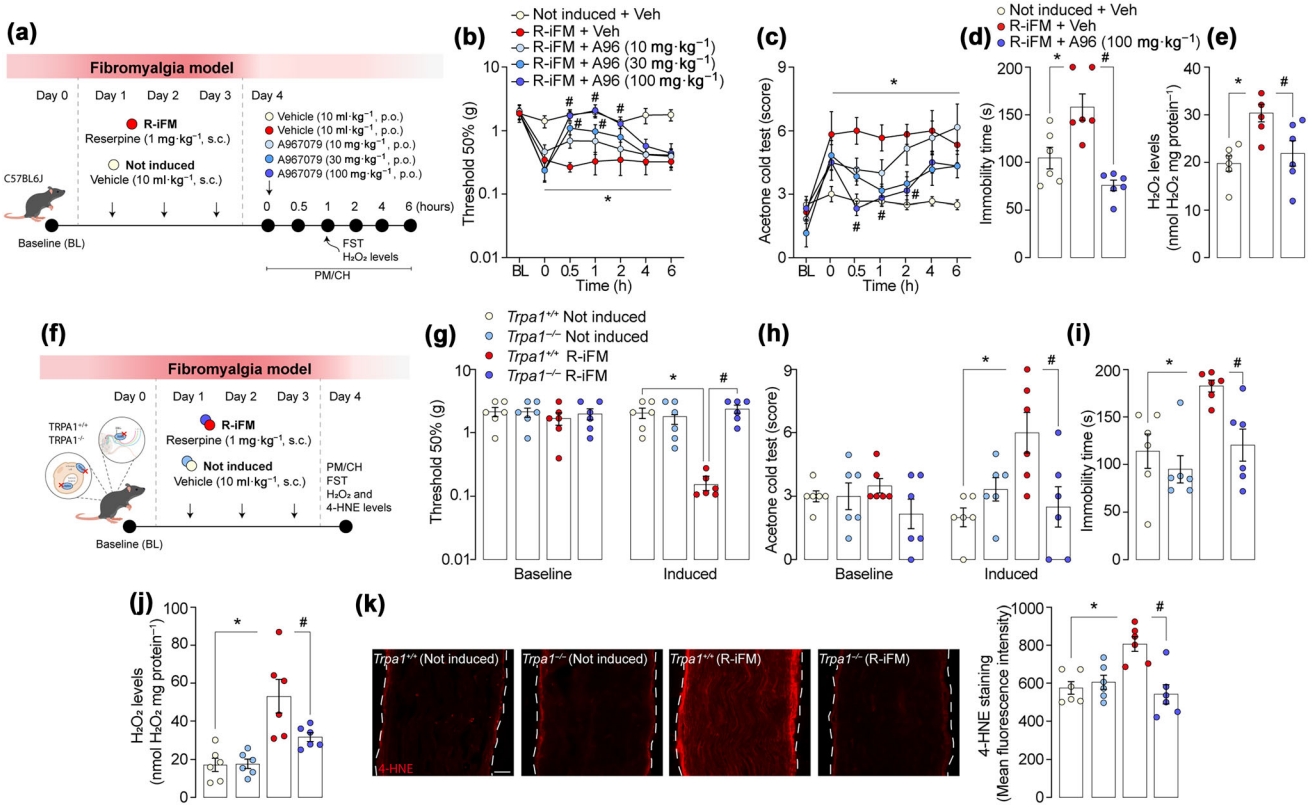

FIGURE 2 TRPA1 activation mediates fibromyalgia (FM) painful and depressive-like behaviours and oxidative stress. (a) Scheme of the TRPA1 antagonist treatment in the FM experimental procedure and timeline. (b) Mechanical allodynia and (c) cold hypersensitivity evoked by reserpine (1 mg·kg^{-1}, subcutaneously, s.c., reserpine-induced fibromyalgia [R-iFM]) or vehicle (Veh; 10 ml·kg^{-1}, s.c., not induced) in C57BL/6J treated with A-967079 (A96; 10, 30 or 100 mg·kg^{-1}, orally, p.o.) or vehicle (10 ml·kg^{-1}, p.o.). (d) Depressive-like behaviour in R-iFM and not induced mice treated with A-967079 (A96, 100 mg·kg^{-1}, p.o.) or vehicle (10 ml·kg^{-1}, p.o.). (e) H_2O_2 levels in sciatic nerve of R-iFM and not induced mice treated with A-967079 (A96, 100 mg·kg^{-1}, p.o.) or vehicle (10 ml·kg^{-1}, p.o.). (f) Scheme of the FM experimental procedure and timeline in *Trpa1*$^{+/+}$ and *Trpa1*$^{-/-}$ mice. (g) Mechanical allodynia, (h) cold hypersensitivity and (i) depressive-like behaviour in *Trpa1*$^{+/+}$ and *Trpa1*$^{-/-}$ R-iFM and not induced mice. (j) H_2O_2 levels in the sciatic nerve of *Trpa1*$^{+/+}$ and *Trpa1*$^{-/-}$ R-iFM and not induced mice. (k) Representative photomicrographs and mean fluorescence intensity of 4-hydroxynonenal (4-HNE) staining in the sciatic nerve of *Trpa1*$^{+/+}$ and *Trpa1*$^{-/-}$ R-iFM and not induced mice (Scale bar: 50 μm) (n = 6 mice per group). Mean ± SEM. $^*P < 0.05$ versus not induced + Veh. $^\#P < 0.05$ versus R-iFM + Veh. Data were analysed by one- or two-way ANOVA followed by the Bonferroni's post hoc test. R-iFM: reserpine-induced fibromyalgia; Veh: vehicle. n = 30 C57BL/6J male mice were used in (a)–(e); n = 12 *Trpa1*$^{+/+}$ and n = 12 *Trpa1*$^{-/-}$ male mice were used in (f)–(k) and Figure 3g.

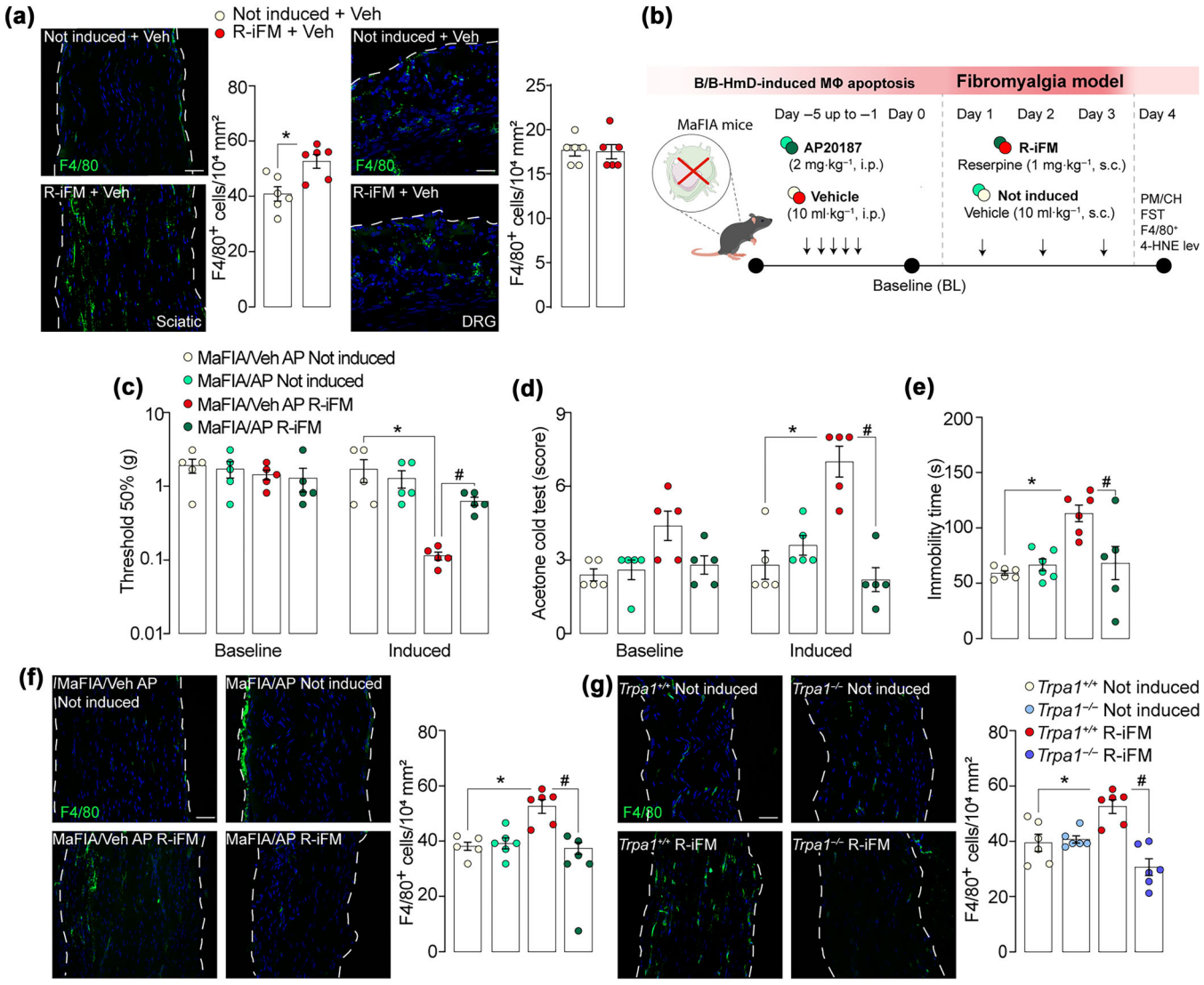

FIGURE 3 Macrophage infiltration in the sciatic nerve drives the development of fibromyalgia (FM) painful and depressive-like behaviours. (a) Representative photomicrographs and the number of F4/80$^+$ cells inside the sciatic nerve and dorsal root ganglia (DRG) of C57BL/6J mice treated with reserpine (1 mg·kg^{-1}, subcutaneously, s.c., reserpine-induced fibromyalgia [R-iFM]) or vehicle (Veh; 10 ml·kg^{-1}, s.c., not induced). (b) Scheme of the FM experimental procedure and timeline in macrophages Fas-induced apoptosis (MaFIA) mice. (c) Mechanical allodynia, (d) cold hypersensitivity and (e) depressive-like behaviour in R-iFM and not induced MaFIA mice treated with AP20187 (2 mg·kg^{-1}, i.p.) or vehicle AP (10 ml·kg^{-1}, i.p.) for five consecutive days. (f) Representative photomicrographs and the number of F4/80$^+$ cells inside the sciatic nerve of R-iFM and not induced MaFIA mice treated with AP20187 or Veh AP. (g) Representative photomicrographs and number of F4/80$^+$ cells inside the sciatic nerve of *Trpa1*$^{+/+}$ and *Trpa1*$^{-/-}$ R-iFM and not induced mice (Scale bar: 50 μm) (n = 5–6 mice per group). Mean ± SEM. $^*P < 0.05$ versus not induced + Veh. $^\#P < 0.05$ versus R-iFM + Veh. Data were analysed by Student's *t* test or one- or two-way ANOVA followed by the Bonferroni's post hoc test. R-iFM: reserpine-induced fibromyalgia; Veh: vehicle. n = 12 C57BL/6J male mice were used in (a); n = 24 MaFIA male mice were used in Figure 2b–f.

The final common pathway originally identified in a neuropathic pain mouse model (De Logu et al., 2017) consists of a TRPA1-mediated calcium-dependent NADPH oxidase 1 (NOX1) activation in Schwann cells. NOX1 by amplifying ROS generation produces a feedforward dual response. On the one hand, it recruits hematogenic macrophages inside the nerve trunk to maintain an increased oxidative stress burden and, on the other hand, it targets TRPA1 in adjacent nociceptors to encode mechanical allodynia (De Logu et al., 2017). Here, the non-selective NOX inhibitor, apocynin (100 mg·kg^{-1}, i.p; Figure 4a), reversed reserpine-evoked intrasciatic H_2O_2 increase, paw mechanical/cold hypersensitivity and depression-like behaviour in C57BL/6J mice (Figure 4b–e). *In vitro* exposure of human Schwann cells to reserpine promoted H_2O_2 release that was attenuated by the NOX1 selective inhibitor ML171 (Figure 4f). Neuroinflammation (F4/80$^+$ cells), 4-HNE staining, H_2O_2 levels, paw mechanical/cold hypersensitivity and depression-like behaviour elicited by reserpine were attenuated by ML171 administration (60 mg·kg^{-1}, i.p; Figure 4a) (Figure 4g–l). Two of the currently recommended drugs for the

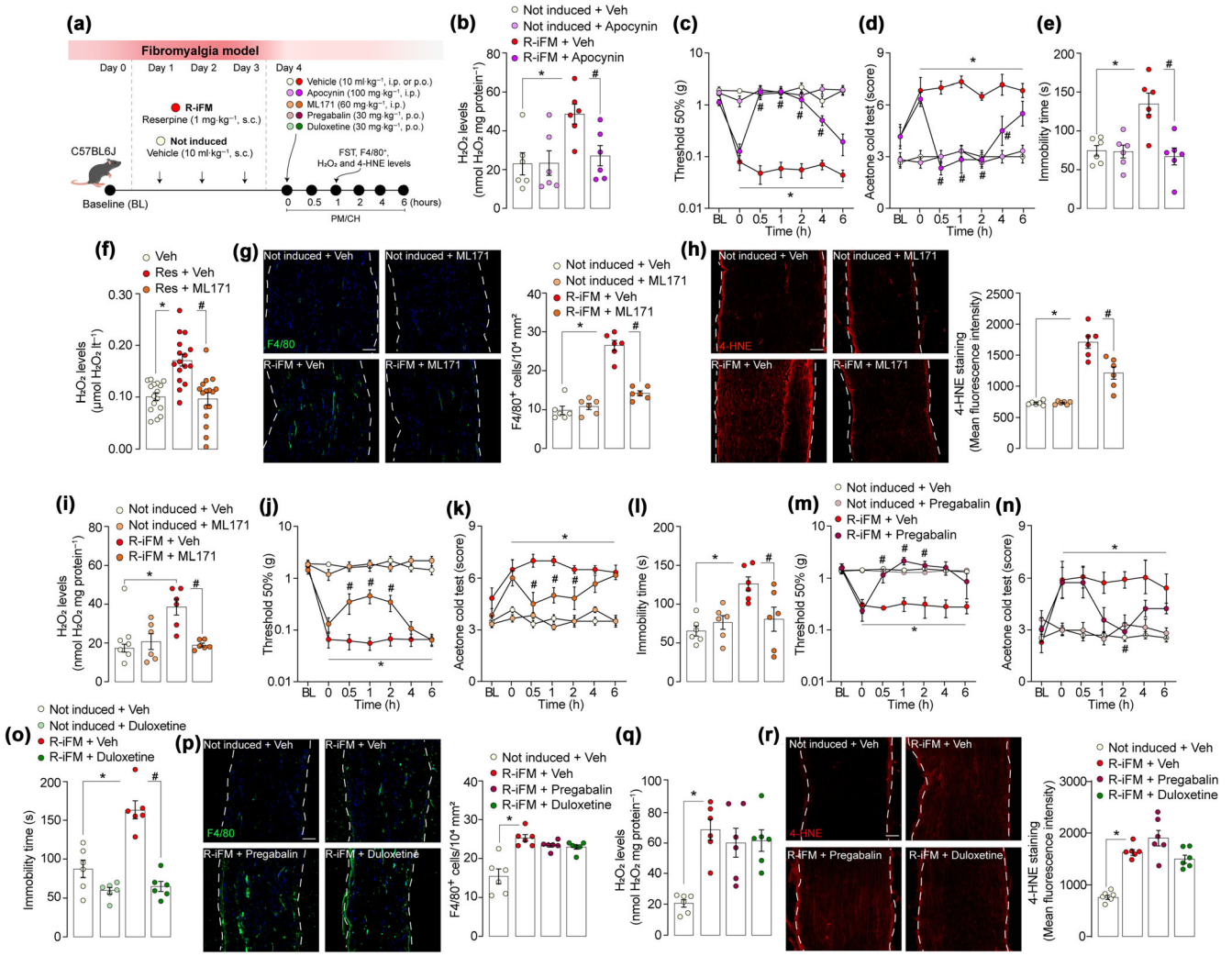

FIGURE 4 Fibromyalgia (FM) painful and depressive-like behaviour are mediated by NADPH oxidase 1-dependent mechanism. (a) Scheme of the NOX inhibitors or reference drugs' treatment in the FM experimental procedure and timeline. (b) H_2O_2 levels in the sciatic nerve of C57BL/6J mice treated with reserpine (1 mg·kg^{-1}, subcutaneously, s.c, reserpine-induced fibromyalgia [R-iFM]) or vehicle (10 ml·kg^{-1}, s.c., not induced) treated with apocynin (100 mg·kg^{-1}, intraperitoneal, i.p.) or vehicle (Veh; 10 ml·kg^{-1}, i.p.). (c) Mechanical allodynia, (d) cold hypersensitivity and (e) depressive-like behaviour in R-iFM and not induced C57BL/6J mice treated with apocynin (100 mg·kg^{-1}, i.p.) or vehicle (10 ml·kg^{-1}, i.p.). (f) H_2O_2 levels in human Schwann cells treated with Res (30 μM), Veh (0.3% DMSO) or ML171 (1 μM). (g) Representative photomicrographs and the number of F4/80$^+$ cells inside the sciatic nerve of R-iFM and not induced C57BL/6J mice treated with ML171 (60 mg·kg^{-1}, i.p.) or vehicle (10 ml·kg^{-1}, i.p.). (h) Representative photomicrographs and mean fluorescence intensity of 4-hydroxynonenal (4-HNE) staining in the sciatic nerve of R-iFM and not induced C57BL/6J mice treated with ML171 (60 mg·kg^{-1}, i.p.) or vehicle (10 ml·kg^{-1}, i.p.). (i) H_2O_2 levels in the sciatic nerve of R-iFM and not induced C57BL/6J mice treated with ML171 (60 mg·kg^{-1}, i.p.) or vehicle (10 ml·kg^{-1}, i.p.). (j) Mechanical allodynia, (k) cold hypersensitivity and (l) depressive-like behaviour in R-iFM and not induced C57BL/6J mice treated with ML171 (60 mg·kg^{-1}, i.p.) or vehicle (10 ml·kg^{-1}, i.p.). (m) Mechanical allodynia and (n) cold hypersensitivity in R-iFM and Not induced C57BL/6J mice treated with pregabalin (30 mg·kg^{-1}, orally, p.o.) or vehicle (10 ml·kg^{-1}, p.o.). (o) Depressive-like behaviour in R-iFM and not induced C57BL/6J mice treated with duloxetine (30 mg·kg^{-1}, orally, p.o.) or vehicle (10 ml·kg^{-1}, p.o.). (p) Representative photomicrographs and number of F4/80$^+$ cells inside the sciatic nerve, (q) H_2O_2 levels and (r) representative photomicrographs and mean fluorescence intensity of 4-hydroxynonenal (4-HNE) staining in the sciatic nerve in R-iFM and not induced C57BL/6J mice treated with pregabalin (30 mg·kg^{-1}, p.o.), duloxetine (30 mg·kg^{-1}, p.o.) or vehicle (10 ml·kg^{-1}, p.o.) (scale bar: 50 μm) (n = 6 mice per group). Mean ± SEM. *P < 0.05 versus not induced + Veh or Veh. $^{\#}P$ < 0.05 versus R-iFM + vehicle or reserpine (Res + Veh). Data were analysed by one- or two-way ANOVA followed by the Bonferroni's post hoc test. R-iFM: reserpine-induced fibromyalgia; Veh: vehicle; Res: reserpine. n = 84 C57BL/6J male mice were used in this figure.

treatment of painful and depressive symptoms in fibromyalgia patients (Clauw, 2014; Sarzi-Puttini et al., 2020), pregabalin and duloxetine (30 mg·kg^{-1}, p.o.; Figure 4a), reduced paw mechanical/cold hypersensitivity and depression-like behaviour (Figure 4m–o), respectively, but not neuroinflammation (increase in F4/80$^+$ cell and H_2O_2 and 4-HNE levels in the sciatic nerve) (Figure 4p–r).

3.3 | Reserpine targets Schwann cell TRPA1 to elicit pain and neuroinflammation

We then investigated the role of Schwann cell TRPA1 in paw mechanical/cold hypersensitivity and neuroinflammation induced by reserpine in *in vitro* and *in vivo* experiments. Human Schwann cells transfected with the H_2O_2 genetically encoded biosensor Hyper 7.2 showed increased fluorescence after stimulation with the TRPA1 agonist AITC. This response was prevented by the TRPA1 antagonist, A-967079, or in a calcium-free medium (Figure 5a). In mice with selective silencing of *Trpa1* in Schwann cells (*Plp1-Cre*$^{ERT+}$/*Trpa1*$^{fl/fl}$ mice) (Figure 5b), paw mechanical/cold hypersensitivity, depression-like behaviour (Figure 5c–e) and increased F4/80^{+} cells and 4-HNE levels in sciatic nerve elicited by reserpine were reduced compared to control mice (Figure 5f,g). To explore whether the Schwann cell/TRPA1/oxidative stress pathway was implicated in macrophage migration, the Boyden chamber assay was used. The presence of human Schwann cells stimulated the migration of LPS-activated human monocytic U937 cells, a response that the TRPA1 antagonist A-967079 inhibited (Figure 5h). The increased H_2O_2 levels in the supernatant were also reduced by A-967079 (Figure 5i). Reported data suggest the possible contribution of Schwann cells TRPA1 to sustain reserpine-induced neuroinflammation and the associated pain-like response.

To explore the contribution of neuronal TRPA1 in reserpine-induced paw mechanical/cold hypersensitivity, we used mice with selective silencing of *Trpa1* in dorsal root ganglia neurons (*Adv-Cre*$^{+}$/*Trpa1*$^{fl/fl}$ mice) (Figure 5j) that displayed reduced paw mechanical/cold hypersensitivity and depression-like behaviour (Figure 5k–m) but not neuroinflammation (F4/80^{+} cell number and 4-HNE levels) in sciatic nerve (Figure 5n,o). These data identify the neuronal TRPA1 as the final target of the Schwann cells/TRPA1/oxidative stress pathway to signal pain paw mechanical/cold hypersensitivity and depression-like behaviour in the present fibromyalgia mouse model.

4 | DISCUSSION

Fibromyalgia is a complex syndrome characterized by an undetermined aetiology and intricate pathophysiology. Nociceptors in fibromyalgia present a reduced threshold to external stimuli, leading to increased sensitivity to pain (Arnold et al., 2016; Littlejohn & Guymer, 2018). Despite the absence of apparent tissue damage or lesions in the somatosensory system, peripheral nociceptors result activated (Kosek et al., 2016; Treede et al., 2019) and fibromyalgia patients commonly experience hypersensitivity to mechanical (Clauw, 2015; Littlejohn & Guymer, 2018) and thermal (cold) stimuli (Clauw, 2014; Larson et al., 2014). However, the central or peripheral origin of the debilitating pain symptoms of fibromyalgia remains uncertain (Martínez-Lavín, 2022). Here, we revealed that TRPA1, expressed by Schwann cell and dorsal root ganglia nociceptors, is a key mediator of pain-like behaviours in a mouse model of fibromyalgia induced by repeated reserpine administration.

The classical response to reserpine administration consisting of monoamine depletion has been associated with sensory changes, including mechanical and thermal hypersensitivity, mirroring symptoms reported by fibromyalgia patients (Brum et al., 2022; Nagakura et al., 2009). We observed that TRPA1 antagonism or global knockout mitigated reserpine-induced fibromyalgia-like behaviours. While TRPA1 is present in a subpopulation of dorsal root ganglia nociceptors to signal spontaneous pain by channel agonists (Hinman et al., 2006; McNamara et al., 2007; Paulsen et al., 2015; Zhao et al., 2020), more recent findings have revealed that Schwann cell TRPA1 sustains prolonged mechanical hypersensitivity (De Logu et al., 2017, 2020, 2021; De Logu, Li Puma, et al., 2019). Here, we found that reserpine in mice encodes this same glial TRPA1 pathway to sustain mechanical and cold hypersensitivity in mice.

Elevated oxidative stress biomarkers have been consistently associated with pain sensitization in various models, including partial nerve injury, ischemia–reperfusion, cancer pain and fibromyalgia (Assavarittirong et al., 2022; De Logu et al., 2017, 2020, 2021). Further support is given by the observation that fibromyalgia patients exhibit an imbalance between oxidants and antioxidants (Akkuş et al., 2009; Altindag & Celik, 2006; Bagis et al., 2005; Cordero et al., 2009, 2010, 2011, 2012; Eisinger et al., 1996; Hung et al., 2020; Ozgocmen et al., 2006). In this study, we observed that repeated reserpine administration increased oxidative stress in sciatic nerve tissue, which is mitigated by treatment with antioxidants, and the reduction correlates with diminished pain-like behaviours. However, despite some supporting studies (Fernández-Araque et al., 2022), because of poor pharmacokinetics or other limiting factors, currently available antioxidants have not provided robust evidence of their benefit in fibromyalgia, to the extent that no guideline recommends these treatments (Ariani et al., 2021; Macfarlane et al., 2017).

Although fibromyalgia patients do not exhibit increased circulating monocytes compared to healthy patients (Taylor et al., 2016), elevated serum levels of M1 macrophage markers, along with various cytokines and chemokines underlines a possible proinflammatory signature of fibromyalgia (Bote et al., 2012; Tripathi et al., 2021). Activated macrophages serve as a major source of oxidative burst (Canton et al., 2021; Valko et al., 2006) at the site of tissue injury. Importantly, macrophages recruited at the site of nerve damage have been reported to sustain neuroinflammation and pain (Chen et al., 2020; De Logu et al., 2017, 2020, 2021; Duque & Descoteaux, 2014; Trevisan et al., 2016). In mouse models of pain evoked by partial sciatic nerve ligation or ischemia/reperfusion, we observed that macrophage recruitment in the injured nerve trunk depends on TRPA1 activation (De Logu et al., 2017, 2020). Here, we show that repeated administration of reserpine facilitates the accumulation of macrophages inside the sciatic nerve tissue. The contribution of macrophages to reserpine-induced neuroinflammation and paw mechanical/cold hypersensitivity was shown using transgenic MaFIA mice, which exhibit a remarkable pharmacological-induced depletion of macrophages (Burnett et al., 2004).

The TRPA1 channel in peripheral nerve tissue has been proposed to act not only as a neuronal sensor for acute painful signals but also

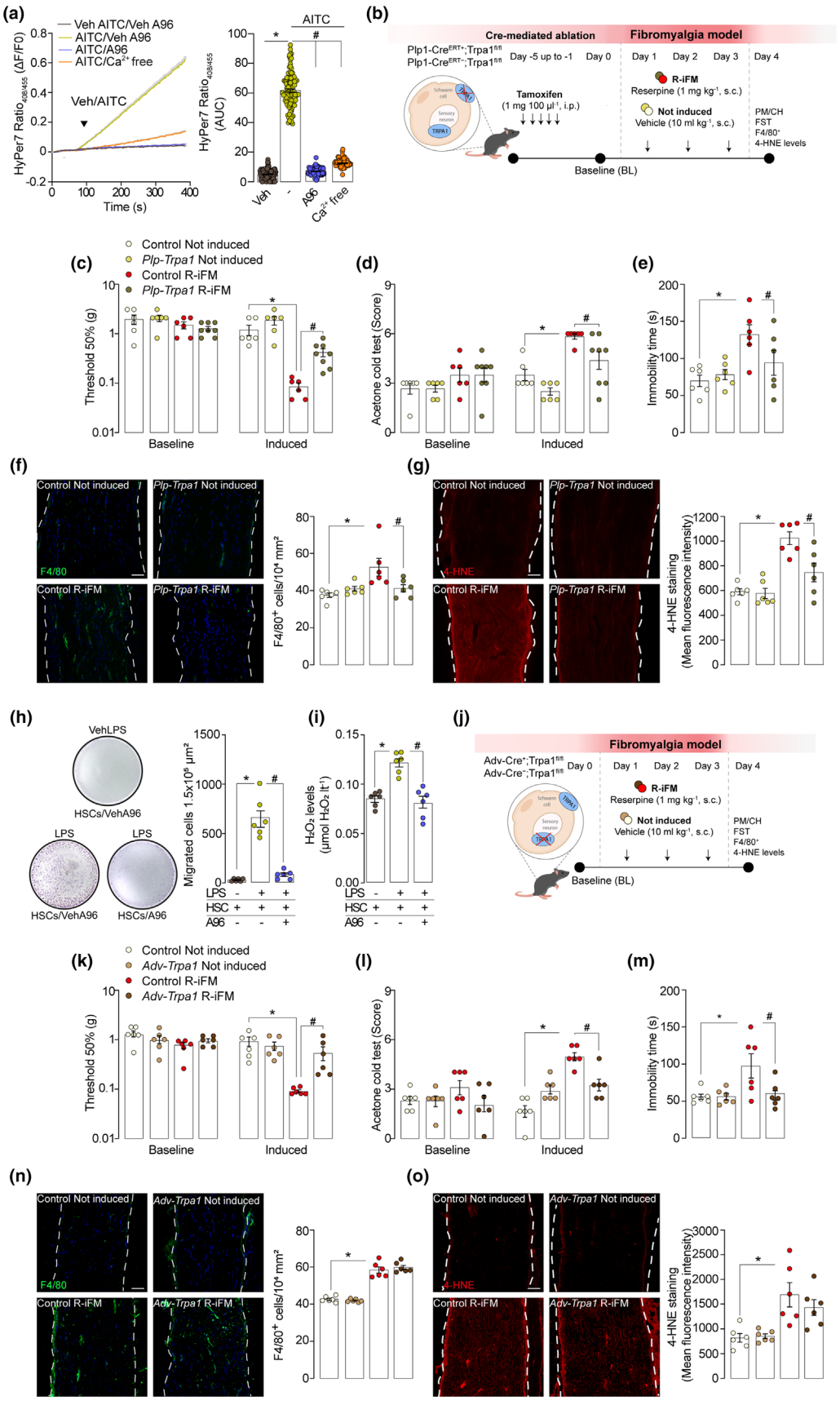

FIGURE 5 Legend on next page.

FIGURE 5 Schwann cell TRPA1 mediate fibromyalgia (FM) painful and depressive-like behaviour and oxidative stress. (a) Typical traces and cumulative data of Hyper7.2 in human Schwann cells (HSCs) exposed to AITC or Veh and in the presence of A-967079 (A96, 30 μM) or in Ca^{2+} medium (cells number: AITC n = 109, A96 n = 105, veh n = 144, calcium-free n = 83; n = 6 independent experiments). (b) Scheme of the FM experimental procedure and timeline in mice lacking TRPA1 in Schwann cells (*Plp1-Cre*$^{ERT+}$;*Trpa1*$^{fl/fl}$). (c) Mechanical allodynia, (d) cold hypersensitivity and (e) depressive-like behaviour evoked by reserpine (1 mg·kg^{-1}, subcutaneously, s.c., reserpine-induced fibromyalgia [R-iFM]) or vehicle (10 ml·kg^{-1}, s.c., not induced) in *Plp1-Cre*$^{ERT+}$/*Trpa1*$^{fl/fl}$ (PLP-TRPA1) or control mice treated with tamoxifen (1 mg·100 μl^{-1}, intraperitoneal, i.p.) for five consecutive days. (f) Representative photomicrographs and number of F4/80^{+} cells inside the sciatic nerve in R-iFM and not induced PLP-TRPA1 or control mice. (g) Representative photomicrographs and mean fluorescence intensity of 4-hydroxynonenal (4-HNE) staining in the sciatic nerve in R-iFM and not induced PLP-TRPA1 or control mice. (h) Representative images and cumulative data of migrating activated (*E. coli* lipopolysaccharide, LPS; 10 μg·ml^{-1}) or not activated (no LPS) human monocytic U937 in the presence or not of human Schwann cells (HSCs) and following A96 (30 μM) or vehicle pre-treatment (n = 6 independent experiments). (i) H_2O_2 levels in HSCs with or without LPS (10 μg·ml^{-1}) or A96 (30 μM). (j) Scheme of the FM experimental procedure and timeline in mice lacking TRPA1 in sensory neurons (*Adv-Cre*$^{+}$; *Trpa1*$^{fl/fl}$). (k) Mechanical allodynia, (l) cold hypersensitivity and (m) depressive-like behaviour evoked by reserpine (1 mg·kg^{-1}, s.c., R-iFM mice) or vehicle (10 ml·kg^{-1}, s.c., not induced) in *Adv-Cre*$^{+}$;*Trpa1*$^{fl/fl}$ (ADV-TRPA1) or control mice. (n) Representative photomicrographs and number of F4/80^{+} cells inside the sciatic nerve in R-iFM and not induced ADV-TRPA1 or control mice. (o) Representative photomicrographs and mean fluorescence intensity of 4-hydroxynonenal (4-HNE) staining in the sciatic nerve in R-iFM and not induced ADV-TRPA1 or control mice (scale bar: 50 μm) (n = 6 mice per group). Mean ± SEM. $^{*}P < 0.05$ versus not induced + Veh. $^{\#}P < 0.05$ versus R-iFM + Veh. Data were analysed by one- or two-way ANOVA followed by the Bonferroni's post hoc test. R-iFM: reserpine-induced fibromyalgia; Veh: vehicle; AITC: allyl isothiocyanate; A96: A-967079; LPS, *E. coli* lipopolysaccharide. n = 12 *Plp1-Cre*$^{ERT+}$;*Trpa1*$^{fl/fl}$ and n = 12 *Plp1-Cre*$^{ERT-}$;*Trpa1*$^{fl/fl}$ male mice were used in (b)–(g). n = 12 *Adv-Cre*$^{+}$;*Trpa1*$^{fl/fl}$ and n = 12 *Adv-Cre*$^{-}$;*Trpa1*$^{fl/fl}$ male mice were used in (j)–(o).

as a sensor and amplifier of oxidative stress (Andersson et al., 2008). TRPA1 mRNA expression has been limited to dorsal root ganglia neurons in a recent study (Patil et al., 2023). However, a series of studies have reported that Schwann cell TRPA1 activation results in ROS release that induces a calcium-dependent activation of NOX1 (De Logu et al., 2017), which amplifies a feedforward mechanism to sustain neuroinflammation (De Logu et al., 2017, 2020, 2021, 2022; De Logu, Li Puma, et al., 2019; Landini et al., 2023). The heightened ROS in the nerve trunk engages the neuronal TRPA1 to sustain mechanical and cold hypersensitivity (De Logu et al., 2017, 2020, 2021, 2022; De Logu, Li Puma, et al., 2019; Landini et al., 2023). In our observation, pregabalin or duloxetine, two centrally-acting drugs used for the treatment of pain and depression in fibromyalgia patients with a presumed effect on the central nervous system (CNS) (Clauw, 2014; Sarzi-Puttini et al., 2020), reduced pain-like and depressive-like behaviour but not neuroinflammation. This suggests that the reserpine model of fibromyalgia involves initially the peripheral nervous system (PNS) with a subsequent CNS involvement.

Recently, the **TRP vanilloid 1 (TRPV1)** channel has been implicated in pain-like behaviours induced by reserpine in Swiss mice (Fischer et al., 2020) or in other models of fibromyalgia, not induced by reserpine (Chen et al., 2014; Fischer et al., 2020; Hsu et al., 2020; Yen et al., 2020; Yüksel et al., 2017). The use of a different mouse strain and of **resiniferatoxin** that inhibits the fuction of TRPV1-positive dorsal root ganglia neurons that also express TRPA1 (Fischer et al., 2020) or different fibromyalgia models (Chen et al., 2014; Fischer et al., 2020; Hsu et al., 2020; Yen et al., 2020; Yüksel et al., 2017) may explain the different results with present findings. In the reserpine-induced model of fibromyalgia, we observed that selective silencing of Schwann cell TRPA1 in mice reproduced the same results obtained in global TRPA1 knockout mice, confirming the key role of Schwann cell TRPA1 in the development and maintenance of neuroinflammation and fibromyalgia-associated pain-like behaviours. Should the reserpine model of fibromyalgia fully model the features of the human disease, our data might propose that TRPA1 targeting could offer a novel therapeutic approach for attenuating fibromyalgia-related painful and comorbid behaviours.

AUTHOR CONTRIBUTIONS

Study concept and design: E. S. Brum, R. Nassini, F. De Logu and S. M. Oliveira. *Acquisition of behaviours data*: E. S. Brum, M. F. P. Fialho, L. Landini, B. L. Kuhn, C. P. Frizzo, P. H. S. Araújo, R. M. Guimarães, T. M. Cunha, C. R. da Silva and G. Trevisan. *Acquisition of cell lines and calcium imaging data*: M. Marini, R. Nassini and F. De Logu. *Acquisition of immunofluorescence data*: E. S. Brum, L. Landini, D. Souza Monteiro de Araújo and M. Titiz. *Analysis and interpretation of data*: E. S. Brum, G. Trevisan, P. Geppetti, R. Nassini, F. De Logu and S. M. Oliveira. *Drafting and revising the content of the manuscript*: E. S. Brum, M. F. P. Fialho, G. Trevisan, P. Geppetti, R. Nassini, F. De Logu and S. M. Oliveira. *Study supervision*: P. Geppetti, R. Nassini, F. De Logu and S. M. Oliveira

CONFLICT OF INTEREST STATEMENT

PG, RN and FDL are founding scientists of FloNext Srl. The authors declare that there are no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the *BJP* guidelines for Design and Analysis, Immunoblotting and Immunochemistry and Animal Experimentation, and as recommended by funding agencies, publishers and other organizations engaged with supporting research.

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