Transcranial direct current stimulation did not revert the increased central and peripheral NGF levels induced by neuropathic pain in rats

Tratamento com estimulação transcraniana por corrente contínua não reverteu o aumento de NGF induzido por modelo de dor neuropática em ratos

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ABSTRACT

BACKGROUND AND OBJECTIVES: The expression of nerve growth factor (NGF) in the large-size neurons may represent a key role in the neuronal synaptic plasticity and re-organization of neuronal function after a nerve injury. Transcranial direct current stimulation (tDCS) is a non-invasive method of cerebral stimulation and represents a promising tool to pain management since it promotes neuroplasticity in the central system, and it can be combined with other interventions. The aim was to investigate the effects of tDCS in the NGF levels in central and peripheral nervous system structures of rats submitted to a neuropathic pain (NP) model.

METHODS: The chronic constriction injury (CCI) of sciatic nerve was used for the induction of NP. For sham surgery, the sciatic nerve was exposed, but without any ligation. The control group did not undergo surgical procedure. After the establishment of NP, treated groups were subjected to tDCS treatment 0.5 mA/20min/day/8 days. NGF levels in cerebral cortex, spinal cord and sciatic nerve were determined by sandwich-ELISA at 48 hours and 7 days after the end of treatment.

RESULTS: The CCI model increased NGF levels in all three structures analyzed at long-lasting time, evidencing the importance of this neurotrophin in neuropathic pain condition. On the other hand, there was no tDCS effect in the central and peripheral NGF levels discarding the participation of this neurotrophin in the analgesic tDCS effect.

CONCLUSION: tDCS modulation effects of nociceptive pathways seem not to be linked to the NGF signaling in this chronic pain model.

Keywords: Nerve growth factor, Pain, Rats, Transcranial direct current stimulation.

RESUMO

JUSTIFICATIVA E OBJETIVOS: A expressão do fator de crescimento neural (NGF) em neurônios de diâmetro largo pode representar um papel importante na plasticidade sináptica neuronal e na reorganização da função neuronal após lesão neural. A estimulação transcraniana por corrente contínua (ETCC) é um método não invasivo de estimulação cerebral e representa uma ferramenta promissora para o manejo da dor, pois promove neuroplasticidade no sistema central, podendo ser combinada com outras intervenções. O objetivo foi investigar os efeitos da ETCC nos níveis de NGF em estruturas do sistema nervoso central e periférico de ratos submetidos a um modelo de dor neuropática (DN).

MÉTODOS: A constrição crônica (CCI) do nervo isquiático foi utilizada para indução do modelo de DN. Na cirurgia sham, o nervo foi exposto, no entanto não houve constrição do nervo. O grupo controle não foi submetido ao procedimento cirúrgico. Após estabelecimento da DN, os grupos tratados foram submetidos a ETCC 0,5 mA/20min/dia/8 dias. Os níveis de NGF no córtex cerebral, medula espinal e nervo isquiático foram mensurados pela técnica de ELISA 48 horas e 7 dias após o final do tratamento.

RESULTADOS: O modelo de dor CCI aumentou os níveis de NGF nas três estruturas analisadas, evidenciando a importância desta neurotrofina na dor neuropática. Por outro lado, não houve

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efeito da ETCC nos níveis de NGF central e periférico, descartando o papel desta neurotrofina no efeito analgésico da ETCC.
CONCLUSÃO: Efeitos da ETCC sobre vias nociceptivas não estão diretamente relacionados com a sinalização do NGF neste modelo de dor crônica.
Descritores: Dor, Fator de crescimento neural, Estimulação transcraniana por corrente contínua, Ratos.

INTRODUCTION

Neurotrophic factors are defined as endogenous substances that act in the development, maintenance, and regeneration of the nervous system[1]. Peripheral nerve injury causes alterations in the expression of neurotrophins levels and their cellular receptors in the peripheral and central nervous systems. Furthermore, changes in the neurotrophin signalling seems to be fundamental in neuropathic pain development, as well as in peripheral nerve regeneration after injury[2]. The Nerve Growth Factor (NGF) is a neurotrophin produced and secreted by cells of the target tissue, then taken up by sympathetic and small sensory fibers through a tyrosine-kinase A receptor (trkA), and retrogradely transported[1,3].

Some studies involving NGF indicate its roles in the early development and in the nociceptive properties of nerves[4]. The primary role of NGF is the neuronal survival in the developing nervous system, however in adults it seems to be more protective through pain modulation[5]. The role of NGF in neuropathic pain has been difficult to define; studies have proposed an exacerbating and alleviating effect of this neurotrophin in rodent models[6-8]. Moreover, the expressing NGF in the large-size neurons may present an essential role in neuronal synaptic plasticity and re-organization of neuronal function after a nerve injury[9]. Recent studies have shown that in pain conditions, NGF expression at the site of tissue injury increases and acts in TrkA on the sensory nerve endings producing conformational alterations and increases the expression of these proteins[10]. These events seem to be related to peripheral and central sensitization in the spinal cord, inducing hyperalgesia and allodynia[3].

In this context, a technique that can modulate focally induced plastic changes in pain-related neural networks, such as transcranial direct current stimulation (tDCS), may have significant therapeutic effects[11]. Previous studies of the present research group showed long-lasting analgesic effects after repeated sessions of anodal tDCS on chronic inflammation[12], hyperalgesia induced by chronic restraint stress models[13], and in the chronic constriction injury of sciatic nerve (CCI)[14]. In addition, studies in animal and cellular models demonstrated that tDCS can modulate synaptic transmission, molecular biosynthesis, and neuronal morphology. Several neurotransmission systems are involved in its effects; however, the main mechanisms of this technique are not completely understood[15]. Considering the importance of non-invasive therapies to management of neuropathic pain conditions and the need to identify the mechanisms of tDCS, the aim of this study was investigated the effects of tDCS in the NGF levels in the central nervous system structures and sciatic nerve of rats submitted to a CCI pain model.

METHODS

A total of 84 adult male Wistar rats, 55-65 days-old; weighing 200–250g, were used. Animals were randomized by weight and housed with three animals per home cages made of polypropylene material (49cmx34cmx16cm) with the floor covered with sawdust. All animals were maintained in a controlled environment (22±2°C), under a standard light-dark cycle (lights-on at 07:00h and lights-off at 19:00h), with water and chow (Nuvital, Porto Alegre/ Brazil) available ad libitum. The study was performed in accordance with the Guide for the Care and Use of Laboratory Animals 8th ed.[16]. The experimental protocol complied with the ethical and methodological standards of the Animal Research: Reporting of In Vivo Experiments (ARRIVE guidelines)[17]. The experiment used the number of animals necessary to produce reliable scientific data.

The rats were habituated to the maintenance room for one week before the experiments begin, and then, divided into seven groups: control (CT); sham neuropathic pain (SN); sham neuropathic pain plus sham tDCS treatment (SNS); sham neuropathic pain plus tDCS treatment (SNT); neuropathic pain model (NP); neuropathic pain plus sham tDCS treatment (NPS); neuropathic pain plus tDCS treatment (NPT). After the establishment of neuropathic pain, rats were subjected to a daily session of tDCS for eight consecutive days (Figure 1). For all procedures, investigators were blinded to avoid and prevent bias.

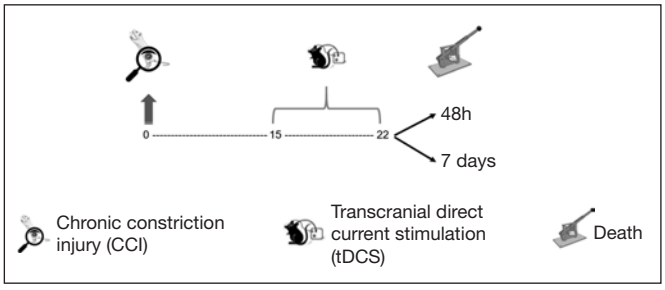

Figure 1. Experimental design

The CCI of sciatic nerve described by study[18] and adapted[14] was used as a model for the induction of neuropathic pain. Rats were anesthetized with isoflurane 5% for induction, 2.5% maintenance and placed in the right lateral decubitus position for the accomplishment of the left thigh trichotomy and skin antisepsis with 2% iodine alcohol. After skin incision of the left hind limb, in the middle third of the thigh to expose the femoral biceps muscle, the common sciatic nerve was exposed and three loose ligatures were tied (Vycril 4.0), separated by an interval of 1 mm, thus, the length of nerve affected was approximately 5.0mm. Ligatures reduced barely the diameter of the nerve but did not interrupt the epineural circulation. Finally, the skin was sutured using Mononylon 4.0 thread. For sham surgery, the sciatic nerve was exposed similarly to the CCI model, but not ligated. After surgery and anesthetic recovery, the animals were allowed in their home cages where they remained until the day of death. The control group did not undergo surgical procedure.

The same investigator performed the ligatures in all rats to ensure an equal level of constriction. As described in a previous study of the present group, the neuropathic pain model using chronic constriction of sciatic nerve is established 14 days after the nerve ligature. Decreased latency and paw withdrawal threshold were observed in the rats submitted to constriction of sciatic nerve, confirming the establishment of the neuropathic pain model[14,19,20].

After the establishment of neuropathic pain, the animals of the active treatment group were subjected to a 20 minutes session of bimodal tDCS, every afternoon for eight consecutive days, as described by study[21]. The direct constant current of 0.5 mA intensity 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. After placement, electrodes were fixed onto the head with adhesive tape (Micropore™) and covered with a protective mesh to prevent removal.

The cathodal electrode was positioned at the midpoint between the lateral angles of both eyes at supraorbital area, and the anodal electrode was placed on the head using landmarks of the neck and shoulder lines as a guide at the anterior and posterior regions in the midline between the two hemispheres of the parietal cortex, as described by autors[22]. This technique mimics humans tDCS protocols to pain treatment[23-26] and has been applied by the present research group showing longer-lasting effects on pain relief, and an antihyperalgesic response in paw inflamed rats[12]. For sham stimulation, the electrodes were placed and fixed in the same positions as for real stimulation; however, the stimulator was turned off during the entire time.

The animals were killed by decapitation 48 hours and 7 days after the last session of tDCS and the tissue samples of cerebral cortex, spinal cord and left paw sciatic nerve were collected. Structures were frozen at -80°C until assays were performed.

The NGF levels on cerebral cortex, spinal cord, and left paw sciatic nerve were determined by sandwich-ELISA using monoclonal antibodies specific for NGF (R&D Systems, Minneapolis, United States). Total protein was measured by Bradford's method using bovine serum albumin as standard.

All experiments and procedures were approved by the Institutional Animal Care and Use Committee (GPPG-HCPA protocol no.120512).

Statistical analyses

Data were expressed as the mean ± standard error of the mean (S.E.M). A three-way analysis of variance (ANOVA) followed by Bonferroni were performed to compare all groups considering NP, treatment, and time as independent factors. P-values less than 0.05 were considered significant. The values were presented as percentage of the control group. SPSS 19.0 for Windows was used.

RESULTS

Regarding the NGF cortex levels, an interaction between NP vs time in the NGF levels (Three-way ANOVA, $p<0.05$, $F(1.69) = 286.380$, figure 2, Panel A) was observed, with a significant increase in the NGF levels at 29th post-surgery day when compared with 24th post-surgery day measure. The NGF spinal cord levels presented main effects of NP and time (Three-way ANOVA, $F(1.68) = 10.776$, $p<0.05$, $F(1.68) = 9.258$ respectively, figure 2, Panel B). All rats displayed increased NGF levels in the spinal cord after the NP; however, its levels were significantly higher at 29th post-surgery day. In the peripheral structure analysed, increased NGF levels in the sciatic nerve after the NP model with main effects of NP and time in this structure were observed (Three-way ANOVA, $F(1.71) = 17.867$, $p<0.05$, $F(1.71) = 7.760$, respectively, figure 2, Panel C).

Panel A. NGF levels in the cerebral cortex (n=4-6 per group). Data are expressed as percentage of control mean ± S.E.M (pg/mg of protein). Groups: (CT) control; (SN) sham neuropathic

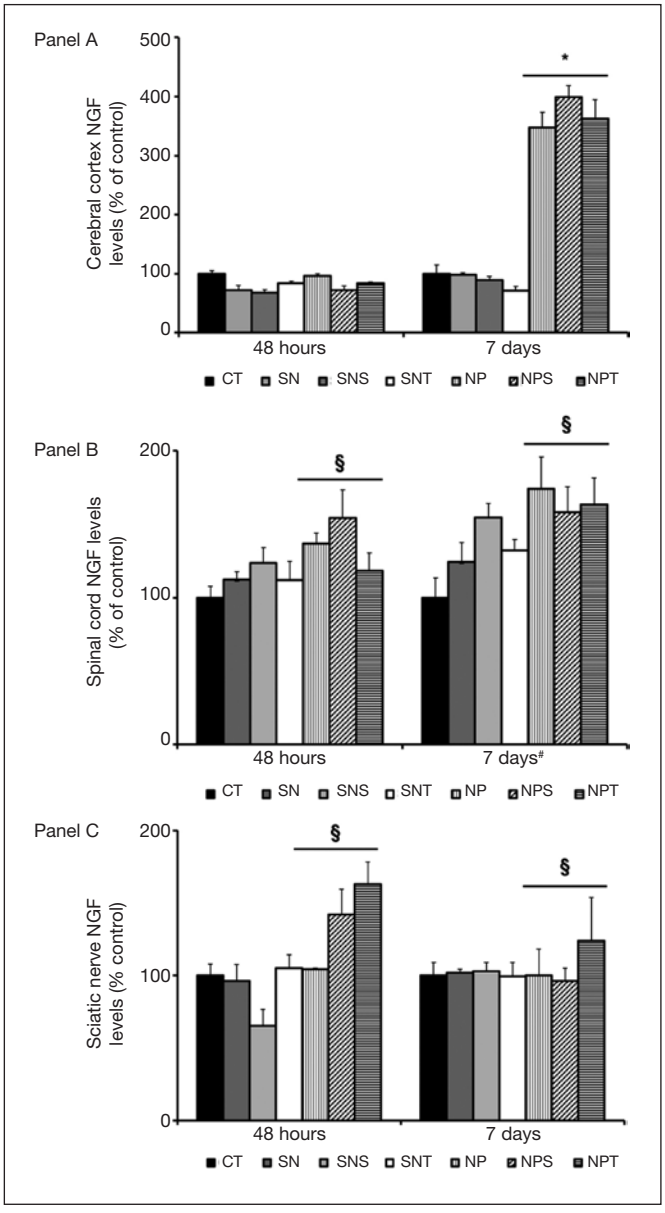

Figure 2. NGF levels in different structures

pain; (SNS) sham neuropathic pain plus sham tDCS treatment; (SNT) sham neuropathic pain plus tDCS treatment; (NP) neuropathic pain model; (NPS) neuropathic pain plus sham tDCS treatment; (NPT) neuropathic pain plus tDCS treatment.
* interaction between pain model vs time (three-way ANOVA/ Bonferroni, p<0.05).

Panel B. NGF levels in the spinal cord (n=4-6 per group). Data are expressed as percentage of control mean ± S.E.M (pg/mg of protein). Groups: (CT) control; (SN) sham neuropathic pain; (SNS) sham neuropathic pain plus sham tDCS treatment; (SNT) sham neuropathic pain plus tDCS treatment; (NP) neuropathic pain model; (NPS) neuropathic pain plus sham tDCS treatment; (NPT) neuropathic pain plus tDCS treatment.
§ significant effect of pain model; # significant effect of time (three-way ANOVA/ Bonferroni, p<0.05).

Panel C. NGF levels in the sciatic nerve (n=4-6 per group). Data are expressed as percentage of control mean ± S.E.M (pg/mg of protein). Groups: (CT) control; (SN) sham neuropathic pain; (SNS) sham neuropathic pain plus sham tDCS treatment; (SNT) sham neuropathic pain plus tDCS treatment; (NP) neuropathic pain model; (NPS) neuropathic pain plus sham tDCS treatment; (NPT) neuropathic pain plus tDCS treatment; § significant effect of pain model (three-way ANOVA/ Bonferroni, p<0.05).

DISCUSSION

In this study, an interesting effect of CCI model with increased levels of NGF in the spinal cord and the cerebral cortex was found. In the sciatic nerve, an increased NGF level at short-term after the CCI model was observed. On the other hand, the analgesic tDCS effects are not linked to modifications in the NGF levels.
Preclinical models of inflammation and neuropathic pain have demonstrated that hyperalgesia displayed by the animals is linked to alterations in the NGF levels[27-29]. Additionally, clinical studies using healthy volunteers support the role of NGF in the induction of abnormal pain states, such as mechanical and thermal hyperalgesia[30,31]. Few evidences show the NGF function in the central nervous system acting in the generation and maintenance of neuropathic pain in CCI pain model[32].
It's interesting to highlight that increased NGF levels at long-lasting time after the CCI model were found. This effect was observed only at approximately 30 days after the neuropathic pain model in the cerebral cortex; however, increased levels were already presented at the 24th day post-surgery with a progressively augmentation at 30th post-surgery in the spinal cord. Previous studies demonstrated the NGF role in some peripheral nerve injury models: after unilateral sciatic nerve crush, higher synthesis of NGF was found in dorsal root ganglia[33] and after CCI model, the neuropathic pain was linked to a glial overexpression of NGF[34-36].
In the central nervous system, the production of NGF is thought to be by glial cells, such as microglia[37] and oligodendrocytes[38]; however, the main source of NGF are the astrocytes[39,40]. Several studies have shown the complex interaction between the immune central system and the neuropathic pain process[41-43]. This group's previous research[14] showed long-lasting increased levels of IL-1β in central nervous structures, such as cerebral cortex and spinal cord; a similar pattern was observed on NGF levels at same structures as demonstrated in the present study. It's suggested that the NGF acts upon the immune axis, since it's characterized by the presence of the TrkA receptor, corroborating the interaction between central nervous and immune systems[44].
Considering the challenge of the treatment to relief the pain symptoms in different neuropathic pain conditions, new therapeutical approaches have been investigated. Studies have shown that the increase of neurotrophins in the spinal cord, such as NGF, seems to be correlated with the incidence of pain[45]. This way, some studies showed that the treatment with anti-NGF in neuropathic pain can prevent and reverse the hyperalgesia and mechanical allodynia[36,46-48]. Particularly in the CCI model, the study[49] suggested that anti-NGF treatment improves chronic neuropathic pain through the reduction of NGF and P-substance in peripheral sites and by the indirect effect in the central sensitization, modulating the descending pathways of pain perception[49].
The present study proposed to understand the mechanisms of a new therapeutic tool for neuropathic pain, which nowadays demonstrates efficacy in clinical[50-52] and preclinical studies[12,13]. Despite the reversion of thermal and mechanical hyperalgesia after the CCI model through repeated bicephalic tDCS[14], a relationship between its effect and NGF pathway in the central nervous system and peripheral nerve was not found.
Despite the increasing evidence of NGF role in the induction and maintenance of neuropathic pain, few studies have shown its central structures levels. The present results demonstrated the importance of NGF levels at cerebral cortex and spinal cord structures. On the other hand, some limitations of the present study need to be noted: i) lack of a time-course of NGF levels since early stage post-surgery; ii) long-lasting evaluation of NGF levels; iii) assessment of NGF levels in the peripheral nerve and different structures of the central nervous system.

CONCLUSION

The CCI model increased NGF levels in cerebral cortex and spinal cord, evidencing the important feature of this neurotrophin in neuropathic pain condition. Furthermore, evidences showed that bicephalic tDCS can modulate nociceptive pathways; this effect is not linked to the NGF signalling. New studies are needed to elucidate the role of NGF central levels in neuropathic pain.

AUTHORS' CONTRIBUTIONS

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Statistical analysis, Data collection, Conceptualization, Project management, Research, Methodology, Writing - Preparation of the original, Writing - Review and Editing.
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Funding Acquisition, Conceptualization, Methodology, Writing - Preparation of the original, Writing - Review and Editing, Supervision.

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