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Transcranial direct current stimulation (tDCS) reverts behavioral alterations and brainstem BDNF level increase induced by neuropathic pain model: Long-lasting effect

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ABSTRACT

Introduction: Neuropathic pain (NP) is a chronic pain modality that usually results of damage in the somatosensory system. NP often shows insufficient response to classic analgesics and remains a challenge to medical treatment. The transcranial direct current stimulation (tDCS) is a non-invasive technique, which induces neuroplastic changes in central nervous system of animals and humans. The brain derived neurotrophic factor plays an important role in synaptic plasticity process. Behavior changes such as decreased locomotor and exploratory activities and anxiety disorders are common comorbidities associated with NP.
Objective: Evaluate the effect of tDCS treatment on locomotor and exploratory activities, and anxiety-like behavior, and peripheral and central BDNF levels in rats submitted to neuropathic pain model.
Methods: Rats were randomly divided: Ss, SsS, SsT, NP, NpS, and NpT. The neuropathic pain model was induced by partial sciatic nerve compression at 14 days after surgery; the tDCS treatment was initiated. The animals of treated groups were subjected to a 20 minute session of tDCS, for eight days. The Open Field and Elevated Pluz Maze tests were applied 24 h (phase I) and 7 days (phase II) after the end of tDCS treatment. The serum, spinal cord, brainstem and cerebral cortex BDNF levels were determined 48 h (phase I) and 8 days (phase II) after tDCS treatment by ELISA.
Results: The chronic constriction injury (CCI) induces decrease in locomotor and exploratory activities, increases in the behavior-like anxiety, and increases in the brainstem BDNF levels, the last, in phase II (one-way ANOVA/SNK, $P < 0.05$ for all). The tDCS treatment already reverted all these effects induced by CCI (one-way ANOVA/SNK, $P < 0.05$ for all). Furthermore, the tDCS treatment decreased serum and cerebral cortex BDNF levels and it increased these levels in the spinal cord in phase II (one-way ANOVA/SNK, $P < 0.05$).
Conclusion: tDCS reverts behavioral alterations associated to neuropathic pain, indicating possible analgesic and anxiolytic tDCS effects. tDCS treatment induces changes in the BDNF levels in different regions of the central nervous system (CNS), and this effect can be attributed to different cellular signaling activations.
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1. Introduction

Neuropathic pain, a condition of complex chronic pain, typically derives from damage to the somatosensory system, which in some cases can paradoxically lead to abnormally increased nerve activity (Treede

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et al., 1999) characterized by hyperalgesia, allodynia, and spontaneous pain (Florio et al., 2009). Anxiety symptoms are in fact frequently reported in patients with neuropathic pain. Studies have shown that a large number of patients with chronic pain suffer from depression or anxiety disorders resulting from severe pain (Edwards, 2005). Animal models of neuropathic pain have demonstrated that the level of mechanical sensitivity is positively correlated with anxiety behaviors (Hasnie et al., 2007) as indexed by increased thigmotaxis in the open-field arena (Wallace et al., 2007). A previous study using neuropathic pain models showed that rats exhibited mechanical hypersensitivity and heightened anxiety-like behavior, which was reduced by treatment with analgesic drugs (Roeska et al., 2009).

Recently, it has been reported that activity-dependent synaptic plasticity in the spinal cord dorsal horn might be a contributive mechanism to the development of chronic pain produced by sciatic nerve ligation in rats (Geng et al., 2010). It has also been proposed that the brain-derived neurotrophic factor (BDNF) has an important role in synaptic plasticity as well as in spinal dorsal horn nociceptive information signaling (Miletic and Miletic, 2002). Likewise, BDNF acts as a modulator in the nociceptive response following spinal cord lesion, playing a key role in the development of neuropathic pain after peripheral nerve injury (Fukuoka et al., 2001). Synapses within each relay are under precise regulation in order to provide appropriate behavioral responses (Zhuo et al., 2011). For example, functional and morphological changes in neuronal circuits during transcranial direct current stimulation (tDCS) seem to require the regulation of BDNF expression (Ganguly and Poo, 2013), and for that reason, this neurotrophin has been used as a biomarker for cortical excitability effects on neuronal activity (Soltész et al., 2014).

Transcranial direct current stimulation is a non-invasive technique that modulates cortical excitability in the human motor (Nitsche and Paulus, 2000) and visual (Antal et al., 2010) cortex. This technique modifies not only the activity of cortical areas located directly under the electrodes, but also from distant areas—probably due to primary interconnections (Lang et al., 2005). The current model of tDCS effects is based on cortico-cortical interactions, with some subcortical components (e.g., anterior cingulate cortex and thalamic nuclei) in those circuits (Kuo et al., 2007). Clinical studies have shown that tDCS can improve cognition performance (Utz et al., 2010) in stroke patients (Hummel and Cohen, 2006) and chronic pain syndromes (DosSantos et al., 2012). Studies using rats have demonstrated the effects of tDCS on memory (Dockery et al., 2011; Li et al., 2011), Parkinson's disease, and focal epilepsy models (Liebetanz et al., 2002). Additionally, data from our research group revealed both immediate and long-lasting effects of repeated sessions of anodal tDCS treatment on the chronic inflammation (Laste et al., 2012) and hyperalgesia induced by chronic restraint stress models (Spezia Adachi et al., 2012). Considering the effects of tDCS on pain and psychiatric disorders, the aim of this study was to evaluate the effect of tDCS treatment on the locomotor and exploratory activities, anxiety-like behaviors, and peripheral and central BDNF levels of rats subjected to the neuropathic pain model over the medium (48 h) and long term (7 days) after tDCS treatment completion.

2. Methods

2.1. Animals

Male Wistar rats (weight, ≥250 g) aged between 55 and 65 days at the beginning of the experiment were used. The animals (n = 144) were randomized by weight and housed in groups of three rats per polypropylene cage (49 × 34 × 16 cm) with sawdust-covered flooring. All animals were maintained in a controlled environment (22 ± 2 °C) under a standard light–dark cycle (lights-on at 0700 h and lights-off at 1900 h), with water and chow (Nuvital, Porto Alegre, Brazil) ad libitum. All experiments and procedures were approved by the Institutional Committee for Animal Care and Use (GPPG-HCPA protocol no. 12-0514) and conformed to the Guide for the Care and Use of Laboratory Animals (8th ed., 2011). The maintenance of the animals followed the Brazilian law 11794, which establishes procedures for the scientific use of animals. The experimental protocol complied with the ethical and methodological standards of the ARRIVE guidelines (Kilkenny et al., 2010). The experiment used the number of animals necessary to produce reliable scientific data.

2.2. Neuropathic pain model: chronic constriction injury (CCI) of the sciatic nerve

The chronic constriction injury (CCI) of the sciatic nerve described by Bennett and Xie (1988) was used as a model for the induction of neuropathic pain. Briefly, the animals were anesthetized using isoflurane (5% for induction, 2.5% for maintenance) and placed in the dorsal position for the left thigh hair shaving and skin antisepsis with 2% alcoholic iodine (Bennett and Xie, 1988). Using aseptic techniques, the left common sciatic nerve was exposed at the middle third of the thigh by removing part of the biceps femoris muscle. Close to the sciatic trifurcation, approximately 7 mm of the first one-third of the nerve was released from adhering tissue and three ligatures (4-0 Vicryl) were tied at 1 mm intervals. Thus, the total length of the nerve involved was approximately 5 mm. Ligations were loose in order to minimize nerve constriction and allow epineural blood flow. To ensure equal level of constriction, the same investigator performed the ligatures in all rats. After the procedure, the surgical incision was closed using 4-0 mononylon. For the sham surgery, the sciatic nerve was exposed similarly to the CCI model, but not ligated. After surgery and anesthetic recovery, the animals were returned to their cages, where they remained until the day of death. The control group did not undergo any surgical procedure.

2.3. Transcranial direct current stimulation (tDCS)

Fourteen days following surgery (CCI or sham), the animals received anodal tDCS therapy, which consisted of a constant low intensity current (0.5 mA) applied for 20 min every afternoon during 8 days, as described by Spezia Adachi et al. (2012). The animals remained under light display after the completion of the application of tDCS. Notably, this model of application required no anesthesia, unlike models used in previous tDCS studies with rats (Liebetanz et al., 2009). In fact, this lack of anesthesia adds strength to the study, because volatile anesthesia (such as isoflurane) has been shown to decrease excitatory transmission and to increase inhibitory transmission (Ouyang and Hemmings, 2005), altering BDNF levels and, consequently, neuroplasticity (Lu et al., 2006). Thus, we removed this confounding factor by adapting the human model using ECG electrodes (Wachter et al., 2011) on the rats.

The direct current was delivered from a battery-powered, constant current stimulator using ECG electrodes with conductive adhesive hydrogel. The heads of the rats were shaved to improve electrode adherence; the electrodes were trimmed to 1.5 cm^2 surface area for better fit. The electrodes were fixed to the head using adhesive tape (Micropore™) and covered with a protective mesh to prevent removal. The cathode was positioned at the midpoint between the lateral angles of both eyes (supraorbital area) and the anode was placed between the ears, on the neck of the rat (parietal cortex). This technique mirrors tDCS protocols for humans (Nitsche et al., 2008) and has been applied by our research group, showing antinociceptive effects (Laste et al., 2012; Spezia Adachi et al., 2012).

For the sham stimulation, the electrodes were placed in the same positions as the real stimulation; however, the stimulator remained in the "off" position throughout the procedure so as to use the same blinding methodology as used in humans.

2.4. Experimental design

The animals were acclimated to the study environment for 1 week before the beginning of the experiment, after which time they were randomly allocated into 6 groups: Sham Surgery (Ss), Sham Surgery + Sham tDCS (SsS), Sham surgery + tDCS (SsT), Neuropathic Pain (Np), Neuropathic Pain + Sham tDCS (NpS), and Neuropathic Pain + tDCS (NpT). Subsequently, the surgical groups received their respective interventions (CCI or sham surgery). Fourteen days later, the hot plate test was conducted to evaluate thermal pain sensitivity (hyperalgesia) as an efficacy control of the pain model whereby the presence of neuropathic pain in the CCI groups was verified. The animals were then treated for 8 days according to the specific protocol for each group (tDCS, Sham-tDCS, or no treatment).

To test the effects of tDCS on the animals' behavior, the Open Field (OF) and Plus Maze (EPM) tests were conducted at the time of tDCS after the seventh session (Behavior test I) and one week after the last treatment day (Behavior test II). For the biochemical assays, each group of animals was further divided into 2 subgroups and killed at different timepoint treatments (at 2 and 7 days post-tDCS). Since the control group of this experiment (naive animals) obtained similar results to the sham surgery animals in behavioral pain tests, with no statistically significant difference between them, we chose to use the sham surgery as the control relative to the other groups.

2.5. Open field test (OF)

The behavioral assessment was performed in a 60 × 40 × 50 cm varnished wooden cage with glass-lined inside walls. The linoleum floor was divided by dark lines into twelve 13 × 13 cm squares. Each trial started immediately after the animals were placed in the back left corner and allowed to explore the surroundings for 5 min (Bianchin et al., 1994; Medeiros et al., 2012). The box was cleaned between trials.

Three measures were evaluated during the test: (1) latency to leave the first quadrant (in seconds); (2) number of total crossings, and (3) number of rearing behaviors (i.e., vertical activity). The number of line crossings (all paws crossing the boundary into an adjacent marked-out area) was taken as a measure of locomotor activity (Roesler et al., 1999). The latency to leave the first quadrant assessed anxiety-like behaviors (Britton and Britton, 1981). The amount of time the animals spent rearing (standing upright on its hind legs) (Wells et al., 2013) was used to assess exploratory activity (Medeiros et al., 2012). Grooming was defined as the licking/washing of the head and/or body and indicated biological functions of caring for the body surface (Silveira et al., 2005).

2.6. Elevated plus-maze test (EPM)

The elevated plus-maze test was used mainly to assess anxiety-like behaviors. The maze was constructed using black PVC and elevated to a height of 50 cm above floor level. The apparatus comprised two open arms and two enclosed arms (50 × 40 × 10 cm), which extended from a common central platform (10 × 10 cm). The animal was placed in the EPM central area facing one of the open arms. During a 5-min session, the following behavioral measures were recorded: (1) number of non-protected head-dipping movements (NPHD); (2) total time spent in the open arms (TOA), and (3) total time spent in the closed arms (TCA). Non-protected head-dipping movements were considered to occur when the animal dipped its head over the sides of the maze while in an open arm. In the EPM, entering a new area was counted when all four paws crossed into the new arm or the central area (Rozisky et al., 2014). After each test, the apparatus was cleaned to remove any scent from the previously tested rat.

2.7. Tissue collection

The animals were killed by decapitation 48 h (Phase I) or 7 days after the completion of the tDCS treatment (Phase II). Central nervous system structures (prefrontal cortex, brainstem, and spinal cord) were removed and frozen at −80 °C for subsequent analysis.

2.8. BDNF assays

Blood samples were collected and centrifuged in plastic tubes for 10 min at 4500 rpm at 4 °C; the serum and plasma were stored at −80 °C. The levels of BDNF were determined by sandwich ELISA using monoclonal antibodies specific for BDNF (R&D Systems, Minneapolis, United States). Total protein was measured by Bradford's method using bovine serum albumin as standard. The spinal cord, cortex, and brainstem specimens were collected and frozen at −80 °C until the time of testing.

2.9. Statistical analysis

Data were expressed as mean ± standard error of the mean. To evaluate behavior parameters (EPM and OF) across groups, one-way ANOVA followed by SNK was performed at different timepoints (24 h and 7 days) after the last tDCS session. One-way analysis of variance (ANOVA) followed by Student–Newman–Keuls (SNK) was also conducted to compare all groups for BDNF levels. P-values of less than 0.05 were reported as statistically significant.

3. Results

Previous data from our group showed that tDCS was able to revert the mechanical and thermal hyperalgesia in neuropathic pain induced by the CCI model (Cioato et al., 2014).

3.1. Open field test

The ANOVA for phase I of the OF test showed a between-group effect on the number of line crossings ($F_{(6,62)} = 3.76$, $P < 0.05$). In addition, there was an increase in this parameter in the NpT group compared to the Np group ($F_{(6,62)} = 3.76$, $P < 0.05$). No effect was found on the other parameters (Fs < 1.60, $P > 0.05$ for all).

Considering phase II of the OF test, the ANOVA showed differences between groups in the total number of crossings ($F_{(5,64)} = 16.06$, $P < 0.05$), the latency to leave the first quadrant ($F_{(5,64)} = 26.06$, $P < 0.05$), and the number of rearings ($F_{(5,64)} = 24.05$, $P < 0.05$). The NpT group had a higher number of line crossings than groups Np ($F_{(5,64)} = 16.06$, $P < 0.05$) and NpS ($F_{(5,64)} = 16.06$, $P < 0.05$). The NpT group showed a shorter latency to leave the first quadrant compared to groups Np ($F_{(5,64)} = 26.06$, $P < 0.05$) and NpS ($F_{(5,64)} = 26.06$, $P < 0.05$). Similarly, rearing activity in the NpT group was greater than that in groups Np ($F_{(5,64)} = 24.05$, $P < 0.05$) and NpS ($F_{(5,64)} = 24.05$, $P < 0.05$). There was no effect on the other parameters (Fs < 2.96. $P > 0.05$ for all) (Fig. 1).

3.2. Elevated plus-maze test

For phase I of the EPM test, one-way ANOVA showed that NpT and NpS spent less time in the closed arms ($F_{(5,53)} = 5.82$, $P < 0.05$) compared to the overall mean for the Np groups. Additionally, the NpT and NpS groups had increased time in the open arms compared to the Np group ($F_{(5,53)} = 6.43$, $P < 0.05$) (Fig. 2). Considering the NPHD movements, the NpT and NpS groups had higher means in relation to the Np group ($F_{(5,53)} = 5.32$, $P < 0.05$). There was no effect on the other parameters (Fs < 4.86; $P > 0.05$ for all) (Fig. 2).

During EPM test II, the NpT group showed increased time in the open arms compared to the Np ($F_{(5,59)} = 5.29$, $P < 0.05$) and NpS groups

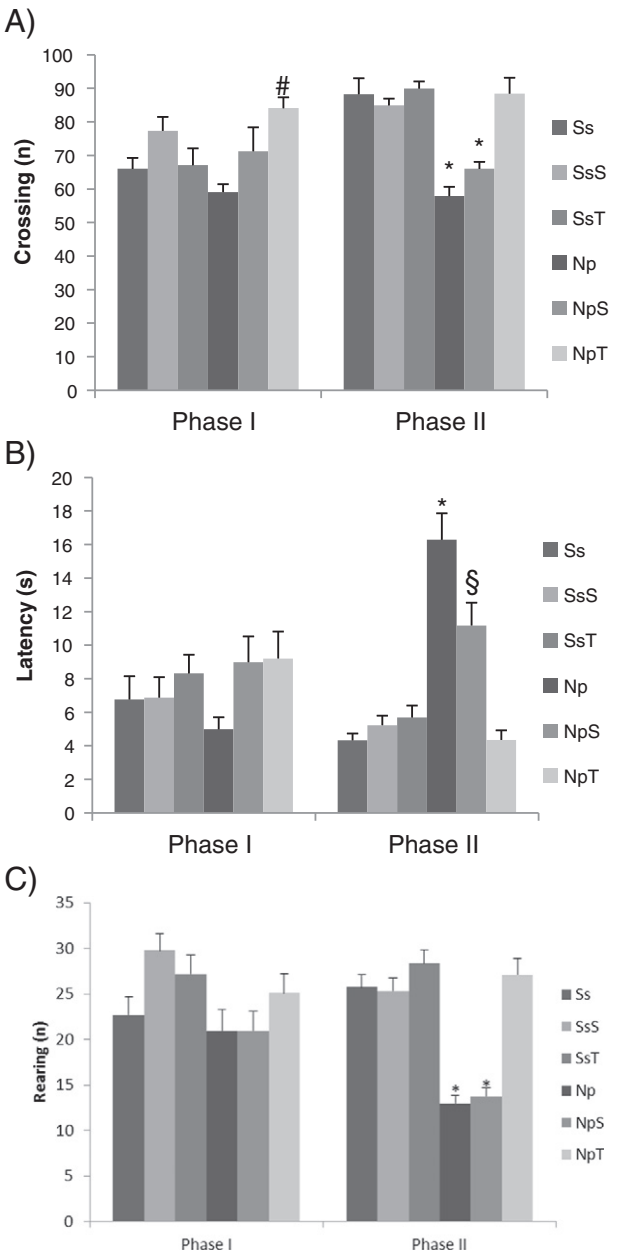

Fig. 1. Open field test. Data expressed as mean ± SEM, n = 21–24 animals/group. Ss (sham surgery); SsS (sham surgery + sham tDCS); SsT (sham surgery + tDCS); Np (Neuropathic pain); NpS (neuropathic pain + sham tDCS); NpT (neuropathic pain + tDCS). Group analysis was performed by one-way ANOVA followed by SNK. Panel A: Number from line crossings. Phase I: #significant difference in relation to Np groups, $P < 0.05$. Phase II: * significant difference in relation to Ss, SsS, SsT and NpT groups, $P < 0.05$. Panel B: Latency to leave first quadrant (seconds). Phase I there is no significant difference $P > 0.05$. Phase II: * significant difference in relation to Ss, SsS, SsT, NpS and NpT groups, $P < 0.05$. § significant difference in relation to SsS, SsT, Np and NpT groups, $P < 0.05$. Panel C: number of rearing behaviors (exploratory activity). Phase I: there was no significant difference, $P > 0.05$. Phase II: * significant difference in relation to Ss, SsS, SsT and NpT groups, $P < 0.05$.

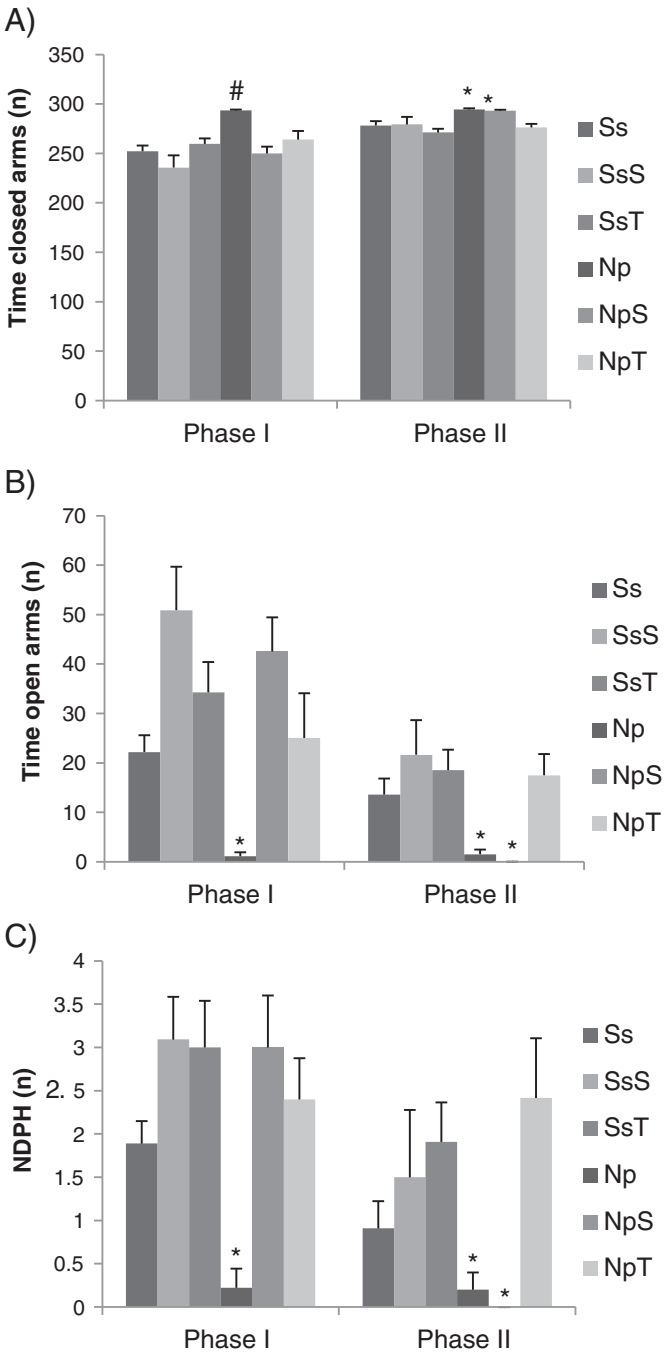

Fig. 2. Plus maze test. Data expressed as mean ± SEM, n = 19–22 animals/group. Ss sham surgery; Ss (sham surgery); SsS (sham surgery + sham tDCS); SsT (sham surgery + tDCS); Np (Neuropathic pain); NpS (neuropathic pain + sham tDCS); NpT (neuropathic pain + tDCS). Group analysis was performed by one-way ANOVA followed by SNK. Panel A: total time spent on the open arms (TOA). Phase I: # significant difference from Ss, SsS, SsT, NpS and NpT groups, $P < 0.05$. Phase II: * significant difference from Ss, SsS, SsT and NpT groups, $P < 0.05$. Panel B: total time spent on the closed arms (s) (TCA). Phase I: # significant difference from Ss, SsS, SsT, NpS and NpT groups, $P < 0.05$. Phase II: * significant difference from Ss, SsS, SsT and NpT groups, $P < 0.05$. Panel C: number NDPH. Phase I: * significant difference from Ss, SsS, SsT, NpS and NpT groups, $P < 0.05$. Phase II: * significant difference from Ss, SsS, SsT and NpT groups, $P < 0.05$.

($F_{(5,59)} = 5.29$, $P < 0.05$). The same effect was noted for other groups compared to Np and NpS (all Fs < 4.69, $P < 0.05$). Further, the NpT group time was decreased in the closed arms compared to Np and NpS ($F_{(5,59)} = 5.25$, $P < 0.05$). No differences were observed between groups NpS and Np ($F_{(5,59)} = 5.29$, $P < 0.05$). The NpT group showed increased frequency of NPHD movements in relation to the Np and NpS groups ($F_{(5,59)} = 3.88$, $P < 0.05$). Interestingly, there was no significant difference between groups Np and NpS compared to all sham groups (Ss, SsS, and SsT) ($F_{(5,59)} = 3.88$, $P > 0.05$). There was no significant difference in the other parameters (all Fs < 4.78; $P > 0.05$) (Fig. 2).

3.3. Peripheral and central BDNF levels

In phase I, serum BDNF levels were not significantly different between groups ($F_{(5,34)} = 0.88$, $P > 0.05$). By contrast, in phase II, serum BDNF levels were decreased in the NpT group compared to the others ($F_{(5,33)} = 2.89$, $P < 0.05$).

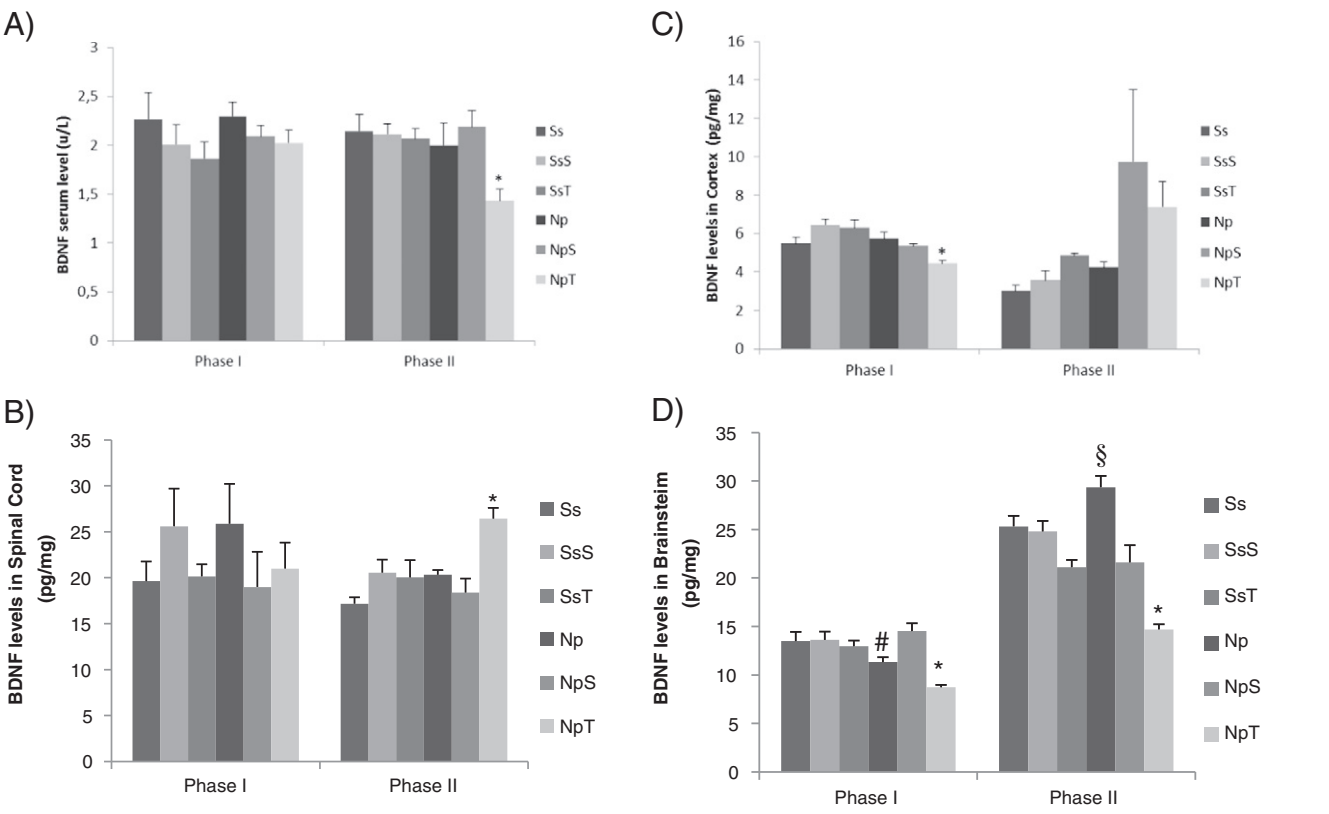

Fig. 3. BDNF levels. Data expressed as mean ± SEM, n = 5–6 animals/group. Ss (sham surgery); SsS (sham surgery + sham tDCS); SsT (sham surgery + tDCS); Np (Neuropathic pain); NpS (neuropathic pain + sham tDCS); NpT (neuropathic pain + tDCS). Group analysis was performed by one-way ANOVA followed by SNK. Panel A: BDNF serum levels. Phase I: there was no significant difference, $P > 0.05$. Phase II: * significant difference from Ss, SsS SsT, Np and NpS groups, $P < 0.05$. Panel B: BDNF spinal cord levels. Phase I: there was no significant difference, $P > 0.05$. Phase II: * significant difference from Ss, SsT, Np and NpS groups, $P < 0.05$. Panel C: BDNF cortex cerebral levels. Phase I: there was no significant difference, $P > 0.05$. Phase II: * significant difference from Ss, SsT, Np and NpS groups, $P < 0.05$. Panel D: BDNF brainstem levels. Phase I: * significant difference from Ss, SsT, Np and NpS groups, $P < 0.05$. # significant difference of NpS group, $P < 0.05$. Phase II: * significant difference from Ss, SsT, Np and NpS groups $P < 0.05$. § significant difference of Ss, SsT and NpS groups, $P < 0.05$.

In the spinal cord, BDNF levels were no different between groups ($F_{(5,27)} = 0.85$, $P > 0.05$) in phase I. However, NpT showed an increase in phase II compared to the other groups ($F_{(5,27)} = 5.82$, $P < 0.05$) (Fig. 3).

In phase I, cerebral cortex BDNF levels were significantly decreased in the NpT group in relation to the other groups ($F_{(5,30)} = 6.12$, $P < 0.05$). However, there was no significant difference between groups in phase II ($F_{(5,30)} = 2.50$, $P > 0.05$).

Brainstem BDNF levels were reduced in phase I in the NpT group compared to the others ($F_{(5,30)} = 9.06$, $P < 0.05$). Furthermore, an increase was noted in the NpS group compared to the other Np groups ($F_{(5,30)} = 9.06$, $P < 0.05$). In phase II, levels were lower in the NpT group relative to the other groups ($F_{(5,30)} = 19.14$, $P < 0.05$) and Np showed an increase compared to the other groups ($F_{(5,30)} = 19.14$, $P < 0.05$). The NpS group exhibited decreased levels in comparison to the Np group ($F_{(5,30)} = 19.14$, $P < 0.05$) (Fig. 3).

4. Discussion

This study shows for the first time the effect of tDCS on the reversal of behavioral alterations induced by the neuropathic pain model. Such effect was indicated by reversion of the decreased locomotor and exploratory activities and increased anxiety-like behavior that had been induced by that model 14 days following CCI surgery. Those particular types of behavior are characteristic of persistent neuropathic pain model (Zurowski et al., 2012).

The CCI animals showed a decrease in locomotor and exploratory activities in phase II, corroborating a study that showed the same results after 3 weeks of the neuropathic pain surgical procedure in rats (Roeska et al., 2008; Seminowicz et al., 2009). In addition, those animals exhibited a longer latency to leave the first quadrant. Walsh and Cummins (1976) suggested that the output latency to leave the first quadrant is a behavior associated with emotional factors, which may indicate an increase in anxiety-like behaviors (Stanford, 2007; Walsh and Cummins, 1976). Most importantly, tDCS treatment reverted those CCI-induced effects; therefore, we can suggest that this effect could have resulted from the action of tDCS on cortical areas involved in the pain matrix, such as the thalamus; anterior cingulate cortex (ACC); insular cortex; frontal, premotor, primary sensory and motor cortices (Moseley, 2003; Vaseghi et al., 2015; Zaghi et al., 2009). Additionally, previous studies from our group using chronic inflammation (Laste et al., 2012) and hyperalgesia induced by chronic restraint stress models (Spezia Adachi et al., 2012) showed an antinociceptive effect of cortical stimulation by tDCS. Beyond the analgesic effect, human studies with tDCS have shown effects on motor control, stress, and depression, which could justify our results (Gonçalves, 2012; Nitsche et al., 2009a; Orban de Xivry and Shadmehr, 2014).

Adequate locomotor activity results from the interaction of several neurotransmitters (Kandasamy, 2000), such as those in the opioid system, which is an important modulator of the descending pathway of pain. The opioid system could be implicated in this behavior as well as in the pain suppression mechanism (Fields, 2004). The involvement of the opioid system is demonstrated by the administration of the opioid receptor antagonist naloxone, which reduces the locomotor and exploratory activities in naive rats (File, 1980). In support of those data, it has been found that μ-opioid receptor knockout mice show less exploratory activity (Yoo et al., 2003). According to Taylor et al. (2012), brain modulation by electrical stimulation can induce changes to the endogenous

opioid system in humans. Similarly, motor cortex stimulation (MCS) induced an increase in endogenous opioid activity in patients with chronic pain (Maarrawi et al., 2007; Taylor et al., 2012), and anodal tDCS enhanced endogenous opioid release in healthy humans (DosSantos et al., 2012). Thus, we can suggest that a single pathway is involved in tDCS behavior modulation effect on the opioid system.

Another hypothesis is that the descending projections of the prefrontal cortex exert excitatory control on the midbrain dopaminergic neurons, triggering dopamine release in the ventral striatum and nucleus accumbens (NAc) (Jackson et al., 2001). Furthermore, neurochemical and electrophysiological studies have demonstrated that prefrontal cortex stimulation increases dopamine release in the NAc and increases burst firing of midbrain dopaminergic neurons, thereby corroborating this interaction (Karreman and Moghaddam, 1996; Murase et al., 1993; Taber and Fibiger, 1995; Tong et al., 1996). The stimulation of dopaminergic neurons increases locomotor activity (Fishman et al., 1983), which proves the importance of these neurons to the modulation of this behavior. The use of tDCS in humans is known to increase the levels of extracellular dopamine (Tanaka et al., 2013). Arguably, then, tDCS treatment modulates the dopaminergic system in the frontal cortex, promoting dopamine release and consequently improving locomotor and exploratory activities of CCI rats.

Neuropathic pain, as a chronic stressor, induces both physiological and psychological changes, which may lead to multiple neuropsychiatric disorders (Asmundson and Katz, 2009). Although the latency to leave the first quadrant as measured by the OF test is not a specific variable to assess anxiety, we noted that neuropathic pain induces increased latency, which corroborates the anxiety-like behavior assessed by the EPM. In animal studies, the EPM is a validated instrument to evaluate anxiety-like behaviors (Hogg, 1996). The treatment with tDCS reverted the CCI-induced increase in this parameter. Animal studies investigating the effect of neuropathic pain in anxiety-like behaviors are inconsistent. These behaviors are observed in the EPM test with rats up to 2 weeks after the CCI procedure (Kontinen et al., 1999); after spared nerve injury, this effect is maintained for several weeks (Seminowicz et al., 2009). Wallace et al. (2007) used the model of HIV-induced peripheral neuropathy and showed anxiety-like behaviors up to 2 weeks after induction. In addition, CCI rats displayed anxiety-like behaviors in the EPM up to 3 weeks after the injury, but these effects were not found using the model of partial sciatic nerve ligation (PSNL) (Roeska et al., 2008; Wallace et al., 2007). In our study, the CCI rats developed anxiety-like behavior up to at least 21 days after surgery; an effect that was reversed after tDCS treatment indicating an anxiolytic effect of tDCS.

After 48 h of tDCS treatment completion, the neuropathic pain animals that received real tDCS (NpT) showed a decrease in anxiety-like behaviors. Nevertheless, the NpS group showed a similar effect. We could suggest that the body restraint necessary for tDCS application may be implicated in this finding, since it is considered a stress model. It is known that stress situations can activate the endogenous opioid system and promote behavioral changes, such as diminished anxiety levels a protective mechanism of body resistance to stress (Colasanti et al., 2011). At 7 days after the end of tDCS treatment, an anxiolytic tDCS effect was also observed. This effect could be related to tDCS action on different neurotransmitter systems, such as the glutamatergic (Radman et al., 2009), GABAergic (Nitsche et al., 2004b), opioid (DosSantos et al., 2012), cholinergic (Kuo et al., 2007), serotonergic (Nitsche et al., 2009b), and catecholamine (Nitsche et al., 2004a). The periaqueductal gray (PAG) and the hippocampus (Drake et al., 1999) show a high density of opioid receptors and have been extensively studied in relation to anxiolytic states. Morphine administered directly into the PAG showed an anti-stress effect, which was antagonized by naltrexone (an opioid antagonist more selective to μ receptors) and mimicked by DAMGO, an agonist of the μ-selective receptor (Anseloni et al., 1999; Drake et al., 1999). The μ-opioid receptors located in the dorsal hippocampus are involved in anxiolytic behavior; in fact, opioid-deficient mice are more anxious than naive animals (Hayase et al., 2006). Therefore, we can also postulate that the reduction in anxiety-like behaviors found in our study may be related to an effect of tDCS treatment on the opioid system.

Interestingly, the NpS group showed a modulatory effect of brainstem BDNF levels. This result could be due to a synergistic effect of pain and stress caused by the restraint induced by the experimental model. It is known that restraint-induced stress modulates BDNF expression (Autry and Monteggia, 2012); thus, our data may indicate a functional impairment in its regulation in the medium term (48 h after tDCS treatment completion), since this effect was reversed over the long term (7 days post-tDCS treatment). These results corroborate the data from Marmigère et al. (2003), who found that restrained rats had a rapid increase in BDNF levels in the CNS (Marmigère et al., 2003).

The present study also demonstrated that CCI decreased BDNF levels in the brainstem after 23 days post-surgery (48 h after the end of tDCS treatment) and increased those levels after 30 days post-surgery (7 days after tDCS treatment completion). Interestingly, tDCS reduced brainstem BDNF levels in both phases (I and II) and in phase I in the cerebral cortex, while in phase II tDCS decreased serum and increased spinal cord BDNF levels.

In some studies, BDNF is used as a biological marker of clinical conditions such as anxiety, depression, fibromyalgia, and schizophrenia (Kurita et al., 2012; Nurjono et al., 2012). However, the relationship between BDNF levels and anxiety disorders is controversial: BDNF levels appear to be either reduced increased, or not significantly altered in patients with anxiety disorders (Bonne et al., 2011; Dell'Osso et al., 2009; Dos Santos et al., 2011; Molendijk et al., 2012; Wang et al., 2011). In an animal study, using genetically engineered mice for BDNF overexpression resulted in elevated anxiety (Govindarajan et al., 2006). Polymorphism of BDNF (Met/Met) was associated with increased anxiety-related behaviors in mice, thus suggesting a key role of genetic predisposition in anxiety and depressive disorders (Chen et al., 2006).

Chronic neuropathic pain situations can be associated directly with high levels of anxiety symptoms (Wallace et al., 2007). Therefore, the analgesic effect of tDCS can contribute to the management of anxiety symptoms in these conditions. The reduction in anxiety-like behaviors may be related to the reversion of brainstem BDNF levels after tDCS treatment. This neurotrophin has also been implicated in the control of nociceptive neurotransmission and in the behavioral pharmacological and physiological process (Kumamaru et al., 2008). BDNF is involved in the synaptic plasticity and survival of neurons (Siniscalco et al., 2011). The underlying effect of tDCS on BDNF regulation mechanisms has not been fully elucidated. The increased in the spinal cord BDNF levels observed in this study can be related to the synaptic plasticity (Baker-Herman et al., 2004). The role of BDNF in pain is still unclear: studies have shown that attenuation in pain behavior is associated with decreased BDNF levels; however, there is evidence that BDNF may play an antinociceptive role in different settings (Coull et al., 2005). Additionally, study recently published by our research group demonstrated that tDCS was able to decrease BDNF levels in the structures involved in the descending systems only in unstressed animals (Spezia Adachi et al., 2015). Interestingly, corroborating our results, increase of BDNF levels has been found in neuroplastic events associated with chronic pain conditions (Ranieri et al., 2012). In this way, tDCS might be an alternative treatment to prevent central neuronal alterations presents in the neuropathic pain.

Increased expression of BDNF regulates NMDA and AMPA receptors and facilitates the induction and maintenance phases of long-term potentiation (LTP) (Caldeira et al., 2007). Another role of BDNF is that it is also responsible for regulating the organization of inhibitory synapses (Swanwick et al., 2006). It has been demonstrated that BDNF also acts on presynaptic terminals to enhance the release of the inhibitory neurotransmitter gamma-aminobutyric acid (GABA), an effect that has been suggested to cause indirect suppression through excitation by GABAergic signaling (Malcangio and Lessmann, 2003). Although tDCS

was able to decrease BDNF levels in the serum (Fig. 3A) and in the cerebral cortex (Fig. 3C) and to increase these levels in the spinal cord (Fig. 3B), such findings may indicate that tDCS activates specific pathways (i.e., descending systems) and does not cause general brain activation; in fact, those alterations were found at specific timepoints. As tDCS treatment induces neuronal changes by activating various neurotransmitters, we believe that this variability in BDNF levels in different CNS structures could be due to its extensive action in neural network circuits that activate a variety of signaling pathways.

5. Conclusion

The present data indicate that tDCS reverses the behavioral alterations induced by chronic neuropathic pain, as indexed by changes in locomotor and exploratory activities and anxiety-like behaviors. It is worth noting that tDCS treatment also reversed the increase in BDNF brainstem levels that had been induced by chronic neuropathic pain. In addition, tDCS treatment alters BDNF levels in serum and in different CNS regions independently of the chronic neuropathic pain. The variability of tDCS effects on the levels of BDNF in different structures is probably due to activation of different signaling pathways. Further studies are warranted to evaluate the neurotransmitter system (opioidergic, dopaminergic, glutamatergic, and GABAergic systems) and thereby provide a better understanding of the tDCS mechanisms in the anxiety disorders induced by the neuropathic pain model.

Conflict of interest statement

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper. There was no financial interest between any of the authors or any commercial interest in the outcome of this study.

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