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Research report

Neurobiological mechanisms of antiallodynic effect of transcranial direct current stimulation (tDCS) in a mice model of neuropathic pain

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ABSTRACT

Background: Neuropathic pain is relatively common and occurs in approximately 6–8% of the population. It is associated with allodynia and hyperalgesia. Thus, non-pharmacological treatments, such as transcranial direct current stimulation (tDCS) may be useful for relieving pain.
Objectives: This study aimed to investigate the antiallodynic effect of tDCS in a mice model of neuropathic pain, and the underlying neurotransmission systems that could drive these effects.
Methods: Male, Swiss mice, weighing 25–35 g, were subjected to partial sciatic nerve ligation (PSNL). Allodynia was assessed using a Von Frey filament (0.6 g). First, the behavioral time-course of these mice was assessed after 5, 10, 15 and 20 min of tDCS (0.5 mA). Second, the mice that underwent PSNL were assigned to either the tDCS (0.5 mA, 15 min) or tDCS sham group, and further assigned to receive either saline or a drug (i.e., naloxone, yohimbine, a-methyl-p-tyrosine, q-chlorophenylalanine methyl ester, caffeine, 1,3-dipropyl-8-cyclopentylxanthine, AM281, AM630, flumazenil, MK-801, or lidocaine).
Results: The antiallodynic effect of tDCS lasted 2 h and 4 h, after 10 min and 15 or 20 min of treatment, respectively ($P < .001$, $P < .01$, and $P < .05$, respectively). The antiallodynic effect of tDCS was associated with all the systems that were analyzed, i.e., the opioidergic ($P < .01$), adenosinergic ($P < .001$), serotonergic ($P < .01$), noradrenergic ($P < .001$), cannabinoid ($P < .001$), GABAergic, and glutamatergic ($P < .001$) systems. Lidocaine did not reverse the antiallodynic effect of tDCS ($P > .05$).
Conclusion: The antiallodynic effect of tDCS was associated with different neurotransmitters systems; the duration of these after-effects depended on the time exposure to tDCS.

1. Introduction

Neuropathic pain is a relatively common process that occurs in approximately 6–8% of the general population (Bouhassira et al., 2008; Torrance et al., 2006). It is a direct consequence of an injury that affects the somatosensory system (Treede et al., 2008). Hyperalgesia and allodynia are common characteristics that are associated with neuropathic pain. In addition, neuropathic pain is often resistant to available treatments (Jensen et al., 2001). Thus, non-pharmacological therapies have been investigated as alternative or adjuvant treatments for this condition.

Previous studies demonstrated that transcranial direct current stimulation (tDCS) reverted mechanical hyperalgesia, which was induced by chronic stress (Spezia Adachi et al., 2012) in an inflammatory pain model (Laste et al., 2012), and neuropathic pain, which was induced by chronic constriction injury (CCI) of the sciatic nerve in rats (Cioato et al., 2016). However, there have been no previous studies applying this technique to treat neuropathic pain in mice. Briefly, tDCS is a central neuromodulatory technique,

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which is low-cost, non-invasive, and non-painful; it comprises the application of a weak direct current through small electrodes positioned on the scalp (Nitsche et al., 2008). A recent meta-analysis indicated that tDCS could produce a moderate effect in reducing pain in patients with neuropathic pain (i.e., spinal cord injury [SCI]) (Mehta et al., 2015). The clinical relevance of the analgesic efficacy of tDCS has been presented in different chronic pain conditions, for example in fibromyalgia (Khedr et al., 2017) and visceral pain (Ibrahim et al., 2017).

According to the literature, the long-lasting effects of tDCS can be linked to the treatment duration and baseline condition of the patient, such as their healthy or acute/chronic condition (Lefaucheur et al., 2017). The effect of dual tDCS has also been described according to its action, where immediate effects were generated by a change in the resting membrane potential, while the long-term effects were dependent on the modulation of the N-Methyl-D-aspartate (NMDA) receptors (Nitsche et al., 2003). Our previous data demonstrated that the after-effects of bicephalic tDCS on nociceptive behavior lasted for 7 days after the last session of tDCS in rats with neuropathic pain (Cioato et al., 2016). Although clinical *meta*-analysis revealed that the analgesic effect of tDCS did not last until the follow-up time point in patients with SCI, which in part, could be related to the lack of follow-up monitoring (Mehta et al., 2015). Additionally, pre-clinical and clinical studies have described differences in the responses to the tDCS effect, according to the baseline status, e.g., tDCS decreased the levels of BDNF in the spinal cord and brainstem structures in unstressed animals alone (Spezia Adachi et al., 2015). Further, healthy subjects presented higher levels of itching sensation, and the intensity of tingling sensation, while patients experienced more headaches after tDCS stimulation (Poreisz et al., 2007).

Although tDCS treatment has been used to treat different pathological conditions, its mechanism has not been full elucidated yet. Animal and cellular model studies demonstrated that tDCS is capable of modulating synaptic transmission, molecular biosynthesis, neuronal morphology, and different neurotransmitters' system (for review see Medeiros et al., 2012). The lack of pharmacological studies that supports the tDCS effects upon neuropathic pain led us to whether tDCS had an antiallodynic effect in a neuropathic pain model in mice and which neurotransmission systems played a role in eliciting the tDCS effects in a neuropathic pain model in mice. Thus, we hypothesized that tDCS would have an antiallodynic effect in a mice model of neuropathic chronic pain. Therefore, we aimed to increase knowledge about the role that different neurotransmitter systems play in eliciting the action mechanisms of tDCS. In addition, we investigated if tDCS has a peripheral influence in the same animal model.

2. Results

2.1. Time course of the effect of tDCS on mechanical allodynia induced by PSNL in mice

The antiallodynic effect of tDCS was assessed at baseline, on the 9th day after PSNL induction, in a mice model of chronic neuropathic, using a Von Frey Filament test (0.6 g). The tDCS session was applied for 5, 10, 15, or 20 min and the time course of tDCS effect was evaluated at 0.5, 1, 2, 3, 4, and 5 h.

The results are presented in Fig. 1A–D. At the baseline, we did not observe any differences in the mechanical threshold between the groups (one-way ANOVA, $P > .05$). However, there was marked and long-lasting mechanical allodynia in a PSNL model, with a higher response frequency on the 9th postoperative day after the PSNL, compared with the baseline response and Sham-PSNL group (one-way ANOVA/Tukey, $P < .001$). Interestingly, the tDCS treatment produced a significant antiallodynic effect, which was maintained for 4 h after the 15- and 20-min tDCS stimulations (one-way ANOVA/Tukey, $P < .01$ and $P < .05$, respectively). The antiallodynic effect of tDCS was observed until 2 h after the 10-min of tDCS treatment ($P < .001$). However, 5 min of tDCS stimulation had no effect on the mechanical allodynia response, i.e., no significant difference from PSNL animals (two-way ANOVA repeated measures/Tukey, $P < .05$).

2.2. Involvement of the opioidergic system on antiallodynic effect of tDCS in a neuropathic pain model

We administrated naloxone, a nonselective opioid antagonist, to different sites (i.p. and i.t.). In both conditions, the systemic and intrathecal administration of naloxone reversed the tDCS effects that were observed, i.e., there was a decrease in the percentage of withdrawal frequency. The group pretreated with naloxone (i.p. or i.t.) presented a significantly greater withdrawal frequency ($P < .01$) than that in the saline-tDCS group, when assessed at 1 h after stimulation (Fig. 2A and B).

2.3. Involvement of the adenosinergic system on antiallodynic effect of tDCS in a neuropathic pain model

We administrated caffeine (a nonselective adenosine receptor antagonist) and DPCPX (a selective adenosine receptor antagonist), at different sites (i.p. and i.t., respectively). The administration of caffeine with the tDCS sham had no effect on the response frequency. The thresholds were like those seen in the saline group (i.p.) (Fig. 3A). Pretreatment with caffeine (i.p.) prevented the decrease in response frequency resulting from tDCS 15′ (one-way ANOVA/Tukey, $P < .001$).

Thereafter we investigated the mechanism by which adenosine A1 receptors (A_1Rs) affect the antiallodynic effect of tDCS 15′ in a neuropathic pain model. We administrated the selective A_1Rs antagonist, DPCPX, to an intrathecal site (Fig. 3B). Pretreatment with DPCPX had no effect on the response frequency. In addition, spinal administration of DPCPX, 15 min before tDCS 15′, prevented a decrease in the response frequency seen following the tDCS treatment (one-way ANOVA/Tukey, $P < .001$). These findings suggest that the central activation of A_1Rs adenosine receptors can contribute to the antiallodynic effect of tDCS 15′.

2.4. Involvement of the cannabinoid system on antiallodynic effect of tDCS in a neuropathic pain model

As seen in Fig. 4A and B, the systemic administration of selective cannabinoid receptors antagonists, CB_1Rs (AM281) and CB_2Rs (AM630), prevented the decrease in the percentage of response frequency resulting from the tDCS treatment. The percentage of withdrawal frequency response of the group that was pretreated with AM281 or AM630 (i.p.) were significantly higher (one-way ANOVA/Tukey, $P < .001$) when compared to saline-tDCS group, 1 h after treatment (Fig. 4A and B).

2.5. Involvement of the monoaminergic system on antiallodynic effect of tDCS in a neuropathic pain model

Pretreatment with AMPT, yohimbine, or PCPA, in combination with the tDCS sham, had no effect on the response frequency; the thresholds were like those of the saline + tDCS sham (Fig. 5A–C). The administration (i.p.) of AMPT, yohimbine, or PCPA before tDCS treatment, however, prevented the decrease in withdrawal frequency resulting from the treatment (one-way ANOVA/Tukey, $P < .001$, $P < .001$, and $P < .01$, Fig. 5A–C, respectively). Thus, we demonstrated that antiallodynic effects of tDCS (15′) could be

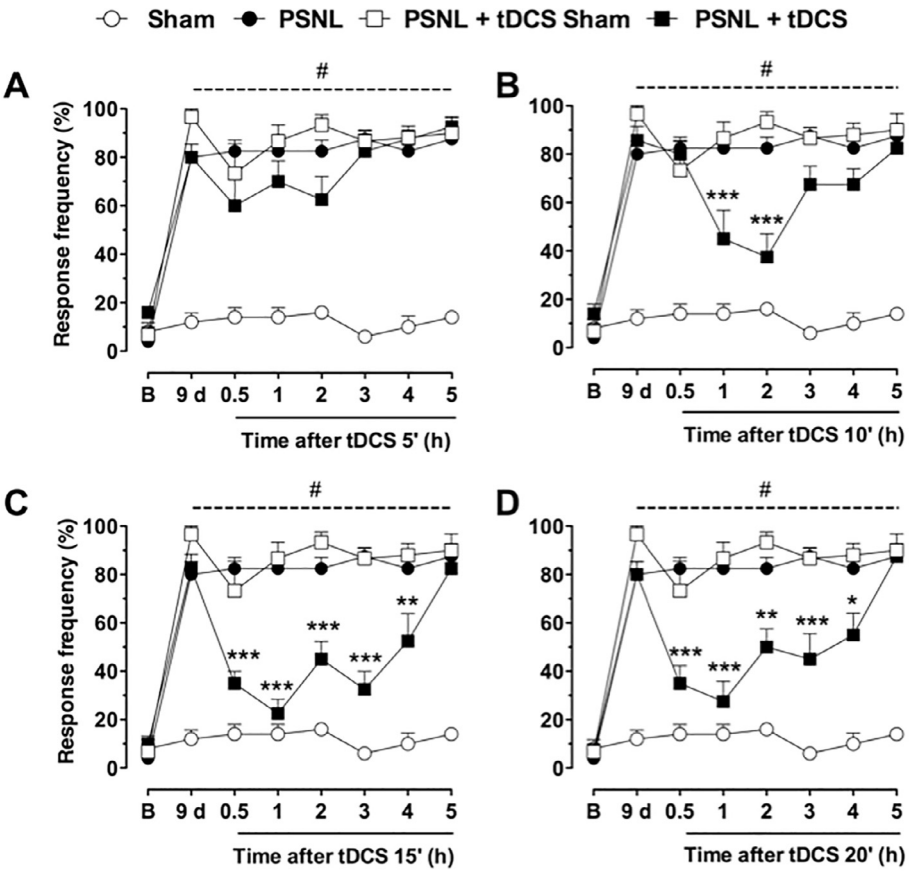

Fig. 1. Time course of the effect of transcranial direct current stimulation (tDCS) on mechanical allodynia induced by PSNL in mice. Panel A. After 5 min of tDCS. Panel B. After 10 min of tDCS. Panel C. After 55 min of tDCS. Panel D. After 20 min of tDCS. #significantly different to the sham, *significantly different to the other groups at the same time-point (two-way ANOVA repeated measures, P < .05), **different to the other groups at the same time-point (two-way ANOVA repeated measures, P < .01), ***different to the other groups at the same time-point (two-way ANOVA repeated measures, P < .001).

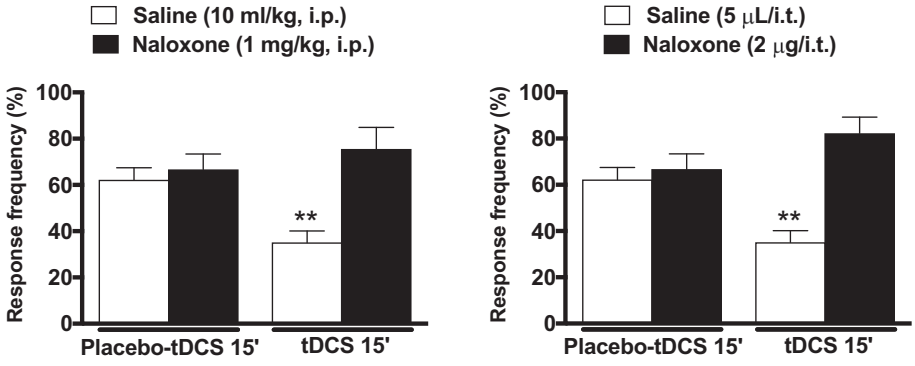

Fig. 2. Involvement of the opioidergic system on antiallodynic effect of tDCS in a neuropathic pain model. Panel A. Naloxone (i.p.). Panel B. Naloxone (i.t.). **significantly different to other groups (one-way ANOVA/Tukey P < .01).

associated with the activation of the noradrenaline and serotonin in the descending inhibitory pathways.

2.6. Involvement of the GABAergic system on the antiallodynic effect of tDCS in a neuropathic pain model

The results presented in Fig. 6 demonstrated that the systemic administration of a GABAergic receptor antagonist (Flumazenil) prevented the decrease in the percentage of response frequency, which occurred after the tDCS treatment. The percentage of withdrawal frequency values in the group pretreated with Flumazenil was significantly higher (one-way ANOVA/Tukey, P < .001) when compared to saline-tDCS group, 1 h after tDCS treatment (Fig. 6).

2.7. The involvement of the glutamatergic system on antiallodynic effect of tDCS in a neuropathic pain model

The results presented in Fig. 7 demonstrated that the systemic administration of an uncompetitive NMDA receptor antagonist prevented the decrease in the percentage of response frequency resulting from the treatment. The percentage of withdrawal frequency values in the group pretreated with MK801 was signifi-

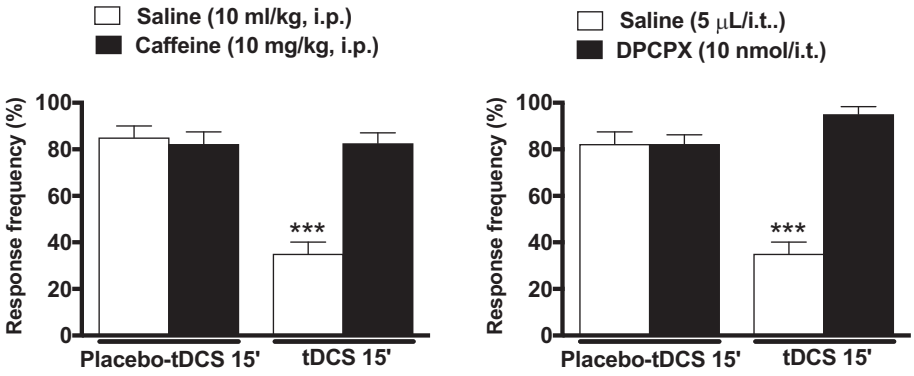

Fig. 3. The involvement of the adenosinergic system on antiallodynic effect of tDCS in a neuropathic pain model. Panel A. Caffeine (i.p.). Panel B. DPCPX (i.t.). ***significantly different to other groups (one-way ANOVA/Tukey P < .001).

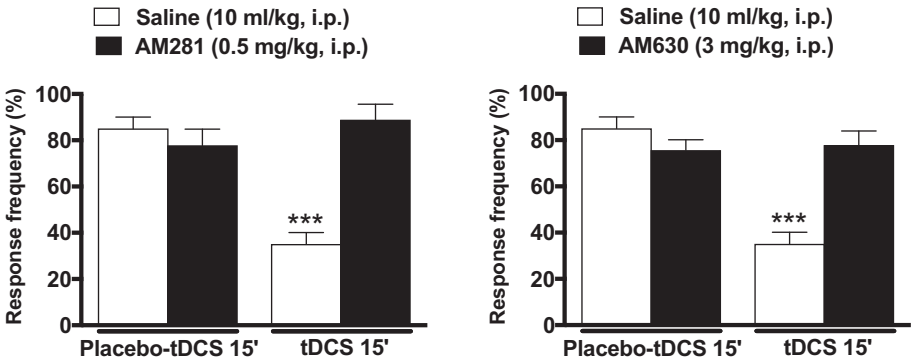

Fig. 4. The involvement of the cannabinoid system on the antiallodynic effect of tDCS in a neuropathic pain model. Panel A. AM281 (i.p.). Panel B. AM630 (i.p.). ***significantly different to other groups (one-way ANOVA/Tukey P < .001).

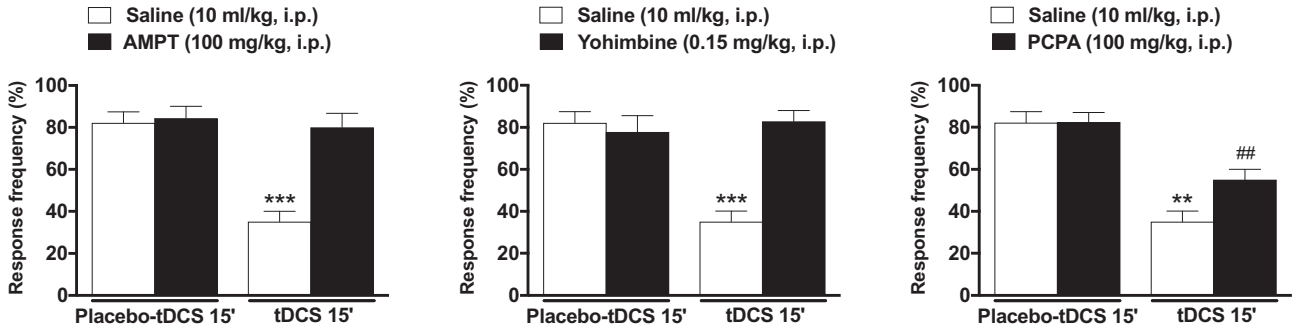

Fig. 5. The involvement of the monoaminergic system on the antiallodynic effect of tDCS in a neuropathic pain model. Panel A. AMPT (i.p.). Panel B. Yohimbine (i.p.). Panel C. PCPA (i.p.). **significantly different to other groups (one-way ANOVA/Tukey P < .01), ***significantly different to other groups (one-way ANOVA/Tukey P < .001), ##significantly different to both placebo-tDCS groups (one-way ANOVA/Tukey P < .01).

cantly higher (one-way ANOVA/Tukey, P < .001) when compared to saline-tDCS group, 1 h after the tDCS treatment (Fig. 7).

2.8. Influence of peripheral pathways on antiallodynic effect of tDCS

Finally, to assess the influence of peripheral pathways on the antiallodynic effect of tDCS 15′, the animals were pretreated with a lidocaine injection. As seen in Fig. 8, treatment with lidocaine, 15 min before tDCS, did not change its analgesic effect. Local anesthesia with lidocaine did not reverse the antiallodynic effect induced by tDCS (15′) compared with the saline-tDCS group (one-way ANOVA/Tukey, P > .05). Interestingly, the antiallodynic effect induced by tDCS 15′ was not linked with local mechanisms.

3. Discussion

Our findings demonstrated that bicephalic tDCS elicited an antiallodynic effect in a PSNL murine model of neuropathic pain. In addition, the time course revealed that the duration of application was directly associated with the after-effects response. A similar duration response, i.e., the antiallodynic effect lasted until 4 h after the tDCS application, was seen following 15 and 20 min of tDCS stimulation. Additionally, we demonstrated the involvement of peripheral and central mechanisms of a wide variety of systems, without local effect of tDCS.

Previous studies demonstrated the ability of anodal tDCS to increase the excitability of neurons. Conversely, cathodal tDCS

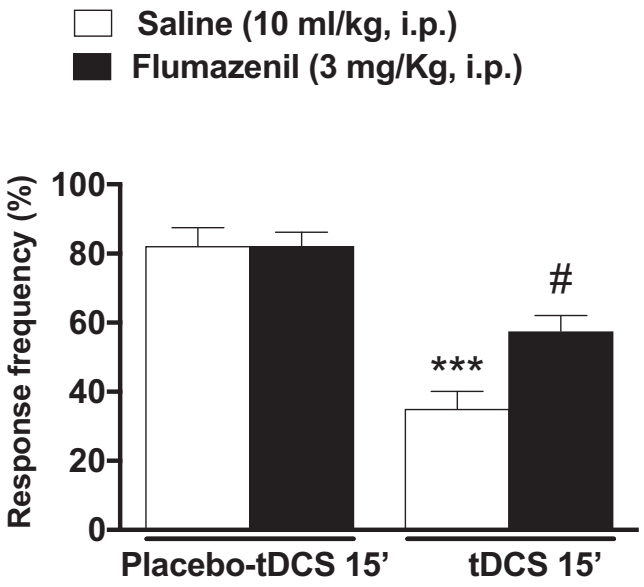

Fig. 6. The involvement of the GABAergic system on antiallodynic effect of tDCS in a neuropathic pain model. ***significantly different to other groups (one-way ANOVA/Tukey P < .001 for both), #different to both placebo-tDCS groups (one-way ANOVA/Tukey P < .001).

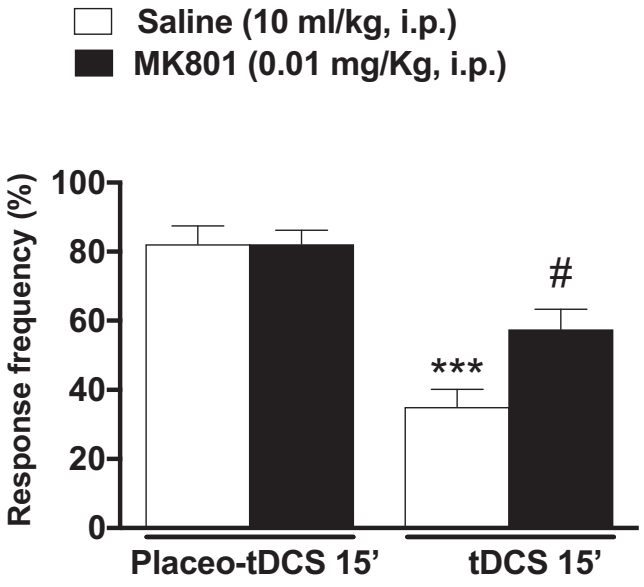

Fig. 7. The involvement of the glutamatergic system on antiallodynic effect of tDCS in a neuropathic pain model. ***significantly different to other groups (one-way ANOVA/Tukey P < .001), #significantly different to both placebo-tDCS groups (one-way ANOVA/Tukey P < .001).

decreases the excitability of neurons (Brunoni et al., 2012). Anodal stimulation is effective in relieving chronic pain in rats (Laste et al., 2012; Spezia Adachi et al., 2012). The effects of both anodal and cathodal tDCS are involved with glutamatergic synapses (Nitsche et al., 2003), while the anodal tDCS is involved with GABAergic neurotransmission (Nitsche et al., 2004). Furthermore, the long-term effects of tDCS are linked to the duration of application, i.e. longer (Nitsche and Paulus, 2001; Nitsche et al., 2003) or repeated sessions (Spezia Adachi et al., 2012) of tDCS increased its long-lasting effects. In the present study, we demonstrated the role the GABAergic and glutamatergic systems play in driving the antiallodynic effects of tDCS. Moreover, we confirmed the intrinsic

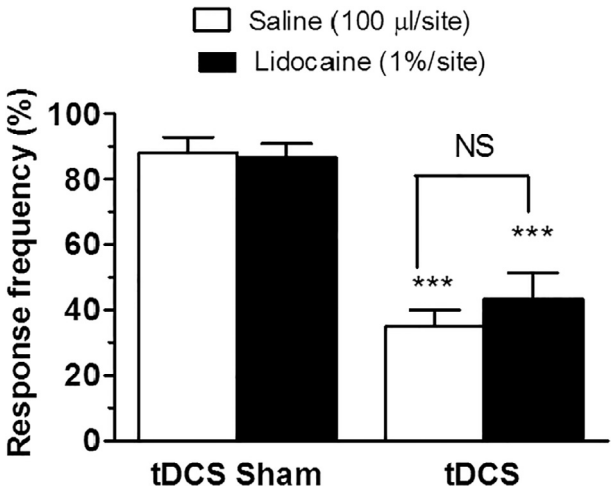

Fig. 8. The influence of peripheral pathways on antiallodynic effect of tDCS. ***significantly different to the tDCS sham group (one-way ANOVA/Tukey P < .001). NS = not significant.

relationship between the duration of tDCS stimulation and long-lasting effects, where the antiallodynic effect was maintained for 4 h after 15 min or 20 min of stimulation. A stimulation of 5 min of tDCS had no effect on the mechanical allodynia response in the PSNL mice.

The results of the current study demonstrated that different pathways are involved in eliciting the antiallodynic effect of bicephalic tDCS in a model of neuropathic pain. One important mechanism of tDCS in the antiallodynic effect was related to the descending inhibitory pathway, including the opioidergic system (Ossipov et al., 2010). Evidence from previous studies showed that PSNL induced sensitization in the opioid pathway (Petraschka et al., 2007; Xu et al., 2004), leading to the inefficacy of opioid treatments for neuropathic pain (Bleeker et al., 2001; Kupers et al., 1991). However, it is interesting to highlight the involvement of the opioidergic system in the action mechanism of tDCS in a neuropathic pain model. We found a total reversion of hypernociceptive behavior of mice after naloxone administration corroborating previous study that suggested a role for the opioid system in the effect of tDCS that was applied to the primary motor cortex (DosSantos et al., 2012).

In addition, the descending inhibitory system is comprised of monoaminergic pathways, including the serotonin (5-HT), norepinephrine, and dopamine neurotransmitter systems (Pertovaara, 2006; Zhao et al., 2007). The presence of serotonin in the spinal cord may modulate the nociceptive transmission in both directions, i.e., have a facilitatory or inhibitory action (Bardin, 2011). The noradrenergic pathway, however, plays an antiallodynic role (Benarroch, 2008). In the present study, we demonstrated that the antiallodynic effect of tDCS was reversed after the administration of serotonin or noradrenaline antagonists.

Similarly, the adenosinergic system plays an important role in the modulation of pain, since the nucleoside adenosine decreases nociception, cellular excitability, and inflammation (McGaraughty and Jarvis, 2006). The adenosine receptor subtype A1 has a modulatory effect on pain transmission at spinal level (Schulte et al., 2003). The antiallodynic effect of tDCS was linked to specific and unspecific adenosine receptors, as demonstrated by the results following the administration of DPCPX and caffeine, respectively. DPCPX is a specific A1 antagonist, while the caffeine is a general antagonist against all types of adenosine receptors (Ribeiro and Sebastião, 2010). We demonstrated the total reversion of hypernociceptive behavior after DPCPX or caffeine administration in

mice. Further, a previous study demonstrated that DPCPX prevented long-term depression (LTD) that was evoked in the somatosensory cortex after cathodal tDCS (Marquez-Ruiz et al., 2012).

An increasing number of studies have focused on the role the cannabinoid system in the nociceptive modulation process (Ashton and Milligan, 2008). In the present study, we showed, for the first time, the involvement of systemic CB1 and CB2 receptors in driving the antiallodynic effect of tDCS, where we observed the complete reversion of the hypernociceptive behavior after the administration of AM281 and AM620 CB1 receptors are localized in the peripheral and central nervous system (Munro et al., 1993), while CB2 receptors are found in immune system cells (Galiegue et al., 1995), and in neuronal and glial cells in the brain (Onaivi et al., 2012). Ligation of the sciatic nerve up regulated the expression of CB2 in the spinal cord (Zhang et al., 2003).

Classically, 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 (Fregni and Pascual-Leone, 2007). However, these effect may also involve projections to more remote areas (Lima and Fregni, 2008), such as the periaqueductal gray area (PAG), which is part of the descending system to the spinal cord (Heinricher et al., 2009). In accordance with these previous studies, Spezia and colleagues (Spezia Adachi et al., 2015) suggested that tDCS delivered to the cerebral cortex could induce neuronal changes in the spinal cord and brainstem by top-down systems. In addition, based on the top-down effects these authors also proposed a modified model for electrical brain stimulation, which integrates spinal and supraspinal circuits (Spezia Adachi et al., 2015).

In addition, the main action mechanism of cathodal tDCS might be an induction of LTD effects, i.e., reducing cortical excitability (Lefaucheur, 2008). The effects of cathodal tDCS involve the hyperpolarization of neuronal soma and desynchronization of neuronal activity. Its long-term effects seem to occur through the modulation of synaptic transmission, subsequently causing LTD in the thalamus cingulate pathway, which appears to be dependent on NMDA and the duration (Chang et al., 2015). Thus, cathodal tDCS seems to promote intracortical inhibition. Conversely, anodal tDCS facilitates synaptic plasticity mediated by a long-term potentiation (LTP)-like mechanism (Monte-Silva et al., 2013). Finally, the mechanisms underlying the effects of tDCS seem to be involved not only in local polarity-related modifications of cortical excitability, but also in more complex inter hemispheric connections (Tatti et al., 2016).

Interestingly, according to a recent meta-analysis, tDCS produced moderate effect in reducing the pain of patients with SCI. However only few studies were included in this meta-analysis, with some limitations (Mehta et al., 2015). Given the maladaptive plasticity inpatients with chronic pain, the use of the neuromodulatory techniques to promote synaptic plasticity might be a putative treatment for neuropathic pain conditions (Naro et al., 2016). In addition, a previous study also highlighted that tDCS might best be combined with other treatments to maximize the overall treatment efficacy for reducing pain and maximizing quality of life in neuropathic pain patients (Ngernyam et al., 2013).

Our technique was different from other tDCS montages used in rats (Kamida et al., 2013) or mice (Cambiaghi et al., 2010), in that it was like tDCS that is applied to humans, where the electrodes are located upon the intact scalp (as shown in the Fig. 1). Given the possible side-effects reported previously (Nitsche et al., 2008), the electrical current applied on the skin can produce local effects, which could have subsequently drive the antiallodynic effect observed after tDCS application. Our findings following the lidocaine administration, however, demonstrated that the local application of bicephalic tDCS did not play a role in eliciting its antiallodynic effect. It is interesting to note that some clinical studies evaluated the effect of local anesthetics and/or analgesics before tDCS application, mainly in relation to its role in reducing side effects (Guarienti et al., 2015; Guleyupoglu et al., 2014; McFadden et al., 2011), and the blinding bias in clinical trials. For example, they showed that pretreatment with ketoprofen reduces erythema (Guarienti et al., 2015), while pretreatment with 6% benzocaine (Guleyupoglu et al., 2014) and topical EMLA (McFadden et al., 2011) reduces discomfort.

It is, however, also important to consider a limitation of the present study. Non-invasive tDCS has not been well established in animal models, since in humans both electrodes are usually placed on a specific area on the head. Although we tried to mimic similar placement positions, the small head size of the mice contributed to a bicephalic stimulation.

3.1. Conclusion

Overall, tDCS is a promising, non-pharmacological therapeutic intervention to treat chronic pain. The exact action mechanism of tDCS, however, is not well understood. Bicephalic tDCS presented a significant antiallodynic effect in a mice model of neuropathy. Multiple pathways, such as the opioidergic, adenosinergic, cannabinoid, monoaminergic, GABAergic and glutamatergic systems, including peripheral and central mechanisms, were involved in eliciting the antiallodynic effects of tDCS. The lack of specificity of the mechanism of action of antiallodynic effect of tDCS may be a positive feature for the effective treatment for neuropathic pain.

4. Material and methods

4.1. Animals

The experiments were conducted in male Swiss mice (25–30 g) that were kept in a room with controlled temperature (22 ± 2 °C), under a 12-h light/dark cycle (lights on at 06:00 h), with free access to laboratory chow and tap water. The animals were acclimatized to the laboratory settings for at least 1 h before testing and were used only once throughout the experiments. All experiments and procedures were approved by the Institutional Animal Care and Use Committee (GPPG-HCPA protocol No. #140078), complied with Brazilian Law (Brazil, Law No. 11.794, 2008; 2013), and conformed to the Laboratory Guide for the Care and Use of Animals (The National Academies Press, Eighth Edition, 2011). The experimental protocol also complied with the ethical and methodological standards of the ARRIVE guidelines (Kilkenny et al., 2013). Vigorous attempts were made to minimize animal suffering and decrease external sources of pain and discomfort, and to limit the number of animals used to a number that was essential to produce reliable scientific data. Mice were habituated to the maintenance room for 1 week prior to the experiments. The number of animals and the intensity of noxious stimuli used were the minimum necessary to demonstrate consistent effects of the drug treatment.

4.2. Drugs

The following substances were used: naloxone, q-chlorophenylalanine methyl ester (PCPA), a-methyl-p-tyrosine (AMPT), yohimbine, caffeine, 1,3-dipropyl-8-cyclopentylxanthine (DPCPX), AM281, AM630, xylazine, and ketamine (purchased from Sigma Chemical Company, St Louis, MO, USA); and isoflurane (Cristália, SP, Brazil); and flumazenil and dizocilpine hydrogen maleate (MK-801) (Sigma). All drugs were dissolved in 0.9% NaCl solution (saline), except for AMPT, which was dissolved in 5% Tween 80.

Appropriate vehicle-treated groups were also assessed simultaneously. Drugs were administered intraperitoneally (i.p.) or intrathecally (i.t.), as indicated in the tests, and a trained professional performed all the administrations.

4.3. Partial sciatic nerve ligation (PSNL)

The mice were anesthetized with an injection of 10 mg/g of xylazine (i.p.) and 80 mg/kg of ketamine (i.p.). A partial ligation of the right sciatic nerve was performed by tying 1/3–1/2 of the dorsal area of the distal part of sciatic nerve, according to the procedure described by Malmberg and Basbaum (Malmberg and Basbaum, 1998). Each group comprised eight animals.

4.4. Assessment of mechanical allodynia

The mice were individually placed in clear Plexiglas boxes (9 × 7 × 11 cm) on an elevated wire mesh platform to allow access to the ventral surface of the right hind paw. The withdrawal frequency was measured from the number of times (out of 10) the animal withdrew the paw in response to the 0.6-g filament (Stoelting, Chicago, IL) (Bobinski et al., 2011; Bortalanza et al., 2002). The animals were acclimatized for at least 1 h before the behavioral test. The frequency of withdrawal responses of naïve mice to mechanical stimuli that was assessed before the PSNL procedure was presented as the baseline in the graphs (B).

4.5. Time course of the effects of transcranial direct current stimulation (tDCS) on mechanical allodynia induced by PSNL in mice

All animals underwent either a PSNL or sham-PSNL procedure. To determine the long-lasting effect of tDCS, the PSNL animals received 5, 10, 15 or 20 min of tDCS. The time course was assessed using a Von Frey filament (0.6 g) at 0.5, 1, 2, 3, 4 and 5 h after the stimulation.

4.6. Transcranial direct current stimulation (tDCS)

First, the trichotomy of the head was performed in all animals, in the region where the electrodes were placed to obtain better adhesion of the electrodes with a constant current of 0.5 mA (Fregni et al., 2006). According Liebetanz et al. (Liebetanz et al., 2006), a constant current with an intensity of 1 mA caused skin lesions on the animal. The lowest current capable of generating an effect, without causing tissue damage, was 0.5 mA. After establishing neuropathic pain, the animals in the real treatment groups underwent a 15-min session of bicephalic tDCS (Fig. 9). The cathode was positioned at the midpoint between the lateral angles of both eyes (supraorbital area), while the anode was placed on the head using 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., 2011).

The animals were subjected to only one session of bicephalic tDCS for 15 min. A time course of responses demonstrated the same antiallodynic effect after 15 and 20 min (described later at Section 2), and thus, 15 min was set as the duration to avoid immobilization the animal. The direct current was generated from a battery containing a constant stimulator connected to electroencephalogram (EEG) electrodes and adapted using a conductive hydrogel. A device connecting to a multimeter showing the electrical current was used to control the current flow. For sham stimulation, the electrodes were placed and fixed in the same position and time (15′) that the actual stimulation, however the stimulator remained in the "off" position throughout the procedure.

4.7. Pharmacological tests to investigate the mechanisms of antiallodynic effects induced by tDCS

The animals were assessed with von Frey filaments on the 10th postoperative day to investigate the neurotransmission systems involved in the antiallodynic action of tDCS. This was considered the baseline measurement. Then, 20 or 15 min after each drug administration, using i.p.or i.t., respectively, the animals underwent a single 15-min session of active or sham tDCS or sham tDCS for 15 min. Mechanical allodynia was assessed using von Frey filaments (06 g) 1 h after the tDCS session, as illustrated in Fig. 10. The following groups were used in subsequent experiments: active t DCS + vehicle, active tDCS + drug, sham tDCS + vehicle and sham tDCS + drug.

4.7.1. Intraperitoneal (i.p.) pathway investigation

4.7.1.1. Involvement of the opioidergic system. The animals were treated with naloxone (1 mg/kg, i.p.; a non-selective opioid receptor antagonist) or saline solution (vehicle, 10 mL/kg, i.p.) (Martins et al., 2012).

4.7.1.2. Involvement of the catecholaminergic system.

a. The animals were treated with α-methyl-*p*-tyrosine (AMPT, 100 mg/kg, i.p., an inhibitor of the enzyme tyrosine hydroxylase) (Kaster et al., 2007), or saline solution (vehicle, 10 mL/kg, i.p.).
b. The animals were treated with yohimbine (0.15 mg/kg, i.p., a selective antagonist of alpha-2 adrenergic receptor) or saline solution (vehicle, 10 mL/kg, i.p.) (Zakaria et al., 2014).

4.7.1.3. Involvement of the serotonergic system. The animals were pretreated with *p*-chlorophenylalanine methyl ester (PCPA, 100 mg/kg, i.p., an inhibitor of serotonin synthesis) or saline (10 mL/kg, i.p.) once a day for 4 consecutive days, from the 6th to the 9th days after surgery (Santos et al., 2005). On the 10th postoperative day, the animals were evaluated using the von Frey filament test; this was considered the baseline measurement. The final dose of PCPA was then administered (on day 4) (Walker et al., 2013).

4.7.1.4. Involvement of the adenosinergic system. The animals were treated with caffeine (10 mg/kg, i.p., a non-selective antagonist of adenosine receptors) or saline (vehicle, 10 mL/kg, i.p.) (Martins et al., 2013).

4.7.1.5. Involvement of the cannabinoid system.

a. The animals were treated with AM281 (0.5 mg/kg, i.p., CB1 receptor antagonist and inverse agonist) or saline solution (vehicle, 10 mL/ kg, i.p.) (Berger et al., 2014).
b. The animals were treated with AM630 (3 mg/kg, i.p., CB2 receptor antagonist and inverse agonist) or saline solution (10 mL/kg, i.p.) (Berger et al., 2014).

4.7.1.6. Involvement of the GABAergic system. The animals were treated with Flumazenil (3 mg/kg, i.p., benzodiazepine receptor antagonist) or saline solution (vehicle, 10 mL/kg, i.p.) (Carballo-Villalobos et al., 2014).

4.7.1.7. Involvement of the glutamatergic system. The animals were treated with dizocilpine hydrogen maleate (MK801, 0.01 mg/kg, i. p., NMDA-selective receptor antagonist) or saline solution (vehicle, 10 mL/kg, i.p.) (Dhir and Kulkarni, 2008).

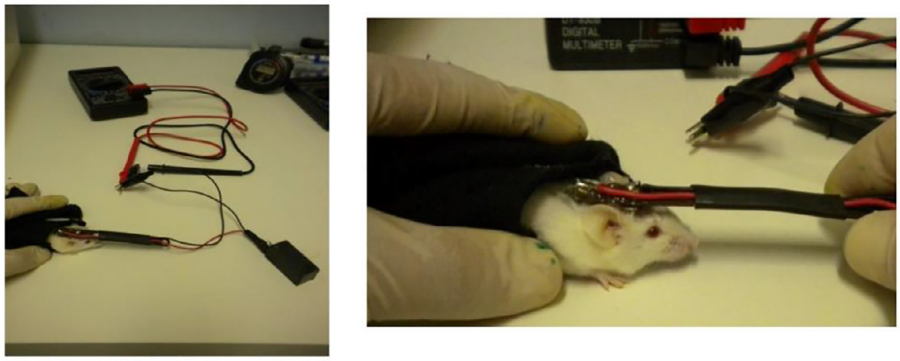

Fig. 9. Demonstrative of electrode positions (author).

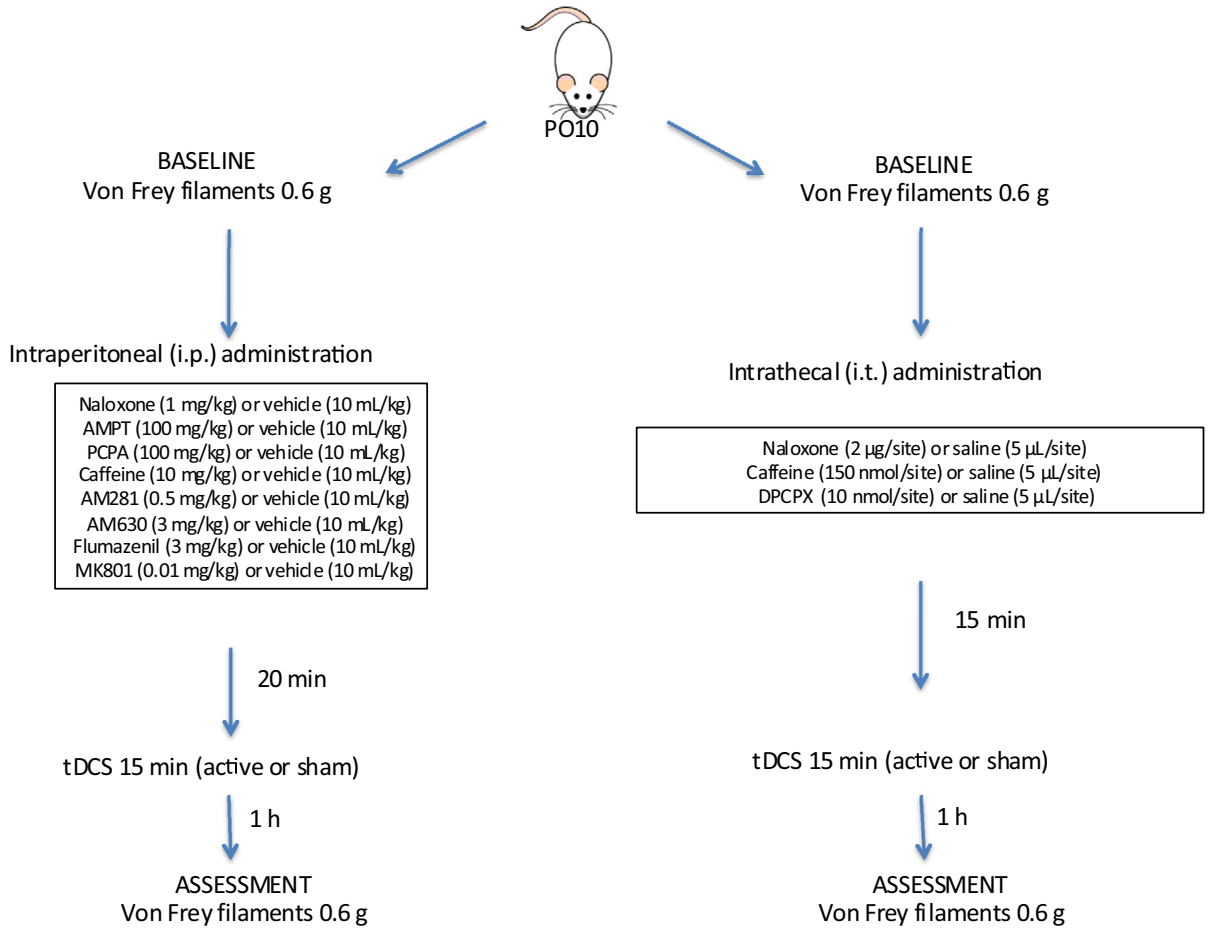

Fig. 10. The experimental design for the pharmacological assays used in the current study. PO: postoperative day, AMPT: α-methyl-*p*-tyrosine, PCPA: *p*-chlorophenylalanine methyl ester; MK-801: dizocilpine hydrogen maleate, DPCPX: 1,3-dipropyl-8-cyclopentylxanthine, tDCS: transcranial direct current stimulation.

4.7.2. Intrathecal (i.t.) pathway investigation

4.7.2.1. Involvement of the opioidergic system. the animals were treated with naloxone (2 µg/site, i.t., µ–selective opioid receptor antagonist) or saline (vehicle 5 µL/site, i.t.) (Martins et al., 2013).

4.7.2.2. Involvement adenosinergic system. The animals were treated with 1,3-dipropyl-8-cyclopentylxanthine (DPCPX, 10 nmol/site, i.t., a selective A1 receptor antagonist) or saline (vehicle 5 µL/site, i.t.) (Martins et al., 2013).

4.7.3. Influence of local anesthesia with lidocaine on antiallodynic effect of tDCS

The animals were pretreated with an injection of lidocaine (1%/site) to assess the role peripheral pathways play in eliciting the antiallodynic effect of tDCS 15′.

4.8. Blinding

The researchers were blinded to the group assignment in all behavioral tests. A team of three researchers conducted the stimu-

lation and application of drug tests, and a fourth researcher performed the von Frey test measurements. The latter researcher was unaware of the type of stimulation (tDCS or tDCS sham) or the administered drug (active or vehicle). Thus, we believed that there was no researcher bias to influence the results.

4.9. Statistical analysis

Data were expressed as mean ± standard error of mean (S.E.M.). The normality of the data was assessed using the Shapiro Wilk Test. If the data were normally distributed, then it was analyzed using a parametric test, one-way ANOVA, or two-way ANOVA repeated measures, followed by Tukey's test. Statistical significance was set at $P < .05$.

Contributors

This work was conducted in collaboration between all the authors. The authors AS, DFM, and LFM designed the study, and performed the experimental assays and statistical analyses; authors CN, TCM, and AS performed the experimental assays and statistical analyses; authors WC and ARSS participated in the design of the study; author ILST coordinated the study, performed the statistical analyses, and helped to draft the manuscript. All authors read and approved the final manuscript.

Conflict of interest

There was no financial interest between any of the authors or any commercial interest in the outcome of this study.

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