UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

FACULDADE DE FARMÁCIA

TRABALHO DE CONCLUSÃO DE CURSO

Correlation between neurotrophic activity and sensitivity to mechanical stimuli in immobilization stress model treated with transcranial direct current stimulation

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Trabalho de conclusão de curso apresentado à Faculdade de Farmácia da UFRGS, pela aluna Carolini da Silva como parte dos requisitos necessários para a obtenção do grau de farmacêutica.

Orientador: Prof. Dr. Alexandre Silva de Quevedo

Coorientadora: Me. Elissa Kerli Fernandes

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ARTIGO

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Correlation between neurotrophic activity and sensitivity to mechanical stimuli in immobilization stress model treated with transcranial direct current stimulation

Running title: Correlation between BDNF and analgesia

Carolini da Silva[a], Elissa Kerli Fernandes[b], Adriana Fernanda Kuckartz Vizuete[c], Jamile Boff[d], John Neubert[e], Carlos Alberto Saraiva Gonçalves[c], Deise Ponzoni[d], Edela Puricelli[d], *Alexandre Quevedo[b]

[a]College of Pharmacy, Federal University of Rio Grande do Sul - UFRGS, Brazil.
[b]Neuroscience Graduate Program, Federal University of Rio Grande do Sul - UFRGS, Brazil [c]Department of Biochemistry – UFRGS. [d]Postgraduate Program in Dentistry - UFRGS, Brazil. [e]Department of Orthodontics, Faculty of Dentistry, University of Florida, USA

***Corresponding author:**

Alexandre S. Quevedo, D.D.S, Ph.D.

Department of Surgery and Orthopedic

School of Dentistry - UFRGS

R. Ramiro Barcelos, 2492 - Santa Cecilia, Porto Alegre - RS, Brazil, 90035-004.

Phone: +51 3308-5010+51

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Conflict of interest

The authors declare that there are no conflicts of interest.

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Abstract

Stress causes biochemical and behavioral changes that can contribute to the genesis of diseases. Transcranial direct current stimulation modulates neuronal excitability. In the present study, the hypothesis that there is a correlation between the effects of tDCS on neurotrophic activity and the ability to increase the threshold for mechanical sensitivity was tested. Male Wistar rats were divided into ten groups: 1) Behavioral control (BeC); 2) Biochemical control (BiC); 3) Immobilization 30 (IMO30); 4) Immobilization 60 (IMO60); 5) Immobilization 120 (IMO120); 6) Immobilization 24h (IMO24h); 7) Immobilization + tDCS 30 (IMO+tDCS30); 8) Immobilization + tDCS 60 (IMO+tDCS60); 9) Immobilization + tDCS 120 (IMO+tDCS120); 10) Immobilization + tDCS 24h (IMO+tDCS24). Afterwards, they were submitted to the von Frey baseline behavioral test. In the following six days, they were submitted to the stress model by immobilization. On the seventh day, they received the last immobilization with the application of 20 minutes of tDCS or false stimulation with disconnected electrodes. Immediately after, they were subjected to the von Frey test 30, 60, 120 minutes and 24 hours after the session, then killed by decapitation with spinal cord collection for biochemical analysis. BDNF levels cord were assessed by Enzyme Linked Immunosorbent Assay (ELISA). There was a negative correlation between BDNF levels and mechanical sensitivity. The result show that tDCS can be an option to prevent effects of stress, however, studies still need to define adequate doses that the increase in BDNF is not exacerbated to the point of reducing the nociceptive threshold.

Keywords - key: **BDNF; Stress; Transcranial direct current stimulation; tDCS.**

1 – Introduction

The stress response can be understood as a set of organic alterations that are triggered in the face of different external or internal stressor stimuli (Lopes, 2015). These changes aim to maintain the homeostasis of the organism, generating responses to the immune, nervous and endocrine systems (McEwen, 1998). The activation of the endocrine axis by repeated stress conditions may exceed the body's ability to maintain homeostasis and predispose the individual to the development of physical and mental diseases (Margis et al., 2003; Schneider et al., 2020). As this response seems to be a universal mechanism of adaptation, animal models are used to better understand their mechanisms and seek new markers to measure the response to therapies (Broekkamp et al., 1984; Fernandes et al., 2012).

Immobilization by movement restriction is one of the most used as a stress model in animals (Buynitsky and Mostofsky, 2009). Its advantages are to be a simple, painless model and not cause lasting debilitation (Buynitsky and Mostofsky, 2009). The application of the technique has already been shown to increase the levels of corticosteroid hormones (Lee et al., 2008), produce antinociceptive effects (Seo et al., 2006), decrease the expression of (Smith et al., 1995b) or also increase them (Smith et al., 1995a; Fukuoka et al., 2001), as well as its receptors (Ueyama et al., 1997).

Neurotrophins are proteins that regulate survival, development, neuronal function and plasticity (Huang and Reichardt, 2001). They comprise four types of structurally proteins: nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophic-3 (NT-3) and neurotrophic-4 / neurotrophic-5 (NT-4 / 5) (McAllister et al., 1999). Due to its wide distribution in the nervous system and its

ability to vary according to the body's response to internal and environmental stressors, BDNF becomes very interesting to understand the effects of new therapies (Qian et al., 2007).

Transcranial direct current stimulation (tDCS) is a technique that a direct current of weak intensities (Ardolino et al., 2005) is administered in the scalp, causing changes in neuronal excitability that may be long-term (Fertonani et al., 2010). First studies in humans aimed to treat or modify psychiatric diseases (Nitsche et al., 2008). There are records of application of electrical currents de of more than 200 years (Priori, 2003). It clinical use has been well accepted, as well as the evolution of the technique as a promising therapeutic tool in various disorders (Liebetanz et al., 2006). In addition, it has shown promising results in the reversal of negative stress-related effects, such as increased sensitivity to pain and neurotrophin alteration (Zortea et al., 2019). Thus, tDCS becomes an important ally in clinical neuroscience as a possible non-pharmacological approach for treating disorders of different etiologies (Priori, 2003).

Previous studies have shown that a single session of tDCS cause alteration in the nociceptive (Dimov et al., 2016) and in neurotrophic activity (Fritsch et al., 2010). This research aims to analyze the correlation between these changes in rats submitted to a stress model by immobilization and treated with a single session of tDCS.

2 - Methodology

The present study was approved by the Institutional Committee for The Care and Use of Animals (CEUA) of the Hospital de Clínicas de Porto Alegre (HCPA) (number 2019-0126).

The behavioral experiments were carried out at the Animal Experimentation Unit (UEA-HCPA). Biochemical analyses were performed at the Calcium Ligand Protein Laboratory of the Department of Biochemistry - Institute of Biological Sciences and Health - ICBS; Federal University of Rio Grande do Sul - UFRGS.

2.1 Animals

The *male Wistar rats* with a mean age of 60 days, weighing between 180 and 230g, were housed in polypropylene cages (49 x 34 x 17.8 cm) in the Animal Experimentation Unit of the Hospital de Clinics de Porto Alegre. The animals were kept under standard experimental conditions, submitted to light-dark cycles of 12 hours, at room temperature (22 °C ± 2 °C) and controlled humidity (40-60%), with free access to food and water.

2.2 Experimental design

On the first day of experiment (D1), the animals were submitted to a von Frey test, with the objective of evaluating them for sensitivity to mechanical stimulus (except the biochemical control group), and decide on staying in the study according to the results. There were no animals that presented abnormal sensitivity, which would imply exclusion from the study. From the second day (D2) to D7, they were submitted to 5 minutes of immobilization daily, wrapped in a towel, with the aid of microporous tape, except for the control groups (behavioral and biochemical). In D8, they were immobilized for 20 minutes with electrodes on the head, at the same time as the true or false tDCS session was performed. Then, the behavioral test was performed and immediately euthanized. To make

the systematic analysis of the effects of this single session of tDCS over time, euthanasia was performed at different times, defining the groups. At that time, both the animals that received the true and false stimulation were also divided into groups according to the time they were euthanized after the session: 30, 60 or 120 minutes and 24 hours.

Therefore, the animals were divided into the following groups:

1) Behavioral control (BeC); 2) Biochemical control (BiC); 3) Immobilization 30 (IMO30); 4) Immobilization 60 (IMO60); 5) Immobilization 120 (IMO120); 6) Immobilization 24h (IMO24h); 7) Immobilization + tDCS 30 (IMO+tDCS30); 8) Immobilization + tDCS 60 (IMO+tDCS60); 9) Immobilization + tDCS 120 (IMO+tDCS120); 10) Immobilization + tDCS 24h (IMO+tDCS24).

Immediately after euthanasia, the total spinal cord was collected for further biochemical tests.

2.3 Immobilization stress model

The immobilization model consisted of maintain the animals wrapped in a piece of towel with the aid of a microporous tape. Only the animal's head was out of the towel used to contain the movements of the (Castagliuolo et al., 1996; Hebert et al., 2005). For six days, they were kept in this condition for 5 minutes daily, and on the seventh day, the containment lasted 20 minutes, a time that lasted the stimulation with tDCS, both false and true (Hebert et al., 2005).

2.4 tDCS session

To the application of tDCS, the animals were trichotomized in the head region, which helps to maintain the adhesion of the electrocardiogram electrodes. The electrodes were positioned on the animals' heads and manteined fixed with

microporous tape. A current of 500 μA was applied for 20 minutes (Fregni et al., 2006). The current comes out of a battery and passes through the electrodes. In the groups that received false stimulation, this battery was turned off.

2.5 Behavioral test

2.5.1 Von Frey Test

The von Frey test was applied to evaluate the sensitivity to mechanical stimuli. Twenty-four hours before the test, the animals were acclimated to the device for ten minutes. During the test, the animals were placed individually inside a cage, whose floor was a metal grid. The stimulus consisted of pushing the rear paw of the animal with a transducer, which has attached a polypropylene cone, until the paw is removed. The force required appears on the display of the appliance.

Data were collected two times. The first test (baseline) was performed at the beginning of the experiment (D1). The second test was performed after the application of tDCS in D8.

2.6 Euthanasia and sample collection

Euthanasia was performed by decapitation, because the action of anesthetics could compromise the analysis of the data, since anesthesia consists of increasing the nociceptive threshold (Borsook and Becerra, 2011) besides interfering with biochemical parameters (Lu et al., 2006). The spinal cord was collected and stored in a freezer at -80ºC for further biochemical analysis.

2.7 Biochemical test

2.7.1 Quantification of BDNF

For the quantification of BDNF in the spinal cord, the FineTest® ELISA kit was used. The manufacturer's instructions were followed for quantification in homogenized nervous tissue. The results were expressed in pg/g of tissue.

2.8 Statistical analysis

Statistical analysis was performed with the IBM SPSS® program Statistics. Data normality was assessed using the Kolmogorov-Smirnov test.

The analysis of the relationship between the mechanical sensitivity threshold and BDNF levels in the spinal cord was performed with the Pearson correlation test. For this, the data collected in the quantification of tissue BDNF and in the von Frey test were normalized, that is, assuming for their respective control groups a value of 100%.

3 - Results

3.1 Correlation between BDNF levels and mechanical threshold

There was a negative correlation between spinal cord BDNF levels and mechanical threshold in stressed animals treated with tDCS ($r = -0.253$; $P = 0.047$) (Fig 1). Among BDNF levels and mechanical sensitivity in the group that received only the stress model with false stimulation, there was no correlation ($r = -0.214$; $P = 0.27$) (Fig 2).

4 - Discussion

In this study, it is hypothesized that sensitivity to mechanical stimuli is related to BDNF levels of the spinal cord. The statistical test performed using the

absolute data collected in the two analyses showed no correlation. This relationship was also evaluated after normalization of the von Frey data, where the baseline test value of each rat was considered 100%, and the value found after the intervention was considered a variation from its own control value (percentage of variation of the baseline test). The data from the BDNF analysis were normalized considering the average of the control groups as 100%. In animals that received only false stimulation, no relationship was found between the data. However, there was a negative correlation between BDNF levels in the spinal cord and the sensitivity threshold in animals treated with true tDCS, which is a strong indication that this finding is due to the current applied to the animals' central nervous system.

Immobilization is the most used model in stress studies (Buynitsky and Mostofsky, 2009). Its use over the last few years has helped to establish that the increase in corticosterone levels is one of the physiological changes most found in studies of repeated stress (Buynitsky and Mostofsky, 2009). In humans glucocorticoids, having these a broad role of stress response action (Munck et al., 1984).

BDNF is a growth factor recognized as an important mediator of neuronal plasticity (Barde et al., 1982), as it produces long-term changes in synaptic structure and function (Lindholm et al., 1994; Thoenen, 1995).

The tDCS is a technique that, despite having been used empirically for many years, still does not have the cellular and molecular mechanisms fully elucidated (Fritsch et al., 2010). It is known that the direct currents provided through the scalp modulate brain activity (Priori, 2003; Fritsch et al., 2010) and induce changes in cortical motor excitability that persist for a long time after its

application (Ardolino et al., 2005). Anodal current is excitatory (depolarization), while the cathodic current is inhibiting (hyperpolarization) (Priori, 2003).

The negative correlation found in this study shows that the higher the tissue level of BDNF in the spinal cord of the animals treated with tDCS, the higher the threshold of sensitivity to mechanical stimuli (Fig. 1). This correlation was not found in animals that were only submitted to the stress model and receive a false stimulation (Fig 2), which suggests that this correlation is related to the direct current applied to stressed animals.

The von Frey test assesses nociceptive sensitivity to wicked or harmful stimuli. Through this technique, stimulation of nerve endings free of highly myelinated fibers is performed, whose transmission speed is fast (fibers Aβ), and of non-myelinated fibers, in which the transmission speed is lower (C fibers) (Lopes, 2014). With this, action potentials are generated that transmit this stimulus from the periphery to the central nervous system. The changes in the BDNF found in this study may be related to the ability of tDCS to prevent the reduction of BDNF levels in the central nervous system in the face of immobilization stress (Fritsch et al., 2010; Regner, 2018). This neurotrophin contributes to the facilitation of the excitation of neurons that are part of the dorsal roots of the spinal cord. It is known that its levels are increased in nerve injuries, as well as in perineural injection models that have hyperalgesia as a results (Kraychete et al., 2008 ; Zhou et al., 2010). Therefore, it is suggested that the main finding of the present study is related to the ability of tDCS to prevent the reduction of BDNF levels to the point of reducing the nociceptive threshold of neurons involved in the transmission of mechanical stimuli from the periphery to the central nervous system (Kraychete et al., 2008; Fritsch et al., 2010). The

mechanisms involved may be related to the ability of growth factors to increase the expression of channels that are related to the transmission of mechanical stimulus. If the correlation found is related to the dose or the period of direct current application, further studies are needed to find the dose and frequency that prevent the negative effects of stress on neurotrophic parameters, but that does not increase BDNF levels to the point of losing the analgesic effect of tDCS, when the reduction of the nociceptive threshold occurs under recurrent stress conditions.

In the modern society, the human being is immersed in stressors in daily life (McEwen, 1998). It is interesting to look for techniques that aim to reduce the harmful effects of this inevitable repeated exposure. In this sense, tDCS has proved to be beneficial in several aspects for the prevention or recovery from damage from stress (Adachi et al., 2012), however, it is necessary to define the appropriate dose and frequency that its beneficial effects can be take advantage of the maximum.

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Figure Legends:

Figure 1: Correlation between BDNF levels and mechanical sensitivity threshold in groups submitted to stress and treated with tDCS. BDNF levels of the spinal cord were negatively correlated with the increase in the threshold of mechanical pain in animals treated by TDCS (r=-0.253; P=0.047).

Figure 2: Correlation between BDNF levels and mechanical pain threshold in animals submitted to stress and false stimulation model. There was no correlation between the data (r = -0.214; P = 0.27).

Figure 1

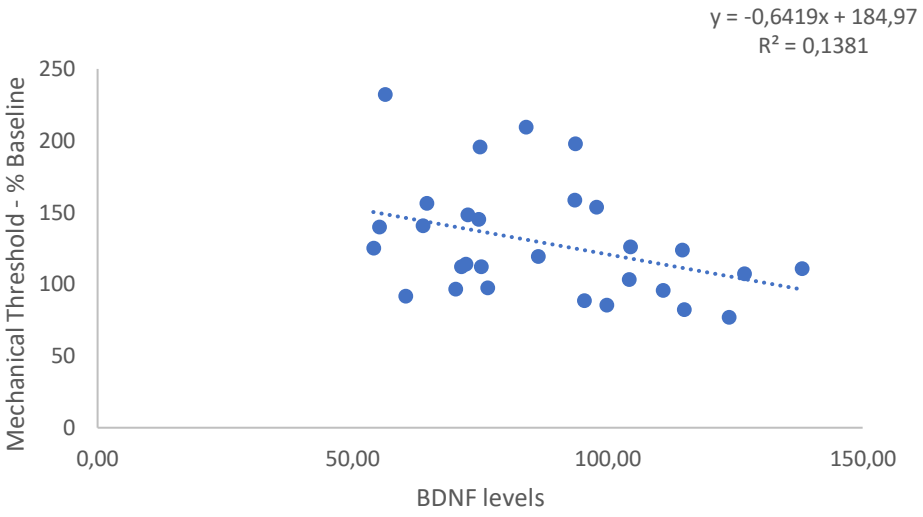
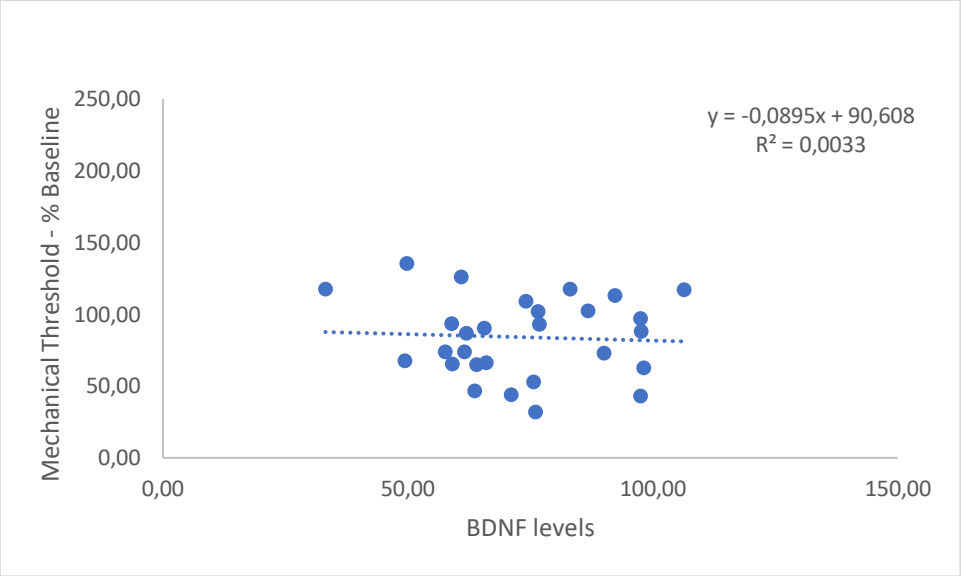

y = -0,6419x + 184,97
R² = 0,1381
Mechanical Threshold - % Baseline
250
200
150
100
50
0
0,00
50,00
100,00
150,00
BDNF levels

Figure 2


y = -0,0895x + 90,608
R² = 0,0033
Mechanical Threshold - % Baseline
250,00
200,00
150,00
100,00
50,00
0,00
0,00
50,00
100,00
150,00
BDNF levels

HOSPITAL DE CLÍNICAS PORTO ALEGRE RS

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GRUPO DE PESQUISA E PÓS GRADUAÇÃO
COMISSÃO DE ÉTICA NO USO DE ANIMAIS

Certificamos que o projeto abaixo, que envolve a produção, manutenção ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto humanos), para fins de pesquisa científica, encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA), e foi aprovada pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA) e pelas áreas de apoio indicadas pelo pesquisador.

Projeto: 160408 **Data de Aprovação do Projeto:** 14/10/2016

Título: Avaliação dos efeitos agudos da estimulação transcraniana com corrente continua (ETCC) na ativação de vias neurais descendentes e de mecanismos sistêmicos relacionados ao controle da dor em ratos naive

Pesquisador Responsável: IRACI LUCENA DA SILVA TORRES

Equipe de pesquisa:

ALEXANDRE SILVA DE QUEVEDO, DEISE PONZONI, ETIANE MICHELI MEYER CALLAI, FELIPE ERNESTO ARTUZI, ISABEL CRISTINA DE MACEDO, VANESSA LEAL SCARABELOT

Submissão	Documento	Espécie/Linhagem	Sexo/Idade	Qtd.	Data Reunião	Situação
10/08/2016	APROVAÇÃO	RATO - WISTAR	M/60dias	100	11/10/2016	APROVADO

Total de Animais: 100

Coordenador
Comissão de Ética no Uso de Animais

- Os membros da CEUA/HCPA não participaram do processo de avaliação onde constam como pesquisadores.
- Toda e qualquer alteração do Projeto deverá ser comunicada à CEUA/HCPA.
- O pesquisador deverá apresentar relatórios semestrais de acompanhamento e relatório final ao CEUA/HCPA.

Attachments