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# Transcranial direct-current stimulation reduces nociceptive behaviour in an orofacial pain model

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## Summary

**Background**: Transcranial direct-current stimulation (tDCS) is a noninvasive method of brain stimulation suggested as a therapeutic tool for pain and is related to the reversal of maladaptive plasticity associated with chronic pain.

**Objectives**: This study investigated the effect of tDCS, a non-pharmacological therapy, on local mechanical hyperalgesia, and remote thermal hyperalgesia in rats submitted to orofacial inflammatory pain model, by facial von Frey and hot plate tests, respectively. In addition, we evaluated levels of BDNF, NGF, IL-10 and IL-6 in the brainstem and blood serum of these animals at 24 hours and 7 days after the end of tDCS treatment.

**Methods**: Rats were subjected to temporomandibular joint pain and treated with tDCS. The animals were divided into control, pain and pain + treatment groups. Mechanical and thermal hyperalgesia were evaluated at baseline, 7 days after administration of complete Freund's adjuvant, and immediately, 24 hours, and 7 days after the tDCS treatment. Neuroimmunomodulators levels were determined by ELISA. Statistical analyses were performed by (GEE)/Bonferroni (behavioural tests), three-way ANOVA/SNK (neurochemical tests) and Kruskal-Wallis (histological analysis).

**Results**: Transcranial direct-current stimulation reduced mechanical and thermal hyperalgesia ($P < 0.01$). We observed interaction between factors (pain and treatment) increasing brainstem BDNF ($P < 0.01$) and NGF ($P < 0.05$) levels. Furthermore, we found an increase in IL-6 and IL-10 levels in the brainstem at 24 hours and 7 days after tDCS, respectively.

**Conclusion**: We showed that tDCS reduces thermal and mechanical hyperalgesia induced by orofacial pain until 7 days after treatment. These findings demonstrate that tDCS was effective in the control of orofacial inflammatory pain.

**KEYWORDS**
hyperalgesia, neuromodulation, orofacial inflammatory pain, tDCS, temporomandibular joint

## 1 | INTRODUCTION

Orofacial pain is a painful condition, that is, associated with head and neck tissues as well as oral structures.[1] Temporomandibular disorders (TMD) are an important type of orofacial pain, which may be accompanied by inflammation.[2] Arthritic TMD can be classified as either low (osteoarthritis) or high inflammatory process type. The low inflammatory type starts in the matrix of the articular surface of the joint, followed by subcondylar bone, and capsule. In contrast, high inflammatory arthritic TMD presents inflammation, primarily in the synovial cells and joint bone, and frequently, is a source of temporomandibular joint (TMJ) pain.[3] In addition, allodynia is common in both TMD and neuropathic orofacial pain due to the release of neurotransmitters that cause peripheral sensitisation.[4]

Temporomandibular joint inflammatory models, as the Freund's Adjuvant (CFA) model, promote releasing of inflammatory mediators that produce long-lasting inflammation[5] and acute and persistent pain[1,6] in rats. Previous study has shown that, in rats, CFA administration into the TMJ induces an increase in the calcitonin gene-related peptide (CGRP), nerve growth factor (NGF), interleukin-1β (IL-1β) and tumour necrosis factor-α (TNF-α) levels, until 6 weeks after administration.[1,7] Inflammation in the TMJ region results in increased excitability of the trigeminal subnucleus caudalis,[8] which is a sensory nucleus with a laminated structure similar to the spinal dorsal horn and, due to this close similarity has been termed the medullary dorsal horn.[72]

The non-steroidal anti-inflammatory drugs have been largely used for treatment of TMJ pain due to inflammatory process. These drugs reduce oedema and other symptoms induced by prostaglandin cascade; however, despite the reduction in local inflammation, the cause of the inflammation is not affected, which could be an overload in the joint or parafunctional habits.[9] The peripheral and central sensitisation resulting from TMJ pain requires different therapeutics approaches.[4] In this context, non-pharmacological therapies could be used along with traditional therapies to increase efficacy and reduce adverse effects.

Transcranial Direct-Current Stimulation (tDCS) is a method of noninvasive brain modulation with significant effects on different types of chronic pain in clinical research and animal models.[10-13] A small, direct-current is applied to the brain, through two electrodes, then polarising the neural tissue and altering the resting membrane threshold, with subsequent changes in the synaptic plasticity.[14] Our previous study has shown that tDCS induces a significant analgesic effect in a rat model of hyperalgesia induced by chronic inflammation.[13] Furthermore, tDCS was able to reverse hyperalgesia and allodynia in a rat model of chronic restraint stress, showing cumulative effect of repeated tDCS treatment that remained until 24 hours after the end of tDCS treatment.[12]

Considering that TMJ pain reduces life quality of patients.[69] and pharmacological treatments are associated to side effects, our aim was investigated the effect of tDCS, a non-pharmacological therapy, on local mechanical hyperalgesia, and remote thermal hyperalgesia in rats submitted to orofacial inflammatory pain model, by facial von Frey and hot plate tests, respectively. In addition, we evaluated the levels of brain-derived neurotrophic factor (BDNF), NGF, IL-10 and IL-6 in the brainstem and peripheral blood serum of these animals at 24 hours and 7 days after the end of tDCS treatment.

## 2 | MATERIAL AND METHODS

### 2.1 | Animals

Male Sprague Dawley rats weighing 250-300 g were used in this study. We opted to use only male rats to avoid possible biases regarding the effect of gonadal hormones on nociceptive responses and biomarker levels. Oestrogen can modulate neurotrophins, including NGF and BDNF, which were evaluated in this study.[51,62] Moreover, the effect of oestrogen on pain threshold involves modulation of receptors localised in the spinal cord and endogenous opioid system that are a target of tDCS treatment.[63] In addition, the use of male and female rats would result in a significant increase in the number of animals needed to study, a potential obstacle to the approval of Institutional Committee for Animal Care and Use. Rats were randomised by weight and were housed three per cage. Cages were made of polypropylene material (49 × 34 × 16 cm), and the floor covered was with sawdust. One hundred and four animals were used for the behavioural tests (52 animals for the von Frey test and 52 for the hot plate test). Rats were divided into two groups that were killed at 24 hours and 7 days after the end of treatment. Rats were collected from different samples for biochemical analysis. However, some samples were lost during processing (homogenisation, centrifugation and pipette step), and for this reason the final n-value for each analysis varies. Then, we used 90 blood serum samples for BDNF and IL-10 analysis and 80 from them for NGF and IL-6 analysis. We collected 80 brainstem samples for BDNF and IL-10 analysis, from these only 70 were used for NGF and IL-6 analysis.

All rats were maintained in a controlled environment (22 ± 2°C), under a standard light-dark cycle (lights-on at 07:00 hours and lights-off at 19:00 hours) with water and chow (Nuvital, Porto Alegre/Brazil) provided ad libitum. All experiments and procedures were approved by the Institutional Committee for Animal Care and Use (GPPG-HCPA protocol No. 12-0104) and conformed to the Guide for the Care and Use of Laboratory Animals 8th ed. 2011. The maintenance of the animals followed law 11.794 (Brazil), which establishes procedures for the scientific use of animals. The experimental protocol complied with the ethical and methodological standards of the ARRIVE guidelines.[15]

### 2.2 | Experimental groups

Rats were randomised into six groups: the total control group (C), which was not manipulated; the saline-sham tDCS group (SS), which received vehicle (saline) injection into the TMJ, and were submitted to sham tDCS treatment; the saline-tDCS group (ST), which received vehicle (saline) injection into the TMJ as well as active tDCS

treatment; the orofacial pain group (O), which received CFA injection into the TMJ and no tDCS treatment; the orofacial pain-sham tDCS group (OS), which received CFA injection into the TMJ and received sham tDCS treatment; and the orofacial pain-tDCS group (OT), which received CFA injection into the TMJ and underwent active tDCS treatment.

## 2.3 | Orofacial inflammatory pain model induced by complete Freund's adjuvant (CFA)

Initially, rats were anesthetised with 5% isoflurane for induction, followed by 2.5% for maintenance. Orofacial inflammation was induced by intra-articular administration of 25 μL complete Freund's adjuvant (CFA, Sigma, St Louis, MO, USA; Mycobacterium tuberculosis) into the left TMJ region. CFA was suspended in a total volume of 50 μL (oil/saline, 1:1). Control rats received 50 μL of a 0.9% saline solution (SAL). This dose was based on previous reports. The TMJ region was identified by palpation, and the injection was delivered manually by advancing a 30-gauge needle through the skin immediately inferior to the posterior border of the zygomatic arch until the needle contacted the mandibular condyle. CFA is considered to be a reliable irritant that produces long-term inflammation at the injection site and promotes intense and persistent pain.[6] Previous studies have shown that this dose of CFA, injected into the TMJ, causes persistent behavioural hyperalgesia.[5,16, 28,29,68]

## 2.4 | Transcranial direct-current stimulation (tDCS)

The anodal tDCS treatment started 7 days after the CFA administration. This period was selected due to a study from Okamoto et al[17] demonstrating that the most intense nociceptive response occurs between the 7th and 14th day after CFA injection, in both von Frey and formalin tests. In the same way, Spears et al[7] showed temporal changes in inflammatory mediator concentrations after CFA injection, with elevated IL-1β, TNF-α and NGF TMJ levels between the 2nd and 14th day after CFA administration. These levels remained elevated until the 6th week after CFA injection, although this increase was not as significant as that in the first 2 weeks. The tDCS treatment was applied using ECG electrodes (1.5 $cm^2$), by a battery-driven, constant current stimulator designed for continuous application of low currents to small mammals, for 20 min/d over 8 days, as described by Spezia Adachi et al,[12] In the present study, the electrodes were placed on the scalp of animal; this placement is similar to that used in human studies of tDCS for pain.[18-20,65] The cathodal electrode was placed at the mid-point of the lateral angle of the eyes (supraorbital area), and the anodal electrode was positioned on the head using the landmarks of the neck and shoulder lines as a guide (the anterior and posterior regions in the midline between the two hemispheres of the parietal cortex) as described by Takano et al,[21] The intensity of the constant current was 0.5 mA for 20 minutes; this intensity did not produce skin lesions, as observed in our previous studies.[12,13] In an earlier study, a constant current of 1 mA intensity produced skin lesions as current density is comparatively much higher than the traditional 1 mA tDCS applied using large pads in humans.[22] The sham stimulation replicated the real stimulation; however, the stimulator was turned off after 30 seconds of stimulation so the animals could maintain continuity of the physical sensation of the actual tDCS conditions.[12,23]

## 2.5 | Von frey test

Mechanical sensitivity of the vibrissal whisker pad was verified using an automatic von Frey aesthesiometer (Insight, São Paulo, Brazil) at baseline, 7 days after pain induction, and immediately, 24 hours, and 7 days after the final tDCS treatment. Rat TMJ nociception was evaluated by measuring the threshold of the force intensity required to be applied to the TMJ region to elicit a reflex response (eg, head withdrawal). All tests were performed by the same investigator, which was blinded for treatment group.

## 2.6 | Hot plate test

We used the hot plate test to evaluate the central sensitisation resulting from orofacial inflammatory pain.[61] It was necessary the use of a nociceptive test that evaluated pain threshold and not local hyperalgesia [for review see Gregory et al][59] This test was performed at baseline, 7 days after pain induction, as well as immediately, 24 hours, and seven days after the final tDCS treatment. This test was used as an indicator of the supraspinal pain process.[12,24] Licking or jumping responses during this test are considered to be the result of supraspinal sensory integration.[25,26] Twenty-four hours prior to testing, the animals were habituated to the apparatus for 5 minutes to avoid analgesia induced by the novelty of the apparatus.[27] The temperature of the plate was maintained at 55°C ± 0.1°C, and the cut-off time was 20 seconds to avoid tissue damage. All tests were performed by the same investigator, which was blinded for treatment group. This test was performed 1 hour after Von Frey test.

## 2.7 | Blood sampling and tissue collection

Rats were killed by decapitation at 24 hours and 7 days after the end of tDCS treatment. Trunk blood was drawn, and blood samples were centrifuged in plastic tubes for 5 minutes at 20,200 x g at room temperature. The brainstem was collected and stored at −80°C for later analysis. We opted to evaluate brainstem neuromodulator levels as the trigeminal brainstem sensory nuclear complex is an important centre for the redistribution of orofacial somatosensory and pain information. The nociceptive neurons in brainstem trigeminal nuclei can be activated by noxious mechanical or thermal stimulation of orofacial tissues, and also by inflammatory irritants applied to these tissues.[68] This stimulation appears to contribute to the hyperalgesia and trigeminal sensitisation associated with peripheral inflammation.[47,68] Animal studies using CFA-induced TMJ inflammation have shown that central sensitisation is associated with alterations in descending modulatory influences.[70]

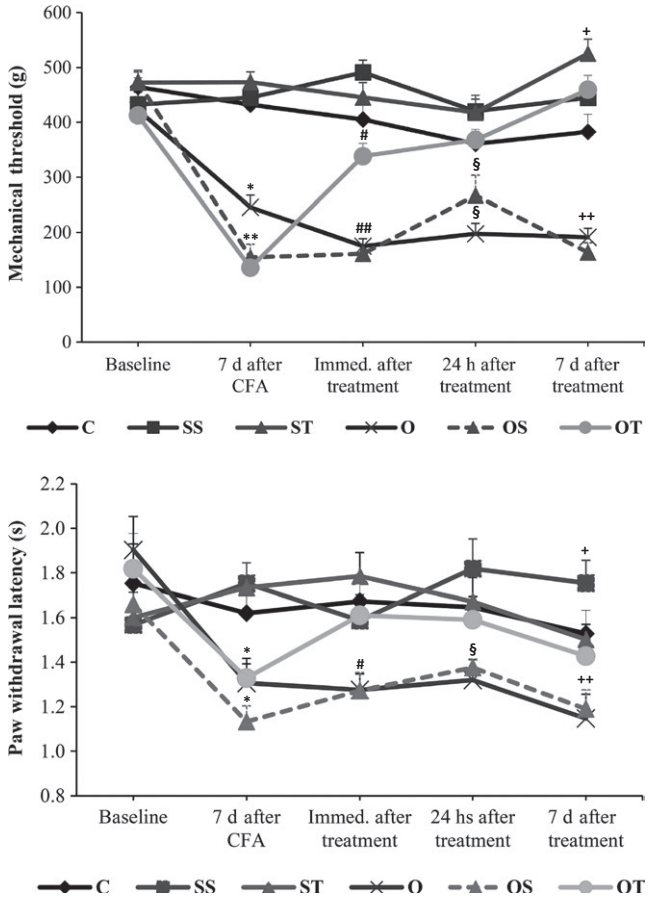


**FIGURE 1** Panel A, Effect of tDCS on the mechanical allodynia response in an orofacial pain model at different timepoints as measured by the Von Frey test. Data are presented as the mean ± SEM (N = 52). Total control group (C); saline-sham tDCS group (SS); saline-tDCS group (ST); orofacial pain group (O); orofacial pain-sham tDCS group (OS); orofacial pain-tDCS group (OT). *Different from all other groups (C, SS, ST, OS and OT) 7 d after administration of CFA; **Different from the C, SS, ST and O groups 7 d after administration of CFA; #Different from the SS, ST, O and OS groups immediately after the last tDCS session; ##Different from the C, SS, ST and OT groups immediately after the last tDCS session; §Different from all other groups (C, SS, ST and OT) 24 h after the last tDCS session; +Different from the C, O and OS groups 7 d after the last tDCS session; ++Different from all other groups (C, SS, ST and OT) 7 d after the last tDCS session; GEE: ($\chi^2$ = 586.51; 20) $P$ < 0.001. Panel B, Effect of tDCS on the thermal hyperalgesia response in an orofacial pain model at various timepoints as measured by the Hot Plate test. Data are presented as the mean ± SEM (N = 52). *Different from all other groups (C, SS and ST) 7 d after administration of CFA; #Different from all other groups (C, SS, ST and OT) immediately after the last tDCS session; §Different from all other groups (C, SS, ST and OT) 24 h after the last tDCS session; +Different from the ST, O, OS and OT groups 7 d after the last tDCS session; ++Different from all other groups (C, SS, ST and OT) 7 d after the last tDCS session; GEE: ($\chi^2$ = 175.24; 20) $P$ < 0.001

In addition, a study from Shimizu et al[47] showed that orofacial inflammatory hyperalgesia was attenuated by injection of IL-10 into the brainstem trigeminal complex.

## 2.8 | Biochemical assays

Brain-derived neurotrophic factor, NGF, IL-6 and IL-10 analyses were performed in brainstem homogenates and blood serum using a commercially available enzyme-linked immunosorbent assay (ELISA) kit for rats (R&D Systems, Minneapolis, MN, United States). The total protein was measured by Bradford's method using bovine serum albumin as the standard. The values were expressed in pg/mg of protein. The brainstem structures were homogenised with a hand-held homogenizer in 1:10 Tris-buffered saline and centrifuged for 20 minutes at 1000 g. The results were expressed as a percentage of the control.

## 2.9 | Histology and histological scoring

Fourteen days after the CFA injection, the TMJs were excised and fixed in 10% buffered formalin for 7 days. The TMJs were then decalcified with 10% nitric acid and fixed in 10% buffered formalin. The tissues were then embedded in paraffin and sectioned. Slides containing the mandibular condyle, disc and retrodiscal area and fossa were prepared and stained with haematoxylin and eosin (HE). A qualitative assessment of the degree of inflammation in the retrodiscal area was made using light microscopy. Scoring was based on a system used by Laste et al,[13]; the percentage of infiltrating mononuclear cells was scored as follows: 0 = absent, 1 = mild (1%-10%), 2 = moderate (11%-50%) and 3 = severe (51%-100%).

## 2.10 | Statistical analysis

The data were expressed as the mean ± standard error of the mean (SEM). An estimating equation (GEE) followed by Bonferroni test was performed to analyse nociceptive behaviour. Three-way analysis of variance (ANOVA) followed by Student-Newman-Keuls (SNK) was performed to compare the biochemical data between groups. The histological analyses were performed by one blinded examiner, and data were presented as median + min; max intervals. Kruskal-Wallis test was used for intergroup comparisons, and differences were considered significant at $P$ < 0.05. IBM SPSS Statistics version 20, Armonk, NY, USA for Windows was used for the statistical analyses.

# 3 | RESULTS

## 3.1 | Nociceptive behaviour

### 3.1.1 | Mechanical hyperalgesia

Transcranial direct-current stimulation treatment increased the mechanical pain threshold of rats exposed to CFA (orofacial inflammatory pain model) compared to O and OS groups, showing an analgesic effect. The generalised estimation equation presented interaction time x treatment ($\chi^2$ = 586.51; 20; $P$ < 0.001). Rats subjected to pain model and tDCS (OT group) showed a significantly increased pain

threshold at immediately, 24 hours, and 7 days after end of tDCS treatment compared to O and OS groups, demonstrating an anti-hyperalgesic effect (Figure 1, Panel A).

### 3.1.2 | Thermal hyperalgesia

We also observed similar results, when we assessed remote thermal hyperalgesia. The response to thermal stimuli was modified by tDCS only in rats subjected to the orofacial pain model. The generalised estimation equation showed interaction time x treatment (Wald $\chi^2$ = 175.24; 20; $P$ < 0.001). The paw latency withdrawal was significantly increased after tDCS treatment (OT group) compared to O and OS groups, demonstrating an anti-hyperalgesic effect (Figure 1, Panel B).

## 3.2 | Biochemical analysis

### 3.2.1 | BDNF levels

The BDNF levels were measured in the brainstem and blood serum. In the brainstem, there was interaction between the independent variables: orofacial pain, tDCS and timepoint (Three-way ANOVA/SNK, $F_{(1,64)}$ = 7.86, $P$ < 0.01). We observed that the orofacial inflammatory pain model (O and OS groups) increased levels of BDNF, which was reversed by active tDCS treatment (OT group) at 24 hours after the last session of tDCS; however, this effect was abolished 7 days after the end of tDCS. BDNF levels in the blood serum levels revealed an interaction between orofacial pain and timepoint ($F_{(2,74)}$ = 3.10, $P$ < 0.01). It was possible to observe a return to baseline levels 7 days after the end of tDCS treatment. (Figure 2, Panels A,B).

### 3.2.2 | NGF levels

Analysis of NGF levels in the brainstem revealed an interaction between the independent variables: orofacial pain and timepoint (three-way ANOVA/SNK, $F_{(2,53)}$ = 4.17, $P$ < 0.05). There was a significant increase in NGF brainstem levels only in rats subjected to orofacial pain (O, OS and OT groups) in relation to others at 7 days after the end of tDCS treatment. No significant effect was observed in serum levels of NGF regarding independents variables (orofacial pain, treatment or timepoint; Figure 3, Panels A,B).

### 3.2.3 | IL-10 levels

Three-way ANOVA showed an interaction between orofacial pain and timepoint in brainstem levels of IL-10 ($F_{(2,66)}$ = 10.72, $P$ < 0.01). In orofacial pain groups, there was an increase in brainstem levels of IL-10 7 days after tDCS treatment compared to the levels 24 hours after treatment. We found effect of timepoint in IL-10 serum levels, which were reduced 7 days after tDCS treatment ($F_{(1,73)}$ = 4.36, $P$ < 0.05), (Figure 4, Panels A,B).

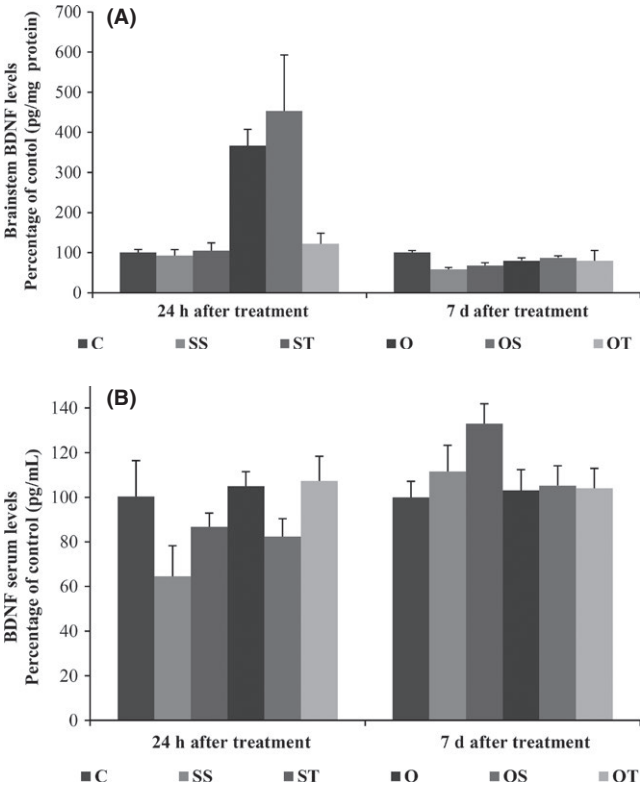


**FIGURE 2** Panel A, BDNF Brainstem levels. Data are presented as percentage of control (Mean ± SEM). Total control group (C), n = 13; saline-sham tDCS group (SS), n = 13; saline-tDCS group (ST), n = 12; orofacial pain group (O), n = 13; orofacial pain-sham tDCS group (OS), n = 12; orofacial pain-tDCS group (OT), n = 13. There was interaction between the independent variables: orofacial pain, tDCS and timepoint (three-way ANOVA/SNK, $F_{(1,64)}$ = 7.86, $P$ < 0.01, N = 76). Panel B, Serum BDNF levels. Data are presented as percentage of control (Mean ± SEM). n per group (C = 15; SS = 14; ST = 14; O = 14; OS = 15; OT = 14).There was interaction between orofacial pain and timepoint (three-way ANOVA/SNK, $F_{(2,74)}$ = 3.10, $P$ < 0.01, N = 86)

### 3.2.4 | IL-6 levels

Analysis of brainstem IL-6 levels revealed an interaction between three independent variables: orofacial pain, tDCS and timepoint (three-way ANOVA/SNK, $F_{(2,48)}$ = 4.84, $P$ < 0.05). tDCS treatment reduced the increase in brainstem IL-6 levels induced by orofacial pain 24 hours after end of tDCS. We also observed an effect of sham tDCS on the OS group. IL-6 serum levels were not modified by any independent variable (three-way ANOVA, $P$ > 0.05; Figure 4, Panels C,D).

## 3.3 | Histological scoring

The histological findings of TMJs are shown in Figure 5. The CFA injection provoked an inflammatory reaction with infiltrating mononuclear cells (macrophages, neutrophils and lymphocytes) in the retrodiscal area (under the synovial membrane). Groups without

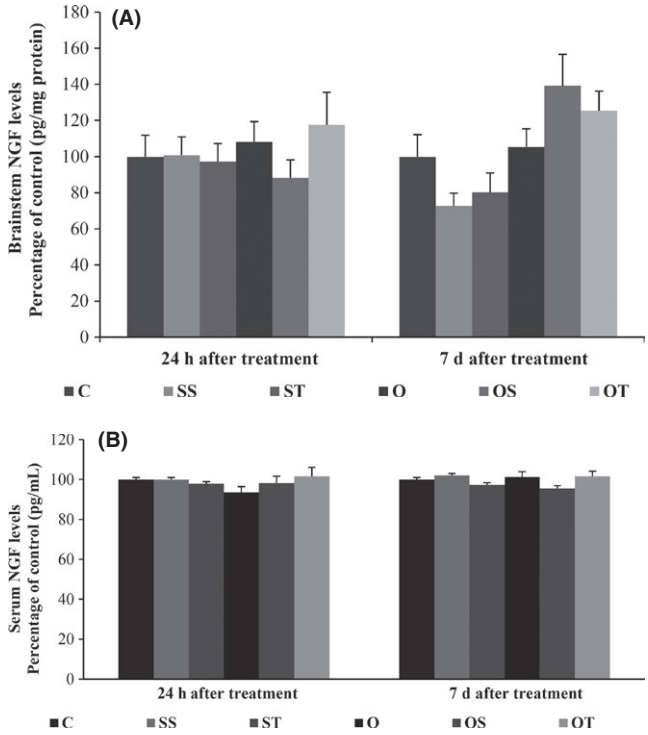


**FIGURE 3** Panel A, NGF Brainstem levels. Data are presented as percentage of control (Mean ± SEM). Total control group (C), n = 11; saline-sham tDCS group (SS), n = 11; saline-tDCS group (ST), n = 11; orofacial pain group (O), n = 11; orofacial pain-sham tDCS group (OS), n = 10; orofacial pain-tDCS group (OT), n = 11. There was interaction between the independent variables: orofacial pain and timepoint (three-way ANOVA/SNK, $F_{(2,53)} = 4.17$, $P < 0.05$, N = 65). Panel B, Serum NGF levels. Data are presented as percentage of control (Mean ± SEM). n per group (C = 13; SS = 13; ST = 12; O = 13; OS = 12; OT = 12).There were no significant effects of the independent variables on serum NGF levels (three-way ANOVA, $P > 0.05$, N = 75)

CFA did not show any signs of inflammation, whereas CFA groups showed inflammatory infiltrate (Table 1). In addition, fibrosis was present along with numerous droplet vacuoles, presumably lipids. CT, SS and SE groups presented normal tissue architecture in the TMJ (Figure 5B,D and F), while the rats that received CFA had highly abnormal histology in the TMJ, with pronounced inflammation in the retrodiscal area (Figure 5A,C and E). The inflammation scores were higher in the CFA groups (Table 1; Figure 5) compared to other groups (Kruskal-Wallis, $P < 0.05$).

## 4 | DISCUSSION

Our innovative study showed analgesic effects of repeated tDCS treatment in TMJ inflammation rat model. tDCS was applied upon motor cortex of rats and reduces mechanical and thermal hyperalgesia induced by CFA injected directly in TMJ. Interestingly, we found this analgesic effect of tDCS until at least 7 days after the end of tDCS, demonstrating a long-lasting analgesic effect of tDCS. Our previous studies had demonstrated the analgesic effect of tDCS up to 24 hours after treatment,[12,13] and also 7 days.[56] In addition, according to our results, we also suggest that the analgesic effect of tDCS can be linked to BDNF levels in brainstem at least at short-term. Also, at short-term, we found that orofacial inflammation model induced by CFA was able to increase the IL-6 brainstem levels and this effect was reverted by sham tDCS and active tDCS.

The main mechanism of action of tDCS remains unclear, although studies suggest that it may involve depolarisation of the neural membrane[43,44] and changes in cortico-striato-thalamo-cortical connectivity.[45] Previous studies suggest that the effect of tDCS depends on the projection of fibres from the motor cortex to other structures involved in pain processing, such as the thalamus and brainstem nuclei, which down regulate processing from sensitised neurons.[42,46,47] The basic tDCS protocol involves application of a weak electrical current to the scalp surface between two electrodes (anode and cathode). The type of stimulation used determines the effect of treatment, anodal stimulation typically depolarised neuronal membrane.[19] The short-term effects of anodal tDCS on motor cortex excitability are associated with changes in the resting neuronal threshold[57]; while, the long-lasting effects of tDCS involve the glutamatergic N-methyl-D-aspartic acid (NMDA) receptors.[22] Considering that tDCS is not a focal technique, it is difficult spatially restricted the treatment to a discrete brain region, particularly in small animals.[31] However, the cerebral cortex is a target of tDCS treatment because it is related to a variety of different activities, including detection, perception and modulation of pain information.[32,33] The top-down effect of tDCS involves projections to areas such as the periaqueductal grey area,[30] thalamus, brainstem and spinal cord.[31,34]

We observed a significant increase in BDNF levels in the brainstem 16 days after orofacial pain induction. BDNF levels have previously been associated with pain sensitivity,[35] and it has a well-documented pronociceptive role in inflammatory and neuropathic pain processes. Previous study suggests that the BDNF-TrkB receptor cascade in the rostral ventromedial medulla (RVM) circuitry is involved in the development of persistent pain after inflammation.[36,37] Furthermore, BDNF down regulates the $K^+$–$Cl^-$ co-transporter ($KCC_2$) that maintains the $Cl_2$ gradient in inhibitory GABA synapses, decreasing GABA inhibition, and resulting in facilitation of pain signalling.[35] Our results demonstrate that tDCS completely reverses the increased levels of BDNF in the brainstem due orofacial pain at short-term, consistent with a previous study from our research group that showed a reduction in the BDNF levels in the spinal cord and brainstem of rats with hyperalgesia induced by chronic stress.[31] We suggest that tDCS modulates these neuroplastic events induced by chronic pain, decreasing the interaction of BDNF-TrkB receptors. We also highlighted that the effects of anodal tDCS involve a cascade of events at the cellular and molecular level associated with modulation of the GABAergic system. However, it is not possible to dismiss the possible involvement of other systems, such as the glutamatergic, dopaminergic, serotonergic and cholinergic systems.[38]

Another important neurotrophin that also plays a role in chronic pain is the NGF.[39] In our study, the orofacial pain model had increased

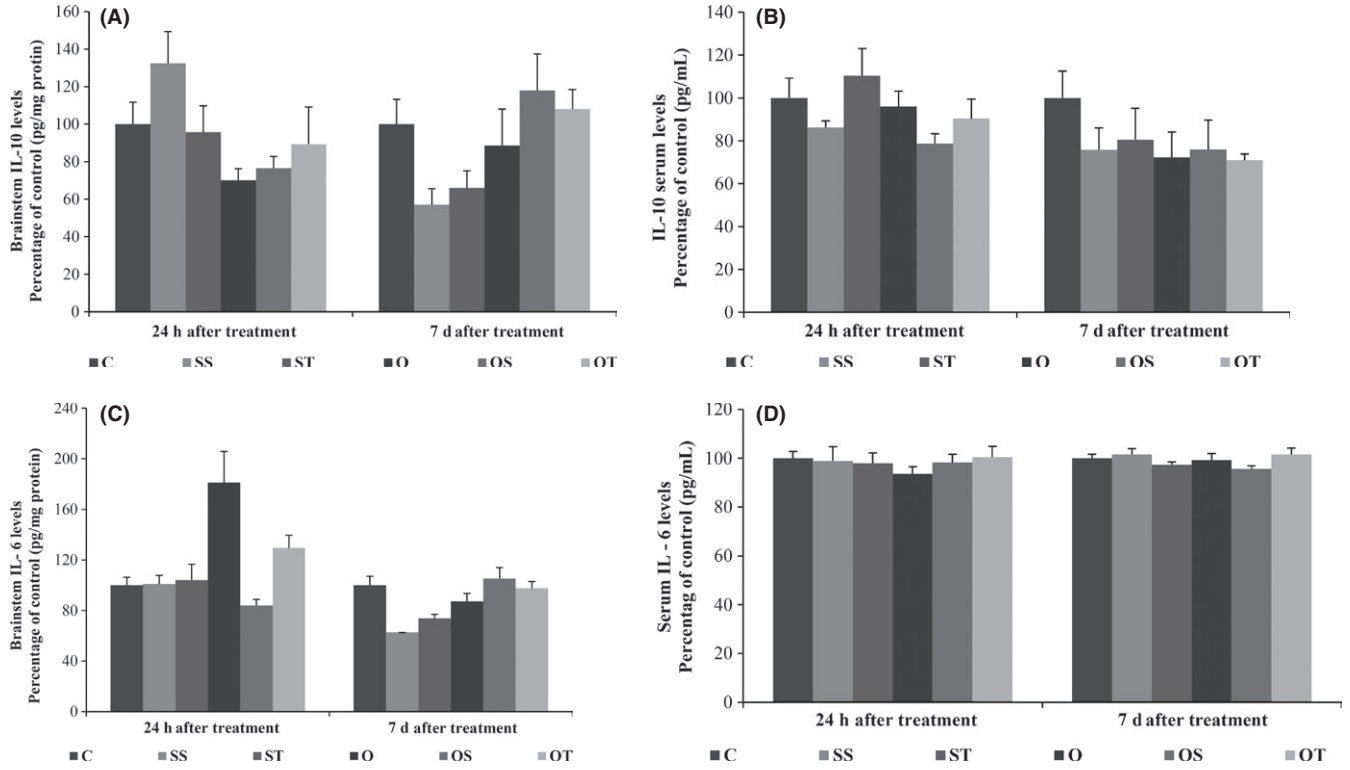


**FIGURE 4** Panel A, IL-10 Brainstem levels. Data are presented as percentage of control (Mean ± SEM). Total control group (C), n = 14; saline-sham tDCS group (SS), n = 13; saline-tDCS group (ST), n = 13; orofacial pain group (O), n = 13; orofacial pain-sham tDCS group (OS), n = 12; orofacial pain-tDCS group (OT), n = 13. There was interaction between orofacial pain and timepoint (three-way ANOVA/SNK, $P < 0.01$, N = 78). Panel B, IL-10 serum levels. Data are presented as percentage of control (Mean ± SEM). n per group (C = 15; SS = 14; ST = 14; O = 14; OS = 14; OT = 14). (*) There was a significant effect of timepoint on serum IL-10 levels (three-way ANOVA/SNK, $P < 0.05$, N = 85). Panel C, IL-6 Brainstem levels. Data are presented as percentage of control (Mean ± SEM). Total control group (C), n = 11; saline-sham tDCS group (SS), n = 09; saline-tDCS group (ST), n = 10; orofacial pain group (O), n = 10; orofacial pain-sham tDCS group (OS), n = 10; orofacial pain-tDCS group (OT), n = 10. There was interaction between orofacial pain, tDCS and timepoint (three-way ANOVA/SNK, $P < 0.01$, N = 60). Panel D, IL-6 Serum levels. Data are presented as percentage of control (Mean ± SEM).n per group (C = 13; SS = 13; ST = 12; O = 13; OS = 12; OT = 12). There were no significant effects of the independent variables on serum IL-6 levels (three-way ANOVA, $P > 0.05$, N = 75)

brainstem levels of NGF, which was not reversed by tDCS. The role of NGF in hyperalgesia is well-studied; previous study showed increased NGF levels in the spinal fluid of patients with chronic headaches.[40] As well as, in an animal model, infusion of NGF intracerebroventricular induces a pain-like response in rats.[41] However, few studies have considered the effects of neuromodulatory techniques upon neurotrophins like BDNF and NGF. Furthermore, Brunoni et al,[42] found no tDCS effects upon NGF plasma levels in patients experiencing an acute major depressive episode.

The tDCS montage used in the current study (with the cathodal electrode placed in the supraorbital area and the anodal electrode placed in the parietal cortex) has been previously used by our research group, with good results in different animal models of pain, such as chronic inflammation,[13] hyperalgesia induced by stress[12] and neuropathic pain.[56] Our recent study, using a neuropathic pain mouse model, showed that the antiallodynic effect of tDCS was related to the descending inhibitory pathway, including the opioidergic and monoaminergic system.[58] Furthermore, we have tested the influence of peripheral pathways on the antiallodynic effect of tDCS. Mice were pre-treated with lidocaine injected directly in the scalp 15 minutes before tDCS, and no changes in the analgesic effect were observed. Local anaesthesia did not reverse the effect induced by tDCS.[58] The effects of tDCS have been attributed to the interactions between prosencephalon regions, such as the primary motor cortex (M1), dorsal lateral prefrontal cortex (DLPFC) and cingulate cortex (2011). However, these effects may also involve projections to more remote areas such as the periaqueductal grey area (PAG), which is part of the descending system to the spinal cord.[30]

Interesting to note that different circuits at the spinal and supraspinal levels where assessed by the behavioural nociceptive tests used in current. The local mechanical hyperalgesia was assessed by von Frey test, while remote thermal hyperalgesia by hot plate test. The trigeminal primary afferents pass into the brainstem and terminate in the trigeminal brainstem sensory nuclear complex (subnuclei interpolaris, oralis and caudalis).[55] The subnucleus caudalis has been specifically implicated in orofacial nociceptive mechanisms, and it is compared to the spinal dorsal horn due to its many analogous morphological and physiological features[68] and now it has been termed the medullary dorsal horn.[53,54,68,72] In these regions, the afferent nociceptive fibres

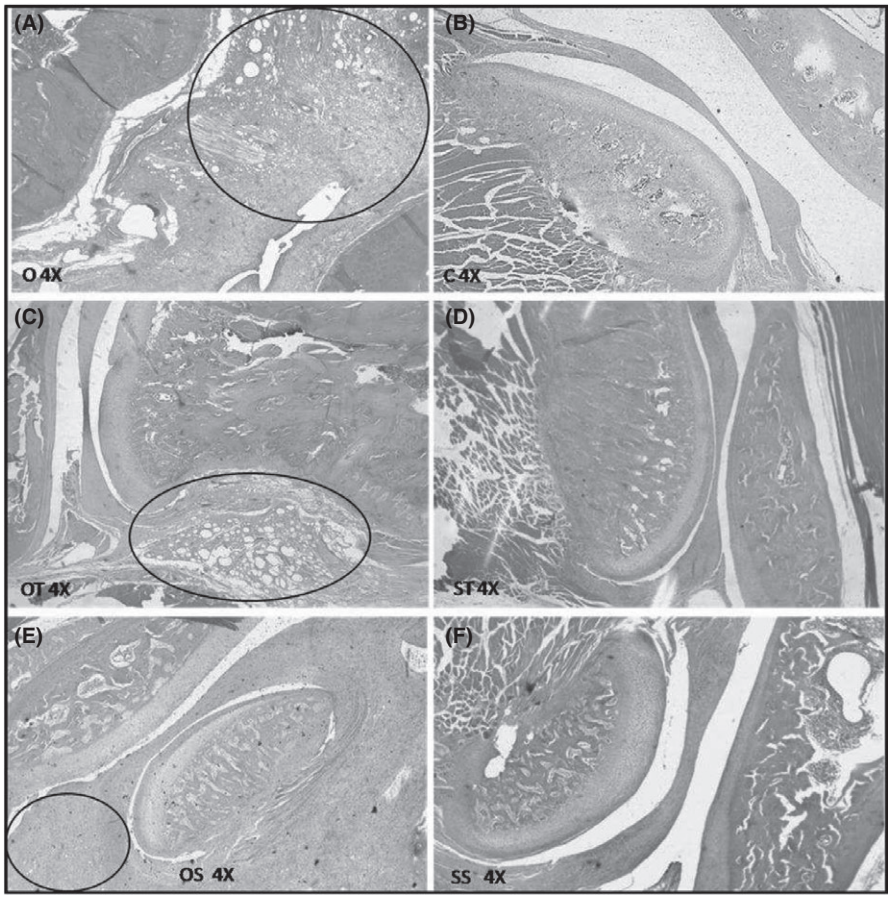


**FIGURE 5** Haematoxylin and eosin stained sections from TMJs. Haematoxylin and eosin stained sections from TMJs. The circles indicate infiltrating mononuclear cells (macrophages, neutrophils and lymphocytes). A,C, and E, severe joint damage with increased inflammation in the retrodiscal area; B,D, and F, normal TMJ architecture. X40

release neuropeptides (substance P and ATP) and excitatory amino acids such as glutamate, that increasingly excite nociceptive neurons (nociceptive-specific, NS; and wide dynamic range, WDR), a process known as central sensitisation. CFA-induced inflammation originating in the TMJ leads to hyperexcitability of medullary dorsal horn neurons and increased responsiveness to thermal and mechanical stimuli. This causes an expansion of peripheral receptive fields, sometimes extending across the midline.[8,16,71]

In addition, clinical evidence in some chronic pain conditions (eg, temporomandibular disorders) reflect a heightened central excitatory state, resulting from a decrease in central inhibitory control mechanisms, and descending modulation from areas in the cerebral cortex, hypothalamus, amygdala and other sites converging on the brain. Periaqueductal grey matter (PAG) modulates nociceptive inputs and pain perception through its interactions with these descending projections, as well as ascending projections from sites such as the spinal dorsal horn and trigeminal brainstem sensory nuclear complex. PAG neuron excitation is associated with inhibition of nocifensive spinal and craniofacial reflexes in the rat.[52,70] Thus, in our study, the reduction in descending modulation due the orofacial pain process could influence the nociceptive response in remote areas, such as in the paw. This would also support that tDCS, through cerebral cortex stimulation, can modulate remote areas of the neuroaxis, such as the brainstem and spinal cord, via top-down modulation across sensory systems.[60,68]

**TABLE 1** Histological score

| Group | Score (Median + min; max) |
|---|---|
| C | 0 (0; 0)[a] |
| SS | 0 (0; 0)[a] |
| ST | 0 (0; 0)[a] |
| O | 3 (2; 3)[b] |
| OS | 3 (3; 3)[b] |
| OT | 3 (2; 3)[b] |

The possible score ranged from 0 = absent, 1 = mild (1%-10%), 2 = moderate (11-50%) to 3 = severe (51%-100%). Significant differences are designated with the matching superscript letters (a, b), analysis by Kruskal-Wallis, $P \leq 0.05$; n = 3-5. C, total control group; O, orofacial pain group; OS, orofacial pain-sham tDCS group; OT, orofacial pain-tDCS group; SS, saline-sham tDCS group; ST, saline-tDCS group.

Additionally, we observed different profiles of neuroimmunomodulators linked to pain conditions and tDCS treatment (BDNF, NGF, IL-6 and IL-10) in the brainstem and blood serum. In craniofacial tissues, these mediators are involved in peripheral sensitisation following inflammation.[52] They are released from mast cells, immune cells, macrophages and injured cells and act on ion channels or membrane receptors on peripheral nociceptive afferent nerve endings and thereby may alter the sensitivity of the endings.[66,52]

Few studies in the literature assess the role of tDCS in the inflammatory process related to pathological conditions as well as to specific central nervous system structures. We evaluated the profile of cytokines associated with orofacial inflammatory pain conditions and tDCS treatment. IL-10 is an anti-inflammatory cytokine released during the resolution phase of inflammation that prevents tissue damage caused by infections and inflammation.[43,44] We observed a decrease in IL-10 levels 7 days after tDCS treatment compared to observed increase 24 hours after the end of tDCS treatment. Also, our previous study showed a decrease in IL-10 levels assessed 7 days after the end of tDCS treatment in a sciatic neuropathic pain model.[56] However, the mechanism for the decrease in IL-10 in the brainstem, the site of the trigeminal first synapse, proposed in this study is nociceptive signalling decrease, since tDCS activated descending pain-inhibitory pathways. Less signalling from these pathways would lead to a decrease in the release of pain-related mediators, including IL-10, resulting in an analgesic effect.[67] Therefore, with less pain, proinflammatory cytokines, neurotrophins leading to neuroplastic maladaptation, and consequently anti-inflammatory cytokines would no longer be needed.

Our IL-10 results at 24 hours after the end of treatment is consistent with previous data that showed an imbalance between pro- and anti-inflammatory cytokines, which stimulates microglia to produce more proinflammatory mediators. This process can lead to neuroinflammation and consequently, neurodegeneration.[46] Shimizu et al,[47] using a CFA-induced inflammation model, generated by CFA injection into the masseter muscle, demonstrated that injection of IL-10 into the subnucleus caudalis of the trigeminal nucleus complex reduces hyperalgesia in the masseter region. Thus, it is possible that in our CFA-induced, orofacial pain model, there is an imbalance in the inflammatory system triggered by decreased IL-10 levels.

Additionally, we observed that groups exposed to orofacial pain had increased brainstem levels of IL-6. This cytokine has known proinflammatory and regulatory effects in neural tissues, immune cells (T and B), macrophages, fibroblasts, microglia and astrocytes. IL-6 is rapidly induced during acute inflammation associated with injury, infection and neuronal death.[48,49] The function of this interleukin includes the promotion of neuronal growth factor synthesis,[49] and interestingly, we found an increase in the levels of IL-6 and NGF in the brainstem. Moreover, sham and active tDCS decreased IL-6 levels in the brainstem, consistent with a study by Brunoni et al[42] that showed a decrease in serum IL-6 levels in depressive patients treated upon tDCS treatment.

We also measured peripheral levels of BDNF, NGF, IL-10 and IL-6 in the blood serum; however, no tDCS effects were observed in their levels. This result does not necessarily mean that tDCS does not induce neuroplastic or neuroimmune effects, but rather that the peripheral blood levels do not reflect the central effects in chronic orofacial pain. Only the main effect observed was interaction between timepoint and orofacial pain on the BDNF levels, with the levels of this mediator returning to baseline levels 7 days after the end of tDCS treatment. Also, a study involving patients with central sensitivity syndrome showed an increase in serum BDNF levels compared to control subjects, suggesting that this neuroplasticity mediator could be a screening tool for pain clinicians.[50]

It is important to highlight that our study has some limitations. First, the sham group received active tDCS for 30 seconds, similar how has been used in clinical research. However, this short application on the small rat's head could modulate remote areas of the neuroaxis, as we observed in the NGF brainstem levels. Seven days after end of treatment, the sham group (OS) showed increased NGF levels compared to control group. Additionally, this increase was state-dependent since this increase was not observed in the no-pain group. Second, as our main objective was to evaluate the therapeutic effect of tDCS after pain model was established, and we have not tested yet the preemptive effects of tDCS on orofacial inflammatory pain. Our recent study has observed that preemptive tDCS prevents stress-induced hyperalgesia[64] and induces analgesia in the post-operative period and contributes to tissue repair, preventing the chronic inflammatory process and fibrosis (Zancanaro, 2017 - *personal communication*).

In summary, we demonstrated that anodal tDCS is effective for relieving short-, medium- and long-term inflammatory pain in rats. tDCS alters neuromodulatory mediators (BDNF, NGF, IL-6 and IL-10) levels in a rat orofacial pain model. These findings suggest that multiple physiologic mechanisms mediate the analgesic effects of tDCS involved in modulating pain processing. Furthermore, we observed an important role of the central immune system in chronic inflammatory pain, which could be involved in secondary hyperalgesia and neuroplastic changes. Our findings also contribute with data about the benefits of tDCS upon chronic pain condition, and an option as a non-pharmacological and noninvasive therapeutic tool. However, additional studies to elucidate the full mechanism of action of anodal tDCS are necessary to comprehend the immediate and delayed effects of this technique in pain processing and relieving.

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## CONFLICT OF INTEREST

None of the authors had any financial or commercial interest in the outcome of this study.

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