Molecular Neurobiology
https://doi.org/10.1007/s12035-023-03237-7

Electrical Stimulation of the Auricular Branch Vagus Nerve Using Random and Alternating Frequencies Triggers a Rapid Onset and Pronounced Antihyperalgesia via Peripheral Annexin A1-Formyl Peptide Receptor 2/ALX Pathway in a Mouse Model of Persistent Inflammatory Pain

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Received: 29 April 2022 / Accepted: 13 January 2023

Abstract
This study evaluated the antihyperalgesic and anti-inflammatory effects of percutaneous vagus nerve electrical stimulation (pVNS) by comparing the effects of alternating and random frequencies in an animal model of persistent inflammatory hyperalgesia. The model was induced by Freund's complete adjuvant (CFA) intraplantar (i.pl.) injection. Mice were treated with different protocols of time (10, 20, or 30 min), ear laterality (right, left or both), and frequency (alternating or random). Mechanical hyperalgesia was evaluated, and some groups received i.pl. WRW4 (FPR2/ALX antagonist) to determine the involvement. Edema, paw surface temperature, and spontaneous locomotor activity were evaluated. Interleukin-1β, IL-6, IL-10, and IL4 levels were verified by enzyme-linked immunosorbent assay. AnxA1, FPR2/ALX, neutrophil, M1 and M2 phenotype macrophage, and apoptotic cells markers were identified using western blotting. The antihyperalgesic effect pVNS with alternating and random frequency effect is depending on the type of frequency, time, and ear treated. The pVNS random frequency in the left ear for 10 min had a longer lasting antihyperalgesic effect, superior to classical stimulation using alternating frequency and the FPR2/ALX receptor was involved in this effect. There was a reduction in the levels of pro-inflammatory cytokines and an increase in the immunocontent of AnxA1 and CD86 in mice paw. pVNS with a random frequency in the left ear for 10 min showed to be optimal for inducing an antihyperalgesic effect. Thus, the random frequency was more effective than the alternating frequency. Therefore, pVNS may be an important adjunctive treatment for persistent inflammatory pain.

Keywords Electrical stimulation · Inflammation · Vagus nerve · Annexin · Hyperalgesia · Frequency

Introduction

A common problem that results in high healthcare costs for governments is persistent or chronic pain of inflammatory origin [1–3]. It is associated with a decrease in quality of life due to the functional impairment that it causes [4, 5]. Inflammatory pain resulting from inflammatory disease has its etiology in the sensitization of primary nociceptive neurons by the direct action of inflammatory mediators [6, 7]. Pro-inflammatory inflammatory cytokines are inflammatory mediators that sensitize primary nociceptive neurons [8–10]. During the inflammatory response, these cytokines modulate phagocytosis, gene expression, and cell apoptosis [9]. The gold standard preclinical model for studying inflammatory pain is the model using complete Freund's adjuvant (CFA) administered intraplantarly (i.pl.) [11, 12]. This model is widely used

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Published online: 06 February 2023

because it causes mechanical hyperalgesia behavior while allowing the quantification of mediators and cells involved in the inflammatory process [13–15]. In the resolution phase, specialized lipid mediators such as annexin A1 (AnxA1) are synthesized to counter the initial insult [16, 17]. AnxA1 synthesis occur through the regulation of the glucocorticoids [18, 19] and its interaction with the formyl peptide receptor 2/ALX (FPR2/ALX) promotes pro-resolving effects, such as reduction of pro-inflammatory cytokines, inhibition of neutrophil infiltration, increased outflow of phagocytes, and macrophage polarization [20–22]. However, if there is no adequate response from the organism, inflammation becomes persistent [23, 24].

Bioelectronic medicine has presented various options for non-pharmacological treatments, such as vagus nerve stimulation (VNS) [25]. VNS has been approved by the International Food and Drug Administration (FDA) in the USA in the clinical conditions: refractory epilepsy and depression [26, 27]. However, there is still research on its therapeutic potential in headaches, arthritis, asthma, pain, fibromyalgia, bipolar disorder, and dementia [26, 28]. In addition, both anti-inflammatory [29–33] and analgesic [30, 34–36] effects caused by electrostimulation of the auricular branch of the vagus nerve (VN) have already been demonstrated in clinical [37] and non-clinical models [38, 39].

Electrostimulation of the auricular branch of VN becomes interesting [40], since it is a minimally invasive method, which promotes greater adherence to treatment VN has already been described as a potent regulator of at least three important pathways in the control of inflammation, such as hypothalamic–pituitary–adrenal (HPA) axis, vago-vagal reflex, and sympathetic-splenic [27, 41, 42]. The HPA axis when stimulated by the VN induces the release of the hormone corticosterone [43], which consequently induces the release of AnxA1 [18, 19]. The electrostimulation parameters of VNS used also have an influence on pain (such as time, ear laterality, and frequency), as observed in a study in humans that compared the frequency of 1 Hz and 25 Hz in chronic migraine and observed that 1 Hz had a longer lasting effect [35]. Further, studies with percutaneous electrical stimulation lack standardization of parameters; however, it is known that the alternating frequency type is already widely used in practice and present preclinical evidence regarding anti-hyperalgesic and anti-inflammatory effects [44, 45], and more recently, the random frequency has shown effects more at the level of modulation of brain areas [46–49]. Based on the above, it became interesting to understand the effects of electrical stimulation of the VN auricular branch on hyperalgesia and the role of the peripheral AnxA1-FPR2/ALX pathway in this effect, in an animal model of persistent inflammatory hyperalgesia.

Material and Methods

Animals

Female Swiss mice were used (weighed between 30 and 45 g), obtained from the Central Animal Facility of the Federal University of Santa Catarina (UFSC). Animals were acclimated to 22 ± 2 °C, in a 12 h light/12 h dark cycle (light from 7 am) with free access to food and water and without environmental enrichment. The criteria for exclusion and euthanasia were as follows: signs of immobility, inability to ingest water/feed, epidermal injury, seizures, or vocalization without being stimulated. On the day of the experiment, animals were randomly distributed among groups, through the automatic generator randimization.com. As a strategy to minimize potential variables that could inferred in data collection, we acclimated the animals in the laboratory for at least 1 h before the tests, in room with controlled temperature and humidity. Protocols, such as adequate restraints during some stages, were also taken to minimize the effects generated by handling the animals.

Evaluators were blinded for the conduction of the experiments. All experiments were performed after approval by the Ethics Committee on the use of Animals (CEUA), under protocol numbers 18.048.2.07.IV and 20.007.4.08.IV. Animals were treated in accordance with the ethical principles established by the Brazilian College of Animal Experimentation [50].

Drugs

The following substances were used: saline (LBS Laborasa Indústria Farmacêutica, São Paulo, SP, Brazil); Tween (1,000,422, Sigma-Aldrich®, St. Louis, MO, USA); isoflurane (1–2%), ketamine (150 mg/kg), xylazine (30 mg/kg), CFA (50%, 20 µL/site), WRW4 (Trp-Arg-Trp-Trp-Trp-NH2, Tocris, Bioscience®, Bristol, UK); peroxidase conjugate second antibody (1:5000, Goat anti-rabbit-HRP; Cell Signaling Technology, Danvers, MA, USA); goat anti-MMR/CD206 polyclonal antibody (1:1000, AF2535, R&D Systems®, Minneapolis, USA); mouse anti-CD86 monoclonal antibody (1:1000, ab213044, Abcan®, Cambridge, MA, USA), rabbit anti-arginase-1 monoclonal antibody (1:1000, 93668S, Cell Signaling Technology, Danvers, MA, USA); anti β-actin-HRP primary antibody (1:45,000, Sigma-Aldrich® Co, St. Louis, MO, USA); anti-iNOS (NOS-2) (1:2000, NBP1-33,780, Novus Biologicals, Centennial, CO, USA); rabbit anti-AnxA1 monoclonal antibody (1:1000, v/v, Cell Signalling Technology, Beverly, MA, USA); anti-FPR2/ALX primary antibody

(1:500, Santa Cruz Biotechnology, Santa Cruz, CA, USA); anti-Bax antibody (1:1000, Biolegend®, USA); ELISA Kits for mice: IL-1β (catalog number DY401-05, R&D Systems®, Minneapolis, MN, USA), IL-4 (catalog number DY404-05, R&D Systems®, Minneapolis, MN, USA), IL-6 (catalog number DY406-05, R&D Systems®, Minneapolis, MN, USA), IL-10 (catalog number DY417, R&D Systems®, Minneapolis, MN, USA).

Induction of Persistent Inflammatory Hyperalgesia

The induction of the animal model of persistent inflammatory hyperalgesia was performed by an intraplantar (i.pl.) injection of 20 µl of CFA 50% (0.5 mg/ml) into the ventral surface of the right hind paw [51, 52].

pVNS Treatment

Animals treated with pVNS were lightly sedated with 2% inhaled isoflurane and 100% oxygen via a nasal mask. After sedation, needling (0.18 mm × 8 mm) was performed in the right ear, or left ear, or both in the A2 and B2 region of the concha cavity (cymba and cavum). Region A2 corresponds to the anteromedial portion of the cymba concha and region B2 to the posteromedial portion of the cavum concha [53]. Each needle was connected to an NKL 608 electrostimulation device cable. Two stimulus programs were used: (1) alternating frequency (mixed) between F1 and F2 and (2) random frequency in an interval between F1 and F2. The alternating frequency, F1 consisted of 2 Hz stimuli held for 5 s and F2 consisted of 10 Hz stimuli held for 5 s. Other parameters were selected: pulse width of F1: 700 µs; F1 "on" time: 5 s; F1 rest time: 0 s and F2 = 10 Hz; non-polarized; F2 pulse width: 200 µs; F2 "on" time: 5 s; F2 rest time: 0 s). Treatment times chosen were 10, 20, and 30 min [44, 54] and the intensity was 0.8 mA. This intensity was selected according to the result of a pilot experiment (data not shown) which revealed that this was the maximal intensity that did not provoke signs of discomfort in the animals behavior, and it was similar to the intensity used in other pre-clinical pVNS studies [38, 39] for both alternating and random frequencies treatment. The CFA + pVNS*off* group (control) underwent the same preparation, sedation, and needle insertion procedures in the same auricular points; however, the device was turned off.

Study Design

At 6 and 96 h after CFA injection in the animals' right hind paw, curves of different treatment times (10, 20, and 30 min), treatment ear (right, left, and both), and different frequencies (alternating and random) were performed to verify the effect on mechanical hyperalgesia, assessed by the von Frey test (vF). After defining the best parameters, we carried out experiments to evaluate the effect of repeated treatment, tolerance (10th day), and spontaneous locomotor activity (11th day) of treatments. The effect of pVNS on paw edema and surface temperature was evaluated 4 h after treatment daily until the 5th day. We used a pharmacological/behavioral test to investigate the involvement of peripheral FPR2/ALX receptors in this effect. Animals were pre-treated with the WRW4 antagonist (i.pl.) and mechanical hyperalgesia was evaluated by the vF test. In addition, at 6 and 96 h after CFA injection, the spleen (only at 96 h) and skin of the right hind paw were collected. The immunocontent for M1 and M2 phenotypes of macrophages, FPR2/ALX receptor, AnxA1, and apoptotic cells were analyzed. In addition, concentrations of pro- and anti-inflammatory cytokines in the skin of the right hind paw and in the spinal cord were measured at 6 h and 96 h after CFA injection (Fig. 1).

Groups used for the experiments were as follows: Naive (without stimulation and without injection of CFA); CFA (without stimulation, only with injection of CFA in the right hind paw); Saline (injection into the right hind paw with

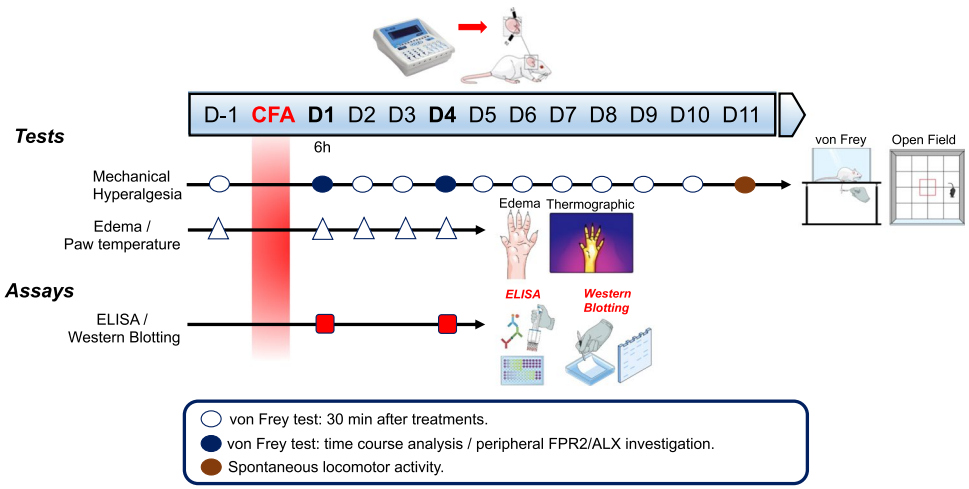

Fig. 1 Schematic representation of the experimental design. CFA, Freund's complete adjuvant; D, days; h, hours

saline and without stimulation); CFA + pVNS*off* (CFA injection in the right hind paw and without electrostimulation, only needling at different times [CFA + pVNS*off* 10 min or pVNS*off* 30 min] and laterality of the ear); CFA + pVNS (CFA injection in the right hind paw, with needling and electrical stimulation at different times and ear laterality); WRW4 (CFA and WRW4 injection in the right hind paw). Treated groups were compared to the CFA + pVNS*off* and the Saline or Naive groups were compared to the CFA group.

Sample Size

The number of animals used was the minimum necessary to demonstrate the effects obtained by the treatment (n/total = 448), requiring the number of 8 animals per group, based on Daniel [55]. The calculation presents the following equation to obtain a confidence coefficient of 95%: $n = \{[(z\ alpha + z\ beta) * s]/sigma\}^2$. The total number of animals per experiment were as follows: curves to evaluate the best time and ear laterality ($n = 168$); antihyperalgesic tolerance and spontaneous locomotor activity ($n = 24$); paw surface edema and temperature ($n = 40$); peripheral mechanism FPR2/ALX ($n = 56$); concentration of pro and anti-inflammatory cytokines at 6 h and 96 h ($n = 80$); analysis of FPR2/ALX, AnxA1, M1, and M2 phenotype of macrophages in immunocontent ($n = 80$).

Assessment of Mechanical Hyperalgesia

Mechanical hyperalgesia was evaluated using a vF monofilament (0.6 g, VFH, Stoelting, Chicago, USA) [13]. Animals were placed individually in an acrylic observation chamber (9 × 11 cm), without a bottom and covered with a lid. The chamber was positioned on a 6 mm wire mesh platform (70 × 40 cm). The frequency of the right hind paw withdrawal response was expressed as percentage values (up to 100%) and interpreted as nociceptive behavior. Time-course experiments were performed evaluating mechanical hyperalgesia to define the best pVNS time (10 min, 20 min, or 30 min) and ear laterality (right, left or both ears) at 6 h and 96 h. To assess analgesic tolerance, animals were treated daily and evaluated before and after treatment until they were tolerant to treatment [56–58].

Assessment of Paw Edema

Edema assessment was performed by measuring the thickness of the right hind paw (medial portion) using a digital micrometer. Data were expressed by the absolute values obtained in micrometers (μm) [44].

Assessment of Paw Surface Temperature

Temperature of the ventral surface of the right hind paw (central region) was evaluated using a thermography camera with an accuracy of ± 0.1 °C and an infrared spectrum range of 7.5 to 13 m, using a cold/hot palette and temperature variation between 20 and 40 °C. Data were expressed as absolute values obtained in degrees Celsius (°C) [44, 59].

Assessment of Spontaneous Locomotor Activity

Spontaneous locomotor activity was recorded on the 11th day after the last intervention of the tolerance experiment. In the test, animals were placed in a square container (40 cm × 40 cm × 40 cm) for a period of 5 min. The total distance traveled the total time spent in the central area [20 cm × 20 cm] and a number of entries in the central zone and full speed were measured using the ANYmaze software (Stoelting, USA). The apparatus was sanitized with ethanol solution (10%) at each test to minimize possible trails induced by animal odors [60, 61].

Administration of the FPR2/ALX Receptor Antagonist

Investigation of peripheral FPR2/ALX receptor involvement was performed at 6 h and 96 h after CFA injection. Animals were pre-treated via i.pl. with WRW4 (antagonist for the FPR2/ALX receptor) at a dose of 10 μg/20 μl/i.pl., while the CFA + pVNS*off* group was pre-treated with saline (20 μl/i.pl.) [45]. After 15 min, animals received the pVNS treatment and mechanical hyperalgesia was assessed 30 min after the treatments.

Procedure for Collecting Tissue Samples

To analyze cytokine concentration and to perform western blotting, 30 min after treatment (of each animal), animals were euthanized and tissue samples were collected by dissection from the following: skin of the right hind paw, lumbar spinal cord (whole segment L3–L5 at 6 h or 96 h), and spleen (at 96 h after CFA injection). Samples were placed inside 2-mL Eppendorf microtubes and stored at a – 80 °C freezer for posterior analysis.

Western Blotting Analysis

Samples were pulverized and incubated in lysis buffer [100 mM Na2VO4, 100 mM phenylmethylsulfonyl fluoride and a 1% protease inhibitor cocktail (P8340—Sigma-Aldrich Co., LLC, St. Louis, MO, USA)] in T-Per (Tissue Protein Extraction Reagent; Thermo Scientific,

Rockford, IL, USA), and then incubated on ice for 30 min. After centrifugation at 6000 × g for 20 min (4 °C), the supernatant was collected, separated, and stored in a − 80 °C freezer. Protein content was measured by the Bradford method [62], using a calibration standard curve with BSA (0.05 to 0.5 mg/mL). Aliquots of total protein (50 µg) were boiled at 95 °C for 5 min at 25% volume in Laemmli buffer (1 M sodium phosphate pH 7.0, 10% sodium dodecyl sulfate (SDS), 10% β-mercaptoethanol, 50% glycerol, 0.1% bromophenol blue). The samples were submitted to polyacrylamide gel electrophoresis. After electrophoresis, the proteins were transferred to a PVDF membrane at a constant voltage of 90 V for 2 h. After 1 h of blocking with 5% skim milk (Molico® Nestlé), the membranes were incubated overnight (4 °C) with the following primary antibodies: anti-AnxA1 (1:1000, v/v, Cell Signaling Technology, Beverly, MA, USA); FPR2/ALX (1:500, Santa Cruz Biotechnology, Santa Cruz, CA, USA); anti-Bax (1:1000, Biolegend, USA); anti-arginase-1 (1:1000, 93668S, Cell Signaling Technology, Danvers, MA, USA); anti-CD86 (1:1000, ab213044, Abcan, Cambridge, MA, USA); anti-MMR/CD206 (1:1000, AF2535, R&D Systems®, Minneapolis, USA); anti-Ly-6G (1:1000, R&D Systems®, Minneapolis, USA); anti-iNOS (NOS-2) (1:2000, NBP1-33,780, Novus Biologicals, Centennial, CO, USA); anti-β-actin-HRP (1:45,000, Sigma-Aldrich Co, St. Louis, MO, USA). After incubation, the membranes were washed for 30 min with TBS-T and incubated with the peroxidase-conjugated secondary antibody (1:10,000, goat anti-rabbit-HRP; Cell Signaling Technology, Danvers, MA, USA) (except for anti-rabbit-actin-HRP) for 1 h at room temperature. After this period, a 30-min wash with TBS-T was performed, followed by exposure of the membranes to the chemiluminescence kit (ECL) and developed using a photodocumentation device (iBright Imaging Systems, Invitrogen/Thermo Fisher Scientific, Waltham, MA, USA). Semiquantitative band analysis were performed by densitometry using the Image Studio Lite program (LI-COR, Biosciences, USA). Values were normalized using the β-actin values and expressed graphically as arbitrary units.

Enzyme-Linked Immunosorbent Assay

For determination of IL-1β, IL-6, IL-4, and IL-10 levels, 100 µL of each sample were used. These cytokine concentrations analyses were performed using the Duo Set Enzyme-Linked Immunosorbent Assay Kits (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions. Values were estimated by interpolating data using a standard curve for each cytokine by a colorimetric assay, which was measured at 450 nm (correction at 540 nm) in a spectrophotometer (Perlong DNM-9602, Nanjing Perlove Medical Equipment Co., Nanjing, China). Values obtained were expressed in picograms per milligram [63, 64].

Statistical Analysis

The data obtained were analyzed using the statistical program GraphPad Prism (version 8.0, La Jolla, CA, USA). The Shapiro–Wilk normality test was performed, and data were identified as parametric. Data were presented as mean ± standard deviation and was compared using one-way analysis of variance (ANOVA) (locomotor activity, cytokines, western blotting data) or two-way ANOVA (pVNS treatment curves, tolerance, edema, temperature of paw surface, peripheral mechanism data) followed by the Bonferroni or Tukey test. In the comparison between the CFA and saline groups, the unpaired *t*-test (cytokines and western blotting, supplementary Figs. 2–6) was used. In all analyses, *p* values less than 0.05 were considered statistically significant. There was no exclusion of data due to sample loss.

Results

pVNS with Alternating Frequency Reduces Mechanical Hyperalgesia 6 h After CFA Injection

Results presented in Figs. 2, 3, 4, and 5 demonstrate that the CFA paw injection induced mechanical hyperalgesia in the right hind paw of the mice, which persisted throughout the evaluation period. Figure 2 illustrates the curves of best time and laterality of ear in animals treated with alternating frequency 6 h after CFA injection. pVNS on right ear for 10, 20, or 30 min significantly ($p < 0.05$ to $p < 0.001$) inhibited mechanical hyperalgesia for 0.5 h (Fig. 2A). pVNS on left ear for 10, 20, or 30 min significantly ($p < 0.001$ to $p < 0.001$) decreased mechanical hyperalgesia for 1 h (Fig. 2B). The pVNS on both ears for 10 min significantly ($p < 0.05$ to $p < 0.001$) reduced mechanical hyperalgesia for 1 h. Treatment for 20 or 30 min was not effective (Fig. 2C). Additionally, the area under the curve (AUC) analysis shows that mechanical hyperalgesia was significantly reduced ($p < 0.05$) by treatment of animals with pVNS on left ear for 10 or 30 min (Fig. 2E). No significant differences were observed in the AUC analysis of treatments with pVNS on right ear or both ears (Fig. 2D and F).

pVNS with Alternating Frequency Decreases Mechanical Hyperalgesia 96 h After CFA Injection

Results presented in Fig. 3 demonstrate the curves of best time and laterality of ear in animals treated with alternating frequency 96 h after CFA injection. pVNS on right ear for 10 min, but not 20 or 30 min,

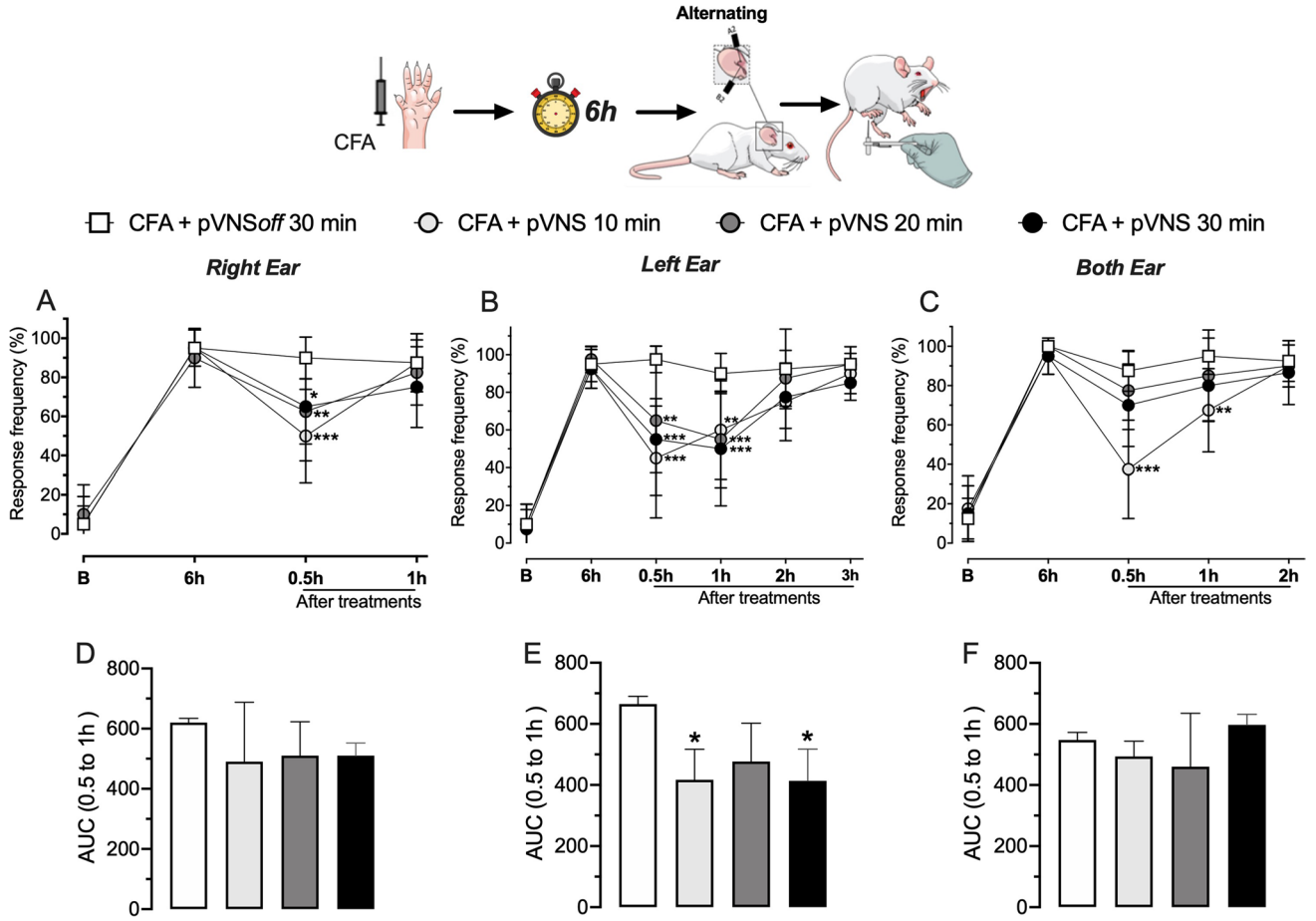

Fig. 2 Time-course analysis of the antihyperalgesic effect of pVNS with alternating frequency performed with different stimulation times in the right ear (**A**), left ear (**B**), or both ears (**C**) at 6 h after CFA injection. *$p<0.05$, **$p<0.01$, and ***$p<0.001$ when compared to the CFA + pVNS*off* 30 min group. Two-way analysis of variance (ANOVA) and analysis of area under the curve (one-way ANOVA, **D**–**F**). pVNS, percutaneous vagus nerve stimulation; AUC, area under the curve; CFA, Freund's complete adjuvant

significantly ($p<0.0001$) inhibited mechanical hyperalgesia for 0.5 h (Fig. 3A). pVNS on left ear for 10 min significantly ($p<0.0101$) decreased mechanical hyperalgesia for 2 h (Fig. 3B) and for 1 h with 30 min ($p=0.0007$, Fig. 3B) and for 0.5 h with 20 min ($p=0.0019$, Fig. 3B) of pVNS. The pVNS on both ears for 10 min significantly ($p<0.01$ to $p<0.001$, Fig. 3B) reduced mechanical hyperalgesia with analgesic effect lasting for 1 h and for 0.5 h with 30 min ($p=0.0016$, Fig. 3C) of pVNS. Treatment for 20 min and with the device turned off was not effective (Fig. 3C). The AUC analysis shows that mechanical hyperalgesia was significantly reduced by treatment of animals with pVNS on left ear or both ears for 10 or 30 min ($p<0.05$ to $p<0.01$, Fig. 3E and F). No significantly differences were observed in the AUC analysis of treatments with pVNS on right ear (Fig. 3D).

pVNS with Random Frequency Attenuates Mechanical Hyperalgesia 6 h After CFA Injection

The data presented in Fig. 4 demonstrate that the pVNS on right ear for 10 or 30 min significantly ($p<0.05$ and $p<0.001$) reduced mechanical hyperalgesia induced by CFA with analgesic effect lasting for 0.5 h (Fig. 4A). pVNS on left ear for 10 min significantly ($p<0.0001$) decreased mechanical hyperalgesia for 3 h (Fig. 4B) and for 1 h with 20 min ($p=0.0047$), and for 0.5 h with 30 min ($p=0.0005$) of pVNS. The pVNS on both ears for 10, 20, or 30 min significantly ($p<0.001$) reduced mechanical hyperalgesia for 0.5 h (Fig. 4C). The AUC analysis shows that mechanical hyperalgesia was significantly reduced by treatment of animals with pVNS on left ear for 10 min ($p=0.0003$, Fig. 4E). No significantly differences were observed in the AUC analysis of treatments with pVNS on right ear or both ears (Fig. 4D–F).

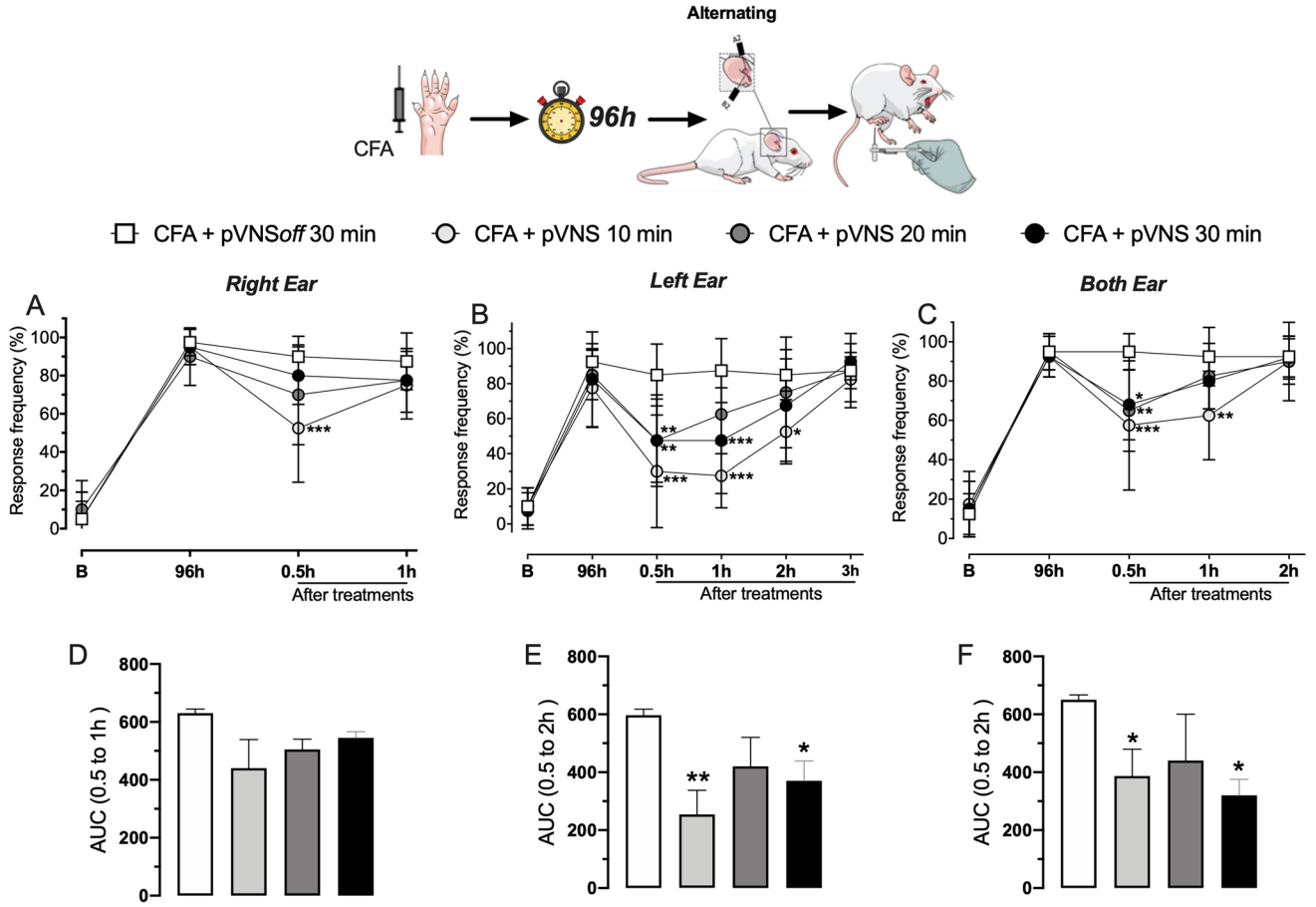

Fig. 3 Time-course analysis of the antihyperalgesic effect of pVNS with alternating frequency performed with different stimulation times in the right ear (**A**), left ear (**B**), or both ears (**C**) at 96 h after CFA injection. $p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ when compared to the CFA + pVNS*off* 30 min group. Two-way analysis of variance (ANOVA) and area under the curve analysis (one-way ANOVA, **D**–**F**). pVNS, percutaneous vagus nerve stimulation; AUC, area under the curve; CFA, Freund's complete adjuvant

pVNS with Random Frequency Inhibits Mechanical Hyperalgesia 96 h After CFA Injection

The data presented in Fig. 5 demonstrate that the pVNS on right ear for 10 or 30 min significantly ($p < 0.001$) inhibited mechanical hyperalgesia induced by CFA with analgesic effect lasting for 0.5 h (Fig. 5A). pVNS on left ear for 10 min significantly ($p < 0.0001$) reduced mechanical hyperalgesia for 2 h (Fig. 5B) and for 1 h with 20 or 30 min ($p < 0.0001$) of pVNS. The pVNS on both ears for 10, 20, or 30 min significantly ($p < 0.0001$) reduced mechanical hyperalgesia for 0.5 h (Fig. 5C). The AUC analysis shows that mechanical hyperalgesia was significantly reduced by treatment of animals with pVNS on the left ear for 10 min ($p = 0.0030$, Fig. 5E). No significantly differences were observed in the AUC analysis of treatments with pVNS on the right ear or both ears (Fig. 5D–F).

Repetitive pVNS Treatments Induces Antihyperalgesia and Tolerance

Results presented showed that the i.pl. injection of CFA induced mechanical hyperalgesia in the right hind paw throughout the evaluation period (up to the 10th day) (Fig. 6). Daily treatments with CFA + pVNS 10 min alternating and CFA + pVNS 10 min Random were effective in reducing mechanical hyperalgesia until the 9th day of treatment, when treated groups were compared to their respective controls. On the 10th day, antihyperalgesic tolerance was observed, as no significant difference was found between the treated groups and the control groups. In addition, during the treatment period, no significant difference ($p > 0.05$, Fig. 6) was observed between the treated groups.

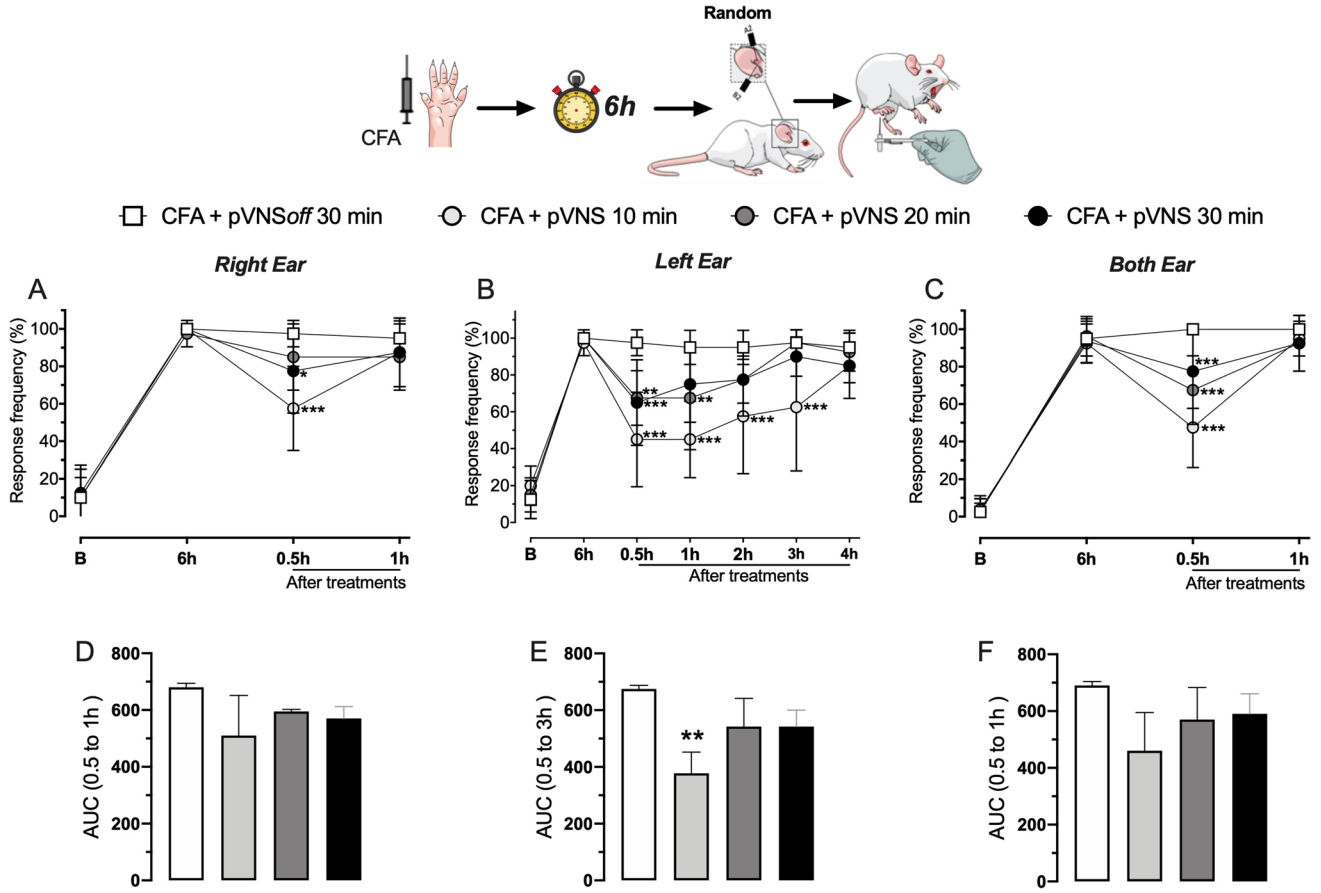

Fig. 4 Time-course analysis of the antihyperalgesic effect of pVNS with random frequency performed with different stimulation times in the right ear (**A**), left ear (**B**), or both ears (**C**) at 6 h after CFA injection. *$p<0.05$, **$p<0.01$, and ***$p<0.001$ when compared to the CFA + pVNS*off* 30 min group. Two-way analysis of variance (ANOVA) and area under the curve analysis (one-way ANOVA, **D**–**F**). pVNS, percutaneous vagus nerve stimulation; AUC, area under the curve; CFA, Freund's complete adjuvant

Repetitive pVNS Reduces Paw Edema and Surface Temperature

On the third day after consecutive treatments, there was a reduction ($p=0.0440$) of paw edema in the CFA + pVNS 10 min Alternating group, when compared to the CFA + pVNS*off* 10 min group (see Supplementary Fig. 1A). A reduction ($p<0.0001$) in the surface temperature of the paw was also observed in the CFA + pVNS 10 min Alternating group and in the CFA + pVNS 10 min Random group, when compared to the CFA + pVNS*off* 10 min group on the second consecutive day of treatment (see Supplementary Fig. 1B).

pVNS Does Not Change Spontaneous Locomotor Activity

No statistical difference was observed in spontaneous locomotor activity between groups in any of the evaluated parameters, such as maximum speed ($p=0.1512$ for Naive vs CFA + pVNS*off* 10 min, $p=0.1728$ for Naive vs CFA + pVNS 10 min Alternating, $p=0.3852$ for Naive vs CFA + pVNS 10 min Random, Fig. 7A); entries in the center ($p=0.8826$ for Naive vs CFA + pVNS*off* 10 min, $p=0.7726$ for Naive vs CFA + pVNS 10 min Alternating, $p=0.9294$ for Naive vs CFA + pVNS 10 min Random, Fig. 7B); time spent in the center ($p>0.9999$ for Naive vs CFA + pVNS*off* 10 min, $p=0.9641$ for Naive vs CFA + pVNS 10 min Alternating, $p=0.9451$ for Naive vs CFA + pVNS 10 min Random, Fig. 7C); and total distance traveled ($p=0.5703$ for Naive vs CFA + pVNS*off* 10 min, $p=0.7295$ for Naive vs CFA + pVNS 10 min Alternating, $p=0.5883$ for Naive vs CFA + pVNS 10 min Random, Fig. 7D).

Involvement of the Peripheral FRP2/ALX and AnxA1 Protein in the Reduction of Mechanical Hyperalgesia Caused by pVNS

Behavioral Pharmacological Assessment

Pretreatment with WRW4 had no effect on withdrawal frequency, and the responses were like those observed on the

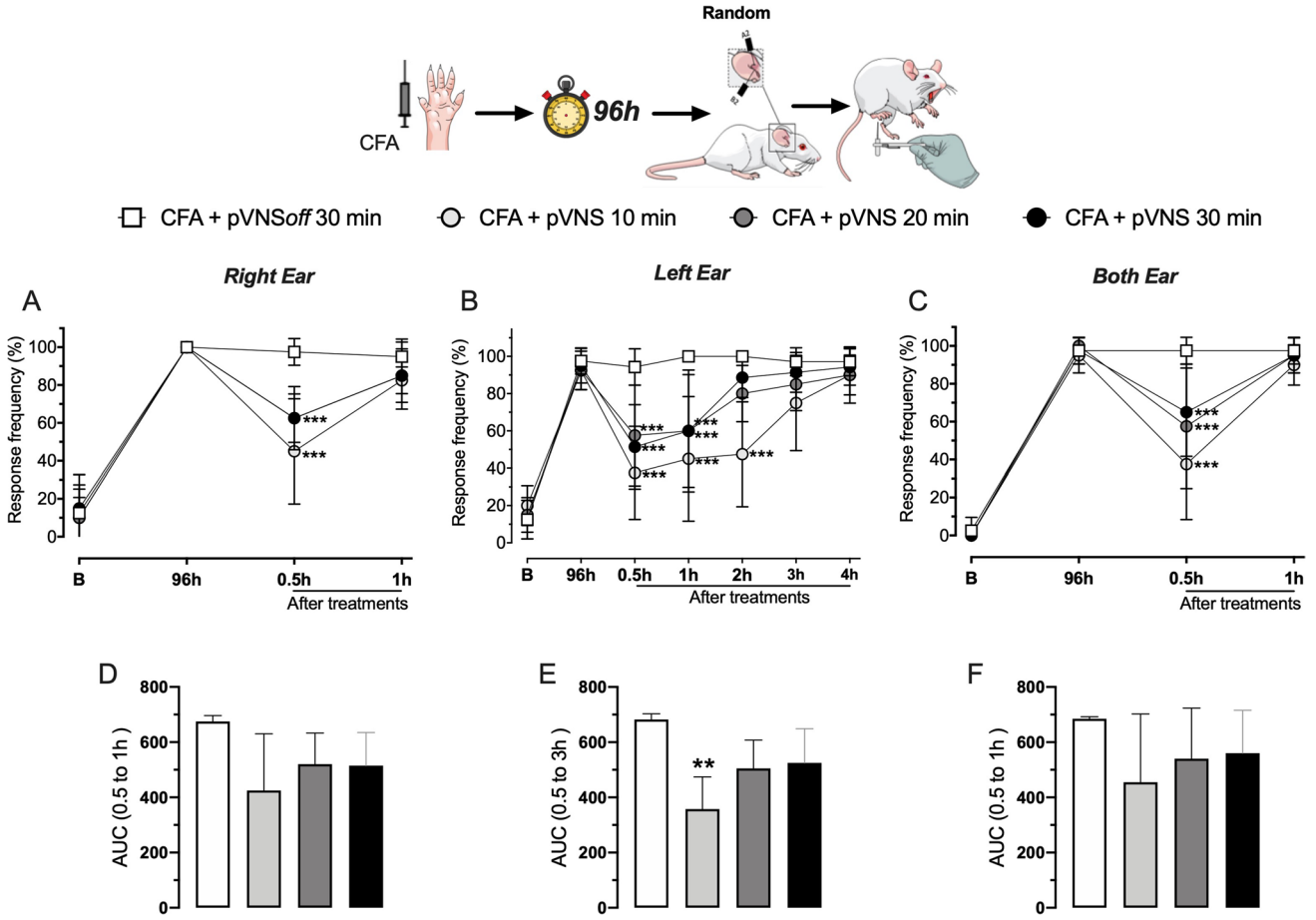

Fig. 5 Time-course analysis of the antihyperalgesic effect of pVNS with random frequency performed with different stimulation times in the right ear (**A**), left ear (**B**), or both ears (**C**) at 96 h after CFA injection. $^{**}p < 0.01$ and $^{***}p < 0.001$ when compared to the CFA + pVNS*off* 30 min group. Two-way analysis of variance (ANOVA) and area under the curve analysis (one-way ANOVA, D–F). pVNS, percutaneous vagus nerve stimulation; AUC, area under the curve; CFA, Freund's complete adjuvant

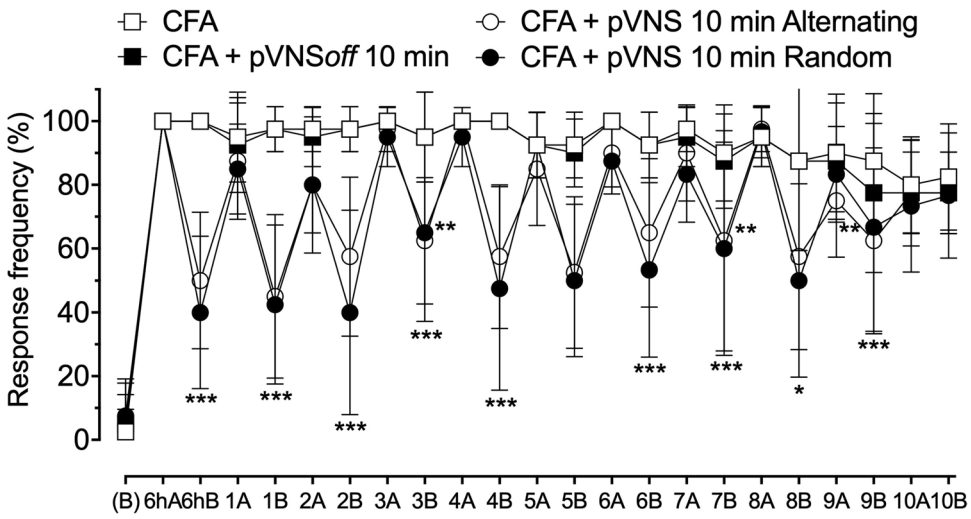

Fig. 6 Effect of pVNS with random and alternating frequencies in the left ear on mechanical hyperalgesia tolerance. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ when compared to the CFA + pVNS*off* 10 min group. Data were analyzed using two-way analysis of variance. CFA + pVNS*off* 10 min group did not receive electrostimulation. pVNS, percutaneous vagus nerve stimulation; AUC, area under curve; CFA, Freund's complete adjuvant; A, after; B, before; (B), basal

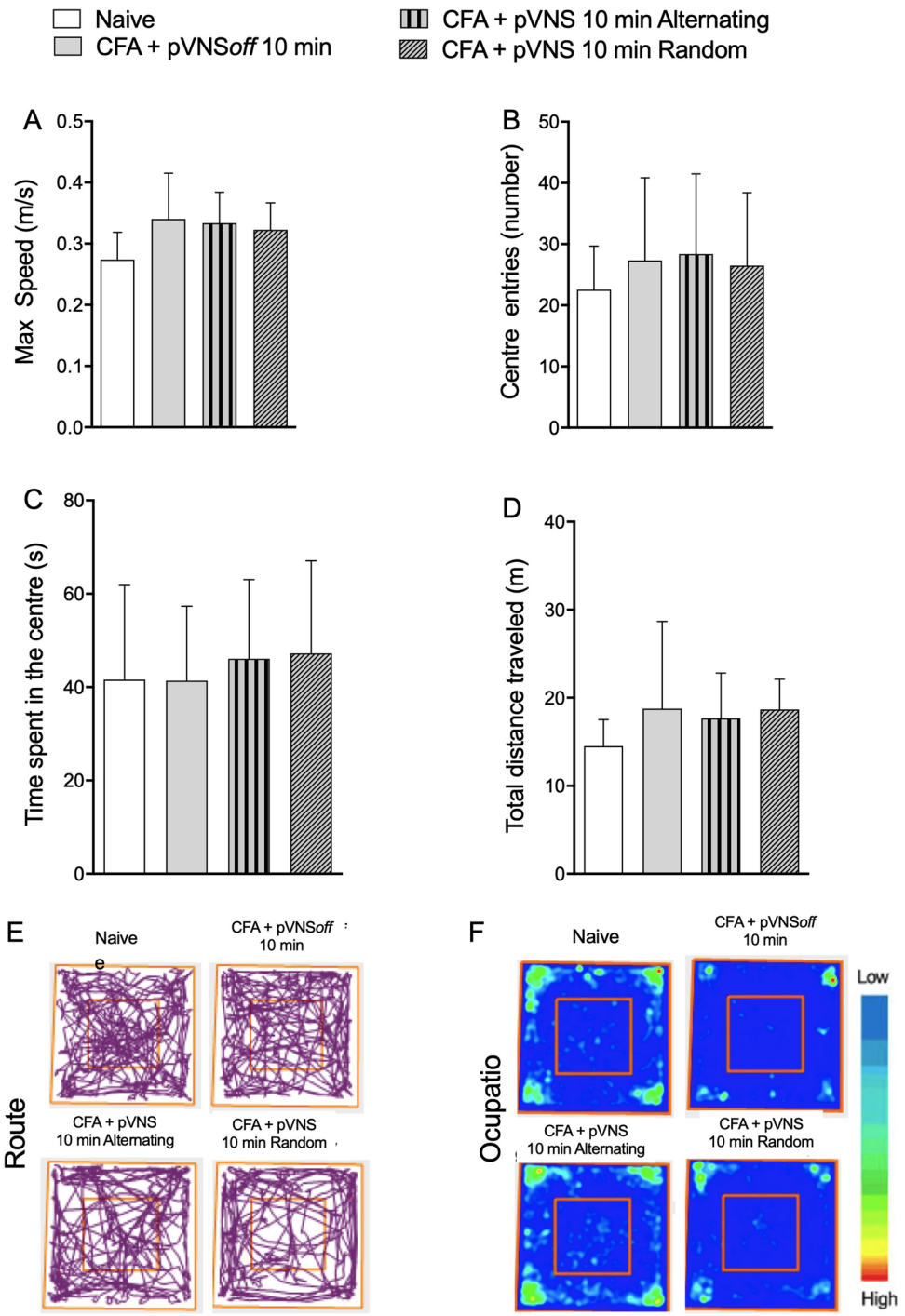

Fig. 7 Effect of pVNS with random and alternating frequencies in the left ear on locomotor activity. In **A** (maximum speed), **B** (entries in the center), **C** (time spent in the center), **D** (total distance traveled), **E** (illustration of groups routes), and **F** (illustration of spaces occupied by groups). Data were analyzed using one-way analysis of variance (ANOVA). pVNS*off* 10 min group did not receive electrostimulation, CFA + pVNS 10 min group received treatment for 10 min, and the Naive group did not receive any type of intervention. pVNS, percutaneous vagus nerve stimulation; CFA, Freund's complete adjuvant

CFA + pVNS*off* 10 min control group (Fig. 8A–D). In addition, the withdrawal frequency values for the groups pretreated with WRW4 were significantly higher compared to those on the CFA + pVNS 10 min Random ($p = 0.0008$) and CFA + pVNS 10 min Alternating ($p = 0.0204$) groups 30 min after treatments (Fig. 8A–D), both at 6 h and 96 h ($p = 0.0009$ for Random and $p = 0.0028$ for Alternating) after CFA injection. Intraplantar administration of WRW4, 15 min before pVNS treatments, prevented the decrease in the withdrawal frequency resulted by the treatment ($p = 0.0017$ for Random and $p = 0.0056$ for Alternating) (Fig. 8B and D) only at 96 h, not on 6 h ($p = 0.2887$ for Random and $p = 0.4496$ for Alternating) after CFA injection.

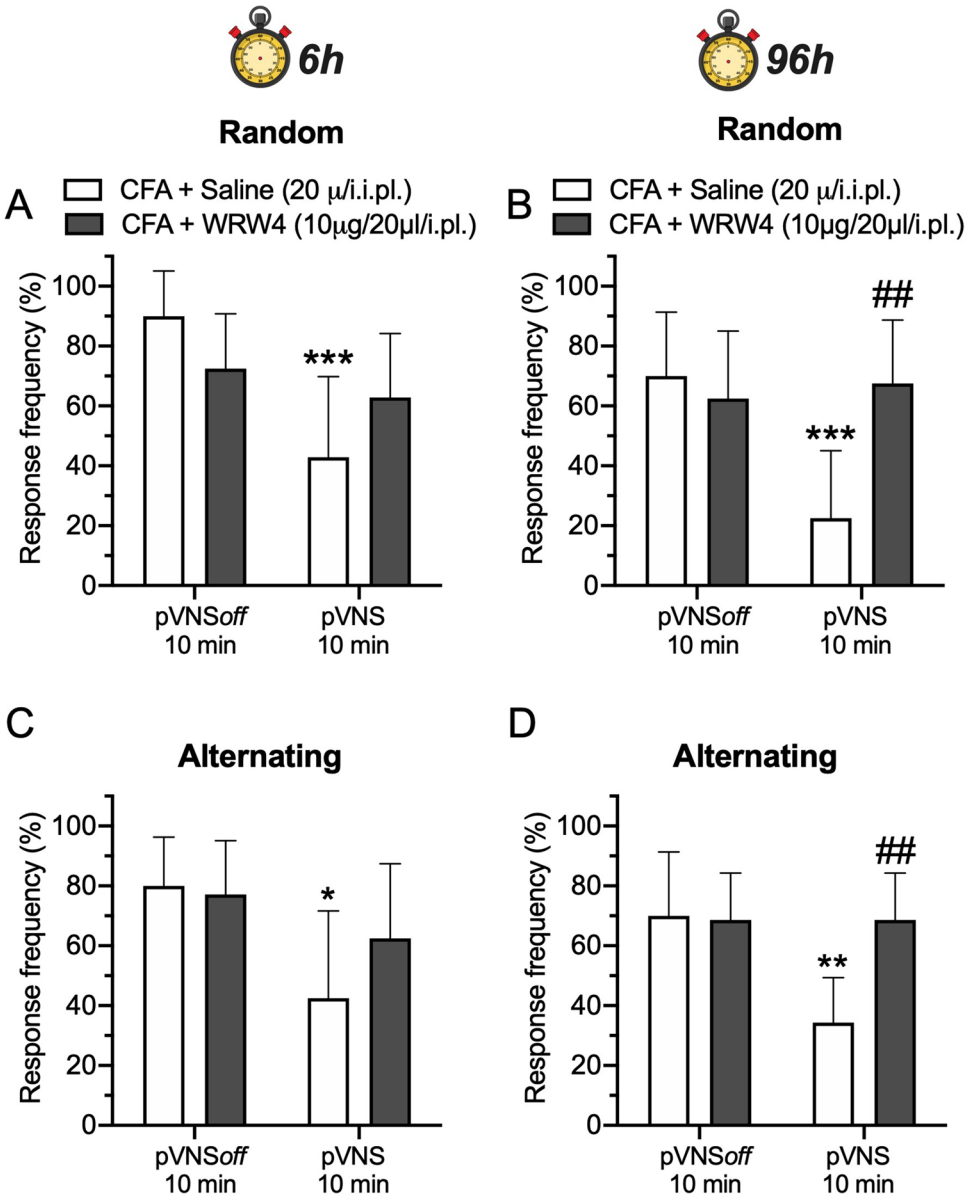

Fig. 8 Intraplantar administration of WRW4 in the reduction of mechanical hyperalgesia caused by pVNS with random vs alternating frequencies in the left ear. $^{*}p<0.05$, $^{**}p<0.01$, and $^{***}p<0.001$ when compared to the pVNS*off* 10 min group. $^{\#\#}p<0.01$ when compared to the pVNS 10 min group. Data were analyzed using two-way analysis of variance (ANOVA). pVNS*off* 10 min group did not receive electrostimulation, pVNS 10 min group received treatment for 10 min, WRW4 group received the FPR2/ALX receptor antagonist i.pl., and the Saline group received saline i.pl. pVNS, percutaneous vagus nerve stimulation; i.pl., intraplantar

Biochemical Evaluation of the Immunocontent of the FPR2/ALX Receptor and AnxA1 Protein

There was an increase in the immunocontent of intact AnxA1 ($p=0.0009$ for 6 h; $p=0.0056$ for 96 h) after CFA injection when compared to the Saline group (see Supplementary Fig. 2A, B). However, CFA injection (6 h or 96 h) did not alter the FPR2/ALX immunocontent in paw skin (see Supplementary Fig. 2C and 2D). The CFA + pVNS 10 min Random group had higher immunocontent of intact AnxA1, both at 6 h ($p=0.0064$) and at 96 h ($p=0.0193$) after CFA injection, when compared to the CFA + pVNS*off* 10 min group (Fig. 9A and B). However, the CFA + pVNS 10 min Random and CFA + pVNS 10 min Alternating groups had higher FPR2/ALX immunocontent in the paw skin only at 6 h after CFA injection ($p=0.0003$; $p=0.0002$, respectively) when compared to the CFA + pVNS*off* 10 min group (Fig. 9C). Treatments did not change the FPR2/ALX immunocontent in the paw skin 96 h after CFA injection (Fig. 9D).

The Effect of pVNS on M1 Macrophages (NOS-2 or CD86), M2 Macrophages (ARG1 or CD206) and Apoptotic Cell (Bax) Markers

There was no change in NOS-2 (M1) immunocontent 96 h after CFA injection (see Supplementary Fig. 3A) between treated groups (Fig. 10A). The CFA group also had ($p=0.0095$, see Supplementary Fig. 3B) higher CD86 (M1) immunocontent when compared to the Saline group. Lower ($p=0.0095$, see Supplementary Fig. 3C) ARG1 (M2)

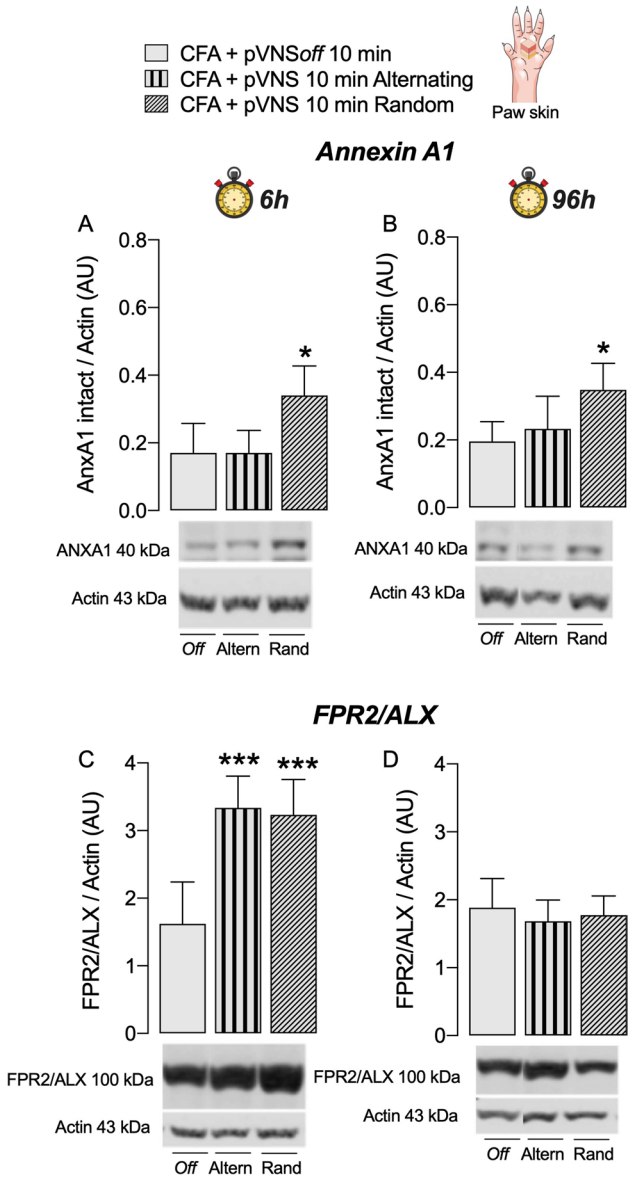

Fig. 9 Effect of random and alternating pVNS frequencies in the left ear on the immunocontent of the FPR2/ALX receptor and the AnxA1 protein in the paw skin. $*p<0.05$ and $***p<0.001$ when compared to the CFA + pVNS*off* 10 min group. One-way analysis of variance (ANOVA) was used to analyze the groups. CFA + pVNS*off* 10 min group did not receive electrostimulation, CFA + pVNS 10 min group received treatment for 10 min. pVNS, percutaneous vagus nerve stimulation; CFA, Freund's complete adjuvant; AnxA1, annexin A1; FPR2/ALX, formyl peptide receptor 2; Off, CFA + pVNS*off* 10 min; Alter, Alternating; Rand, Random

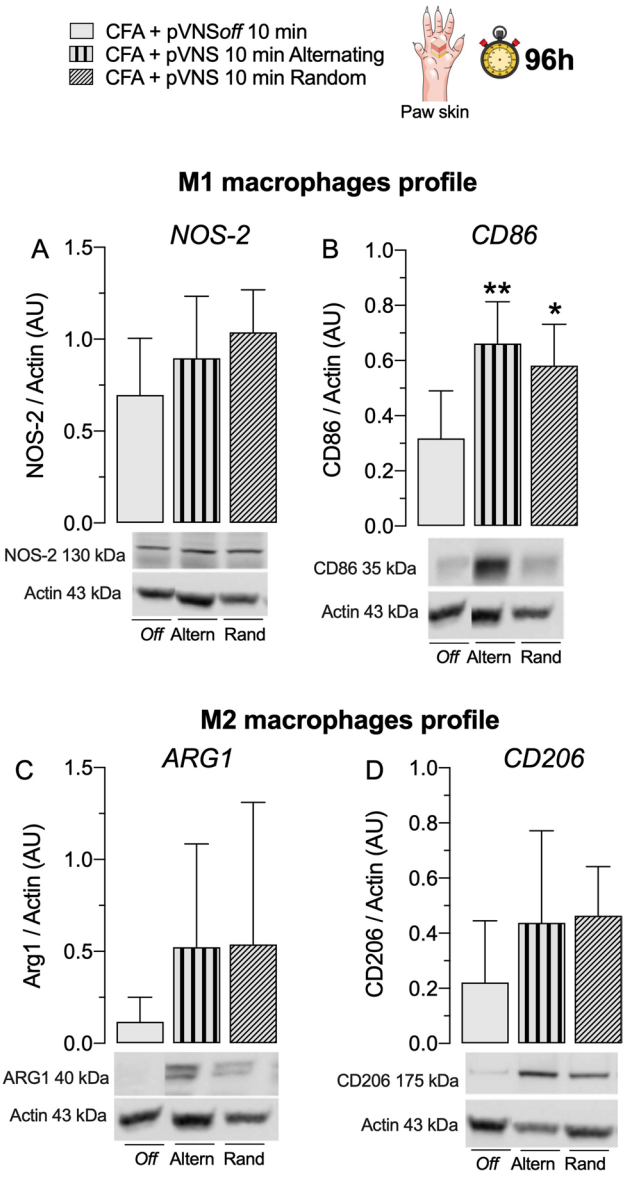

Fig. 10 Effect of pVNS with random and alternating frequencies in the left ear on the expression of macrophages M1 and M2 at 96 h in the paw skin. $p<0.05$, $**p<0.01$ when compared to the CFA + pVNS*off* 10 min group. One-way analysis of variance (ANOVA) was used to analyze the groups. CFA + pVNS*off* 10 min group did not receive electrostimulation, CFA + pVNS 10 min group received treatment for 10 min. pVNS, percutaneous vagus nerve stimulation; CFA, Freund's complete adjuvant; NOS-2, induced nitric oxide synthase 2; Arg 1, arginase 1; CD206, cluster of differentiation 206; CD86, cluster of differentiation 86; *Off*, CFA + pVNS*off* 10 min; Alter, Alternating; Rand, Random

and CD206 (M2) immunocontent ($p=0.0001$; see Supplementary Fig. 3D) were observed in the CFA group when compared to the Saline group. Treated groups CFA + pVNS 10 min Alternating ($p=0.0079$) and CFA + pVNS 10 min Random ($p=0.0305$) had higher CD86 (M1) immunocontent when compared to the CFA + pVNS*off* 10 min group (Fig. 10B).

pVNS Did Not Change Neutrophil and Apoptotic Cell Immunocontent in Paw Skin

In Supplementary Fig. 4C-D, higher neutrophil immunocontent is observed in the paw skin of the group that received CFA, when compared to the Saline group, both

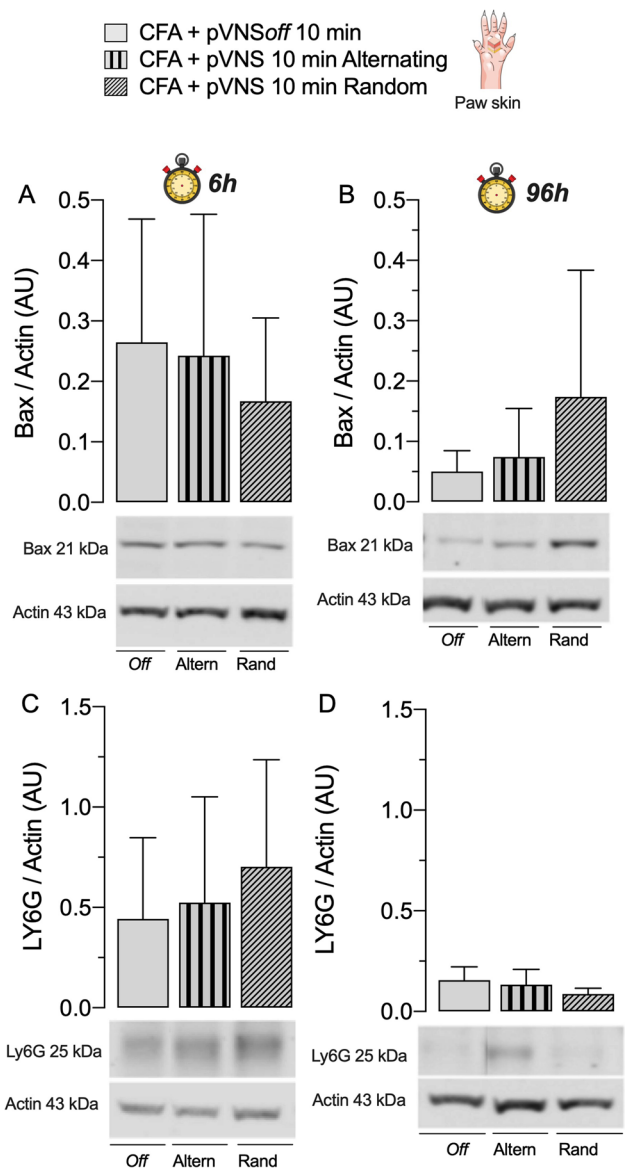

Fig. 11 Effect of pVNS with random and alternating frequencies in the left ear on the expression of neutrophils and apoptotic cells at 6 h and 96 h in the paw skin. One-way analysis of variance (ANOVA) was used to analyze the groups. pVNS*off* 10 min group did not receive electrostimulation, CFA + pVNS 10 min group received treatment for 10 min. pVNS, percutaneous vagus nerve stimulation; CFA, Freund's complete adjuvant; *Off*, CFA + pVNS*off* 10 min; Alter, Alternating; Rand, Random

at 6 h ($p = 0.0407$) and at 96 h ($p = 0.0014$) after CFA injection. However, no difference was observed between groups treated at 6 h and 96 h after CFA injection (Fig. 11C and D). Furthermore, we found no changes in the immunocontent of apoptotic cells at 6 h or 96 h (see Supplementary Fig. 4A an 4B) in the paw skin samples between the experimental groups (Fig. 11A and B).

pVNS Did Not Change Immunocontent of AnxA1, FPR2/ALX, M1 Macrophages (NOS-2 or CD86), M2 Macrophages (ARG1 or CD206) and Apoptotic Cells (Bax) in the Spleen

Ninety-six hours after CFA injection, we observed only a lower immunocontent of M1 macrophages (CD86) in the spleen of the CFA injected animals, when compared to the animals that received saline (see Supplementary Fig. 5B). In markers such as AnxA1, FPR2/ALX, NOS-2, ARG1, CD206, and Bax, no statistically significant changes were found in the spleen, when compared to animals that received saline (see Supplementary Fig. 5) or pVNS treatments (Fig. 12A–G).

pVNS Reduces Interleukin 1β and IL-6 at 6 h in Animal Model of Persistent Inflammatory Hyperalgesia

Six hours after the administration of CFA in the paw of mice, we observed that the CFA group had higher concentrations of IL-1β ($p = 0.0037$) and IL-6 ($p = 0.0001$), as well as lower ($p < 0.0001$) IL-10 and IL-4 concentrations in the paw skin, when compared to the Saline group (see Supplementary Fig. 6A-D). Furthermore, only the IL-1β concentrations of the CFA group were higher ($p = 0.0021$) in the spinal cord when compared to the Saline group at 6 h after CFA administration (see Supplementary Fig. 6E). Interestingly, the CFA + pVNS 10 min Random group had lower concentrations of IL-1β ($p = 0.0478$, Fig. 13A) and IL-6 ($p = 0.0107$ Fig. 13B) in the paw skin, when compared to the CFA + pVNS*off* 10 min group. However, we found no changes in cytokine concentrations in the spinal cord among treated groups (Fig. 13E–H).

Discussion

Percutaneous or transcutaneous vagus nerve stimulation (VNS) are non-invasive alternatives to avoid the risks and costs associated with surgical implantation [40] and battery replacement of the invasive VNS. Those methods have quickly gained popularity. Auricular and cervical stimulation are the most common forms of non-invasive VNS. Both non-invasive strategies show promising results, but few studies have explored the effects of the different parameters and mechanism of action of these methods. To maximize clinical benefits, it is important to optimize the method of application. In the present study, we investigated in an unprecedented way the effects of pVNS in animals with peripheral inflammation. We evaluated the main parameters used in clinical practice and the mechanism of action.

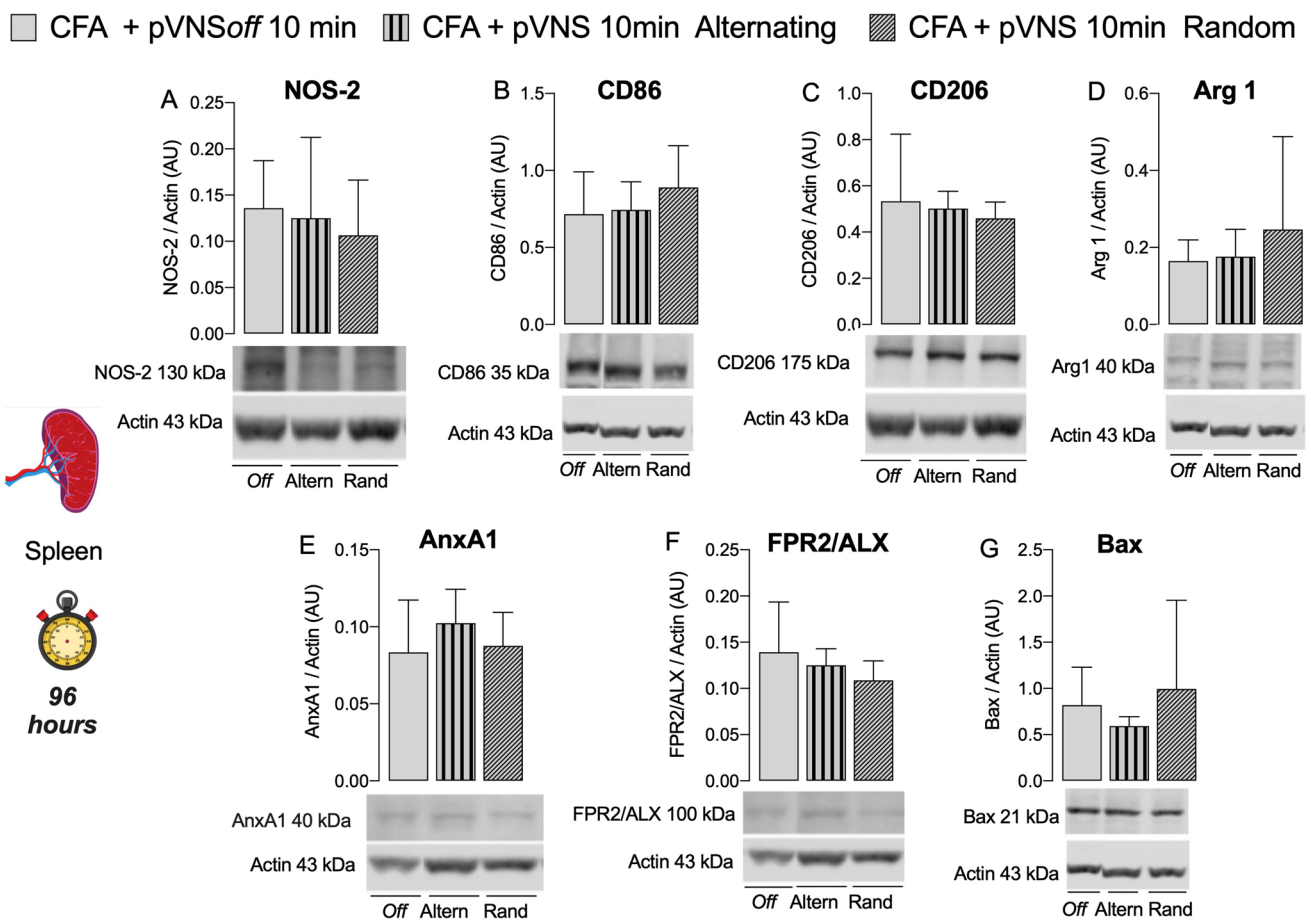

Fig. 12 Effect of pVNS with random and alternating frequencies in the left ear on the expression of M1 (**A**, **B**) and M2 (**C**, **D**) macrophages, AnxA1 (**E**), FPR2/ALX (**F**), and apoptotic cells (**G**) at 96 h in the spleen. One-way analysis of variance was used to analyze the groups. pVNS*off* 10 min group did not receive electrostimulation, pVNS 10 min group received treatment for 10 min. pVNS, percutaneous vagus nerve stimulation; NOS-2, induced nitric oxide synthase 2; Arg 1, arginase 1; CD206, cluster of differentiation 206; CD86, cluster of differentiation 86; AnxA1, annexin A1; FPR2/ALX, formyl peptide receptor 2; *Off*, CFA + pVNS*off* 10 min; Alter, Alternating; Rand, Random

Our first findings related to the primary outcome (hyperalgesia), comparing current frequencies (Alternating vs Random-frequency), showed that the random frequency for 10 min in the left ear had a more prolonged antihyperalgesic effect, both at 6 h and 96 h after i.pl. injection of CFA. This is in line with findings that VNS reduces pain (mechanical hyperalgesia) in pre-clinical models of diabetic neuropathy [65], chronic headache model [66], as well as other clinical studies with experimental pain [67], functional abdominal pain disorders [68], chronic migraine [35, 69], and rheumatoid arthritis [70]. The random-frequency is a new waveform being used clinically that employs a non-repetitive, non-sequential frequency output that allegedly has analgesic superiority when compared to alternating-frequency. Although, few scientific reports have been carried out describing the effects of this random frequency (RF) output. Chao et al. [71] compared the efficacy and duration of the analgesic effect between classic Low-frequency dense-and-disperse electroacupuncture (DD-EA) and non-repetitive and non-sequential frequency (random frequency (RF)-EA at ST36 (*Zusanli*) and GB34 (*Yanglingquan*) in neuropathic rats. The RF-EA reversed hyperalgesia (for 24 h) and allodynia (for 8 h), showing a longer analgesic effect than DD-EA. Our findings corroborate with this previous pilot study in demonstrating the superiority of RF efficacy when compared to classical stimulation models.

Our study also evaluated the effect of repeated treatment and the development of analgesic tolerance caused by both current frequencies [56–58]. We found that both AF and RF showed antihyperalgesic effect for 9 days and antihyperalgesia tolerance, with no significant difference between treatments. These findings are important for the clinical setting as they demonstrate a long-term hyperalgesic effect of the two current frequencies. Although, RF superiority would be expected in a longer time for the development of antihyperalgesic tolerance [71].

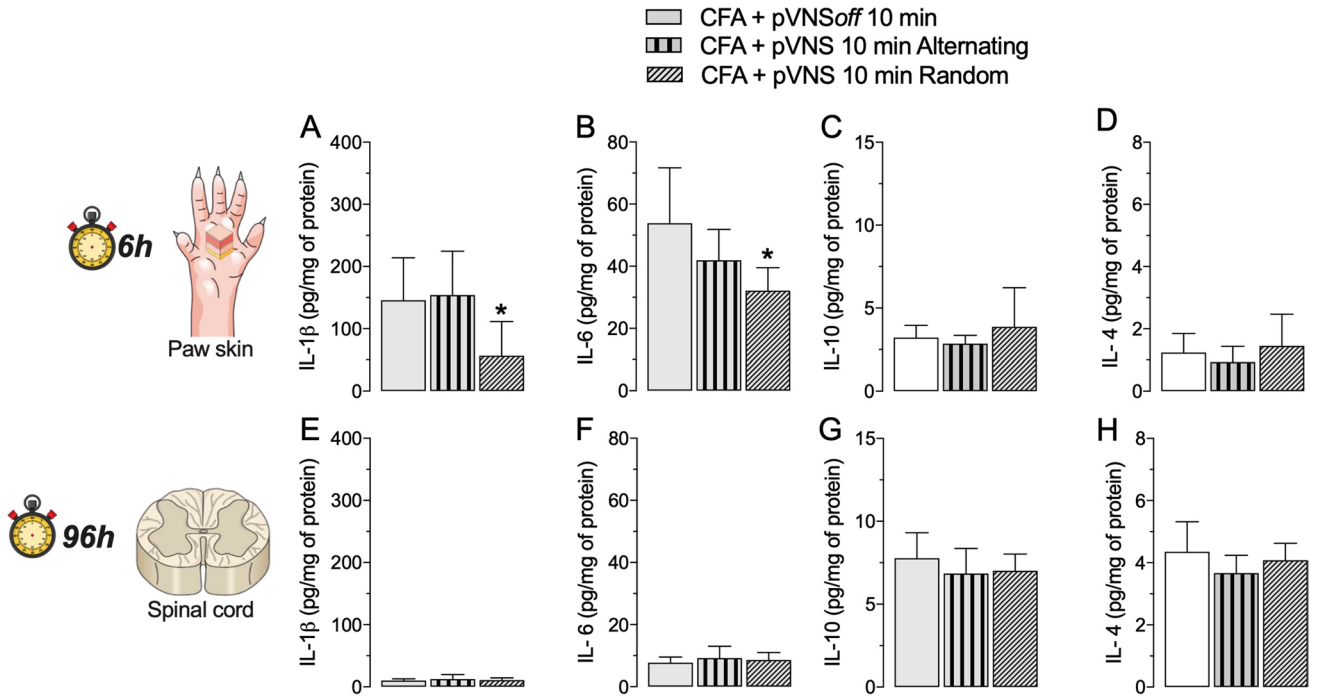

Fig. 13 Effect of pVNS at 6 h and 96 h with random and alternating frequencies in the left ear on inflammatory and anti-inflammatory cytokine levels in the paw skin and spinal cord. $*p < 0.05$ when compared to the CFA + pVNS*off* 10 min group. One-way analysis of variance was used to analyze the groups. CFA + pVNS*off* 10 min group did not receive electrostimulation, CFA + pVNS 10 min group received treatment for 10 min. pVNS, percutaneous vagus nerve stimulation; CFA, Freund's complete adjuvant; IL, interleukin

Chronic painful inflammatory conditions are usually sustained by peripheral or central sensitization [72–74]. These events are caused and sustained by the action of pro-inflammatory cytokines that alter the function of neuronal ion channels, leading to hyperreactivity [73, 75, 76]. Pro-inflammatory cytokines such as IL-1β and IL-6 play an important role in the genesis of neuronal sensitization and consequently in the pathophysiology of chronic painful conditions. Normally, counteracting the deleterious effects of inflammation, there is the release of anti-inflammatory cytokines, such as IL-10, IL-14, and IL-13, followed by the release of pro-inflammatory cytokines [77]. In the present study, we found among the pro-inflammatory cytokines, an increase in the concentrations of IL-1β and IL-6 in the paw skin and of IL-1β in the spinal cord. Among the anti-inflammatory cytokines, a reduction in IL-10 and IL-4 concentrations in the paw skin caused by CFA injection were also observed. These findings agree with previous studies that demonstrated the importance of the peripheral inflammatory process (peripheral sensitization) in the mechanical hyperalgesia observed in the CFA model [45, 78–80]. Interestingly, only pVNS with random-frequency reduced IL-1β and IL-6 concentrations peripherally in the paw skin, but not in the spinal cord. pVNS with alternating-frequency did not change cytokine concentrations. The fact that RF is more effective than AF is corroborated by the pilot study by Chao et al. [71] who found that RF showed a longer analgesic effect than AF in rats with peripheral neuropathy. We hypothesized that the superior effect observed in the group that received RF stimulation may be related to the stochastic resonance (noise) effect [81] caused by the non-repetitive and non-sequential–random–frequency output nature [82, 83].

In addition to the reduction in mechanical hyperalgesia, a reduction in paw edema and temperature (cardinal signs of inflammation) was also observed in parallel with the reduction of pro-inflammatory cytokines. It is well known that IL-1β and TNF can contribute directly and indirectly to the occurrence of edema [84, 85]. By activating cyclooxygenase 2 (COX-2), IL-1β increases the production of PGI2, that has a vasodilating action [86]. In addition, TNF and some other pro-inflammatory cytokines have been found to stimulate inducible NO synthase expression in macrophages and some other leukocytes [87]. Since there is a direct correlation between the production of pro-inflammatory cytokines and the formation of edema; we believe that the pVNS reduced edema by affecting the concentrations of pro-inflammatory cytokines.

One way to develop new approaches to treat chronic inflammatory diseases is to explore the biology of inflammation resolution. Evidence from human studies [70, 88–91] and experimental animal models [22, 92, 93]

provide support that the vagus nerve controls both the majority of acute inflammation and the resolution phase [22, 70, 88–92], Furthermore, it is well established that the inflammatory process is governed by interrelated neural and humoral reflex pathways [94]. Known as the "inflammatory reflex," this concept suggests that a neural circuit (mediated by VN) provides regulatory feedback on the inflammatory state of the body, thus favoring a rapid regulatory immune response [95, 96]. Interestingly, VN is mainly involved in three reflex pathways with a well-defined anti-inflammatory role: (i) the hypothalamic–pituitary–adrenal anti-inflammatory axis; (ii) the anti-inflammatory vago-vagal reflex; (iii) the splenic sympathetic anti-inflammatory pathway. Specifically, in the reflex pathway (i) pro-inflammatory cytokines or endotoxins activate the VN afferent fiber endings that detect the level and location of the lesion/infection, with somatotopic mapping activated in the NTS. Subpopulations of hypothalamic neurons activate the release of adrenocorticotropin hormone by the pituitary gland, stimulating the release of glucocorticoids (GC) by the adrenal glands, decreasing peripheral inflammation. So, this pathway drew our attention to the annexin A1 protein, which is a protein induced by GC [17], also known as lipocortin 1. This protein is important in the pro-resolving process that may play its role in modulating inflammatory pain via the FPR2/ALX receptor [97].

Concerning the mechanism through which pVNS exerts its antihyperalgesic action, the present study shows that the AnxA-FPR2/ALX pathway is likely involved. This conclusion derives from the fact that pre-treatment of animals with the WRW4 (antagonist for the FPR2/ALX receptor), at a dose that produced no significant effect on CFA-induced mechanical hyperalgesia, significantly reversed the antihyperalgesia caused by pVNS. Furthermore, we have recently reported that peripheral AE with alternate frequency also activates the AnxA1-FPR2/ALX pathway [45]. Our biochemical assays also found that pVNS with random frequency had higher AnxA1 immunocontent in paw skin tissue at 6 h and 96 h after CFA injection. Treatment with both random and alternating frequencies had higher FPR2/ALX receptor immunocontent only at 6 h after CFA, when compared to the control. These data corroborate the pharmacological/behavioral findings of the involvement of the AnxA-FPR2/ALX pathway in the antihyperalgesic action of pVNS. Previous studies showed that i.pl. injection of CFA in mice increased the expression of AnxA1 in the DRGs (L4/L5) in 7 days. And that AnxA1 produced its antinociceptive effect on peripheral inflammatory hyperalgesia through the FPR2/ALX receptor present in the DRG [97].

In line with those findings, a relatively high concentration in small sensory neurons throughout the DRG, with a perinuclear distribution has been found. However, authors point out that although AnxA1 is restricted to the neuronal body, it may also be present in the peripheral terminal which connects to skin and muscle or to other tissues, such as free nerve endings or in association with specialized connective tissue or epithelial cells that constitute the sensory afferent [98]. Thus, future studies should analyze the expression of AnxA1 and FPR2/ALX receptor on the peripheral terminal of the primary nociceptive neuron. Such findings would be interesting and would expand our current data. The increase or peripheral/local release of AnxA1 and its action on the FPR2/ALX receptor easily explains the effects of pVNS that we observed. However, AnxA1 is present in biological fluids such as plasma and it is expressed in various cell types (leukocytes, neutrophils, macrophages, T cells, fibroblasts, epithelial cells, endothelial cells and in skeletal muscle). In the central nervous system is expressed in cells of the hypothalamus and pituitary, brain structures that are involved in the anti-inflammatory hypothalamic–pituitary–adrenal axis active by the VN. In this sense, AnxA1 originated from pVNS may originate in other tissues or cells, in addition to the skin of the paw.

Activation of the FPR2/ALX receptor leads to the regulation of several intracellular signaling pathways, which culminate in the inhibition of NF-kB and PLA_2 activity. Thus, AnxA1 prevents the formation of inflammatory precursors of arachidonic acid, induces the formation of anti-inflammatory factors, inhibits the formation of COX-2, iNOS, the activity and migration of neutrophils and the synthesis and release of inflammatory factors [99]. In addition, the externalization of AnxA1 activates a defense mechanism promoting the removal of apoptotic cells and inhibiting the secretion of pro-inflammatory factors by macrophages [100]. Another signaling pathway regulated by the FPR2/ALX receptor activation that may also explain the results found in the present study on inflammatory hyperalgesia, is the molecular pathway of tonic opioid release from neutrophils [15]. It was observed that the activation of human neutrophils with ANXA1 induced a more than fivefold increase in Met-enkephalin secretion and that this effect has been blocked by the specific antagonist for the FPR2/ALX receptor [101]. In summary, the release of opioids in neutrophils and the inhibition of NF-kB and PLA2 activity by ANXA1 through FPR2/ALX activation could be one of the adjacent mechanisms to the antihyperalgesic and anti-inflammatory effects of pVNS, respectively.

Despite the inhibitory effect of VNS on M1 macrophages has been well described the role of macrophage polarization in regulating the anti-inflammatory action of pVNS has not been investigated [102]. Macrophages are key components of the innate immune system; thus, balancing the M1/M2 macrophage ratio and modulating cytokine levels in the inflammatory environment are important therapeutic goals

[102]. In addition, the FPR2/ALX receptor, when activated in the M1 macrophage, induces a phenotype change to M2 macrophage, suggesting a consequent release of anti-inflammatory cytokines [103, 104]. In this sense, as a secondary outcome measures, we also evaluated the effect of pVNS (alternating vs random-frequency) on the immunocontent of M1 (NOS-2 and CD86) and M2 (ARG-1 and CD206) macrophages in the paw skin, because these cells play an important role in inflammatory pain [105]. In the present study, we observed that both pVNS with alternating and random frequency showed higher CD86 (M1 macrophage) immunocontent and did not change the other markers for M2 macrophages. These findings rule out a possible effect of pVNS—under the conditions described here—on macrophage polarization in regulating the anti-inflammatory action, despite inhibiting (IL-1 and IL-6) cytokines associated with the pro-inflammatory macrophage M1.

Finally, in the present study, we also evaluated the polarization of macrophages and other markers of inflammation in the spleen, since other VN reflex pathways, such as (ii) the anti-inflammatory vago-vagal reflex and (iii) the splenic sympathetic anti-inflammatory pathway influence inflammatory cells in this organ [27, 95, 106]. However, we did not find alterations in the immunocontent of the inflammatory biomarkers analyzed in the spleen.

In conclusion, the present results provide convincing evidence that pVNS with alternating and random frequency exerts a rapid onset, relatively long-lasting and pronounced antihyperalgesia against CFA model of pain in mice at parameters that does not interfere with the spontaneous motor activity. However, the effect of random pVNS was superior to classical stimulation using alternating frequency. Furthermore, the implications of this study, the antihyperalgesic effect of pVNS with alternating and random frequency is possibly mediated, at least in part, by AnxA1-FPR2-ALX pathway. Thus, our results suggest that pVNS auricular may be a useful approach to treat inflammatory pain.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s12035-023-03237-7.

Author Contribution DFM: design and supervision of all experiments; DCS, FT, BHO, JM, JKF, VVH, FB, ARD, ECF, NHD, DDL: execution of experiments; DCS, DFM: conduction of the experiments; DFM, DCS, JMM: data analysis and/or tabulation; DFM: data interpretation; DFM, DCS, APP, AOM, LMM, ASIS, BG, EBB: writing of the paper; all authors read and discussed the paper.

Funding This study was supported by grants from the Research and Innovation Support Foundation of Santa Catarina State (FAPESC) grant no. 2020TR732 and grant no. 001. Coordination for the Improvement of Higher Education Personnel (CAPES); Dr. Daniel Fernandes Martins is supported by research fellowships from CNPq (310128/2020–0) and Anima Institute (AI).

Data Availability The datasets generated during and/or analyzed during the current study are not publicly available due to why data are not public but are available from the corresponding author on reasonable request.

Declarations

Ethics Approval The animal study was reviewed and approved by the Ethics Committee on the Use of Animals (CEUA, numbers 18.048.2.07.IV e 20.007.4.08.IV) of University of South Santa Catarina, Brazil.

Consent to Participate Not applicable.

Consent for Publication Not applicable.

Conflict of Interest The authors declare no competing interests.

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