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Research Report

Reversal of chronic stress-induced pain by transcranial direct current stimulation (tDCS) in an animal model

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ABSTRACT

Transcranial direct current stimulation (tDCS) has been suggested as a therapeutic tool for pain syndromes. Although initial results in human subjects are encouraging, it still remains unclear whether the effects of tDCS can reverse maladaptive plasticity associated with chronic pain. To investigate this question, we tested whether tDCS can reverse the specific behavioral effects of chronic stress in the pain system, and also those indexed by corticosterone and interleukin-1β levels in serum and TNFα levels in the hippocampus, in a well-controlled rat model of chronic restraint stress (CRS). Forty-one adult male Wistar rats were divided into two groups control and stress. The stress group was exposed to CRS for 11 weeks for the establishment of hyperalgesia and mechanical allodynia as shown by the hot plate and von Frey tests, respectively. Rats were then divided into four groups control, stress, stress+sham tDCS and stress+tDCS. Anodal or sham tDCS was applied for 20 min/day over 8 days and the tests were repeated. Then, the animals were killed, blood collected and hippocampus removed for ELISA testing. This model of CRS proved effective to induce chronic pain, as the animals exhibited hyperalgesia and mechanical allodynia. The hot plate test showed an analgesic effect, and the von Frey test, an anti-allodynic effect after the last tDCS session, and there was a significant decrease in hippocampal TNFα levels.

Abbreviations: tDCS, Transcranial direct current stimulation; CRS, chronic restraint stress; HPA, hypothalamic-pituitary-adrenal axis; HD-tDCS, High-definition tDCS; DC, direct current; IL1β, interleukin-1β; TNFα, tumor necrosis factor-α; BDNF, brain-derived neurotrophic factor; LTP, long-term potentiation

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These results support the notion that tDCS reverses the detrimental effects of chronic stress on the pain system and decreases TNFα levels in the hippocampus.

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1. Introduction

Several pain syndromes, such as fibromyalgia, chronic back pain, and neuropathic pain, are associated with significant effects on neuroplasticity in pain-related neural circuits, which, in turn, lead to significant effects on the sensory and affective-emotional domains, such as hyperalgesia, allodynia, anxiety and depression (Staud, 2006; Staud and Rodriguez, 2006). In most cases, these conditions are associated with psychiatric disorders, absenteeism, and high costs of chronic treatment or poor outcomes despite treatment (Jensen et al., 2007; Van Hanswijck de Jonge et al., 2008). Pain syndromes are associated with chronic stress, as chronic exposure to pain produces suffering, which activates the hypothalamic-pituitary-adrenal (HPA) axis, thus stimulating the production of corticosterone, the hormone released in stress conditions (for a review, see Martenson et al., 2009). It is known that serum corticosterone levels in rats subjected to chronic stress do not show a significant increase in comparison to control animals; however, this increase is statistically significant when rats are subjected to acute stress (Park et al., 2012; Torres et al., 2001a).

Unlike acute stress, which has been associated with a reduction in pain sensitivity, probably mediated by brain stem pain modulation (for a review, see Martenson et al., 2009), chronic stress has been associated with decreased pain thresholds. Indeed, chronic stress is associated with hyperalgesia (enhanced response to noxious stimuli) (Gamaro et al., 1998; Torres et al., 2001a; Bardin et al., 2009) and allodynia (pain induced by non-noxious stimuli) (Bardin et al., 2009). In the previous study, we demonstrated that chronic stress-induced hyperalgesia remained for 28 days after discontinuation of treatment (Torres et al., 2003). Interestingly, the analgesic response to acute restraint stress (i.e., inhibition of pain) was re-established only after 14 days of discontinuation of chronic stress (Torres et al., 2003).

Although the underlying mechanisms of long-lasting hyperalgesia after chronic stress are still elusive, some studies have advanced understanding of this topic. Human studies have shown that a reduction in pain threshold after long-term psychoemotional stress probably occurs due to a reduction in the activity of the brain's opioid system (Ashkinazi and Vershinina, 1999). Previous data from our group also suggest involvement of the opioid system in the hyperalgesic response induced by prolonged restraint stress (Torres et al., 2001b, 2003; Dantas et al., 2005) Furthermore, activation of stress-related circuitry in the hypothalamus activates pain-facilitating neurons in the rostral ventromedial medulla to produce hyperalgesia (for review, see Martenson et al., 2009), suggesting possible changes in brain activity. Another possibility is increased expression of pro-inflammatory cytokines, such as interleukin-1β and tumor necrosis factor (TNFα), in brain tissue and blood due to stress conditions. These cytokines are closely related to painful and inflammatory diseases, and their release is increased under stressful conditions (for review, see Goshen and Yirmiya, 2009).

In view of the neuroplastic effects of chronic stress on pain-related neural circuitry, deactivation of the stress-induced pain-related neural changes would be best achieved with techniques to induce neuroplasticity (Brunoni et al., 2011). One simple but powerful technique is transcranial direct current stimulation (tDCS). This technique produces modulation of neural activity via small electrical currents that, when applied as a direct current (DC) component, polarize neural tissue, inducing significant changes in the resting membrane threshold (Zaghi, 2010) and subsequent changes in synaptic plasticity, as recently shown in an elegant animal model in mice brain slices DC stimulation (Fristch et al., 2010). In addition, it carries little risk and produces little discomfort, and, with repeated sessions, may produce enduring effects (Poreisz et al., 2007). Previous studies have shown that excitability-enhancing anodal tDCS is effective in reducing pain in patients with fibromyalgia (Fregni et al., 2006a) and spinal cord injury (Fregni et al., 2006b). In addition, anodal and cathodal tDCS of the primary motor cortex and dorsolateral prefrontal cortex have been associated with significant changes in experimental pain in healthy subjects (Reidler et al., 2012; Grundman et al., 2011) Finally, the neuromodulatory effects of tDCS have also been consistently demonstrated in animals, such as in rat models of focal epilepsy (Liebetanz et al., 2006), memory (Dockery et al., 2011), Parkinson's disease (Li et al., 2011), and acute stroke (Wachter et al., 2011)

Given the importance of chronic pain and the variability in its pathophysiology, investigation of techniques that can modulate neural mechanisms is relevant to the development of more rational therapies. Non-invasive stimulation techniques, such as tDCS, may be suitable for treatment of chronic pain. Thus, we investigated whether tDCS reverses the hyperalgesia and allodynia induced by chronic restraint stress. We also measured its effect on serum levels of corticosterone and interleukin-1β, as well as TNFα levels in the hippocampus. The importance of this study lies in the fact that it provides, for the first time, evidence that tDCS can reverse the detrimental effects of a specific causal factor of pain on the pain system. Because such a controlled study (i.e. one including control of level of exposure, timing of application of intervention in relation to exposure, and certain measures in the hippocampus) would not be possible in humans due to ethical issues, this study provides invaluable data for the development of tDCS as a therapeutic tool in chronic pain.

2. Results

2.1. *Basal measure after chronic stress and effects of tDCS on allodynia after the end of tDCS treatment as measured by the von Frey test*

When the stress group was divided into the stress, stress+-sham tDCS, and stress+active tDCS groups, we again observed

a significant difference between baseline measurements in the control group and the other groups (C, 65.71±3.39 g; S, 49.07±2.63 g; SS, 45.36±3.34 g; SN, 53.10±2.23 g; one-way ANOVA/Tukey's test, $F(P=0.001$, $n=9$–12/group, Fig. 1). We tested whether tDCS treatment was associated with a significant change in allodynia as compared with the other no-tDCS groups. We conducted an ANOVA testing group differences immediately and 24 h after treatment adjusting for baseline values (including pre-tDCS as the covariate in this ANOVA model). We did not find a significant effect of time ($F(1,44)=0.05$, $P=0.82$), neither in the interaction time*group ($F(3,44)=1.89$, $P=0.14$), suggesting that after treatment, there was no differences in group behavior over time. But, we found a significant effect of group ($F(3,44)=3.87$, $P=0.015$) considering results after treatment. Post hoc analysis confirmed that SN group showed significant differences as compared with SS group ($P=0.028$). Interestingly, the difference between SN and C that we observed at baseline disappeared after tDCS treatment, confirming that after tDCS, animals' behavior were similar to the non-stress control group. Although there was also a difference between S and C ($P=0.012$), there was no difference between S and SN ($P=0.28$), suggesting likely a lack of power for this later analysis.

2.2. *Basal measure after chronic stress and effects of tDCS on nociception immediately and 24 h after the end of tDCS as measured by the hot plate test*

We then performed similar analysis for the hot plate test. We initially tested whether tDCS treatment was associated with a significant change in hyperalgesia as compared with the other no-tDCS groups (C, 5.75+0.41 s; S, 2.70±0.15 s; SS, 3.08±0.90 s; SN, 3.62±0.59 s; one-way ANOVA/Tukey's test, $F(P=0.000$, $n=9$–12/group, Fig. 2). Same ANOVA controlled for baseline differences disclosed similar findings: no significant effect of time ($F(1,44=3.90)$, $P=0.054$) and no significant interaction time*group ($F(3,44)=0.31$, $P=0.7320$, suggesting that after treatment, there was no differences in group

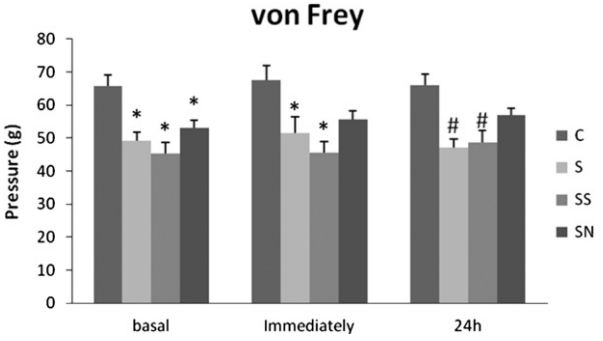

Fig. 1 – Basal measure, immediately and 24 h after the end of tDCS treatment on alodynia induced by chronic stress evaluated with the von Frey test. Data presented as mean±SEM of withdrawal response in grams (g). Groups: C, control; S, stress; SS, stress+sham; SN, stress+neuromodulation. *Significant difference versus control group (C) (one-way ANOVA/Tukey, $P<0.05$, $n=9$–13). #Significant difference versus the control (C) and stress+tDCS (SN) groups (one-way ANOVA/Tukey, $P<0.05$, $n=9$–13).

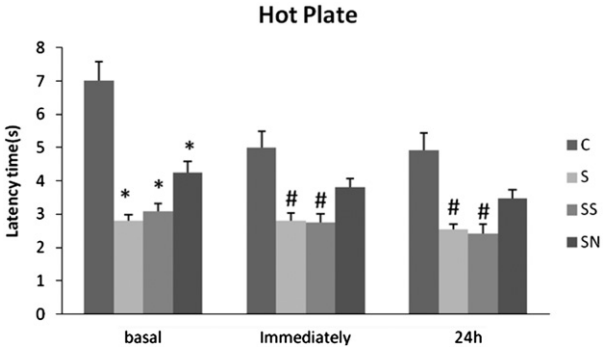

Fig. 2 – Basal measure, immediately and 24 h after the end of tDCS treatment on hyperalgesia induced by chronic rats evaluated with the hot-plate test. Data presented as mean±SEM of response latency (time to onset of paw-licking or jumping) in seconds (s). Groups: C, control; S, stress; SS, stress+sham; SN, stress+neuromodulation. *Significant difference versus control group (C) (one-way ANOVA/Tukey, $P<0.05$, $n=9$–13). #Significant difference versus the control (C) and stress+tDCS (SN) groups (one-way ANOVA/Tukey, $P<0.05$, $n=9$–13).

behavior over time. But, we found a significant effect of group ($F(9,42)=7.08$, $P=0.0000$) considering results after treatment. Here post hoc analysis confirmed that SN group showed significant differences as compared with SS group ($P=0.000$) and S group ($P=0.002$). Similarly the difference between SN and C that we observed at baseline also disappeared after tDCS treatment ($P=1.000$), confirming that after tDCS, animals behavior was similar to the non-stress control group.

2.3. *Effects of tDCS on serum corticosterone and interleukin-1β levels after the end of tDCS treatment*

No effect of stress or tDCS treatment was observed in serum levels of corticosterone (C, 385.90±171.54 nmol/L; S, 295.73± 158.72 nmol/L; SS, 418.02 ±89.90 nmol/L; SN, 424.85±102.17 nmol/L; one-way ANOVA/Tukey's test, $P>0.05$, $n=6$–7, Fig. 3A) or interleukin-1β (C, 46.76±4.93 pg/L; S, 51.22±11.85 pg/L; SS, 58.38±7.45 pg/L; SN, 42.21±3.90 pg/L; one-way ANOVA/Tukey's test, $P>0.05$, $n=3$–6, Fig. 3B).

2.4. *Effects of tDCS on hippocampal TNFα levels after the end of tDCS treatment*

We observed a significant between-group difference in TNFα levels in the hippocampus. The active tDCS group showed decreased levels of TNFα in hippocampus in comparison to the other groups (C, 128.76±28.65 pg/L; S,126.77±13.00 pg/L; SS, 123.26±5.22 pg/L; SN, 52.50±2.00 pg/L one-way ANOVA/Tukey's test, $P\leq0.05$, $n=3$–4, Fig. 4).

3. Discussion

In this study, we demonstrated that tDCS stimulation effectively reversed the hyperalgesia and allodynia induced by the

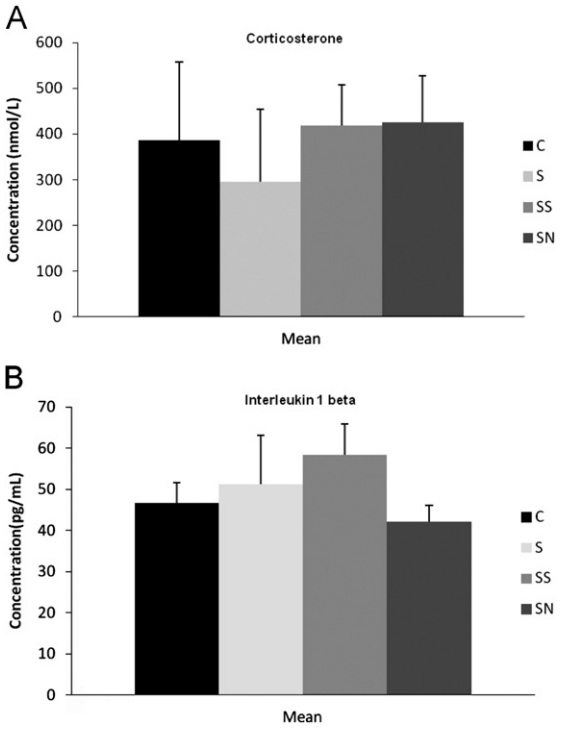

Fig. 3 – Panel A: Evaluation of serum corticosterone levels of chronic stressed rats 48 h after the end of tDCS treatment. Data presented as mean ± SEM of serum corticosterone level in nmol/L. Groups: C, control; S, stress; SS, stress+sham; SN, stress+neuromodulation. There were no significant between-group differences (one-way ANOVA, $P > 0.05$, $n = 3–4$). Panel B: Evaluation of serum interleukin-1β levels of chronic stressed rats 48 h after the end of tDCS treatment. Data presented as mean ± SEM of serum interleukin-1β level in pg/mL. Groups: C, control; S, stress; SS, stress+sham; SN, stress+neuromodulation. There were no significant between-group differences (one-way ANOVA, $P > 0.05$, $n = 6–3$).

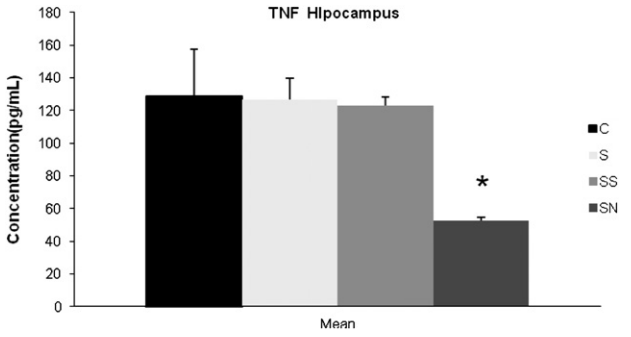

Fig. 4 – Evaluation of hippocampal TNFα levels of chronic stressed rats 48 h after the end of tDCS treatment. Data presented as mean ± SEM of hippocampal TNFα levels in pg/mL. Groups: C, control; S, stress; SS, stress+sham; SN, stress+neuromodulation. *Significant difference in relation to other groups (one-way ANOVA/Tukey, $P < 0.05$, $n = 3–4$).

chronic restraint stress rat model. This result persisted for at least 24 h, which demonstrates the cumulative effects of repetitive tDCS treatment, as, in the previous study, the antinociceptive effect of one session of transcranial eletrostimulation in rats disappeared within 15 min after cessation of electrical stimulation (Nekhendzy et al., 2004). The hyperalgesic effect was assessed by two behavioral components on hot plate (paw licking and jumping), both considered supraspinally integrated responses. This constitutes, at least in part, the rationale for testing of the antihyperalgesic effect of tDCS. Given our electrode montage, it is conceivable that most of the effects found in this study were due to cortical modulation. In this scenario, it is likely that effects of transcranial stimulation on pain relief depend on the projection of fibers from cortical structures to other neural areas involved in pain processing, such as the thalamus and brainstem nuclei, which could activate non-nociceptive neurons (Drouot et al., 2002; Lefaucheur et al., 2006). Thus, we can suggest that stimulation activates descending inhibitory pathways, suppressing pain through a top–down modulation mechanism (Lima and Fregni, 2008).

Although anodal tDCS has been shown to induce pain relief in human studies (for a review, see Mylius et al., 2012), this study fills a critical gap in the knowledge of the field, as we show that consecutive sessions of tDCS can reverse chronic stress-induced pain. In our study, we were able to control the source of pain, thus providing a homogeneous sample in terms of chronic pain mechanisms and demonstrating the effects of tDCS in this condition. In this context, we will briefly review the putative mechanisms involved in the development of hyperalgesia after repeated restraint stress. Previous studies have suggested that this phenomenon could be related to changes in central or peripheral opioid activity (Torres et al., 2001b, 2003; Dantas et al., 2005). The absence of novelty-induced antinociception in these animals supports this theory (Torres et al., 2001b). Exposure of rats to a novel environment is known to be followed by mild, naloxone-reversible antinociception (Siegfried et al., 1987). Opioid receptors can be highly plastic, as reflected by their susceptibility to modifications by various pharmacological and behavioral manipulations (for a review, see Drolet et al., 2001). Dantas et al. (2005) showed decrease in binding of opioid receptors in the hippocampus and cerebral cortex. Additionally, Torres et al. (2003) demonstrated that animals subjected to chronic restraint stress for 6 weeks needed high doses of morphine to exhibit an analgesic response, suggesting that prolonged stress could lead to longer-lasting changes in the neural systems involved in nociceptive modulation. On the other hand, in acute stress, the opiate system seems to be modulated in the opposite direction. In fact, the previous study has demonstrated that animals subjected to acute stress show an increase in the magnitude and duration of the analgesic effect to some opiate agonists (Calcagnetti and Holtzman, 1992).

Other important finding of this study was that corticosterone and interleukin-1β levels in serum did not present statistically significant changes by the tDCS sessions and/or chronic restraint stress. These results are consistent with the literature, which has shown that chronic restraint stress leads to disorganization and deregulation of HPA axis stress responses (for a review, see Goshen and Yirmiya, 2009). In addition, we showed that hippocampal TNFα levels were not increased by chronic restraint stress, unlike the

previous study, which reported increased TNFα level in the hippocampus after 40 days of variable stress (Tagliari et al., 2011). This result was due to the long period of stress used in this study—almost twice cited in the Tagliari paper. Therefore, this reaction was probably reestablished by an adaptive response. On the other hand, hippocampal TNFα levels were significantly decreased in the group that received tDCS as compared with other groups. As TNFα is a proinflammatory cytokine, this could be related to the effects of tDCS on reversal of maladaptative changes in the pain system induced by chronic restraint stress. Hence, one possible mode of action of anodal tDCS is by decreasing hippocampal TNFα levels, causing an anti-inflammatory and anti-hyperalgesic response, even considering normal baseline (pre-stimulation) TNFα levels in the hippocampus.

Although the mechanisms underlying tDCS-mediated pain regulation have yet to be elucidated, its mechanisms of action involve changes in the neuronal electrical membrane potential and modifications in the synaptic microenvironment. Changes in synaptic strength are NMDA receptor-dependent or can alter GABAergic activity (Liebetanz et al., 2002; Nitsche et al., 2003a; Stagg et al., 2009). The tDCS also interferes with brain excitability through modulation of intracortical and corticospinal neurons (Nitsche et al., 2005; Ardolino et al., 2005). The effects of tDCS might be similar to those observed in long-term potentiation (LTP), as demonstrated in an animal study that used anodal motor cortex stimulation (Fritsch et al., 2010). Experiments with spinal cord stimulation have shown that the effects of tDCS are also non-synaptic, possibly involving transient changes in the density of protein channels located below the stimulating electrode (Cogiamanian et al., 2008) or due to glial changes (Radman et al., 2009). Given that a constant electric field displaces all polar molecules and that most neurotransmitters and receptors in the brain have electrical properties, tDCS might also influence neuronal function by inducing prolonged neurochemical changes (Stagg et al., 2009; Cogiamanian et al., 2008).

In addition to neurochemical changes, it is known that tDCS also has a significant effect on current blood flow. Some experiments combining tDCS and transcranial laser Doppler flowmetry (LDF) in a rat model demonstrated that tDCS induces sustained changes on current blood flow. These changes were polarity-specific; anodal tDCS leads to an increase, whereas cathodal tDCS leads to a decrease in current blood flow (Wachter et al., 2011). Whether increased metabolic activity in the experimental model of chronic pain is involved in the reversal of hyperalgesia has yet to be determined.

According to Fertonani et al. (2010), the long-term effects of tDCS also involve glutamatergic NMDA receptors, and synaptic plasticity is also dependent on NMDA receptors. D-cycloserine, a partial NMDA agonist, has been shown to selectively potentiate the duration of motor cortical excitability enhancements induced by anodal tDCS, but not the decrease in excitability induced by cathodal stimulation. A patient with chronic pain was successfully treated with repeated applications of tDCS over the motor cortex combined with D-cycloserine and dextromethorphan administration to prevent recurrence of pain (Antal and Paulus, 2011). The analgesic effect of tDCS could be mediated by modulatory effects in pain sensation in several neurotransmitter systems, including opioid, adrenergic, substance P, glutamate and neurokinin receptors (Morgan et al., 1994; Wu et al., 2000). It leads to a cascade of events resulting in the modulation of synaptic neural chains that include several thalamic nuclei, the limbic system, brainstem nuclei, and the spinal cord (Lima and Fregni, 2008).

It has been demonstrated that pain relief induced by invasive cortical stimulation is also mediated by activation of the endogenous opioid system. In fact, motor cortex stimulation produces activation of the cortical segment and acts on intracortical interneurons. Stimulation of these fibers spreads to different areas thalamic cortical projections, cortical–cortical lateral projections and local cortical connections (Lima and Fregni, 2008). We can hypothesize that the results obtained might depend on the aforementioned mechanisms. However, we did not measure the duration of the antihyperalgesic effect observed.

Viewed as a whole, our findings support the hypothesis of an antihyperalgesic and antiallodynic effect of tDCS. Although the mechanisms underlying this effect remain unclear, the evidence suggests that they include non-synaptic and synaptic mechanisms alike. The non-synaptic mechanism would include changes which, apart from reflecting local changes in ionic concentrations, could arise from alterations in transmembrane proteins and from electrolysis-related changes in H(+), induced by exposure to a constant electric field (Ardolino et al., 2005). The synaptic mechanisms would involve neuroplastic alterations, such as changes in the strength of connections, representational patterns, or neuronal properties, either morphological or functional (Antal et al., 2006). tDCS induces prolonged neuronal excitability and activity changes in the human brain via alterations in neuronal membrane potential, resulting in the prolonged synaptic efficacy changes.

One important question that has yet to be fully elucidated is optimal electrode placement for induction of analgesic effects (Fregni, 2010). It is not clear whether the effects are mainly due to anodal stimulation of frontal areas (including M1) or associated with cathodal stimulation of the contralateral area, although there is extensive evidence showing that modulation of M1 is critically involved with pain modulation, as shown by modeling studies (Mendonca et al., 2011; DaSilva et al., 2012) and high-definition-tDCS(HD-tDCS) (Borckardt et al., 2012). Finally, another important issue is the association between electrode montage and shunting. Although our montage may be associated with shunting, it has previously proved effective, such as in the Takano et al. (2011) study. These authors examined the effectiveness of tDCS using functional magnetic resonance imaging (fMRI) and the signal intensities of fMRI in the frontal cortex and nucleus accumbens, and found significant increases in activity after anodal tDCS exposure in rats. In addition, in silicon finite element model studies have shown that even with close electrodes, such as those used in HD-tDCS, a significant amount of current is injected and reaches cortical areas (Minhas, 2010; Datta, 2009). On the basis of these considerations, we decided to use a cephalic montage as this has been the most widely used method in humans. In fact, a recent study in humans showed that extra-cephalic montages were less effective to

provide pain relief (Mendonca et al., 2011). Another important limitation, also discussed in a recent review, is extrapolation of these results to humans (Volz et al., 2012). In this context, this study, to the best of our knowledge, was the first to show that tDCS can reverse the effects of maladaptive plasticity as expressed by behavioral changes and measured by TNFα levels. On the other hand, one limitation of the study was the lack of difference between one of the analysis for von Frey test – S vs. SN – probably because of less sensitivity of this measurement as compared to hot plate test and also because of differences what these measurements index such as hot plate related to hyperalgesia and von Frey related to allodynia.

In summary, we showed that tDCS was able to reverse completely the detrimental effects of chronic stress on the pain system, as expressed by hyperalgesia and allodynia, and that this effect continued for 24 h. Serum levels of corticosterone and interleukin-1β were not changed by tDCS sessions or chronic restraint stress, but hippocampal TNFα levels decreased. Given that, in this study, animals were exposed to the same level of stress under the same conditions, our findings support further exploration of tDCS as a therapeutic tool early in the exposure to stressful situations that may lead to chronic pain, such as post-traumatic stress disorder, and demonstrate one possible pathway of anodal tDCS treatment. Future studies should also consider assessing other outcomes of stress response, including other behavioral outcomes, as well as measurement of other biochemical variables, such as PCPA (inhibitor of serotonin synthesis), AMPT (inhibitor of tyrosine hydroxylase) and naloxone, to provide a better understanding of the effects of chronic restraint stress on mood and anxiety and further elucidate and optimize this intervention into a potential clinical tool for stress-related conditions.

4. Experimental procedures

4.1. Animals

Sixty-day-old male Wistar rats weighing 180–230 g were used. Experimentally naive animals were housed in groups of five in 49 × 34 × 16 cm polypropylene home cages. All animals were kept on a standard 12-hour light/dark cycle (lights on at 07:00 a.m. and lights off at 07:00 p.m.) in a temperature-controlled environment (22 ± 2 °C). Animals had access to water and chow *ad libitum*. All experiments and procedures were approved by the Institutional Committee for Animal Care and Use (GPPG-HCPA protocol No. 100.381) and performed in accordance with the Guide for the Care and Use of Laboratory Animals 8th edition (2011). Animal handling and all experiments were performed in accordance with International Guidelines for Animal Welfare and Measures were taken to minimize animal pain and discomfort. The experiment used the minimum number of animals required to produce reliable scientific data.

To control the possible effect of outliers, we excluded rats which did not present any response on behavioral testing. All the experimenters were blinded to condition (active or sham tDCS) during post-treatment behavioral testing.

4.2. Chronic restraint stress

The animals were subjected to 1 h of restraint daily, 5 days a week for 11 weeks. Restraint was applied by placing the animal in 25 × 7 cm plastic bottle with a 1-cm hole at the far end for breathing (Ely et al., 1997 with modifications). The animal was unable to move. The control group was not subjected to restraint. These procedures were always performed between 08:00 h and 09:00 h. Restraint sessions continued during the behavioral test period and during tDCS sessions, which were carried out in the afternoon. The animals were divided into four groups (n=12–13): control (C), stress (S), stress+sham tDCS (SS) and stress+tDCS (SN). After 11 weeks of chronic stress exposure, behavioral tests were performed in the afternoon.

4.3. Pain outcome I: von Frey test

Mechanical allodynia was assessed before, immediately and 24 h after the end of tDCS treatment using an automatic von Frey esthesiometer (Insight, São Paulo, Brazil). This is an adaptation of the classical von Frey filaments test in which pressure intensity is recorded automatically after paw removal (Vivancos et al., 2004). It has been proposed that tactile hypersensitivity is likely to be the consequence of a change in function and a phenotypic switch in primary afferent neurons innervating the inflamed tissue and the pattern of excitation they produce in spinal neurons. This assumption was partially confirmed by the finding that a subpopulation of A beta primary afferent neurons came to express substance P after conditioning inflammation, thereby enhancing synaptic transmission in the spinal cord and exaggerating the central response to innocuous stimuli (Ma and Woolf, 1996; Neumann et al., 1996).

Rats were placed in 12 × 20 × 17 cm polypropylene cages with wire grid floors and acclimatized for 15 min, 24 h prior to the test, as the novelty of the apparatus itself can induce antinociception (Netto et al., 2004). For testing, a polypropylene tip was placed perpendicularly underneath the mesh floor and applied to one of the five distal footpads with a gradual increase in pressure. A tilted mirror below the grid provided a clear view of the animal's hind paw. The test consisted of poking the hind paw to provoke a flexion reflex followed by a clear flinch response after paw withdrawal. The intensity of the stimulus was automatically recorded when the paw was withdrawn. Three successive von Frey readings were averaged, and these averages were used as the final measurements. The paw withdrawal threshold was expressed in grams (g) (Vivancos et al., 2004).

4.4. Pain outcome II: hot plate

The hot plate test was carried out to assess the effects of tDCS on the thermal nociceptive threshold (Woolfe and Macdonald, 1944). This test was assessed before, immediately and 24 h after the end of tDCS treatment. We used the hot-plate test to determine changes in latency as an indicator of modifications of the supraspinal pain process (Ossipov et al., 1995), as licking or jumping responses during this test are

considered to be the result of supraspinal sensory integration (Caggiula et al., 1995; Rubinstein et al., 1996).

The hot plate was pre-heated and kept at a temperature of 55±0.5 °C. All rats were acclimated to the hot plate for 5 min, 24 h prior to testing, as, again, the novelty of the apparatus itself can induce antinociception (Netto et al., 2004). Rats were placed in glass funnels on the heated surface and the nociceptive threshold was assessed recording to the time taken to first response (foot licking, jumping, or rapidly removing paws), as described by Minami et al. (1994). Response was recorded in seconds (s) and a cutoff time of 20 s was used.

4.5. *Transcranial direct current stimulation (tDCS)*

After 11 weeks of chronic stress exposure, the rats of SN were subjected to a 20-min session of anodal tDCS every afternoon for 8 days. This period was established because tDCS has been shown to modify cortical excitability for up to 1 h after one session of stimulation (Nitsche and Paulus, 2000; Nitsche et al., 2003b). However, repetitive tDCS application has demonstrated better and longer-lasting effects on pain relief, and in recent study our group showed antihyperalgesic response in paw inflamed rats with this treatment period (Laste et al., 2012). The direct current was delivered from a battery-driven, constant current stimulator using ECG electrodes with conductive adhesive hydrogel. Rats' heads were shaved for better adherence and the electrodes were trimmed to 1.5 cm^2 for better fit. After placement, electrodes were fixed onto the head with adhesive tape (Micropore™) and covered with a protective mesh to prevent removal (Fig. 5A).

The anodal electrode was positioned between the ears, from the neck of the rat (parietal cortex) (Fig. 5B) (Takano et al., 2011 with modifications), so as to mimic anodal placement in human pain studies (Mendonca et al., 2011; Dasilva et al., 2012). The cathodal electrode was positioned at the midpoint of the lateral angle of the eyes (supraorbital area). The electrodes were placed on the skin in a similar manner to that used in human studies of tDCS for pain (Nitsche et al., 2008; Antal and Paulus, 2011; Rosen et al., 2009; Fregni et al., 2006c).

A constant current of 0.5 mA intensity was applied for 20 min (Fregni et al., 2006b; Dockery et al., 2011; Wachter et al., 2011; Liebetanz et al., 2006). According to an earlier study (Liebetanz et al., 2009), a constant current of 1 mA intensity causes skin lesions, as current density is comparatively much higher than the traditional 1 mA tDCS using large pads in humans. We therefore chose to use 0.5 mA, an intensity that has also been used in other animal studies. In addition, in our study, electrodes were fixed onto the skin. We did not observe any lesions with montage and current intensity.

An important point to consider was that this model required neither anesthesia nor surgery, unlike models used in the previous tDCS studies in rats (Dockery et al., 2011; Wachter et al., 2011; Liebetanz et al., 2006). In fact, this represents a strength in this study, as volatile anesthesia (such as isoflurane) has been shown to decrease excitatory and increase inhibitory transmission (Gomez and Guatimosim, 2003; Ouyang and Hemmings, 2005), altering BDNF expression and thus neuroplasticity (Lu et al., 2006; Head et al., 2009). We were thus able to remove this confounding factor in our study by adapting a human model using ECG electrodes (Fregni et al., 2006c).

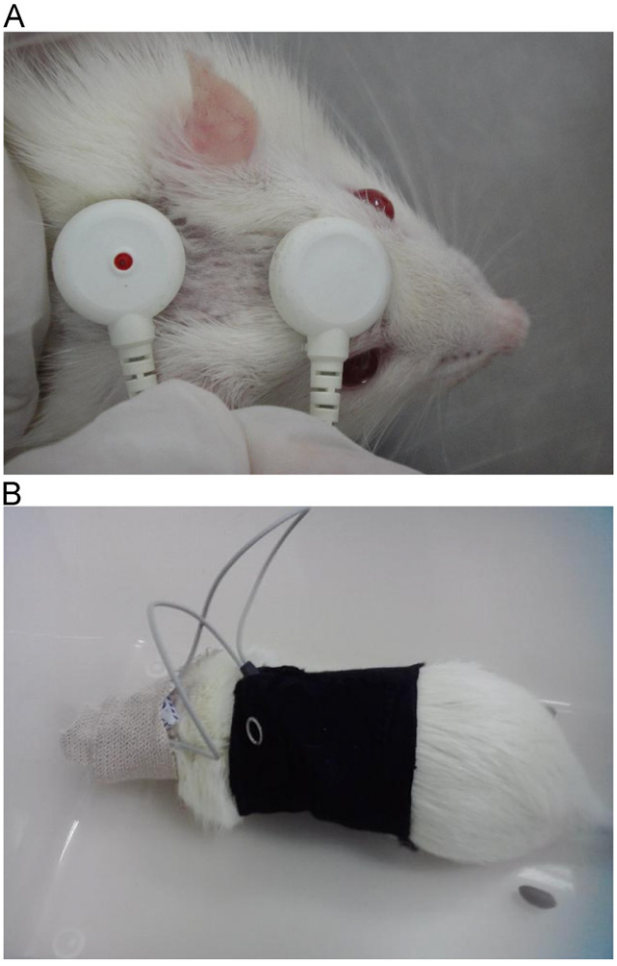

Fig. 5 – Panel A: tDCS electrode placement. The cathodal stimulus electrode was positioned at the midpoint of the lateral angle of the eyes, and the anodal electrode is positioned over the neck and shoulder areas. Panel B: tDCS stimulation procedure. The stimulator was placed onto the thorax with a corset and the electrodes were fixed onto the rat's head.

For sham stimulation, the electrodes were placed in the same positions as for real stimulation; however, the stimulator was turned off after 30 s of stimulation so the animals could maintain continuity of the physical sensation of real tDCS conditions (Gandiga et al., 2006).

4.6. *Blood sampling and tissue collection*

Forty-eight hours after tDCS treatment, the animals were killed by decapitation. Trunk blood was collected and centrifuged at 5000 *g* for 5 min at room temperature. Animals were killed by an experienced investigator. Serum and hippocampus were frozen at −70 °C for subsequent analysis.

4.7. *Analyses of corticosterone and interleukin-1β serum levels*

Serum interleukin-1 and corticosterone levels were determined using commercially available enzyme-linked immunosorbent

assay (ELISA) kits for rat interleukin-1 (Uscn Life Science Inc.) or corticosterone (IBL Corticosterone Kit), according to manufacturer instructions. The results are expressed in pg/mL and nmol/L, respectively.

4.8. *Analysis of TNFα immunocontent*

TNF analysis was performed on hippocampus homogenates. TNF levels were measured by a commercially available enzyme-linked immunosorbent assay (ELISA) kit for rat tumor necrosis factor-alpha (Uscn Life Science Inc.), according to manufacturer protocols. The results are expressed in pg/mL.

4.9. *Statistical analysis*

The results are presented as the mean ± standard error of the mean (SEM). As data were normally distributed, we assessed the difference between groups using one-way ANOVA with Tukey's test when necessary. *P*-values less than 0.05 were considered significant.

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