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Research article

Bimodal transcranial direct current stimulation reduces alcohol consumption and induces long-term neurochemical changes in rats with neuropathic pain

Daniela Silva Santos [a,b,e], Liciane Fernandes Medeiros [a,b,f], Dirson João Stein [a,b,e], Isabel Cristina De Macedo [a,e], Diego Evandro Da Silva Rios [a,e], Carla De Oliveira [a,e], Roberta Ströher Toledo [a,c,e], Felipe Fregni [d], Wolnei Caumo [b], Iraci L.S. Torres [a,b,c,d,*]

[a] Laboratory of Pain Pharmacology and Neuromodulation: Preclinical Investigations – Hospital de Clínicas de Porto Alegre, Porto Alegre, RS, Brazil
[b] Postgraduate Program in Medical Sciences, School of Medicine, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil
[c] Postgraduate Program in Biological Sciences: Pharmacology and Therapeutics, Institute of Basic Health Sciences, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil
[d] Laboratoryof Neuromodulation, Department of Physical Medicine & Rehabilitation, Spaulding Rehabilitation Hospital and Massachusetts General Hospital, Harvard University, Boston, MA, USA
[e] Animal Experimentation Unit and Graduate Research Group, Hospital de Clínicas de Porto Alegre, Porto Alegre, RS, Brazil
[f] Postgraduate Program in Health and Human Development, Universidade La Salle, Canoas, RS, Brazil

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ABSTRACT

This study aimed to evaluate the effects of repeated bimodal transcranial direct current stimulation (tDCS) on alcohol consumption and immunohistological and neurochemical parameters in nerve-injured rats. Forty-eight adult male Wistar rats were distributed into six groups: control, neuropathic pain (NP) + sham-tDCS, NP + alcohol + sham-tDCS, alcohol + sham-tDCS, alcohol + tDCS, and NP + alcohol + tDCS. NP is induced by chronic sciatic nerve constriction (CCI). The rats were exposed to a 10% alcohol solution by voluntary consumption for 14 days. From the 16th day after surgery, bimodal tDCS was applied for 20 min/day for 8 days. Brain structures were collected to evaluate the number of neuropeptide Y (NPY)-positive neurons, neurites, and argyrophilic grains by immunohistochemistry, and brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), interleukin (IL)-6, and IL-10 by ELISA. Nerve-injured rats showed a progressive increase in alcohol consumption compared to the non-injured rats. In addition, there was a reduction in voluntary alcohol consumption over time induced by tDCS. Alcohol exposure, chronic pain, and tDCS treatment modulated the central NPY immunoreactivity. tDCS increased the cerebellar levels of IL-6 and IL-10, and CCI and/or tDCS reduced striatal BDNF levels. The current data suggest that tDCS could be a promising non-pharmacological adjuvant to treat patients with chronic pain who use alcohol to relieve their symptoms.

1. Introduction

Alcohol provides an accessible means for chronic pain patients to relieve their symptoms, despite its potential consequences for long-term health, such as alcohol dependence and alcohol withdrawal syndrome. Approximately 25% of patients with chronic pain consume alcohol to relieve their symptoms [1]. However, chronic alcohol intake is known to induce selective neuronal damage [2] and exacerbates pain, with marked increases in sensitivity occurring after a period of abstinence [3,4]. Both pain and chronic alcohol consumption result in the interaction of a broad range of physiological mechanisms in the nervous system [5,6]. Several biochemical markers seem to be involved in both chronic alcohol consumption and the development of chronic pain.

The development of alcohol dependency induces an adaptive process in the amygdala-NPY system, as well as in the levels of brain-derived neurotrophic factor (BDNF) in areas such as the hippocampus,

Abbreviations: tDCS, transcranial direct current stimulation; NP, neuropathic pain; NPY, neuropeptide Y.

* Corresponding author at: Laboratory of Pain Pharmacology and Neuromodulation: Preclinical Investigations – Hospital de Clínicas de Porto Alegre, Porto Alegre, RS, Brazil.
E-mail address: iltorres@hcpa.edu.br (I.L.S. Torres).

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cerebral cortex, and striatum [7]. In rodents, NPY regulates negative affective states, anxiety-like behavior, nociception, and reward [7]. Furthermore, through its action on the dopaminergic system, NPY may modulate reward circuitry in animals subjected to alcohol consumption [7]. Preclinical studies have shown significant and temporally dynamic changes in the central and peripheral cytokines under pain conditions [8,9] and alcohol exposure [10].

Considering the complexity of chronic pain and alcohol dependence, it is important to search for new therapeutic options. Neuromodulatory techniques have been used to relieve chronic pain and drug cravings, including alcohol dependence [11]. Particularly, transcranial direct current stimulation (tDCS) is a safe and low-cost tool that can be easily applied to such therapeutic options [12]. Our previous preclinical studies have shown short- and long-term effects on nociceptive parameters after tDCS treatment in different chronic pain models, altering central cytokine (IL-1β, IL-10, TNF-α), and neurotrophin (BDNF) levels [8,13–17]. However, there have been no studies on the effects of tDCS on chronic pain associated with alcohol exposure. In this way, it has been suggested that noninvasive brain stimulation may represent a promising alternative treatment when these conditions are associated.

Thus, in the current study, considering that chronic pain can trigger alcohol consumption in individuals to relieve their symptoms and induce alcohol dependence, we aimed to evaluate the effects of repeated bimodal transcranial direct current stimulation (tDCS) on alcohol consumption and immunohistological and neurochemical parameters in nerve-injured rats.

2. Materials and methods

An expanded version of all methods is available in the Supplementary Methods section.

2.1. Animals

Forty-eight adult male Wistar rats (200–250 g) from the Center for Reproduction and Experimentation of Laboratory Animals at Universidade Federal do Rio Grande do Sul/Brazil were used in this study. Initially, animals were randomized by weight and maintained in groups of four per cage (49 × 34 × 16 cm). Rats were housed in a vivarium under standard environmental conditions under a 12-hour light/dark cycle (lights on at 7 a.m.). The animals had *ad libitum* access to water and rodent chow. All experimental procedures were approved by the Institutional Animal Care and Use Committee (GPPG-HCPA#15.0501). The experimental protocol complied with the ethical and methodological standards of the ARRIVE guidelines [18].

2.2. Experimental design

The rats were acclimated to the vivarium for 15 d. The rats were randomly assigned by weight to six groups (n = 8/group): control (CT) – no manipulation; neuropathic pain (NP) – chronic sciatic nerve constriction injury (CCI) plus sham tDCS; NP + alcohol (NPAL) – CCI and alcohol administration plus sham-tDCS; alcohol (AL) – alcohol administration plus sham-tDCS; AL + tDCS (ALtDCS) – alcohol administration and tDCS treatment; and NP + AL + tDCS (NPALtDCS) – CCI, alcohol administration, and tDCS treatment. The sequence of steps and experimental group sizes are elaborated in the experimental design (Fig. 1). All investigators were blinded to the treatment so that the bias between the groups receiving active or sham tDCS treatment could be diminished. Three researchers analyzed the results; importantly, these evaluators were unaware of the experimental protocol. Rats were killed by decapitation 7 days after the end of the tDCS treatment and alcohol withdrawal.

2.3. Neuropathic pain model

NP was induced by CCI of the sciatic nerve according to the method described by Bennett and Xie [19].

2.4. Model of alcohol exposure

The protocol for voluntary ethanol consumption in the two-bottle choice model was adapted from Carnicella et al. [20].

2.5. Transcranial direct current stimulation (tDCS)

Rats were subjected to bimodal tDCS (0.5 mA) under immobilization for 20 min per day for 8 consecutive days, starting on the 16th day after CCI surgery (Fig 2.) [14,8,17].

2.6. Immunohistochemistry

Two animals were used for evaluating the brain structures (PFC, amygdala, and striatum) that had been fixed in 10% buffered formaldehyde, processed, and embedded in paraffin. An average of 16 fields were used to obtain the immunoreactivity of NPY in neurons, neurites,

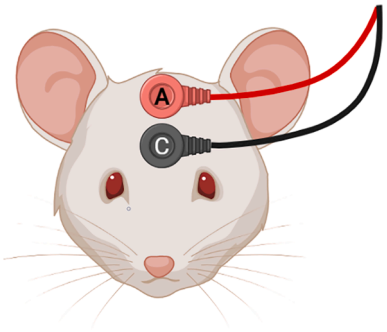

Fig. 2. Electrode placement on the rat scalp during tDCS sessions. A = Anode; C = Cathode. Created with BioRender.com.

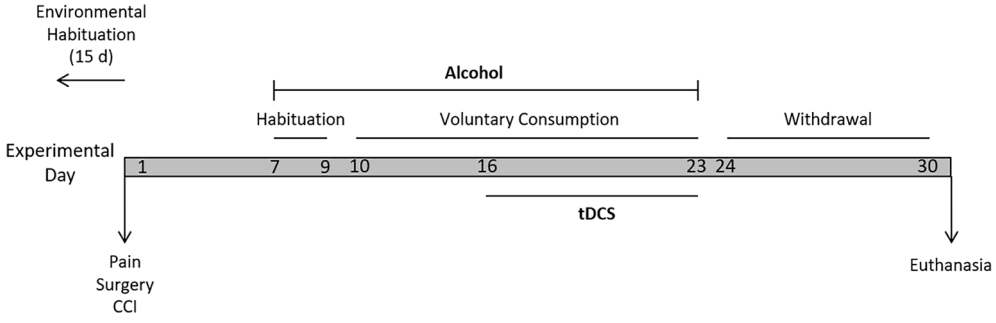

Fig. 1. Experimental timeline. CCI: chronic constriction injury; tDCS: transcranial direct current stimulation.

and argyrophilic grains per field per rat, and the average was obtained between two rats per group. Numbers indicate an increase or decrease of staining in comparison to the control group (% of control of immunoreactive cells/field) (Table 1). Expressive changes were conventionally considered when there was an increase or decrease in NPY staining in over 40% of the control group. The results were calculated as the percentage of control and expressed as delta variation of control (value of group −100).

2.7. Determination of biomarker central levels

The levels of BDNF, NGF, IL-6, and IL-10 were determined using a sandwich enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Minneapolis, USA), which uses specific monoclonal antibodies.

2.8. Statistical analysis

Data are expressed as the mean ± standard error of the mean (SEM). NP establishment was detected by the von Frey test at baseline and 14 days after surgery, and alcohol consumption analysis was carried out using generalized estimated equations (GEEs) followed by Bonferroni's correction. The levels of biomarkers were analyzed using one-way ANOVA followed by the Student–Newman–Keuls (SNK) method. The immunohistochemical detection of NPY was calculated as the percentage of control of the number of immunoreactive cells per field and expressed as delta variation of the control. Statistical significance was set at $P < 0.05$. Statistical analysis was performed using SPSS version 26.0.

3. Results

Data from the nociceptive tests were reported in the Supplementary Results section (Supplementary Fig. 1).

3.1. Alcohol consumption

There was an interaction between group and time (GEE: Wald $\chi^2 = 9929.081$, $P < 0.001$). On day 11, nerve-injured rats consumed less alcohol than non-injured rats. On days 13 and 15, alcohol consumption decreased in all the groups. On day 17, a peak of alcohol consumption was observed in all groups, except in the non-injured groups subjected to tDCS. On day 19, there was a decrease in alcohol consumption in all groups; however, the consumption of injured rats increased from this point on. On day 23, the nerve-injured rats exposed to alcohol (NPAL) increased their consumption, while both groups subjected to tDCS (ALtDCS and NPALtDCS) showed less alcohol consumption, and the AL group presented stabilized levels of alcohol consumption. It is interesting to note that rats in pain increased alcohol consumption over time, while pain-free rats showed decreased alcohol consumption (Fig. 3; Supplementary Fig. 2; Supplementary Table 1).

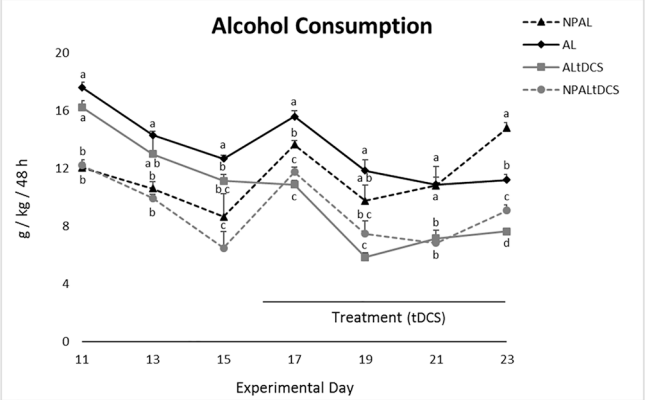

Fig. 3. Measurement of alcohol consumption. Data were expressed as mean ± S.E.M. Different letters show significant differences between groups. There was a significant effect of time (GEE, $P < 0.05$) and group (GEE, $P < 0.001$). There was an interaction between group and time (GEE, $P < 0.001$). AL: alcohol group; NPAL: neuropathic pain plus alcohol group; ALtDCS: alcohol plus tDCS group; NPALtDCS: neuropathic pain plus alcohol plus tDCS group. n = 8 / group.

3.2. Neuropeptide Y

Alcohol exposure, nerve injury, and tDCS treatment modulated central NPY immunoreactivity (Table 1). Analysis of the PFC showed that the CCI induced an increase in the immunoreactivity for NPY in the argyrophilic grains (169.38%); the NPAL group showed an increase in neurites (91.14%) and argyrophilic grains (205.26%); and the AL group showed increased immunoreactivity in neurites (100.24%) and argyrophilic grains (255.98%). The rats subjected to alcohol consumption plus tDCS treatment (ALtDCS group) showed an increase in NPY immunoreactivity in argyrophilic grains (184.78%); the NPALtDCS group showed increased NPY in neurites (54.73%) and argyrophilic grains (96.17%) (Fig. 4; Table 1).

The amygdala analysis showed that the NP group presented an increase in the number of NPY-positive neurons (117.29%), neurites (115.66%), and argyrophilic grains (66.97%). The NPAL group showed an increase in NPY immunoreactivity in neurites (129.38%); however, the increase in neurons induced by CCI was reversed when the rats were exposed to alcohol (a decrease of 12.91%), while the argyrophilic grains (an increase of 12%) were similar to control levels. The AL group showed an increase in immunoreactivity in neurites (89.40%), while the NPALtDCS group showed a decrease in neurons (46.19%), reverting the pain effect, and an increase in the immunoreactivity for NPY in neurites (136.70%) and argyrophilic grains (42.71%) (Fig. 5; Table 1).

The striatum analysis showed that NPY immunoreactivity decreased in the NP group (56.46% in neurons; 61.19% in neurites), AL group (57.21% in neurons), and ALtDCS group (60.36% in neurons; 56.72% in neurites). The animals exposed to CCI, alcohol, and tDCS presented a

Table 1
Central NPY immunodetection.

Experimental Groups	Brain Structure *Variable (NPY location)*								
	Prefrontal Cortex			Amygdala			Striatum		
	Neurons	Neurites	Grains	Neurons	Neurites	Grains	Neurons	Neurites	Grains
Neuropathic Pain (NP)	−14.55	6.19	**169.38**	**117.29**	**115.66**	**66.97**	**−56.46**	**−61.19**	−36.19
Neuropathic Pain + Alcohol (NPAL)	−19.69	**91.14**	**205.26**	−12.91	**129.38**	12.00	−33.33	−37.31	−3.81
Alcohol (AL)	2.68	**100.24**	**255.98**	14.34	**89.40**	29.03	**−57.21**	−31.34	−12.38
Alcohol + tDCS (ALtDCS)	26.39	18.33	**184.78**	−39.24	5.22	21.02	**−60.36**	**−56.72**	−23.81
Neuropathic Pain + Alcohol + tDCS (NPALtDCS)	−17.90	**54.73**	**96.17**	**−46.19**	**136.70**	**42.71**	−16.67	−13.43	−28.67

Data were calculated as % of control (CT) of immunoreactive cells/field and expressed as delta variation of control (value of control − 100). NPY = Neuropeptide Y; tDCS = Transcranial Direct Current Stimulation. The results were calculated by % of immunoreactive cells/field of the control group and expressed as delta variation of control (value of group −100). Expressive changes (in Bold) were considered: a decrease or an increase of over 40% of control. n = 2 rats / group.

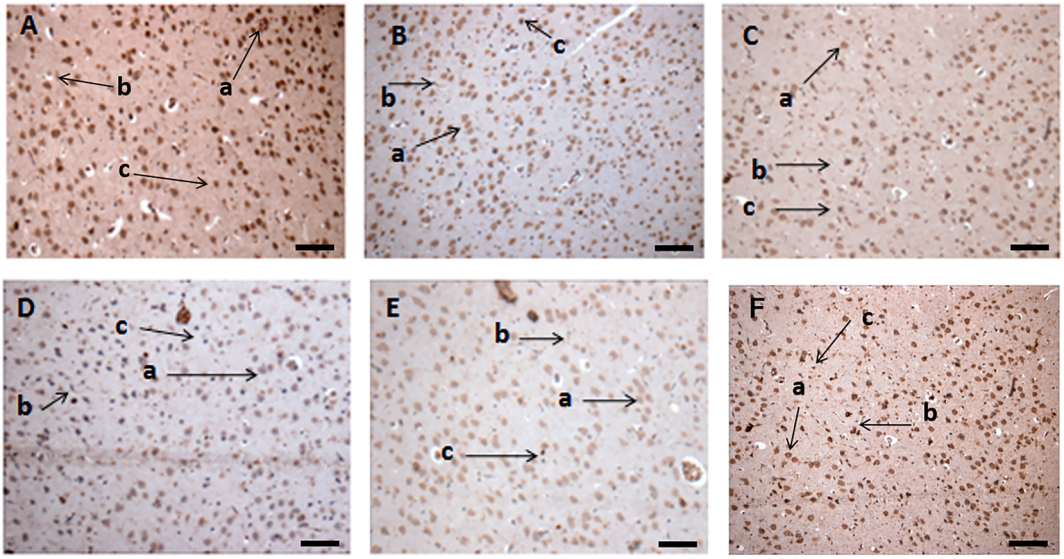

Fig. 4. Representative images of NPY immunodetection in neurons (a), neurites (b), and argyrophilic grains (c) in the Prefrontal Cortex (Image-Pro Plus 7.0, Media Cybernetics, Rockville, MD, USA). (A) CT: control group; (B) NP: neuropathic pain group; (C) NPAL: neuropathic pain plus alcohol group; (D) AL: alcohol group; (E) ALtDCS: alcohol plus tDCS group; (F) NPALtDCS: neuropathic pain plus alcohol plus tDCS group. Magnification: 40X; Scale bars: 500 μm.

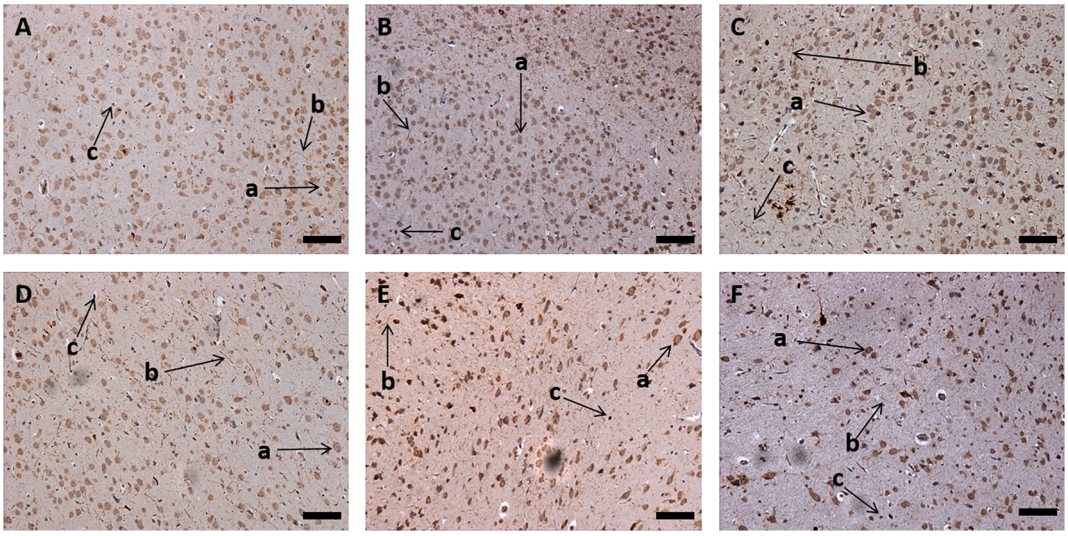

Fig. 5. Representative images of NPY immunodetection in neurons (a), neurites (b), and argyrophilic grains (c) in the amygdala (Image-Pro Plus 7.0, Media Cybernetics, Rockville, MD, USA). (A) CT: control group; (B) NP: neuropathic pain group; (C) NPAL: neuropathic pain plus alcohol group; (D) AL: alcohol group; (E) ALtDCS: alcohol plus tDCS group; (F) NPALtDCS: neuropathic pain plus alcohol plus tDCS group. Magnification: 40X; Scale bars: 500 μm.

reversal of these decreases in immunoreactivity (Fig. 6; Table 1).

3.3. BDNF, NGF, IL-6, and IL-10 levels in the cerebellum and striatum

In the cerebellum, the ALtDCS group presented an increase in IL-6 levels compared to the NPAL group (one-way ANOVA/SNK, $F(5,42) = 2.61$, $P < 0.05$), and IL-10 levels compared to the other groups (one-way ANOVA/SNK: $F(5,42) = 2.92$, $P < 0.05$). There were no differences in cerebellar BDNF and NGF levels between groups (one-way ANOVA: $P > 0.05$, for both) (Table 2).

In the striatum, the NPAL, ALtDCS, and NPALtDCS groups showed lower BDNF levels than the CT group (one-way ANOVA/SNK, $F(5,42) = 5.24$, $P < 0.05$). In addition, the NP group showed a significant decrease in the CT and AL groups (one-way ANOVA/SNK: $F(5,42) = 5.24$, $P < 0.001$). The striatal NGF, IL-10, and IL-6 levels were not different between groups (one-way ANOVA, $P > 0.05$, for all) (Table 2).

4. Discussion

In the current study, we showed that tDCS decreases alcohol consumption, an effect that is more pronounced in non-injured rats. The alcohol consumption level is dependent on the state of the animal; while the non-injured animals consumed more alcohol initially, during the treatment, it was the injured animals that consumed more alcohol, as observed on day 23 (NPAL group). The peak of alcohol consumption was observed on day 17, except for the non-injured rats subjected to tDCS treatment. Alcohol exposure, CCI, and tDCS treatment modulated NPY immunoreactivity in the PFC, amygdala, and striatum. Moreover, only the association between alcohol withdrawal and tDCS treatment increased the cerebellar levels of IL-6 and IL-10. The striatal BDNF levels were decreased by CCI and tDCS treatment, demonstrating the long-

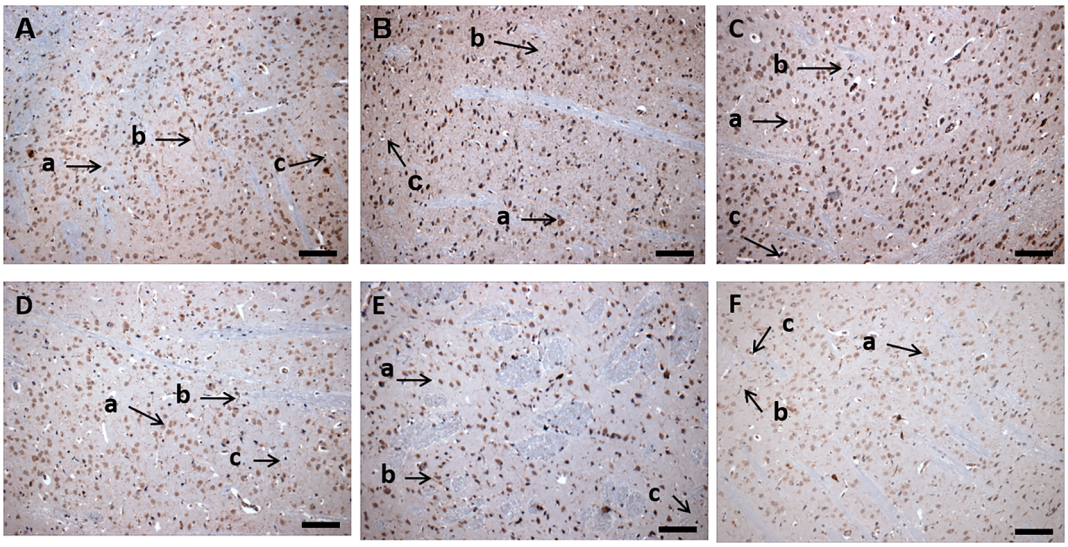

Fig. 6. Representative images of NPY immunodetection in neurons (a), neurites (b), and argyrophilic grains (c) in the striatum (Image-Pro Plus 7.0, Media Cybernetics, Rockville, MD, USA). (A) CT: control group; (B) NP: neuropathic pain group; (C) NPAL: neuropathic pain plus alcohol group; (D) AL: alcohol group; (E) ALtDCS: alcohol plus tDCS group; (F) NPALtDCS: neuropathic pain plus alcohol plus tDCS group. Magnification: 40X; Scale bars: 500 μm.

Table 2
Neurotrophin and cytokine levels in the brain.

Experimental Groups	**Brain Structure** *Variable*							
	Cerebellum				**Striatum**			
	BDNF	*NGF*	*IL-6*	*IL-10*	*BDNF*	*NGF*	*IL-6*	*IL-10*
Control (CT)	18.64 ± 1.01	2.60 ± 0.15	99.72 ± 17.31	1.68 ± 0.21	49.18 ± 5.70	14.13 ± 2.63	159.53 ± 20.18	17.87 ± 3.09
Neuropathic Pain (NP)	19.14 ± 2.06	2.58 ± 0.16	92.51 ± 21.08	1.72 ± 0.15	**18.88 ± 1.15** **	10.05 ± 1.53	124.24 ± 13.89	10.72 ± 1.25
Neuropathic Pain + Alcohol (NPAL)	14.42 ± 1.23	2.25 ± 0.08	52.36 ± 10.42	1.63 ± 0.17	**26.80 ± 1.90** *	10.12 ± 1.31	165.39 ± 13.38	16.31 ± 1.60
Alcohol (AL)	16.34 ± 0.34	2.44 ± 0.11	88.71 ± 8.09	1.90 ± 0.17	38.22 ± 7.90	14.22 ± 2.89	168.42 ± 18.96	15.99 ± 3.68
Alcohol + tDCS (ALtDCS)	19.04 ± 2.04	2.95 ± 0.28	**115.69 ± 13.26 #**	**2.92 ± 0.55 +**	**28.26 ± 4.09** *	12.93 ± 2.11	168.55 ± 13.74	14.26 ± 2.78
Neuropathic Pain + Alcohol + tDCS (NPALtDCS)	18.72 ± 1.60	2.54 ± 0.18	71.53 ± 5.73	1.74 ± 0.25	**26.44 ± 3.87** *	8.67 ± 1.05	124.76 ± 25.71	10.83 ± 1.95

Data are expressed as mean ± S.E.M of pg/mg of protein. IL-6: # significant difference between ALtDCS and NPAL groups in cerebellum (one-way ANOVA / SNK, $P < 0.05$). IL-10: + significant difference from other groups in cerebellum (one-way ANOVA / SNK, $P < 0.05$). BDNF: * significant difference compared to CT group in striatum (one-way ANOVA / SNK, $P < 0.05$); ** significant difference compared to CT and AL groups in striatum (one-way ANOVA / SNK, $P < 0.05$). n = 6 rats / group.

term effect of tDCS (7 days after the end of treatment).

The increased alcohol consumption over time induced by CCI may be related to its analgesic effect, as suggested previously [21], and corroborated by a systematic review suggesting alcohol-inducing pain relief [22]. A study showed an inverse relationship between drinking levels and hyperalgesia over four weeks in rats with inflammatory pain, without an increase in the absolute value of alcohol consumption over time, corroborating the analgesic effect [23]. However, the mechanisms of action remain unclear [24]. Currently, it is believed that the neurotransmitter systems and inflammation are common pathways involved in both pathologies: chronic pain [8] and alcohol withdrawal [25]. In addition, previous studies have shown that the affective and sensory dimensions of pain may be important factors in alcohol abstinence syndrome, and the relief of negative emotional states can trigger alcohol consumption [26,27]. Our results corroborate clinical studies, where repeated tDCS applied bilaterally over the PFC is a promising adjunctive tool to reduce alcohol craving and relapse, facilitating alcoholism cessation [28,29]. We have also shown that tDCS inhibits food cravings in rats [30]. Both drugs and food are known to develop addiction by activation of brain circuitries involved in reward, motivation, and decision-making processes [31].

The current study showed that CCI promoted a more pronounced decrease in striatal BDNF levels than other conditions, and alcohol consumption and tDCS increased the cerebellar levels of IL-6 and IL-10. Like cytokines, BDNF is an important regulator of synaptic plasticity and memory formation [32]. Our previous study showed an increase in PFC BDNF levels and IL-10 levels in the hippocampus, PFC, and brainstem in rats even after 11 days of alcohol withdrawal [25]. Proinflammatory cytokines, such as IL-6, play a major role in initiating and sustaining inflammatory events, and their roles are tissue-specific and can be altered by alcohol [33], and by tDCS [8,9,13,14]. The striatum and cerebellum participate in the accessory motor system, acting in fine-tuning motor movements, which can be related to the tDCS effects and alcohol abstinence observed in the current study. A previous study showed that patients abstaining from alcohol had reduced inhibitory control and higher trait impulsivity, which characterized a dysfunction in the neural inhibitory ability during movement preparation [34]. Thus, we suggest that tDCS over the cerebral cortex of rats can modify inhibitory processes, decrease alcohol consumption, and alter biomarkers.

To the best of our knowledge, this is the first study to demonstrate the effects of tDCS treatment on NPY-immunoreactivity in alcohol consumption/withdrawal. NPY has been shown to modulate both alcohol consumption and aversion-resistant intake, which may be a secondary effect of prolonged alcohol consumption [35]. PFC, amygdala, and striatum were chosen by their involvement in the rewarding system [36], and drug addiction [37]. NPY is associated with both alcohol consumption/withdrawal and chronic pain [38,39]. We observed that CCI modulated the NPY-immunoreactivity in the PFC and in the amygdala, increasing its levels, and decreasing it in the striatum; however, the association with alcohol reversed this effect in the amygdala. In addition, tDCS reversed the increased NPY-immunoreactivity by alcohol consumption/withdrawal in the PFC, demonstrating the modulatory effect of tDCS. Moreover, the association between alcohol and tDCS induced a decrease in NPY immunoreactivity in the amygdala. These results suggest a modulatory effect of tDCS and alcohol consumption in a maladaptive state induced by CCI.

In the PFC, alcohol, CCI, and tDCS treatment altered NPY immunoreactivity, characterizing a greater sensitivity of argyrophilic grains to these interventions, and cells' specific NPY response. The PFC participates in decision-making, executive function, and reward circuitry [40,41,42]. In addition, individuals with chronic pain have been hypothesized to present deficits in PFC functioning and may be more susceptible to alcohol misuse and poor pain management [26]. Considering that argyrophilic grains are granular or punctuate deposits related to neurodegenerative disorders [43], the increase in NPY immunoreactivity in the grains from the PFC may be involved in these harmful effects.

Finally, some limitations of our study should be pointed out: (1) we did not evaluate the cause-effect relationship between the central NPY immunoreactivity and alcohol exposure or tDCS; (2) the groups of non-injured rats did not undergo surgery, anesthesia, or analgesic procedures. Thus, the possible effect on alcohol consumption cannot be discarded; (3) there is no sham-tDCS group; however, to replace this, we opted to immobilize rats from all groups except for the control.

5. Conclusion

Our results show that the level of alcohol consumption is dependent on the state of the animal, and bimodal tDCS treatment decreases alcohol consumption independent of the presence of CCI in rats. Considering our results, and given that the changes in the NPY and BDNF levels were modulated in the striatum, we believe that an investigation of the relationship between NPY and BDNF may clarify aspects of pain and tDCS effects. Thus, these results suggest that tDCS can be a non-pharmacological adjuvant for treating alcohol consumption or withdrawal symptoms in patients with chronic pain who use alcohol to relieve their symptoms.

CRediT authorship contribution statement

Daniela Silva Santos: Conceptualization, Data Curation, Formal analysis, Investigation, Methodology, Visualization, Writing - original draft, Writing - review & editing. **Liciane Fernandes Medeiros:** Formal analysis, Methodology, Visualization, Writing - original draft, Writing - review & editing. **Dirson João Stein:** Formal analysis, Visualization, Writing - original draft, Writing - review & editing. **Isabel Cristina De Macedo:** Conceptualization, Data curation, Formal analysis, Methodology, Visualization, Writing - review & editing. **Diego Evandro Da Silva Rios:** Investigation, Writing - review & editing. **Carla De Oliveira:** Investigation, Methodology, Writing - review & editing. **Roberta Ströher Toledo:** Investigation, Methodology, Writing - review & editing. **Felipe Fregni:** Conceptualization, Writing - review & editing. **Wolnei Caumo:** Conceptualization, Writing - review & editing. **Iraci L. S. Torres:** Conceptualization, Formal analysis, Funding acquisition, Project administration, Resources, Supervision, Validation, Visualization, Writing - original draft, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Author contributions

DSS, ICM, and ILST were responsible for the study concept and design. DSS, DESR, CO, and RS contributed to the acquisition of the data. DSS, DJS, LFM, and ILST were responsible for data analysis. DSS, DJS, LFM, FF, WC, and ILST drafted the manuscript. All authors revised and edited the manuscript and approved the final version.

Author statement

All authors revised and edited the manuscript and approved the final version.

Study area/sample collection

Pharmacology and Neurochemistry.

Ethical approval

Ethical Committee Approval Number**:** GPPG-HCPA#15.0501.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neulet.2021.136014.

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