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Authors

Lemes, Julia Borges Paes

Malange, Kaue Franco

Carvalho, Nathalia Santos

et al.

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Original Research

Blocking Pannexin 1 Channels Alleviates Peripheral Inflammatory Pain but not Paclitaxel-Induced Neuropathy

Julia Borges Paes Lemes[1,*,§], Kaue Franco Malange[1,§], Nathalia Santos Carvalho[1], Amanda Ferreira Neves[2], Marcia Urban-Maldonado[3], Paula Regina Gelinski Kempe[4], Catarine Massucato Nishijima[1], Cecilia Costa Fagundes[1], Celina Monteiro da Cruz Lotufo[5], Sylvia Ottile Suadicani[3], Carlos Amilcar Parada[1,*]

[1]Department of Structural and Functional Biology, Institute of Biology, University of Campinas (UNICAMP), 13083-970 Campinas, SP, Brazil
[2]Department of Medicine, University of Miami Miller School of Medicine, Miami, FL 33136, USA
[3]Department of Urology, Albert Einstein College of Medicine and Montefiore Medical Center, Bronx, NY 10461, USA
[4]Laboratory of Nerve Regeneration, University of Campinas (UNICAMP), 13083-970 Campinas, SP, Brazil
[5]Institute of Biomedical Sciences, Department of Physiology, Federal University of Uberlandia (UFU), 38408-102 Uberlandia, MG, Brazil
*Correspondence: juliabplemes@gmail.com (Julia Borges Paes Lemes); caparada@unicamp.br (Carlos Amilcar Parada)
[§]Current address: Department of Anesthesiology, University of California San Diego, La Jolla, CA 92093, USA.
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Abstract

Background: Pannexin1 (Panx1) is a membrane channel expressed in different cells of the nervous system and is involved in several pathological conditions, including pain and inflammation. At the central nervous system, the role of Panx1 is already well-established. However, in the periphery, there is a lack of information regarding the participation of Panx1 in neuronal sensitization. The dorsal root ganglion (DRG) is a critical structure for pain processing and modulation. For this reason, understanding the molecular mechanism in the DRG associated with neuronal hypersensitivity has become highly relevant to discovering new possibilities for pain treatment. Here, we aimed to investigate the role of Panx1 in acute nociception and peripheral inflammatory and neuropathic pain by using two different approaches. **Methods**: Rats were treated with a selective Panx1 blocker peptide (^{10}Panx) into L5-DRG, followed by ipsilateral intraplantar injection of carrageenan, formalin, or capsaicin. DRG neuronal cells were pre-treated with ^{10}Panx and stimulated by capsaicin to evaluate calcium influx. Panx1 knockout mice (Panx1-KO) received carrageenan or capsaicin into the paw and paclitaxel intraperitoneally. The von Frey test was performed to measure the mechanical threshold of rats' and mice's paws before and after each treatment. **Results**: Pharmacological blockade of Panx1 in the DRG of rats resulted in a dose-dependent decrease of mechanical allodynia triggered by carrageenan, and nociception decreased in the second phase of formalin. Nociceptive behavior response induced by capsaicin was significantly lower in rats treated with Panx1 blockade into DRG. Neuronal cells with Panx1 blockage showed lower intracellular calcium response than untreated cells after capsaicin administration. Accordingly, Panx1-KO mice showed a robust reduction in mechanical allodynia after carrageenan and a lower nociceptive response to capsaicin. A single dose of paclitaxel promoted acute mechanical pain in wildtype (WT) but not in Panx1-KO mice. Four doses of chemotherapy promoted chronic mechanical allodynia in both genotypes, although Panx1-KO mice had significant ablation in the first eight days. **Conclusion**: Our findings suggest that Panx1 is critical for developing peripheral inflammatory pain and acute nociception involving transient receptor potential vanilloid subtype 1 (TRPV1) but is not essential for neuropathic pain chronicity.

Keywords: DRG; Panx1; inflammatory pain; neuropathy; capsaicin

1. Introduction

Pannexin 1 (Panx1) is a cell surface single-channel expressed in different types of cells in the nervous system, including nociceptive neurons, macrophages, and satellite cells [1–3]. They can be activated by mechanical stimulation, intracellular calcium, high extracellular potassium concentration, and N-methyl-D-asparate receptor (NMDA) activation [2,4–6]. Studies have shown an interactive network between Panx1 channels and ATP signaling in the dorsal root ganglion (DRG), where these channels trigger inflammatory pain cascades through caspase-1 maturation, interleukin-1β release, and later activation of pyrin domain-containing protein 3 (NLRP3) (nucleotide-binding domain, leucine-rich–containing family, pyrin domain–containing-3) inflammasome [2,7]. These mechanisms depend on calcium influx through a dynamic channel pore formed between Panx1 and P2X7R (purinergic ionotropic receptors) [8–10]. Recent evidence demonstrated an increase of Panx1 protein expression in DRG and Schwann cells after nerve constriction, and using pharmacological blockage or cell-specific knockdown of Panx1 effectively reduced mechanical and heat sensitization [11,12]. Although valuable efforts have been made in studying the implications of Panx1 channels in nociceptive processing, studies of mechanisms by which Panx1 at the DRG level maintains pain phenotypes are still scarce.

The DRG is an essential site for the transduction of nociceptive signals. Within the ganglia reside the cell bodies of neurons that innervate a vast portion of the peripheral tissue of our body. Each neuron is enveloped by a layer of glial cells, forming units interspersed by macrophages [13]. Besides sharing space in the DRG, this group of cells also exchanges molecules and neurotransmitters that have been proven critical for developing and maintaining pain. Our research group demonstrated that the communication between neurons and satellite glial cells in the DRG mediated by ATP is essential for inflammatory pain development and acute nociceptive response [14,15]. These findings indicate that DRG is a promising target for pain modulation. Taking the sustained link between Panx1 and ATP/inflammation signaling at the DRG, we investigated the implications of these channels in modulating inflammatory pain induced by carrageenan, nociception by capsaicin or formalin, and neuropathic pain induced by chemotherapy.

2. Materials and Methods

2.1 Ethical statement

Studies were conducted using male rats and female or male mice. Animals were housed on a 12 h light/dark cycle with controlled humidity and temperature (22 $\pm$ 1 °C). Food and water were available *ad libitum*. All animal care, handling, and behavioral experiments were performed as per the International Association for the Study of Pain (IASP) guidelines for using animals in pain research [16]. All efforts were made to ensure minimal animal use. Number of animals for each experimental group was established according to statistical calculations for power analysis adopted by the CEUA/UNICAMP (https://www.ib.unicamp.br/comissoes/ceua_principal) and the CONCEA (http://www.mctic.gov.br/mctic/opencms/institucional/concea), as follows: n = 1 + [2C $\times$ (s/d)2] $\rightarrow$ C = (zα + zβ)2 $\rightarrow$ C = (1.96 + 1.282)2 $\rightarrow$ C = 10.51. Thus, n = 1 + [21.02 $\times$ (0.2/0.5)2] $\rightarrow$ n = 1 + (21.02 $\times$ 0.16) $\rightarrow$ n = 1 + 3.3632 $\rightarrow$ n = 4.3632, where: "C" = depends on the strength of statistical test (90% for "zβ") and the level of significance (95%, with "α" = 5%), "z" = values found in statistical books, "s" = estimated standard deviation (20%), and "d" = expected difference between groups (50%) [17]. All animal procedures were reviewed and conducted under the approval of the Albert Einstein College of Medicine Animal Care and Use Committee (IACUC) (protocol number 00001131) and by the Committee on Animal Research CEUA-UNICAMP (protocol number 4566-1/2017, 4566-1(A)/2017).

2.2 Rats

Male Wistar rats (200–250 grams) were used for behavior assessments and primary DRG neuronal cell culture. Rats were acquired from the Multidisciplinary Center for Biological Research (CEMIB) of University of Campinas, Campinas, Brazil, and maintained under standard housing conditions in the Laboratory of Pain Studies animal facility at the Department of Functional and Structural Biology of State University of Campinas, Campinas, Brazil.

2.3 Panx1$^{tm1a(KOMP)Wtsi}$ Knockout Mouse Model

Adult female or male mice (6 months old) with global deletion of Panx1$^{tm1a(KOMP)Wtsi}$ (Panx1-KO) and age-matched wildtype (WT) controls were used in this study for pain behavior evaluation. Panx1-KO and WT mice were maintained and generated in IACUC-approved animal facilities at the Albert Einstein College of Medicine (Bronx, NY, USA) by breeding heterozygous Panx1$^{tm1a(KOMP)Wtsi}$ mice purchased from the Knockout Mouse Project (KOMP) at University of California Davis (Davis, CA, USA), as previously described [18].

2.4 Intra-Ganglionar Drug Administration

Drug inoculation into the DRG of rats was performed using the direct injection method as previously described [19]. Briefly, animals were anesthetized with 2.5% isoflurane (Cristalia, Itapira, Brazil), and the fur was shaved over the lower back region at the level of the iliac crest. Animals were placed in a prone position over a cylinder, causing the lower back to become hyper flexed. A guide cannula (hypodermic needle, 25 $\times$ 10, 19 G) was inserted across the skin along the right side of the spine and down towards the caudal region. An adapted gingival needle was then inserted through the guide cannula towards the intervertebral space between the fifth and sixth lumbar vertebra, reaching the lateral vertebral bony process. With fine movements, the gingival needle was moved until it touched the right DRG, producing a characteristic ipsilateral paw reflex that indicates penetration of the needle tip into the DRG of the fifth lumbar spinal nerve. A total volume of 5 µL of the Panx1 blocker (^{10}Panx, #3348, Tocris, Bioscience, Bristol, UK) or the vehicle (Saline) was then injected into the L5-DRG. The ^{10}Panx concentrations used were 30, 50, 100, and 500 µM.

2.5 Inflammatory Pain Model

The subcutaneous injection of the inflammatory agent carrageenan (Cg) was administered into the central plantar region of the right hind paw, which corresponds to the peripheral field of the L5 DRG [20]. For rats and mice, the dosage used was as follows: λ-Carrageenan at 100 µg/paw (Cg; #9064-57-7, Sigma Aldrich, St. Louis, MO, USA). The injected volume was 50 µL in rats and 25 µL for mice.

2.6 Chemotherapy-Induced Peripheral Neuropathy (CIPN) Model

Panx1-KO and WT mice received a single systemic injection of paclitaxel (i.p., 2 mg/kg, #33069-62-4, Cayman, Ann Arbor, MI, USA) or four injections of the same drug, at the same concentration, on alternate days (1, 3, 5, 7 days) [21]. The control group received systemic administration of vehicle solution: 0.1% Dimethyl sulfoxide (DMSO) (#67-68-5, Sigma Aldrich) in Saline, at the same time points as the treated group.

2.7 Behavioral Tests

2.7.1 Mechanical Withdrawal Threshold: Von Frey Test

Electronic von Frey (for rats: Insight, #EFF301, Ribeirão Preto, Brazil; and for mice: Bioseb, #BIO-EVF-WRS, Pinellas Park, FL, USA) was used in this study to measure the mechanical withdrawal threshold of the hind paw, as previously described [22]. This test was applied to the following groups: paclitaxel and Cg treatments. Rats and mice were kept in a quiet room for 1 hour prior to the experiment to acclimate to testing environment. Measurements were taken blindly by the same experimenter between 9:00 AM and 5:00 PM. The polypropylene pipette tip (10 µL, #T-300, Axygen, Corning, NY, USA) connected to hand-held force transducer was applied perpendicularly to the central region of the animals' hind paw (L5 peripheral field) and pressure was gradually increased until a paw withdrawal or flinch was observed. The stimulus was interrupted at this point. The intensity of allodynia was quantified as the variation of the pressure Δ obtained by subtracting the value obtained after treatment from the basal measurement obtained before treatments. For each animal, three measurements were recorded per paw at each time point. In rats treated with Cg, measurements were performed before (baseline) and 3 hours after injection. In mice treated with Cg, measurements were taken at baseline, 1, 2, 3, 4, and 5 hours after treatment. The group treated with a single injection of paclitaxel, or its vehicle-treated control group, had the paw mechanical thresholds evaluated before and then for a period of 5 hours after treatment. For chronic paclitaxel groups (Panx1-KO and WT mice), the von Frey test was applied before any treatment and daily during an 8-day period. On the days of the injections (1, 3, 5, 7 day), the paw threshold measurement was conducted 3 hours after the systemic administration. After the induction period, von Frey measurements were carried out at 13- and 21-days post-induction.

2.7.2 Capsaicin and Formalin Test

Capsaicin (#M2028, Sigma Aldrich) at 10 µg/paw or vehicle (saline) was injected into the central plantar region of the right hind paw of mice (25 µL) or rats (50 µL). The number of flinches and licking were recorded for a period of 5 minutes after injection. The amount of 50 µL of 2.5% formalin (diluted from a 4% of Paraformaldehyde, #30525-89-4, Sigma Aldrich in phosphate-buffered saline, #P4417, Sigma Aldrich) was injected intradermally on the right hind paw of rats. Immediately after injection, we counted the nociception response for one hour, separated into blocks of 5 minutes each. As previously described, the nociceptive response is expressed as the total number of flinches and licks (3 seconds of licking is considered one flinch) [14].

2.8 Primary Dorsal Root Ganglion (DRG) Cell Culture

Male rats were euthanized under deep isoflurane anesthesia followed by decapitation. Thoracic and lumbar DRGs were harvested and transferred to Hank's buffered saline solution (H2387, Sigma Aldrich). DRGs were incubated in 0.28 U/mL collagenase (type II; #C6885, Sigma Aldrich) for 50 min, followed by incubation with 0.25% trypsin (T0303, Sigma Aldrich) for 10 min. Enzymatic dissociation steps were carried out at 36 °C. Ganglia were washed three times with DMEM (Dulbecco′s Modified Eagle′s Medium, #D5523, Sigma Aldrich) containing 10% of fetal bovine serum and carefully dissociated passing through a glass Pasteur pipette using up and down movements [23]. The cells were then plated in glass bottom dishes coated with 0.02 mg/mL of Poly-d-Lysine (#P6407, Sigma Aldrich) plus 0.016 mg/mL of Laminin (#L2020, Sigma Aldrich) and maintained in DMEM containing 10% of fetal bovine serum and penicillin (10 KU), streptomycin (10 mg/mL) (#P4333, Sigma Aldrich). Cultures were maintained in the incubator at 37 °C with a 5% CO_2 atmosphere for 48 hours, with one media change after 24 hours.

2.9 Intracellular Calcium Imaging

DRG cell cultures were loaded with the Ca^{2+} indicator Fluo-4 AM (5 µM; #F23915, Thermo Fisher Scientific, Waltham, MA, USA) in Hank's buffered saline solution and incubated for 30 minutes. Cells were then treated with ^{10}Panx (50 µM) or vehicle (Hank's buffered saline solution, # H9269, Sigma Aldrich) and maintained at 37 °C for 30 minutes. Serial Ca^{2+} imaging was performed for 5 seconds before and 55 seconds after capsaicin (5 µM) addition using an epifluorescence inverted Leica DMI 600B microscope coupled to a DFC360FX camera and Leica fluorescent light source CTR7000HS (Leica Microsystems, Wetzlar, Germany). At the end of each experiment, KCl (50 mM) was administered to confirm cellular viability. Neuronal Ca^{2+} responses were analyzed using ImageJ software (National Institutes of Health (NIH), Bethesda, MD, USA) and calculated as the mean gray value variations recorded from Regions of Interest (ROIs) placed on each individual cell. Data are presented as $\Delta F/F0$, in which F0 is the baseline.

2.10 Statistical Analysis

Data are expressed as mean $\pm$ Standard Error of the Mean (SEM), and statistical differences were determined using one-way or two-way variance analysis (ANOVA) followed by Tukey's multiple comparison test. The Prism v.8 (GraphPad Software Inc., La Jolla, CA, United States) was used to generate graphs, and for statistical analyses, means were considered significantly different when $p < 0.05$.

3. Results

3.1 Blockade of Panx1 in DRG of Rats Reduces Inflammatory Pain and Acute Nociception

Panx1 peptide blocker (^{10}Panx) at doses of 30, 50, 100, or 500 µM was injected directly into the DRG (L5-right) of rats 30 minutes prior to Cg injection (100 µM/paw, ipsilateral) to investigate the effect of the local blockage of Panx1 in Cg-induced allodynia. All doses except the

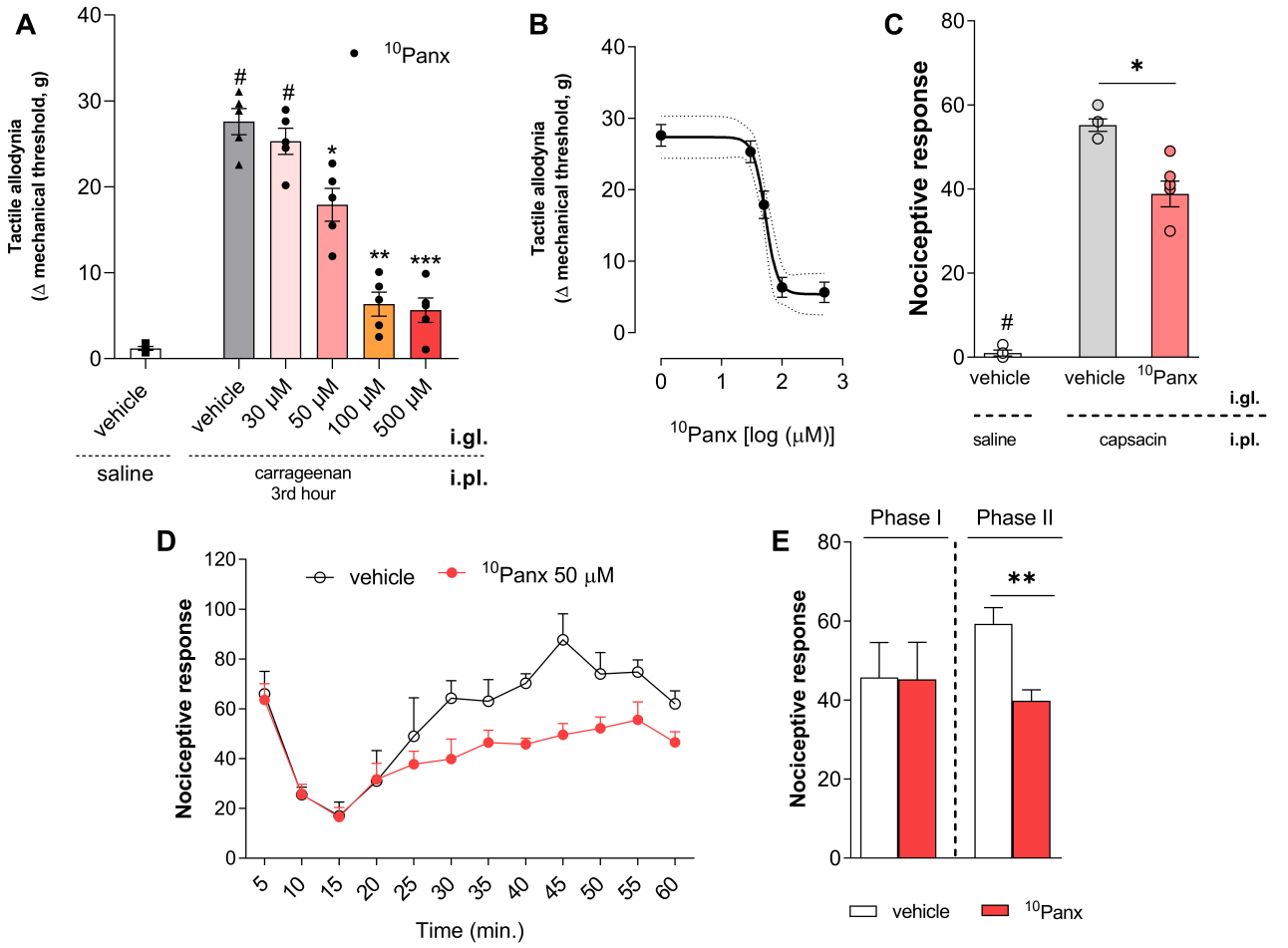

Fig. 1. Panx1 blockage in the L5-DRG of rats reduced inflammatory pain after carrageenan and nociception response after formalin or capsaicin injection. (A) Tactile allodynia of hind paw at the 3rd hour after Cg (100 µg/paw); (data expressed as Δ: mechanical threshold at 3rd hour subtracted by basal measurement). ^{10}Panx 30, 50, 100, 500 µM or vehicle were injected into the right L5-DRG of rats 30 minutes prior Cg. (B) Dose-response curve (Log dose concentration) at the third hour, 50 µM represents the submaximal dose. (C) Nociceptive response to intraplantar injection of capsaicin (10 µg/paw) by rats pretreated with intra-ganglionar (L5) administration of ^{10}Panx (50 µM) or vehicle. (D) Nociceptive response after formalin 2.5% (50 µL) administration in vehicle and treated rats (^{10}Panx 50 µM; i.gl.). (E) Data comparison of Phase 1 (0 to 15 minutes) to Phase 2 (16 to 60 minutes); columns represent the sum of flinches in each phase per group. Results are expressed as mean ± SEM. One-way ANOVA for (A,C,E). #compared to control groups (i.gl vehicle + i.plsaline) and *compared to vehicle i.gl + Cg i.pl or vehicle group. *, #$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ (n = 4–5). Panx1, Pannexin 1; DRG, dorsal root ganglion; Cg, carrageenan; SEM, Standard Error of the Mean; ANOVA, analysis of variance.

30 µM of ^{10}Panx reduced mechanical sensitivity 3 hours after Cg (Fig. 1A) compared to the control group (vehicle + Cg). The doses of 100 and 500 µM of ^{10}Panx presented the highest reduction in pain intensity, and the 50 µM dose corresponded to the submaximal effect (Fig. 1B). In another group of animals, capsaicin (10 µM) or formalin (2.5%) was injected in the paw of rats 30 minutes after intraganglionar injection of ^{10}Panx (50 µM) or vehicle (saline). The treated rats showed lower nociceptive response to capsaicin (Fig. 1C) and to phase 2 of formalin (Fig. 1D). Formalin induces one hour of spontaneous pain response with two characterized phases: Phase 1 consists of the first 10 minutes, where it notices a high number of finches followed by a substantial drop. Phase 2 starts at 16 to 60 minutes (Fig. 1E).

3.2 Panx1 Modulates TRPV1$^+$ Neuron Activation

In vitro capsaicin administration (5 µM) to DRG cell cultures induced an immediate calcium influx in small diameter DRG neurons, as indicated by the Fluo-4AM fluorescence intensity increase in these cells (Fig. 2A-I; Fig. 2A-II; Fig. 2B). Pre-incubation with Panx1 channel blocker (^{10}Panx 50 µM) for 30 minutes significantly reduced the levels of capsaicin-induced intracellular calcium in transient receptor potential vanilloid subtype 1 (TRPV1)$^+$ neurons, as shown by lower fluorescence intensity in treated dishes compared to untreated (Fig. 2A-III; Fig. 2A-IV; Fig. 2B; Fig. 2C). Although Panx1 blockage decreased the calcium influx induced in neuronal DRG cells by capsaicin administration, the number of responding cells was not significantly different between the different treat-

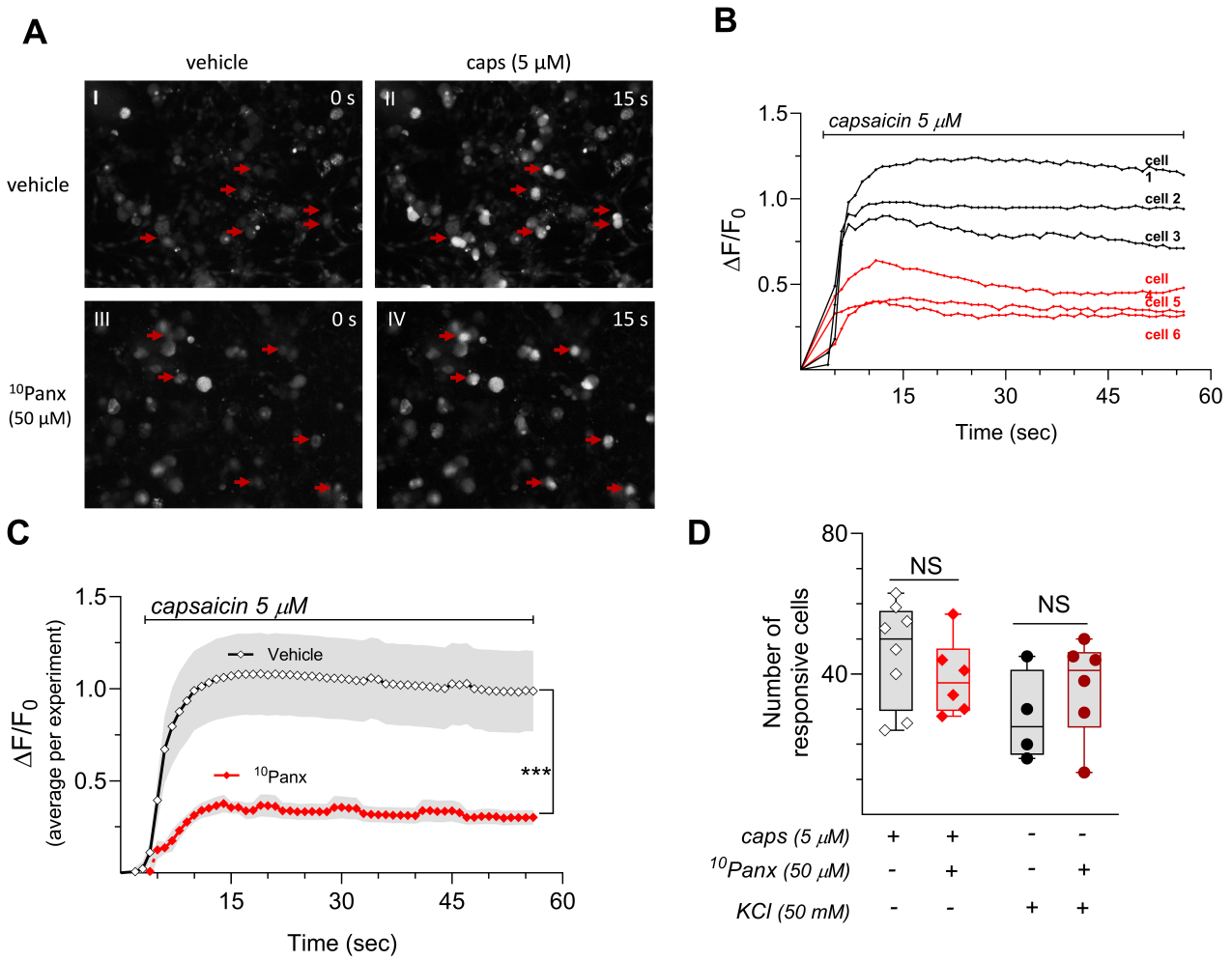

Fig. 2. Panx1 inhibition in DRG cell culture decreases calcium influx induced by capsaicin in neurons. (A) Representative primary DRG cell culture images before and after capsaicin and in two conditions. (I) Basal Fluo-4AM fluorescence emission by neurons (red arrows) of a control dish (previously incubated 30 minutes with Hank's buffered saline solution). (II) Increased fluorescence intensity in the same neurons from image (I), indicating increase in intracellular calcium levels, at 15 seconds following capsaicin (5 µM) administration. (III) Basal Fluo-4AM fluorescence emission by neurons (red arrows) in culture pre-treated 30 minutes with ^{10}Panx (50 µM). (IV) Increased fluorescence intensity in the same neurons from image (III) after 15 seconds of capsaicin (5 µM) administration. (B) Representative intensity of fluorescence of neuronal cells after capsaicin 5 µM from control and treated (^{10}Panx) group administration. (C) Quantification intensity of fluorescence in neurons, maximum values of ΔF/F0 induced by capasacin (5 µM) on DRG cell cultured pre-treated with ^{10}Panx (50 µM) or vehicle (Hank's buffered saline solution). (D) Number of responsive neuronal cells after KCl (50 mM) administration on different conditions. Results are expressed as mean ± SEM. For (C) One-way ANOVA of the average peak amplitude of each group. Three experiments were done on separate days (2 animals per experiment, a total of six rats). For each experiment n = 5–8 dishes (vehicle and ^{10}Panx). Number of analyzed cells was >25 per dish. ***$p < 0.001$, NS means non-significant.

ments (Fig. 2D). In addition, in Panx1 blocker-treated and untreated cultures, the number of cells responding to direct bath administration of buffer enriched with KCl (50 mM) was also not different (Fig. 2D).

3.3 Absence of Panx1 in Mice Prevents Mechanical Allodynia Induced by Carrageenan and Nociception Response to Capsaicin

After verifying the role of Panx1 expressed in the primary afferent neuron of rats in inflammatory pain and nociception response, we performed pain behavior assessments in Panx1 global knockout mice (Panx1-KO), aiming to investigate whether non-neuronal cells (immune cells) that express Panx1 can also show a participation at pain phenotypes. Capsaicin (10 µg/paw) was administrated into the peripheral tissue of WT and KO mice via intraplantar injection. The number of flinches was recorded for 5 minutes in both groups. As shown in Fig. 3A, the nociceptive behavior induced by capsaicin in Panx1-KO mice was remarkably lower than observed in WT, with values similar to the control group which did not receive capsaicin.

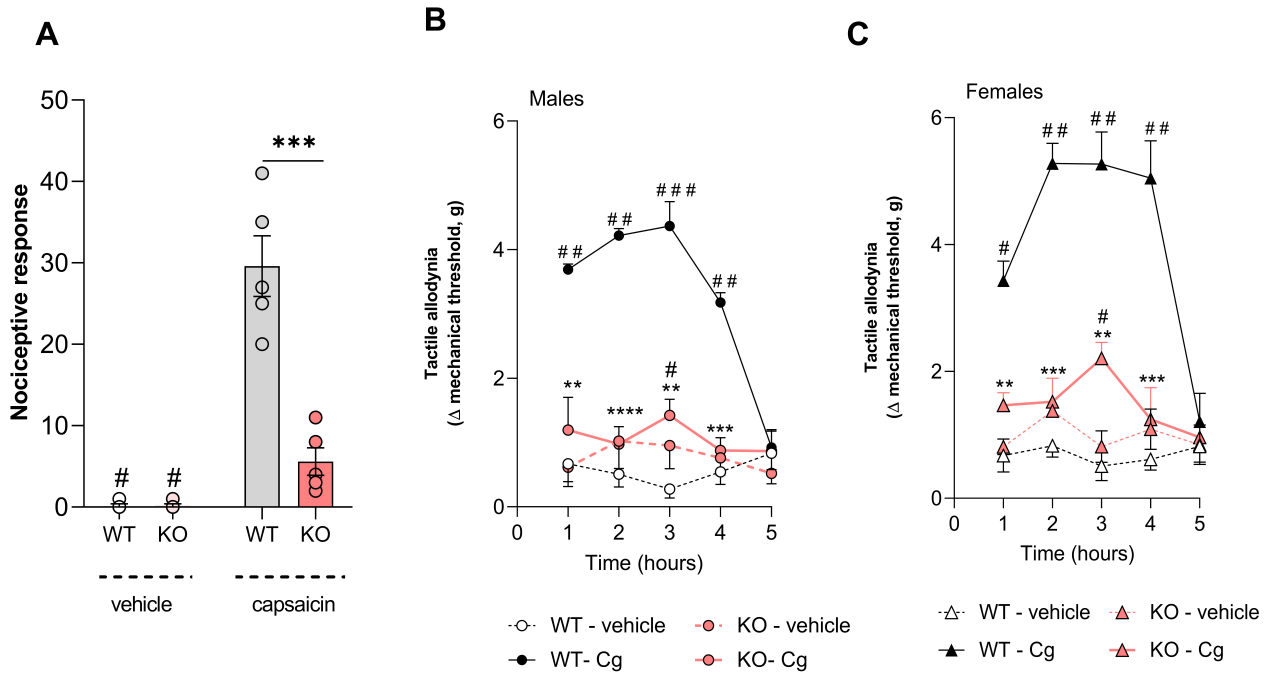

Fig. 3. Panx1-KO mice showed lower nociceptive response to capsaicin and reduced Cg-induced mechanical allodynia. (A) Nociceptive response to intraplantar injection of capsaicin (10 µg/paw) by WT and Panx1-KO mice. (B) Tactile allodynia (data expressed as Δ: mechanical threshold at each hour evaluated subtracted by basal measurement) of hind paw in male WT or Panx1-KO mice treated with Cg (100 µg/paw), or vehicle (saline) (n = 4). (C) Tactile allodynia (data expressed as Δ: mechanical threshold at each hour evaluated subtracted by basal measurement) of hind paw in female WT or Panx1-KO mice treated with Cg (100 µg/paw), or vehicle (Saline, i.pl.). Results are expressed as mean ± SEM. One-way ANOVA for A, ***$p < 0.001$ (WT- capsaicin compared to KO-capsaicin). Two-way ANOVA followed by Tukey's multiple comparison test for (B,C). # compared to control groups (WT/KO vehicle) and * compared to WT-Cg group. #$p < 0.05$, **, ##$p < 0.01$, ***, ###$p < 0.001$ (n = 4-5), ****$p < 0.0001$. WT, wildtype; KO, knockout.

The single dose of Cg (100 µg/paw) in WT mice induced mechanical allodynia in either male or females when compared to controls (saline) (Fig. 3B,C). The sensitization lasted up to 4 hours, returning to the baseline threshold 5 hours after injection. Male or female Panx1-KO mice Cg group (100 µg/paw) presented mechanical sensitivity only at the 3rd hour after injection, and in all other time points, no significant differences were detected between KO Cg-injected and its control group. Even though the KO-treated group showed significant allodynia at the 3rd hour, the intensity was significantly lower than the WT-treated group (Fig. 3B,C). Regarding the baseline tactile sensitivity between the strains, no significant difference was detected on von Frey between WT and Panx1-KO mice and between male and female at naïve conditions (data not shown).

3.4 Absence of Panx1 in Mice does not Prevent Long-Lasting Pain Induced by Paclitaxel

Subsequently, we investigated the participation of Panx1 channels in chemotherapy-induced peripheral neuropathy (CIPN). A single systemic injection of paclitaxel (2 mg/kg; i.p.; Fig. 4) induced short-lasting mechanical allodynia in male and female WT mice that resolved 7 hours after the injection (Fig. 4B,C). WT-female mice presented higher intensity of allodynia than WT-male mice at the five-hour time point (male mean: 2.265; female mean: 5.065, *$p < 0.05$; Fig. 4D). In contrast to those observed in WT mice, both male and female Panx1-KO mice did not develop mechanical hypersensitivity in any time point evaluated (Fig. 4B,C), suggesting that Panx1 channels participate in promoting the acute pain induced by paclitaxel.

Knowing that paclitaxel has been applied for cancer patients' treatment in more than one dose and frequently in women with ovary cancer [24], we performed four systemic administrations of paclitaxel in female WT and Panx1-KO mice (Fig. 5A). The injections of paclitaxel (2 mg/kg/i.p./injection; every other day) promoted chronic mechanical pain in both genotypes that lasted more than 21 days (Fig. 5B). After the first dose of paclitaxel, KO mice did not show mechanical allodynia; however, chronic pain was indeed developed from the second and onward doses. Even so, the intensity of allodynia until the 8th day was significantly lower in the Panx1-KO group compared to the WT-treated group (Fig. 5B), suggesting a partial role for Panx1 in mechanisms underlying the initial peripheral neuronal sensitization.

4. Discussion

The current study demonstrates that Panx1 in the DRG plays a critical role in mechanisms of peripheral inflamma-

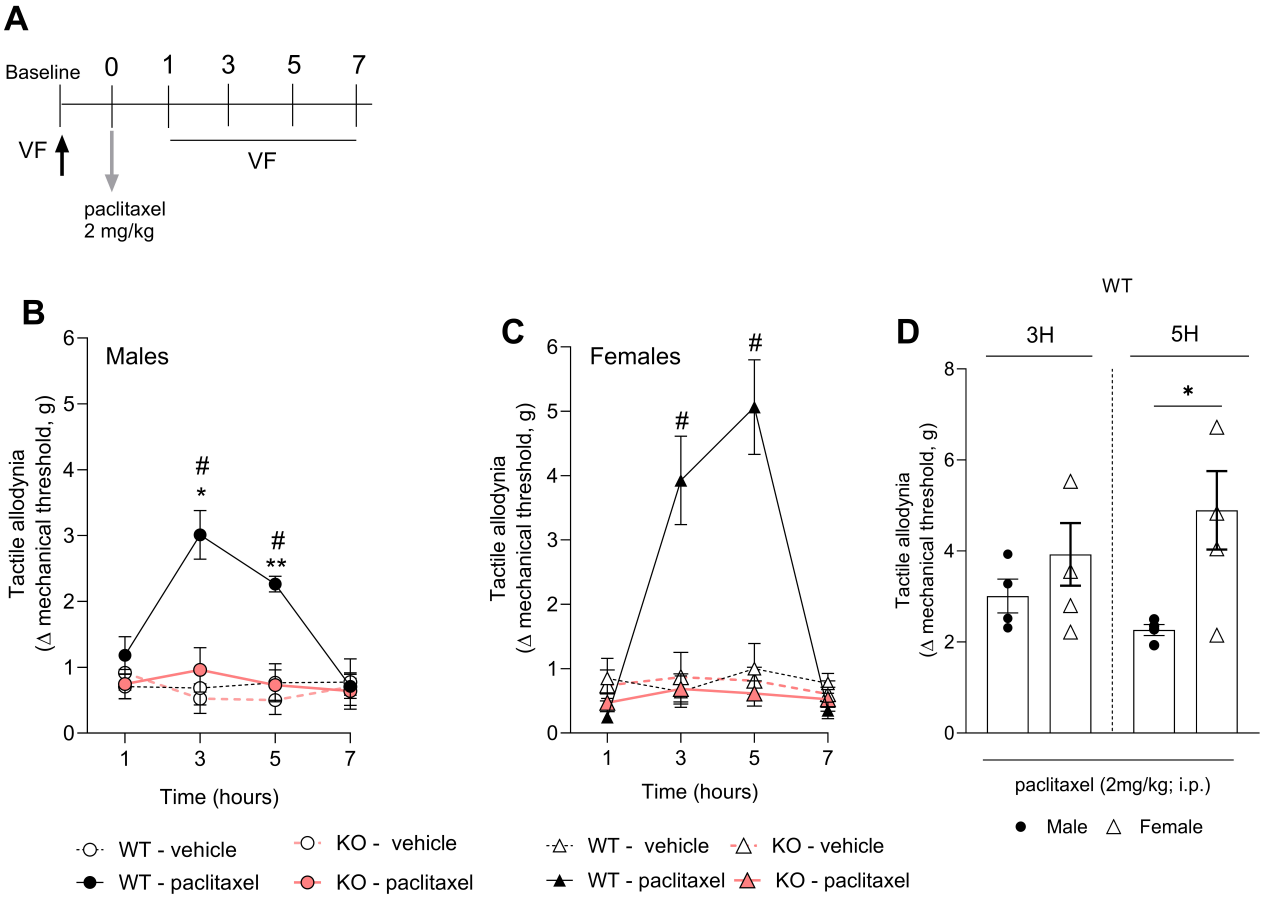

Fig. 4. A single dose of paclitaxel induced acute pain in WT mice. (A) Experimental design of behavior assessment in mice, VF means von Frey test. (B) Tactile allodynia (data expressed as Δ: mechanical threshold at each hour evaluated subtracted by basal measurement) of hind paw in male mice at 1, 3, 5, 7 hours after a single i.p. paclitaxel injection. (C) Tactile allodynia (data expressed as Δ: mechanical threshold at each hour evaluated subtracted by basal measurement) of hind paw in female mice at the same time point. (D) Comparations between WT male with WT female after i.p. paclitaxel injection at 3, 5 hours in von Frey test. Results are expressed as mean ± SEM. *$p < 0.05$, ** $p<0.01$ for WT-Paclitaxel compared to KO-Paclitaxel; #$p < 0.05$ for WT Paclitaxel compared to WT vehicle. Two-way ANOVA followed by the Tukey multiple comparison test (n = 4). VF means von Frey test.

tory pain and nociception transmission involving activation of TRPV1$^+$ nociceptor. In contrast, Panx1 was shown not to be required for paclitaxel-induced neuropathy since the absence of these channels did not preclude the long-term establishment of allodynia in treated mice.

The mechanism(s) by which Panx1 acts on the peripheral nervous system to change the nociceptor threshold directly or indirectly is still poorly understood. In the periphery, these channels are present in the sensory ganglia and in different compartments of nerves, such as axons and myelin [25]. Following nerve injury, an upregulation of Panx1 protein expression in the DRG and Schwann cells was observed, which was accompanied by mechanical and thermal sensitivity [11,12]. The inflammatory orofacial pain model induced by Complete Freund's Adjuvant (CFA) injection into the submandibular skin promoted an increase of Panx1 levels in trigeminal ganglia that was responsible for the maintenance of a hyper-excitable state [7]. Here, we performed subcutaneous administration of an inflammatory agent, carrageenan, in rat and mouse paws to induce peripheral inflammation. Cg injection promotes local interleukin 1 beta (IL-1β) release that stimulates the synthesis of prostaglandin E_2 (PGE_2) via activation of isoenzyme cyclooxygenase-2 (COX-2) [26]. PGE_2 ultimately binds to prostanoid (EP) receptors in the nociceptors triggering a cascade of intracellular signal that promotes changes in neuronal membrane channels expression, such as Nav channels [27]. These changes result in neuronal sensitization and, consequently, mechanical pain development [26,28]. We have now proved for the first time that Panx1 is involved in the development of peripheral inflammatory pain induced by Cg. The pre-treatment with Panx1 blocker directly into the L5-DRG of rats or genetic depletion in mice significantly reduced mechanical allodynia after injection of Cg. It has been shown that upon activation, Panx1 channels release ATP and participates in NLRP3 inflammasome

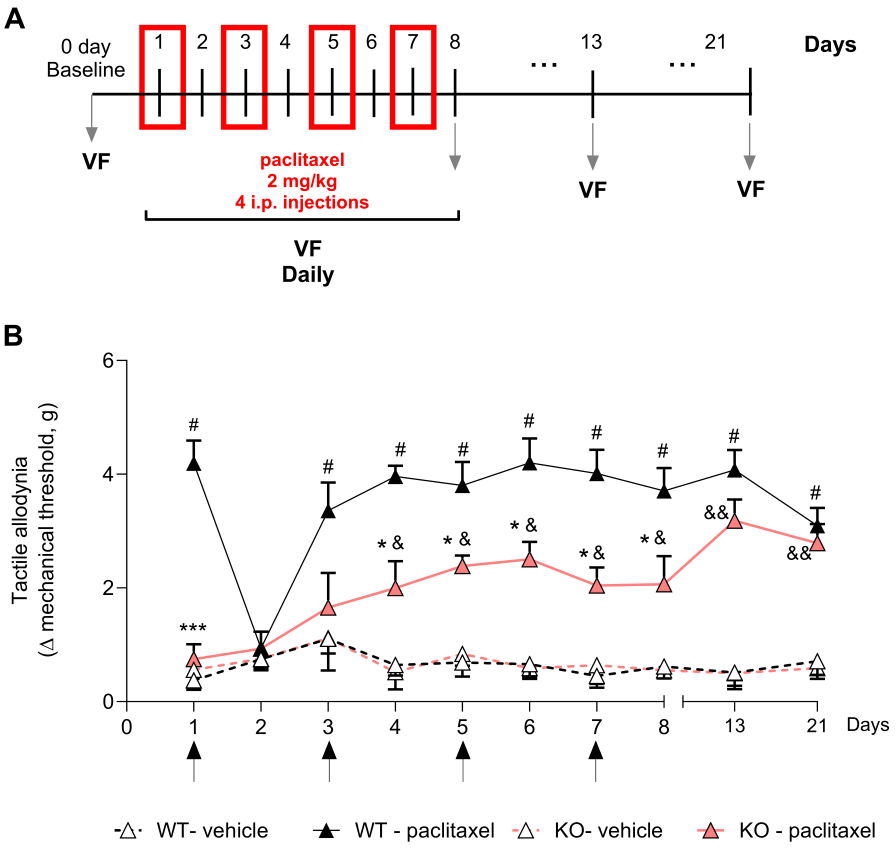

Fig. 5. Panx1-KO mice developed chronic Paclitaxel-induced Neuropathy although with lower intensity in the initial phase. (A) Experimental design and timeline: paclitaxel intraperitoneal injection were performed on days 1, 2, 5 and 7 and von Frey test on baseline and day 8 until 21, daily. (B) Tactile allodynia of female mice on right paw along 21 days. From day 4 to day 8, KO-paclitaxel group was significant diffrent to WT-paclitaxel group ($^{\#}p < 0.05$). Results are expressed as mean ± SEM. Two-way ANOVA followed by Tukey's multiple comparison test $^{\#}p < 0.001$ compared with WT-vehicle; $^{*}p < 0.05$, $^{***}p < 0.001$ compared with WT-paclitaxel; $^{\&}p < 0.05$, $^{\&\&}p < 0.01$ compared with KO-vehicle, n = 4 per group and genotype. VF means von Frey test.

activation, leading to caspase-1 cleavage and IL-1β production [7,9]. In this context, blockage of Panx1 would interrupt or reduce the activation of this inflammatory cascade, resulting in less mechanical pain, as seen in the present study. In addition, there is strong evidence showing the direct interaction of Panx1 with purinergic receptors (ATP-activated receptors), such as P2X7 receptors, during inflammatory and pain states [29]. Our research group previously demonstrated that local blockade of P2X7 receptors in the DRG also reduces the mechanical allodynia induced by carrageenan [15]. Accordingly, in trigeminal ganglion cultures, the autocrine and paracrine ATP signaling triggered by activation of the P2X7-Panx1-P2X complex induced long-lasting biphasic inward currents in neurons, which seems to contribute to orofacial pain [30]. Besides the interaction with other receptors, Panx1 may mediate the interaction between glial cells and neuronal cell bodies within sensory ganglia, which might reduce the input signal to the secondary neuron. Thus, the local blockage of Panx1 in the DRG could promote a reduction in the intensity of the input signal to the spinal cord, thereby resulting in an analgesic effect that would explain the reduced mechanical hypersensitivity in animals treated with carrageenan and ^{10}Panx.

The formalin test presents a stereotyped behavior with two well-defined phases. Phase 1 lasts ±10 minutes, followed by a short period of remission; phase 2 starts at 15 minutes and is sustained for one hour. The initial phase is attributed to a direct excitation of nociceptive neurons, named as algogenic effect, whereas in phase 2, local endogenous mediators such as histamine and serotonin are released [31–33]; for this reason, it can be considered an inflammatory phase. Our data showed that Panx1 expressed in the DRG was important for nociceptive response in phase 2 but not in phase 1, which collaborates with the Cg result, reaffirming the role of Panx1 in peripheral inflammatory process.

Studies that we conducted *in vivo* and *in vitro* using capsaicin disclosed a potential functional interaction between Panx1 and TRPV1 channels in peripheral mechanisms of pain modulation. Capsaicin stimulates nociceptive neurons by activating the TRPV1 channel, a cationic chan-

nel selectively expressed in nociceptive c-fiber [34]. Activation of TRPV1 induces acute nociception initially by mediating Na^{+} and Ca^{2+} influx and depolarizing peripheral c-fiber [35]. Our data revealed that the Panx1 blockade at the DRG level reduced the number of paw flinches and licking of the rat's paw injected with capsaicin. Likewise, Panx1-KO mice showed a lower nociceptive response to capsaicin injection than the WT group. Consistently with the behavior data, *in vitro* blockade of Panx1 channels in DRG cell cultures decreased the amplitude of the calcium transients evoked by capsaicin in neurons, suggesting a functional interplay between TRPV1 activation and Panx1 channels. One hypothesis for this relationship is that both channels physically interacted in the neuronal membrane, and blocking Panx1 channels would reduce TRPV1 activity.

Different Panx1 blockers, such as Carbenoxolone, Probenecid, Mefloquine, and Brilliant Blue dye, were previously used in other pain studies; however, according to recent works, some do not show selectivity; Carbenoxolone can also block gap junctions. For this reason, ^{10}Panx is still the best option for more precise and accurate data regarding the role of Panx1 [8].

In the CIPN model, we observed that Panx1 channels are not required for the chronic establishment of paclitaxel-induced neuropathic pain. However, Panx1-KO-treated mice displayed a two-day delay in the development and reduced mechanical allodynia up to 8 days post-treatment compared to WT-treated mice. Strikingly, one single injection of paclitaxel did not induce acute mechanical pain in Panx1-KO mice. These results suggest that Panx1 plays a partial role in the initial phase of neuronal sensitization in the paclitaxel model. However, other elements begin to emerge and contribute to establishing chronic pain. Nevertheless, in the spared nerve injury (SNI) model, global Panx1-KO mice did not develop chronic pain [36] and intrasciatic injection of Panx1 blocker ten days after chronic constriction injury (CCI) effectively reduced mechanical and heat hypersensitivity [12]. Although it seems contradictory to our results, the neuronal sensitization process caused by chemotherapy drugs might involve different mechanisms compared to a surgical model. Previous studies revealed that paclitaxel causes deficits in mitochondrial bioenergetics, macrophage infiltration, and increased sodium channels in DRG [21,37,38]. More recently, there have been reports of increased Toll-like receptor 4 (TLR4), lipid rafts, and TRPV1 protein expression in the DRG after treating male mice with paclitaxel [39]. All these factors may explain why the absence of functional Panx1 receptor expression did not prevent chronic neuropathic pain induced by paclitaxel. Yet, a group of researchers observed an increase of glutamate release in the cerebrocortical synaptosomes of Oxaliplatin-treated rats which was mediated by functional recruitment of Panx1-P2X7R. By using Panx1 inhibitors they demonstrated reversal on neuropathic pain induced by oxaliplatin [40].

Another aspect that can be involved in Paclitaxel-induced neuropathy is the type of nociceptors. A study revealed that Paclitaxel induces A-fiber hypersensitization, contributing to mechanical allodynia [41]. In our data, Panx1 is shown to be associated with TRPV1 receptors, which are expressed in c-fibers neurons. This can be the reason that Panx-1 KO mice stayed allodynic after four injections of Paclitaxel, as WT mice.

It is important to mention that studies involving genetically modified animals can present some limitations. In Panx1-KO mice, the mRNA expression that leads to Panx1 protein translation is mostly but not completely abolished in different cell types and tissue. Thus, they are considered functional knockout [18]. Also, there might be compensatory effects on the expression and/or function of non-depleted channels, which can result in modifications of animal physiology. The sensory system is not excluded from these changes. In animals with genetic deletion for Panx1, it was seen an auditory sensory loss [42] and an increase in the expression of *Panx3* channels in sensory nerve fibers of the nasal epithelial layer [43]. However, no study has yet shown changes in receptor expression in nociceptive neurons of Panx1-KO mice. Besides that, we observed here a deficient spontaneous pain response (acute nociception) in Panx1-KO mice at the capsaicin test compared to WT group, indicating a potential modification of TRPV1 protein expression or activity in these genetically modified mice.

5. Conclusion

The data presented in this work brings attention to the critical role of Panx1 channels in developing peripheral inflammatory pain and acute nociception involving TRPV1 activation and the lack of involvement of these channels in CIPN induced by paclitaxel. Considering the DRG is located outside of the blood-brain barrier, targeting Panx1 channels expressed in the DRG may provide a novel and exciting approach to inflammatory pain treatment.

Availability of Data and Materials

All data points generated or analyzed during this study are included in this article and there are no further underlying data necessary to reproduce the results

Author Contributions

Conceived and designed the experiments: JBPL, CAP, CMdCL, SOS. Performed the experiments: JBPL, KFM, NSC, AFN, MU-M, CMN. Analyzed the data: JBPL, KFM, AFN, CCF, PRGK. Wrote the paper: JBPL, KFM, CCF. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

All experimental protocols were approved by the Albert Einstein College of Medicine Animal Care and Use Committee (IACUC) (protocol number 00001131) and by the Committee on Animal Research CEUA-UNICAMP (protocol number 4566-1/2017, 4566-1(A)/2017).

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Conflict of Interest

The authors declare no conflict of interest.

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