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NEUROSCIENZE COGNITIVE

EFFETTI DELLA tDCS ANODICA APPLICATA ALLA DLPFC SINISTRA SULLE RISPOSTE DI STRESS IN SOGGETTI SANI

EFFECTS OF ANODAL tDCS APPLIED TO THE LEFT DLPFC ON STRESS RESPONSES IN HEALTHY SUBJECTS

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ABSTRACT

Extended or repeated activation of the stress response can have detrimental effects on mental and physical well-being. The prefrontal cortex, specifically the dorsolateral prefrontal cortex (DLPFC), is believed to have influence on the neuroendocrine stress response system and consequently on emotional state and subjective experience of stress. To explore this hypothesis, we conducted a study in which we enhanced DLPFC excitability using transcranial direct current stimulation (tDCS) in 36 healthy participants (17M; 19F), which were randomly assigned to receive either excitatory tDCS (anodal stimulation) or a sham stimulation over the left DLPFC just before and during the exposure to a psychosocial stress test. We assessed parameters related to the neuroendocrine system (salivary cortisol levels), emotional affect state (positive and negative affect schedule, PANAS) and self-reported experience of stress (visual analogue scale, VAS). The results revealed that a single session of excitatory tDCS over the left DLPFC had an influence on the self-reported experience of stress but no effects in emotional affect and salivary cortisol concentration. Significant differences were found for all the parameters investigated over time both in the active and sham group, indicating that the stress-producing paradigm used has measurable effects in the chosen markers. This study provides initial evidence that a single session of excitatory tDCS over the left DLPFC can attenuate the subjective experience of psychosocial stress confirming the importance of the left DLPFC, a target for non-invasive brain stimulation in depression treatment, in promoting coping strategies in psychosocial stressful situations.

1. Introduction

Stress is a common occurrence in our daily lives and can have negative impacts on both mental and physical health, especially for those who are vulnerable. Research has shown that frequent exposure to stress or persistent activation of the body's stress response system can lead to disease, since this stress response system involves changes in the nervous system and hormone levels, including decreased heart rate variability and increased cortisol secretion (Cohen et al., 2007; McEwen et al., 2008).

The prefrontal cortex plays a role in regulating this response, by inhibiting limbic structures that suppress the body's natural relaxation response, however, in individuals with mood and anxiety disorders, there may be a decrease in prefrontal cortex activity and an increase in limbic activity, leading to persistent activation of allostatic stress responses (Thayer JF et al., 2009).

Transcranial direct current stimulation (tDCS) is a non-invasive technique used to stimulate the brain. It involves applying a low-intensity electric current to the scalp to either increase (anodal tDCS) or decrease (cathodal tDCS) the excitability of the targeted brain area.

The dorsolateral prefrontal cortex (DLPFC) is a popular target for tDCS research, particularly for its relevance to psychiatric conditions. Studies have shown that anodal tDCS may play a role in controlling autonomic and neuroendocrine stress responses through top-down regulation (Brunoni et al., 2013; Carnevali et al., 2019).

1.1. tDCS

Transcranial direct current stimulation (tDCS) is a non-invasive brain stimulation technique in which a constant low-intensity direct current is applied to the brain via sponge electrodes on the head. It was derived from the results of Bindman et al, 1964. showing that direct current modulates spontaneous neuronal activity in the rat brain in a polarity-dependent manner, followed by several studies using direct current in animals and humans in the 1960s and 1970s. These early studies from the 1960s suggested some efficacy of DC in reducing symptoms of depression, but conflicting results and the development of psychotropic drugs led to the early abandonment of this technique (Nitsche et al., 2009). This line of research was reactivated at the beginning of the 21st century by Nitsche and Paulus (Nitsche et al., 2000) with the investigation of different patterns of motor cortex excitability after anodic and cathodic polarization.

Unlike the transcranial magnetic stimulation (TMS), where single magnetic impulses result in action potentials that exceed the depolarization threshold of neurons, tDCS does not cross the threshold due to constant weak polarization. In this model, tDCS shifts neuronal resting membrane potentials toward depolarization after anodic sensing (= excitatory) and toward hyperpolarization after cathodic sensing (= inhibitory; tonic changes in resting membrane potential).

Depending on the polarization, this ultimately leads to a facilitation or inhibition of the neural firing rate (Nitsche et al., 2003), therefore the effect of tDCS is considered to be neuromodulatory. The after-effects of excitatory tDCS last from a few minutes to an hour and a half and can be measured by amplitude changes in motor evoked potentials (MEP) by single TMS pulses on the cortical representational area of the abductor digitorum, minimus, abductor brevis, or interosseous muscle of the first finger (Nitsche et al., 2001). The change in

MEP amplitudes is a surrogate for a change in neuroplasticity, i.e. the activity-dependent change in neuronal information processing.

During a tDCS session, a small battery-powered stimulator delivers a weak, direct current to the electrodes, which in turn pass the current through the underlying brain tissue (Figure 1.1). The electrodes used in tDCS are typically placed over specific regions of the scalp, depending on the research question or clinical application. The anode (positive electrode) is placed over the area of the brain to be stimulated, while the cathode (negative electrode) is placed over a nearby area. The anode and the cathode are known to have opposite effects on the targeted brain region: Whereas anodal tDCS enhances cortical excitability, cathodal stimulation reduces it (Nitsche and Paulus 2000). The direction of the electrical current flows from the anode to the cathode indicating that tDCS can be applied in several ways. The most common protocol is to apply a steady, low-intensity electrical current (usually between 1 and 2 milliamperes) for 20-30 minutes. This protocol is often referred to as "conventional tDCS", alternatively a "sham" protocol may be used, in which the electrical current is applied for only a few seconds and then turned off, to control for placebo effects.

The mechanism of action of tDCS is still not fully understood, it modulates the excitability of neurons in the brain inducing changes in brain activity that can last after the end of the stimulation session. tDCS is generally considered to be safe and well-tolerated, although there are some potential side effects such as skin irritation, mild discomfort, and headache. tDCS is a promising tool for understanding brain function and treating a variety of neurological and psychiatric conditions. The use of tDCS has grown rapidly in recent years, with an increasing number of publications utilizing this technique for both research and clinical applications.

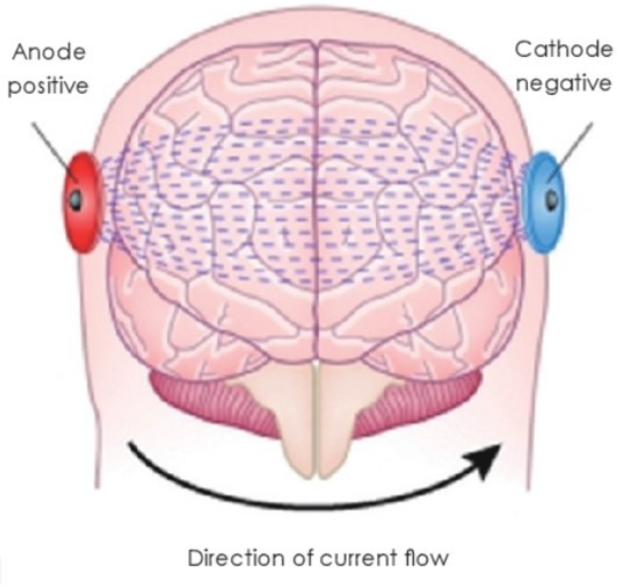

Figure 1.1. *tDCS schematic view (Rosa and Lisanby, 2012)*

1.2. Stress, anxiety and depression

"Grief has limits, whereas apprehension has none. For we grieve only for what we know has happened, but we fear all that possibly may happen" (Pliny the Younger, 61-112 CE).

The acute stress response is embodied in the "fight or flight response" that triggers an anxiety state. When the stress is chronic, depression can develop insidiously under the guise of ongoing anxiety symptoms, depression inhibits stress management and thus a "vicious circle" sets in, depression increases stress and vice versa (Wheatley, 1997) (Figure 1.2).

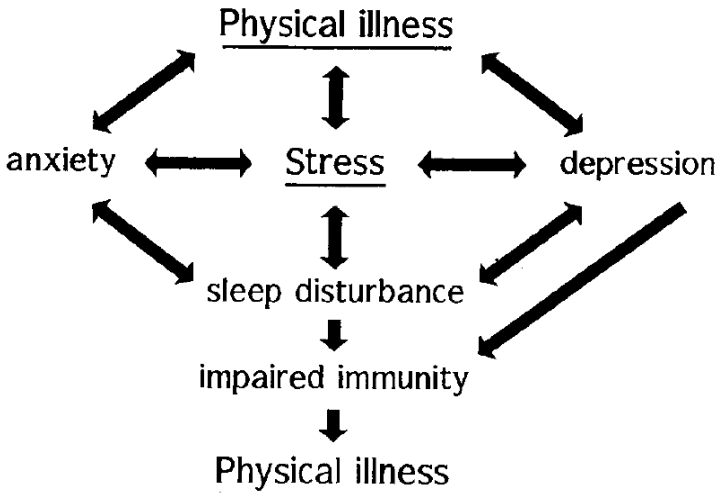

Figure 1.2. *The vicious circle (Wheatley, 1997)*

All higher beings are programmed with the “fight-or-flight” response to stress. In the wild constant fear of predatory attacks creates an existence full of omnipresent fear, it's fair to say it's the “stress jungle” animal similar to the human “fear jungle” (Wheatley, 1990). Dangerous stress is not often encountered by people, but has been replaced by others more subtle pressures that has similar effects on the mental process. The "fight or flight" response is inappropriate for today's human life and can drive to adverse mental and psychological effects, such as: fear, panic disorders, phobias and depression.

For middle-aged adults, the majority of working class, daily life is more stressful compared to other ages, and particularly so for people who are socioeconomically disadvantaged. Furthermore the changes in perceived stress across the decades from 1990 to 2010 were evaluated in a large cohort study of Americans and it was found that generally adults in the 2010s reported experiencing a greater number of daily stressors, and they reported these stressors as being more severe and posing a greater risk to their finances and to their future compared to the reports of same-aged adults in the 1990s (Almeida et al., 2023).

Psychological stressors activate a series of neuroendocrine mechanisms that prepare the body to face the stressful situation. Among these mechanisms, the effects of the hypothalamic-pituitary-adrenal (HPA) axis and the sympathetic-adrenomedullary system are particularly prominent (Figure 1.3). When the body is exposed to a stressful stimulus, the hypothalamus, a small structure in the brain, releases a hormone called corticotropin-releasing factor (CRF). CRF stimulates the pituitary, a gland located at the base of the brain, to produce adrenocorticotropic hormone (ACTH), which in turn stimulates the adrenal glands, located above the kidneys, to release cortisol.

Cortisol has several effects on the body. First, it stimulates glycogenolysis (the conversion of glycogen into glucose) thereby increasing blood sugar levels to provide energy for the body during stressful situations (Kadmiel et al., 2013). Furthermore, cortisol has immunosuppressive effects (Webster et al., 2001) and it influence bone and muscle metabolism (Chiodini, 2011),

The HPA axis is therefore a feedback circuit involving the hypothalamus, the pituitary gland, and the adrenal glands. When exposed to stressors, the hypophysiotropic neurons located in the paraventricular nucleus (PVN) of the hypothalamus produce the CRF and vasopressin (AVP), CRF is released into hypophysial portal vessels, which then access the anterior pituitary gland. CRF binds to the CRF type 1 receptor (CRFR1) on pituitary corticotropes, triggering the activation of cyclic adenosine monophosphate (cAMP) pathway events that lead to the release of adrenocorticotropic hormone (ACTH) into the bloodstream. In the presence of CRF, AVP can also have synergistic effects on ACTH release through the vasopressin V1b receptor. ACTH circulates and binds to the melanocortin type 2 receptor (MC2-R) in the adrenal cortex, stimulating the synthesis and secretion of glucocorticoids into the bloodstream. Glucocorticoids regulate various physiological events and also inhibit further activation of the HPA axis through intracellular receptors present in the brain and

peripheral tissues. Other signaling molecules such as inositol triphosphate (IP3) and diacylglycerol (DAG) are also involved in HPA axis regulation.

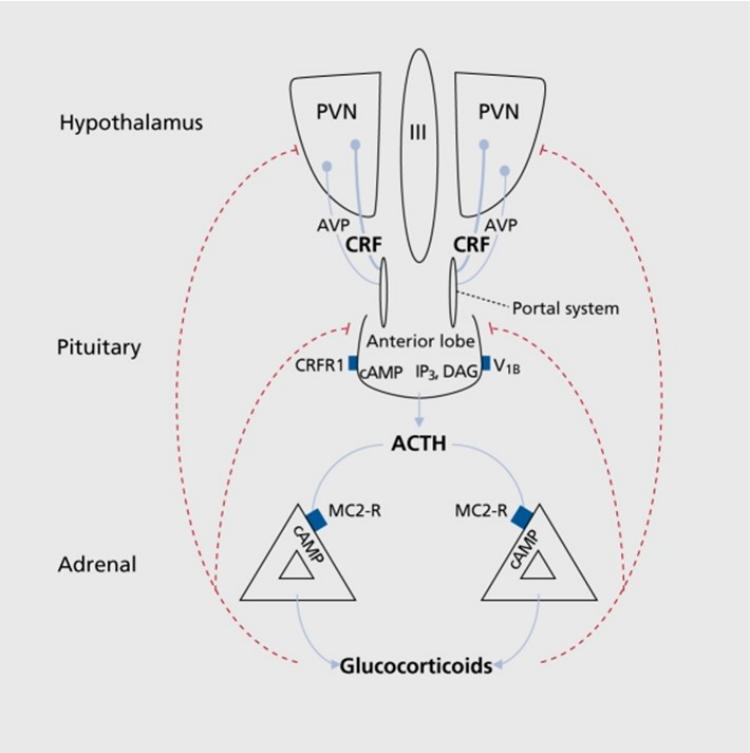

Figure 1.3. *The hypothalamic-pituitary-adrenal axis in neuroendocrine responses to stress. (Smith SM, 2006)*

In addition to the HPA axis, the sympathetic-adrenomedullary system is activated during stress. This leads to increased secretion of adrenaline and norepinephrine from the adrenal glands, which increases heart rate, blood pressure and respiration rate, preparing the body to respond to the stressful situation.

Together, these neuroendocrine mechanisms help prepare the body for the stress response, but if the activation of these mechanisms becomes chronic or excessive, they can contribute to the

development of stress-related conditions such as depression, anxiety, heart disease and diabetes (Wheatley, 1997).

Examining longitudinal changes in daily stress across 20 years of adulthood, results show that stress frequency and stress severity remained relatively stable across the period, whereas stressor diversity increased. Specifically, there are greater variety of stressors over time, although the overall level of stress did not increase indicating that stress levels may be relatively stable within individuals over time, but stressor diversity may be an important factor to consider in understanding stress trajectories over time (Almeida et al., 2023).

1.3. Role of the DLPFC in stress

The dorsolateral prefrontal cortex (DLPFC) is a region of the prefrontal cortex located in the frontal lobe of the brain. It is involved in a wide range of cognitive processes, including working memory, decision-making, planning, problem-solving, and attentional control. The DLPFC is situated in the lateral part of the prefrontal cortex and is one of the largest and most complex regions of the human brain. It is comprised of several subregions, including the middle frontal gyrus, the inferior frontal gyrus, and the superior frontal gyrus.

Research has shown that the DLPFC plays a critical role in a variety of higher-order cognitive functions, including:

- working memory (D'Esposito et al., 2007) - the DLPFC is involved in the temporary storage and manipulation of information, which is essential for many cognitive tasks.
- decision-making (Krawczyk et al., 2012; Fellows et al., 2007) - the DLPFC is critical for making decisions based on multiple factors and can be involved in selecting and implementing the best option.

- planning (Koechlin, 2007; Bahlmann et al., 2009) - the DLPFC is essential for planning and executing complex behaviors, including those involved in problem-solving and goal-directed behavior.
- attentional control (Arnsten 2009; Fuster 2008) - the DLPFC is responsible for controlling attention, allowing us to focus on relevant information and filter out distractions.

The DLPFC is thought to be involved in the cognitive and emotional regulation of stress responses, specifically it plays a role in downregulating the activity of the hypothalamic-pituitary-adrenal axis, which is the major neuroendocrine system that regulates the body's response to stress.

Previous research has shown that psychosocial stress leads to increased activity in the DLPFC, this activity is positively correlated to stress measures as skin conductance response (SCR) and self-reported stress levels, indicating that DLPFC activity plays a significant role in regulating the autonomic nervous system (ANS) and in perceived stress, suggesting the involvement of the DLPFC in emotional processes related to the stress response (Dedovic et al., 2009).

The DLPFC may modulate the response to stress by acting on the amygdala, a brain region involved in the processing of emotional information: the activity of these two regions seems to be positively correlated in the emotional response to psychosocial stress in healthy subjects (Orem et al., 2019). The amygdala plays a critical role in the neural circuitry that regulates the expression of peripheral emotional responses and individual differences in the emotional response to stressors may be mediated by amygdala function (Dedovic et al., 2009).

Studies suggest that stress can impair the structure and function of the DLPFC, which can lead to deficits in cognitive and emotional regulation (Arnsten, 2009) and a damage to the

DLPFC is associated with heightened stress reactivity and reduced stress resilience (Koenigs et al., 2008). Other studies have shown that interventions aimed at enhancing the function of the DLPFC, such as cognitive-behavioural therapy and mindfulness meditation, can improve stress regulation and reduce the negative effects of stress on mental and physical health, founding that mindfulness-based stress reduction (MBSR) is associated with increased activity in the DLPFC and reduced stress reactivity (Hölzel et a., 2011).

Overall, these findings suggest that the DLPFC plays a key role in the regulation of stress responses, and that interventions aimed at enhancing its function may be effective in promoting stress resilience and improving mental and physical health.

1.4. Effects of non-invasive brain stimulation on stress reactivity

Non-invasive brain stimulation (NIBS) refers to a group of techniques that can modulate brain activity without the need for surgical procedures. These techniques aim to influence the neural excitability and connectivity in specific brain regions through external stimulation, those methods are considered safe and are widely used in research and clinical settings to investigate brain function and potentially treat various neurological and psychiatric disorders.

NIBS techniques have been shown to influence emotional processing and stress responses by targeting specific brain regions, specifically non-invasive stimulation of the left DLPFC can reduce emotional stress reactivity, improve emotion regulation, decreased negative affect and reduced physiological stress responses (Smits et al., 2020). NIBS techniques over the left DLPFC can also modulate cortisol reactivity to acute stress and may have a stronger influence when applied before or during the initiation phase of the stress response (Vignaud et al., 2023). Other factors that may modulate the effects of NIBS on stress reactivity include the

type of stress task used, participant characteristics (e.g. healthy individuals vs. suffering from pathologies), and specific parameters of the NIBS technique (e.g. stimulation intensity, duration). The heterogeneity of results in previous research and the influence of various factors underscore the need for optimize the application of NIBS in stress-related contexts (Smits et al., 2020; Vignaud et al., 2023).

1.4.1. Transcranial magnetic stimulation (TMS) of the DLPFC

Transcranial magnetic stimulation (TMS) is a non-invasive brain stimulation technique that uses a magnetic field to induce electrical currents in the brain. During a TMS session, a small electromagnetic coil is placed on the scalp, and a brief and highly focused magnetic pulse is delivered to the underlying brain tissue (Figure 1.4). This pulse creates a rapidly changing magnetic field that penetrates the skull and induces a small electrical current in the targeted brain region.

There are two main types of TMS: single-pulse TMS and repetitive TMS (rTMS). Single-pulse TMS delivers a single magnetic pulse to the brain, which is typically used to study the function of specific brain areas, such as in neuromuscular stimulation. In contrast, rTMS delivers a series of magnetic pulses over time, which can be used to induce longer-lasting changes in brain activity, such as temporary inhibition.

TMS can be delivered to different regions of the brain by changing the position of the TMS coil. The most commonly targeted brain region in TMS studies is the DLPFC, which is involved, as described above, in a range of cognitive processes such as working memory, attention, and decision-making.

The stimulation of the DLPFC has been shown to have therapeutic effects for depression and other neuropsychiatric disorders (Lefaucheur et al., 2014), to improve symptoms of depression in patients who had not responded to other treatments (Philip et al., 2021), to improve working memory, attention, and other cognitive processes (Miniussi et al., 2011).

High-frequency repetitive transcranial magnetic stimulation (rTMS) on the DLPFC was also used for treating post-traumatic stress disorder (PTSD) with a greater reduction in PTSD symptoms (Concerto et al., 2022). Moreover, the effectiveness of rTMS on the DLPFC showed a greater reduction in panic disorder symptoms in patients with panic disorder and comorbid major depression (Liu et al., 2017).

TMS is generally considered to be safe and well-tolerated, although there are some potential side effects such as headache, scalp discomfort, and muscle twitching.

1.4.2. Transcranial direct current stimulation (tDCS) of the DLPFC

tDCS is also non-invasive and has been used to stimulate the DLPFC for various purposes, such as improving working memory or reducing symptoms of depression (Boggio et al., 2006).

tDCS has been investigated for its potential to modulate the activity of the DLPFC and other brain regions involved in stress regulation, it can reduce subjective feelings of stress and anxiety in healthy subjects (Kekic et al., 2014) as well as symptoms of depression and anxiety in patients with major depressive disorder (Brunoni et al., 2014).

Effects of tDCS applied to the left DLPFC on stress regulation have been demonstrated using heart rate variability (HRV) as a stress marker during a laboratory task (Carnevali et al., 2018). HRV is a measure of the variation in time between successive heart beats and is an important indicator of cardiac health. tDCS of the left DLPFC significantly increased HRV

during the stress episode in the active group compared to the sham group, specifically active tDCS increased the high-frequency (HF) component of HRV, which is thought to reflect parasympathetic nervous system activity and is associated with better cardiovascular health. In contrast, there were no significant effects of tDCS on the low-frequency (LF) or very low-frequency (VLF) components of HRV (Carnevali et al., 2018). These findings have implications for the potential use of tDCS as a non-pharmacological intervention for stress-related disorders, such as anxiety and depression, which are associated with altered HRV.

Prefrontal transcranial direct current stimulation has effects on autonomic and neuroendocrine responses to psychosocial stress simulated by a laboratory task (Carnevali et al., 2019), where the participants were asked to answer a series of questions about how they behave and feel in different social contexts (adapted version of the TSST). After completing the questionnaire participants were given a mental arithmetic task, in which they had to solve a series of math problems within a limited time frame. Active tDCS group had significantly lower heart rate and time dependent salivary cortisol levels in response to the stressor task, indicating that the effects of tDCS on cortisol levels depended on the time point of measurement. Specifically, at the 30-minute time point after the stressor task, participants who received tDCS had significantly lower cortisol levels compared to those who received sham stimulation. However, at other time points (immediate post-stressor and 15 minutes after the stressor), there were no significant differences between the tDCS and sham groups in cortisol levels. Additionally, the active tDCS group had higher heart rate variability which is indicative of better autonomic modulation at the level of the heart, showing effects of prefrontal tDCS on neuroendocrine responses to psychosocial stress that could be attributed to changes in prefrontal cortical activity showing that tDCS may enhance prefrontal cortical activity, which in turn may lead to increased inhibition of the hypothalamic-pituitary-adrenocortical (HPA) axis and sympathetic-adrenomedullary (SAM) system responses to stress.

The tDCS of the left DLPFC effects on cortisol responses to a stressor task is supposed to be mediated by changes in the activity of the amygdala, a brain region involved in emotional processing (Zhang et al., 2019) and similar results were also found by stimulating the left ventromedial prefrontal cortex (VMPFC) (Wang et al., 2019), suggesting that tDCS may have potential for modulating stress responses through its effects on key brain regions involved in stress regulation.

1.5. Aim

The aim of the present study was to characterize the effects of anodal tDCS applied to the left DLPFC on stress responses in healthy subjects by measuring the individual's emotional state, subjective experience of stress and salivary cortisol levels before, during and after the controlled administration of a stress-producing paradigm.

2. Methods

2.1. Study design

A randomized, sham-controlled, double-blind design with parallel groups was used. The experimental study was implemented through the assignment of a code for each subject and a double-blind randomization with the aim of evaluating the reactivity to stress during the application of tDCS. The independent variable was the active tDCS condition (n = 18) or sham (n = 18) while the three dependent variables were: salivary cortisol levels, subjective stress experience (VAS), and reported effects on affectivity (PANAS). Cortisol levels were measured at five time points, data on the experience of stress and affectivity were instead sampled six times.

The study was approved by the ethics committee of the Charité – Universitätsmedizin Berlin, compliance with the safety guidelines for tDCS (Antal et al., 2017) was ensured.

2.2. Sample description

The inclusion criteria were: subjects between 18 and 35 years old who did not receive psychotherapeutic or psychiatric treatment in the last 12 months.

The following exclusion criteria were also defined: presence of metal in the head area; presence of a pacemaker, drug infusion pump or hearing aids; heart disease, brain tumours, epilepsy, or stroke; hydrocortisone-based drug therapies; smoking more than 10 cigarettes per day.

At the end of the recruitment process 36 healthy adults participated, 17 males and 19 females aged 22.5±2.8 years old (mean±S.D.). The recruitment took place both within the Medical School of Berlin via an online portal offered by the University (Sona Systems, Ltd) and externally via flyers and information material.

Data collection took place in November and December 2022 at the university laboratories. The subjects recruited outside the Medical School of Berlin did not receive any compensation, while the students of the University were credited with 3.5 hours of laboratory activity during their career.

Before starting the experiment, each participant signed an informed consent for the study and for the protection of personal data, according to the European data protection regulation and the ethic approval given by MSB.

2.3. Experimental setup

A telephone screening was carried out the day before the test to identify any reasons for exclusion from the study. Furthermore, in order to avoid distortions in salivary cortisol levels, during the phone call the subjects were instructed to avoid the actions that affect cortisol levels described above.

Due to the cortisol fluctuations related to the circadian rhythm the acquisition time window was defined from 11 am to 18 pm, the complete session lasted about 90 minutes and each subject was tested individually.

Two adjacent laboratories were used for data acquisition (Figure 2.1), both equipped with table and upholstered chairs.

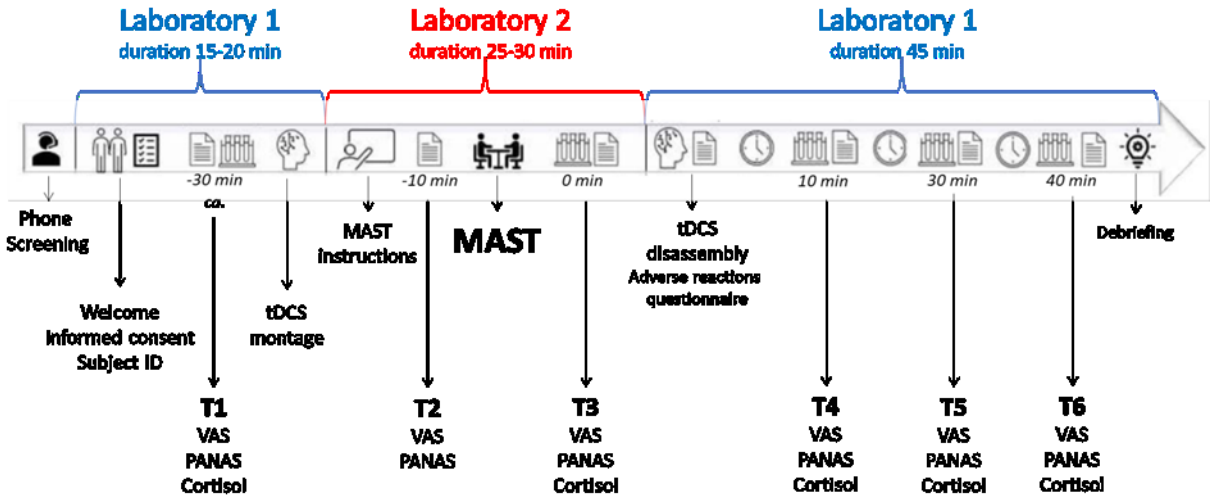

Figure 2.1. *Data acquisition timeline from subject screening to the debriefing*

In the first laboratory (Laboratory 1) there was an experimenter who had the task of welcoming the subjects, collecting informed consent, psychometric data and saliva samples at timepoint T1 (cortisol, VAS, PANAS), assign the subjects identification code, mounting the tDCS instrumentation and turning it on in the "active" or "sham" modality (Figure 2.2 a-b).

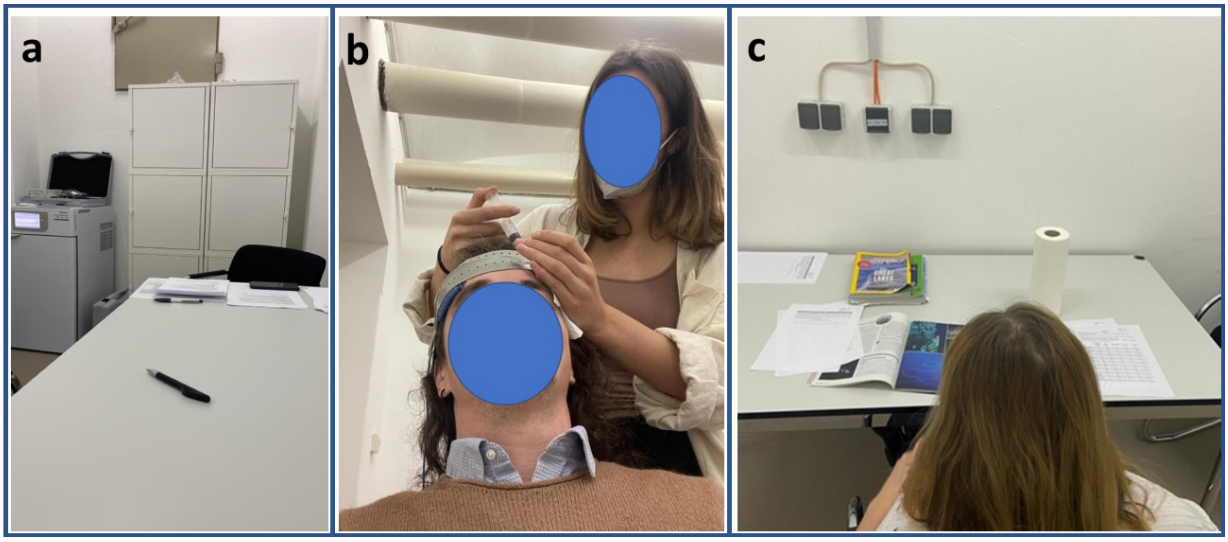

Figure 2.2. *Laboratory 1: sbj view during the welcome and signature of inform consent (a); tDCS montage (b); recovery phase after the stress-producing paradigm occurred in Laboratory 2 (c).*

Finished the first phase in the Laboratory 1 the experimenter accompanied the subject to the second laboratory (Laboratory 2) that was separate from the first by an insulating door and was dedicated to the administration of the stress paradigm. There were, in addition to a table and upholstered chairs, a workstation for the presentation of the paradigm, a basin of cold water and a video camera (Figure 2.3) and other two experimenters. Here he invited the subject to sit down at the table and left the subject in the Laboratory 2 with the assigned investigators to the administration of the MAST.

Figure 2.3. *Laboratory2: insulated access door (a); station for the administration of the stress-producing paradigm (b)*

Subject was then informed that images would be acquired to analyse his bodily expressions and that notes on his behaviour would be taken.

The two experimenters were dislocated one seated in front of the subject, the other standing behind him. The subject received detailed instructions for carrying out the stress paradigm (MAST) through a PowerPoint presentation, both before the start of the paradigm and during

the paradigm itself. Furthermore, the experimenter repeated the instructions verbally before the start of the stress paradigm. Prior to the start of the MAST, they were asked if there were any questions or concerns regarding the procedure.

Once ascertained that the instructions were clear a second measurement (T2) of subjective stress (VAS) and affectivity (PANAS) was then performed.

The experimenter started the MAST on PowerPoint which automatically gave indications to the subject at predefined intervals, the timing was the same for all the subjects. During the stress induction phase, a second experimenter pretended to take notes on the subject's performance and behaviour.

Once the MAST was completed, salivary cortisol, subjective stress experience (VAS) and affectivity survey (PANAS) were immediately acquired (T3).

After completion of the stress phase and measurements in the Laboratory 2, the subject was escorted back to the Laboratory 1 for disassembly of the tDCS equipment and detection of any side effects.

Here magazines with a neutral stimulus function (gardening, naturalistic topics) were made available to the subjects and any conversations were avoided (Figure 2.2 c). In this condition were measured the subjective stress experience (VAS), affectivity survey (PANAS) and salivary cortisol concentration at timepoints T4 (after 10 minutes from the measurement of T3), T5 (after 20 minutes from the measurement of T4) and T6 (after 10 minutes from the measurement of T5).

These last three measurements were made without modifying the environmental conditions and without interacting with the subject.

At the end of the experiment, the experimenters held a debriefing in which the subject was informed about the true purpose of the study and about the falsehood of the recording of

images and notes taken by the experimenters with the aim of increasing the subject's stress levels.

2.3.1. tDCS montage

Left DLPFC tDCS was performed using a portable tDCS device (NeuroConn, DC - Stimulator PLUS).

In transcranial direct current stimulation tDCS a small direct current flows between two electrodes, whereby neuronal excitability can be modulated while the anode electrode moves the membrane potential into the positive field and facilitates the triggering of an action potential (Nitsche et al.,2000). In this study we opted for a bifrontal assembly with positioning of the anode in correspondence with the F3 location and the cathode in F4 of the international 10-20 system (Nitsche et al.,2008) used for EEG recordings, so as to stimulate left DLPFC (Figure 2.4) (Fregni et al., 2005).

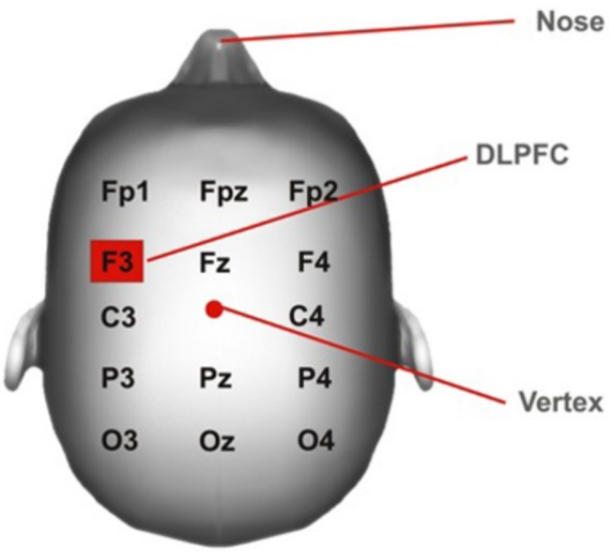

Figure 2.4. *Anode position used to stimulate the left DLPFC according to the 10-20 system.*

The protocol to locate F3 was the following: location of the vertex by measuring and dividing by two the distance between the inion and nasion and, for sagittal centering, the distance between the biauricularis landmarks was measured and divided by two; localization of the primary motor cortex (C3 and C4 locations of the 10-20 system) measuring 20% of the total biauricular distance from the vertex in the auricular direction; the DLPFC is located approximately 5cm in front of the motor cortex. At each step, points on the subject's scalp were marked with a cosmetic pencil.

The electrodes (5x7 cm) were fixed with the rubber bands supplied by the instrument manufacturer, after inserting them in the appropriate sponges soaked in physiological water (0.9% sodium chloride).

The electrical stimulation took place by randomly assigning the subjects to the sham (30 seconds of initial and final stimulation) or active (20 minutes of stimulation without interruption) group, this approach had already demonstrated in previous studies as a reliable method for investigations in double-blind (Gandiga et al., 2006). Since it is possible that the subjects initially feel a slight tingling or itching in the area of the electrodes, by supplying current for a short time even to the subjects of the sham group, perceptual differences between the groups were avoided.

The stimulation took place with a current intensity equal to 1 mA, since even low intensity currents (0.5-1 mA) have been defined as capable of influencing neuronal excitability (Stagg et al., 2011).

2.3.2. Stress-producing paradigm

The Maastricht Acute Stress Test (MAST) is a widely used laboratory stress test designed to induce acute psychological stress in participants (Smeets et al., 2012; Shilton et al., 2017).

The MAST involves a combination of physical and cognitive stressors, is designed to be a simple, quick, and non-invasive procedure aimed at activating the human stress system. The MAST has been developed by combining elements from two of the most common experimental paradigms measuring stress, the Trier Social Stress Test (TSST) (Allen et al., 2016) and the Cold Pressor Test (CPT) (Wirch et al., 2006) (Figure 2.5).

In this experiment, after a baseline rest period the participant was asked to perform a mental arithmetic task in the presence of two experimenters and in front of a videocamera, which involves counting backwards from 2043 in steps of 17. During the arithmetic task, the participant receives auditory feedback indicating whether his/her answers were correct or incorrect and the wet hand was placed on the towel next to the bowl. The test subject was asked to be fast and precise in the backward calculation, every error was meant to start again from the beginning.

Alternating with the arithmetic task, the participant was asked to immerse one hand in ice-cold water (0-2°C). The two stress conditions were alternate in time in block of the minimum duration of 45 sec and maximum duration of 90 sec (Table 2.1).

Table 2.1. *the MAST alternated blocks for hand immersion and arithmetic task.*

N	Test	Duration (s)
1	Hand Immersion	**90**
2	Mental Arithmetic	**45**
3	Hand Immersion	**60**
4	Mental Arithmetic	**60**
5	Hand Immersion	**60**
6	Mental Arithmetic	**90**
7	Hand Immersion	**90**
8	Mental Arithmetic	**45**
9	Hand Immersion	**60**

The test subject was instructed by the experimenter to immerse his hand completely in the ice water without touching the bottom of the basin or clenching his fist.

The water temperature was monitored by an immersion thermometer (Voltcraft DT-300 SE) (Figure 2.5 a)

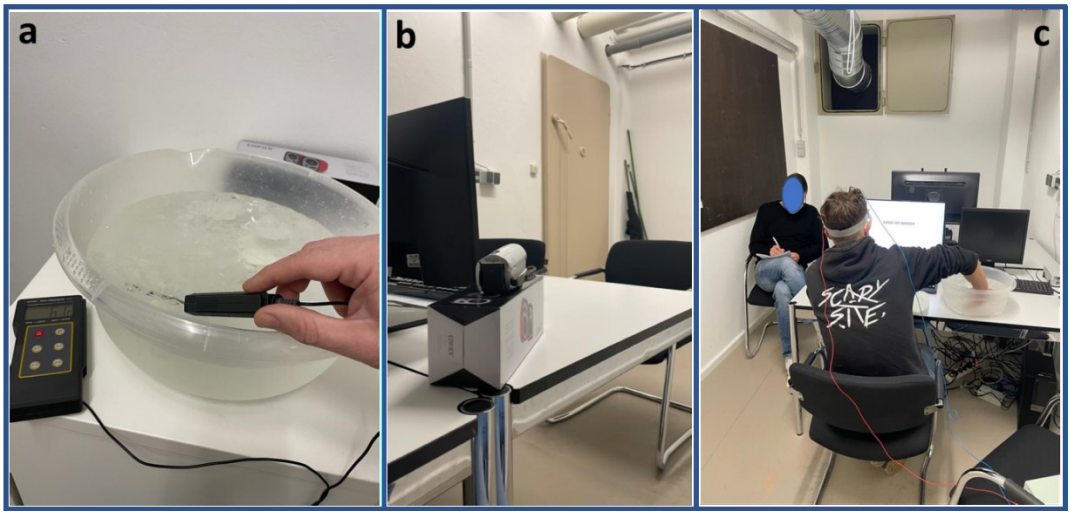

Figure 2.5. *MAST environment elements: cold water monitored with a professional thermometer (a); camera that frames the subject (b); experimenter taking notes during the paradigm (c).*

2.3.3. Objective measure of stress

Salivary cortisol concentration, an index of HPA axis activation, was used as a reliable marker of stress (Hellhammer et al., 2009). Usually, salivary cortisol concentration returns to baseline levels 30 minutes after the acute stressful event (Kudielka et al., 2007; Dickerson et al., 2004) and has fluctuations related to the circadian rhythm, with maximal cortisol levels upon awakening, a decrease during the day and a very low concentration during sleep (Clow et al., 2010).

Cortisol levels were measured using the enzyme-linked immunosorbent assay (Salivette® Cortisol tubes, Sarsted Inc.) according to manufacturer's directions. Subjects remove swabs from the tubes, put them in their mouths and chew them for 60 seconds. Then, they put them back in the test tubes and seal them with the stoppers.

In order to avoid alterations in cortisol levels in preparation for the exam, the subjects were also asked: not to practice extreme sports for at least one day before the exam, nor to make any physical effort, such as cycling, one hour before the exam; not to eat, consume caffeine, sleep and brush their teeth at least half an hour before the exam.

Additionally, subjects were asked to keep cell phones turned off throughout the entire test.

The cortisol samples collected were stored in a dedicated freezer located in the Laboratory 1 (EWALD Innovationstechnik GmbH) at a temperature between -17°C and -18°C (Figure 2.6) until they were collected by the personnel of the analysis laboratory. The freezer was equipped with an acoustic alarm to signal any rise in internal temperature, in addition to recording temperatures over time. Each tube was marked with dedicated labels before being stored.

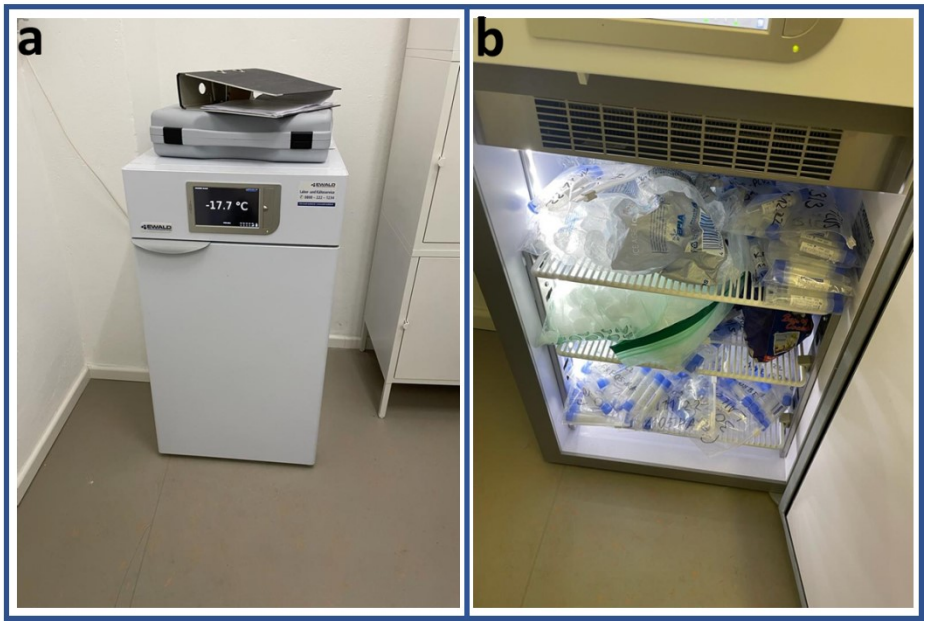

Figure 2.6. *Monitored storage of the saliva samples from outside (a) and inside (b)*

2.3.4. Subjective measure of stress

The subjective experience of stress was measured using the short version of the visual analogue scale (VAS) and the and reported effects on affectivity (PANAS).

The VAS is a self-assessment questionnaire, i.e. the subjective evaluation of a situation recently experienced and, in the present study, consists of placing a cross on a linear segment measuring 100 mm: the evaluation scale is between 0 mm ("I don't agree") and 100 mm ("I totally agree") in relation to the statement: "The situation is stressful for me" (Figure 2.7).

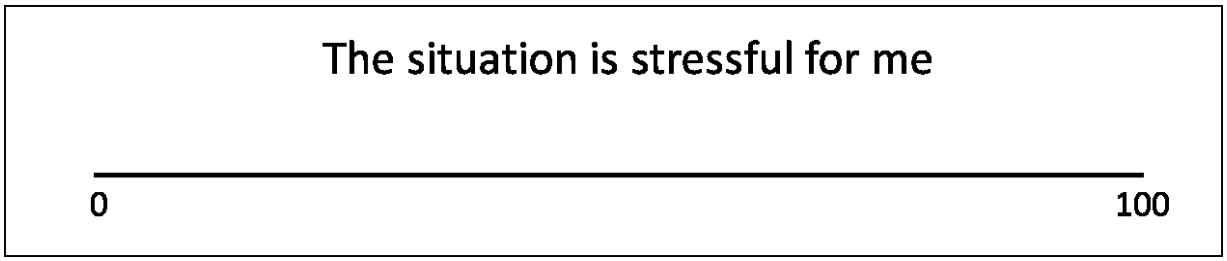

Figure 2.7. *The Visual Analogue Scale.*

The validity of this tool for measuring stress and its reproducibility have been previously described (Lesage et al., 2011).

The Positive and Negative Affect Schedule (PANAS) (Watson et al., 1988; Breyer and Bluemke, 2016) is a questionnaire that measures two dimensions of emotional experience: positive affect (PA) and negative affect (NA). It is a self-report questionnaire that consists of two 10-item scales aimed at measuring both PA and NA affect. Each item consists of one adjective and is rated on a 5-point Likert scale from 1 (not at all) to 5 (very much). The PA scale rates the presence and intensity of positive emotions such as joy, happiness and excitement, while the NA scale rates the presence and intensity of negative emotions such as sadness, anxiety and irritation. The PANAS questionnaire is often used to assess an

individual's emotional state at a given time or to measure changes in mood before and after an experience or intervention.

The questionnaire has primarily been used as a research tool in cohort studies, but it is validated to be used within clinical and non-clinical samples (Crawford et al., 2004).

2.4. Statistical analysis

The normality of data distribution was tested with Shapiro-wilk. Accordingly to the results, continuous variables were compared between the active and placebo groups through a t-test or a Mann-Whitney/Wilcoxon test. Categorical variables as gender were compared with a chi-square test.

As for the longitudinal testing of variables that were evaluated at various timepoints, a mixed ANOVA with repeated measures was performed. The following assumptions for ANOVA were tested: presence of outliers (defined as values above Q3+1.5IQR or below Q1-1,5IWR, where Q1 and Q3 are the first and third quartiles respectively, and IQR is the interquartile range), normality of the distribution (Shapiro Wilk test), homogeneity of variance (Levene's test), homogeneity of covariances of the between-subject factor, sphericity (Mauchly's test). Since most of the considered variables did not satisfy the ANOVA assumptions, a robust version based on Wilcox' WRS functions was used (specifically, as implemented in the WRS2 Package of R). In case of significance, post-hoc tests were performed with pairwise Wilcoxon signed rank test and the p-values were adjusted for multiple comparisons with Bonferroni.

A baseline correction was also considered by taking the difference, for each variable, between its value at T3 (immediately after stress induction) and the minimum between values at T1

and T2 (the baseline before stress induction), then comparing these differences between active and placebo groups. Significance level was considered with p-value <= 0.05.

The statistical analyses were performed with the software R (https://www.r-project.org/ version 4.2.3).

3. Results

Shapiro-wilk test was significant for almost all the tested variables, consequently non-parametric statistics were used.

The two groups, active and placebo, were well matched as for both gender (active M/F 10/8, placebo M/F 7/11, p = 0.5043) and age (active 22.8±3.2, placebo 22.1±2.4 years, p = 0.404).

3.1. VAS score

The descriptive statistics of the subjective scores VAS in the two groups is reported in Table 3.1.

Table 3.1. *Descriptive statistics for VAS scores in active and placebo groups at the different timepoints.*

		Active						Placebo					
		T1	T2	T3	T4	T5	T6	T1	T2	T3	T4	T5	T6
VAS	*median*	29	42	61.5	11	4.5	6	16.5	41.5	69.5	8.5	3	2.5
	iqr	18	26	16.2	10.2	6.75	7.75	23.5	22.8	28.2	19.2	6.75	3
	min	8	8	0	2	0	0	3	8	16	0	0	0
	max	71	83	82	66	57	25	67	87	100	68	20	38

No significant interaction between group and time ($F(5, 14) = 1.0574$, $p = 0.4218$) nor significant effect of group were observed ($F(1, 21) = 0.4061$, $p = 0.5305$), while a significant effect of time was found ($F(5, 14) = 51.5826$, $p < 0.0001$) (Figure 3.1).

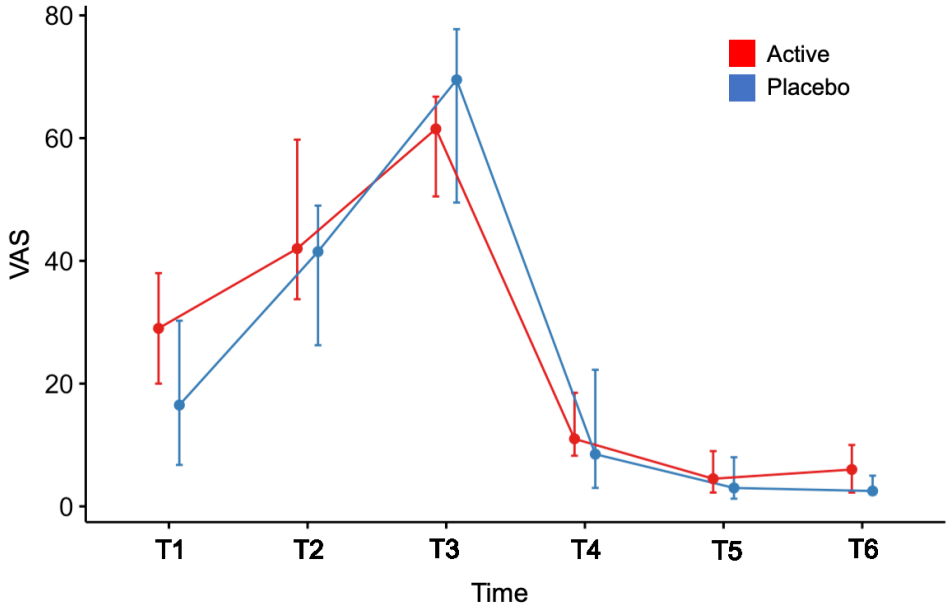

Figure 3.1. *Plot of VAS scores in the active and placebo groups at the different timepoint (median and iqr are shown).*

Given the only significant main effect of time, pairwise tests for the time variable, ignoring group, were performed and showed statistically significant differences between all the different timepoints pairs except for T5-T6 (Table 3.2).

Table 3.2. *VAS results of pairwise post-hoc testing for the time variable.*

VAS			
Timepoints		**p-value**	
T1	**T2**	<0.0001	***
T1	**T3**	<0.0001	***
T1	**T4**	0.006	**
T1	**T5**	<0.0001	***
T1	**T6**	<0.0001	***
T2	**T3**	0.017	*
T2	**T4**	<0.0001	***
T2	**T5**	<0.0001	***
T2	**T6**	<0.0001	***
T3	**T4**	<0.0001	***
T3	**T5**	<0.0001	***
T3	**T6**	<0.0001	***
T4	**T5**	0.00035	***
T4	**T6**	0.0004	***
T5	**T6**	1	

Since the group effects was no significant, no post-hoc comparisons between active and placebo were performed. However, to get a better idea of the phenomenon in the two groups, the Friedman test was performed within the active and the placebo groups separately.

When the active group was considered, the VAS score was statistically significantly different at the various time points (Friedman test, $X2(2)=59.2$, $p<0.0001$, effect size large with Kendall's W 0.580). Post-hoc testing revealed statistically significant differences between multiple timepoint pairs (Table 3.3, Figure 3.2).

As for the placebo group, the VAS score was statistically significantly different at the various time points (Friedman test, $X2(2)=41.9$, $p=0.00069$, effect size moderate with Kendall's W 0.411). Post-hoc tests revealed statistically significant differences between various timepoint pairs (Table 3.3, Figure 3.2).

Table 3.3. *VAS results of pairwise post-hoc testing for the active and placebo groups separately.*

VAS

ACTIVE				PLACEBO			
Timepoints		**p-value**		**Timepoints**		**p-value**	
T1	**T2**	0.007	**	**T1**	**T2**	0.014	*
T1	**T3**	0.009	**	**T1**	**T3**	0.007	**
T1	**T4**	0.005	**	**T1**	**T4**	1	
T1	**T5**	0.003	**	**T1**	**T5**	0.058	
T1	**T6**	0.003	**	**T1**	**T6**	0.097	
T2	**T3**	1		**T2**	**T3**	0.091	
T2	**T4**	0.000114	***	**T2**	**T4**	0.05	*
T2	**T5**	0.003	**	**T2**	**T5**	0.003	**
T2	**T6**	0.003	**	**T2**	**T6**	0.005	**
T3	**T4**	0.004	**	**T3**	**T4**	0.004	**
T3	**T5**	0.00023	***	**T3**	**T5**	0.003	**
T3	**T6**	0.005	**	**T3**	**T6**	0.000114	***
T4	**T5**	0.007	**	**T4**	**T5**	0.15	
T4	**T6**	0.012	*	**T4**	**T6**	0.123	
T5	**T6**	1		**T5**	**T6**	1	

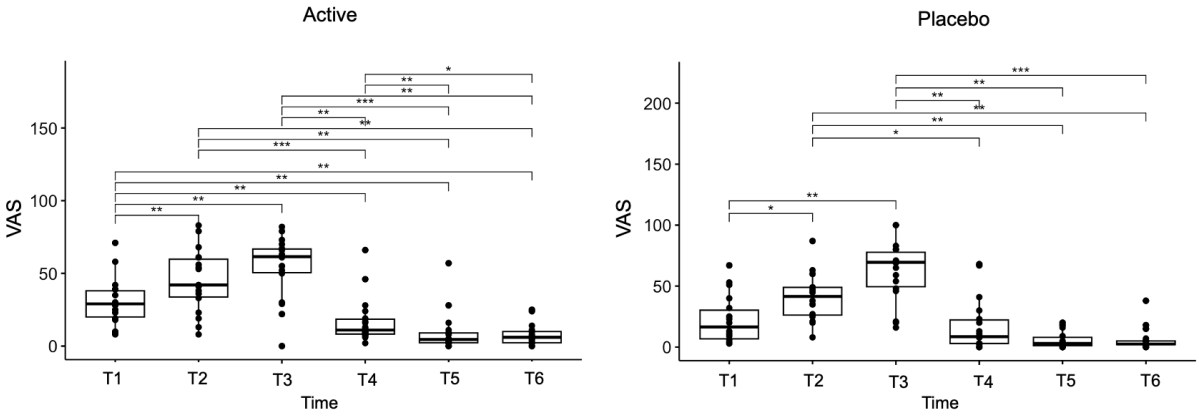

Figure 3.2. *Box-plot of VAS scores in the active and placebo groups at different timepoints. The significant post-hoc tests are reported in the plot (* p<0.05, ** p<0.01, ***p<0.001).*

When the baseline correction was considered to explore the different effects on the stress reactivity as evaluated with VAS score, a significant difference between active and placebo groups was found (p = 0.034).

3.2. Salivary cortisol concentration

The descriptive statistics of cortisol levels in the two groups is reported in Table 3.4.

Table 3.4. *Descriptive statistics for cortisol levels in active and placebo groups at the different timepoints.*

		Active						Placebo					
		T1	T2	T3	T4	T5	T6	T1	T2	T3	T4	T5	T6
Cortisol levels	*median*	3.88	-	4.34	5.68	3.9	3.34	2.53	-	3.2	4.22	3.04	2.99
	iqr	2.78	-	3.7	5.45	4.09	3.27	4.88	-	3.01	5.03	4.38	4.03
	min	1.1	-	1.36	1.92	1.41	1.44	0.36	-	0.48	0.71	0.75	0.76
	max	7.82	-	10.2	13.2	10.2	8.08	10.5	-	8.74	12.2	8.99	8

No significant interaction between group and time ($F(4, 16) = 1.0627$, $p=0.4055$) nor significant effect of group were observed ($F(1, 21) = 0.8966$, $p=0.3542$), while a significant effect of time was found ($F(4, 16) = 7.4653$, $p<0.0012$) (Figure 3.3).

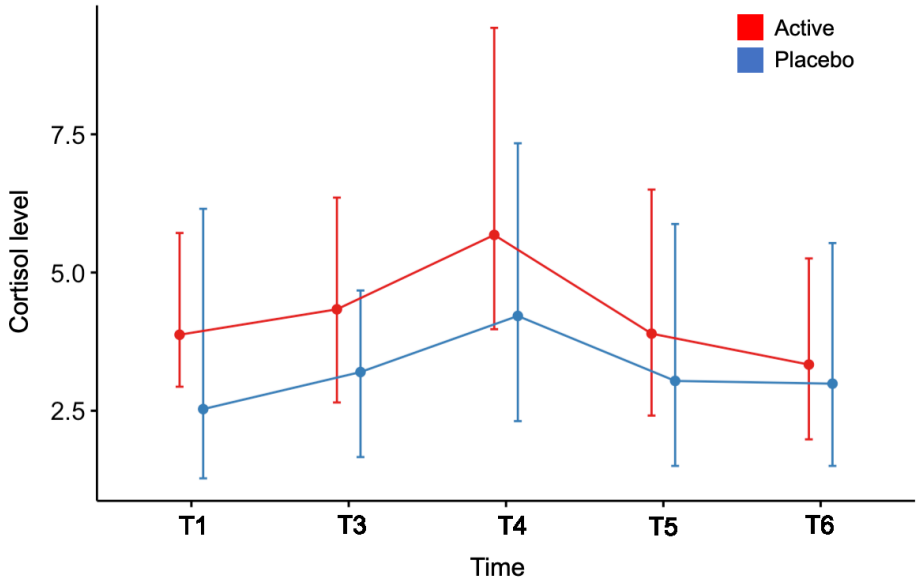

Figure 3.3. *Plot of cortisol levels in the active and placebo groups at the different timepoints.*

Given the only significant main effect of time, pairwise tests for the time variable, ignoring group, were performed and results are reported in Table 3.5

Table 3.5. *Cortisol level results of pairwise post-hoc testing for the time variable.*

Cortisol			
Timepoints		**p-value**	
T1	**T3**	1	
T1	**T4**	0.022	*
T1	**T5**	1	
T1	**T6**	1	
T3	**T4**	0.00013	***
T3	**T5**	1	
T3	**T6**	0.219	
T4	**T5**	<0.0001	***
T4	**T6**	<0.0001	***
T5	**T6**	0.003	**

Since the group effects was no significant, no post-hoc comparisons between active and placebo were performed. However, to get a better idea of the phenomenon in the two groups, the Friedman test was performed within the active and the placebo groups separately.

In the active group, cortisol levels were significantly different at the various time points (Friedman test, X2(2) = 67.9, p = <0.0001, large effect size Kendall's W 0.799). Pairwise testing between groups revealed statistically significant differences between T3-T4, T4-T5, T4-T6 and T5-T6 p=0.002 (Table 3.6, Figure 3.4).

In the placebo group, the cortisol levels were statistically significantly different at the various time points (Friedman test, X2(2) = 69.8, p = <0.0001, effect size large Kendall's W 0.821). Pairwise Wilcoxon signed rank test between groups revealed statistically significant differences between T3-T4 and T4-T5 (Table 3.6, Figure 3.4).

Table 3.6. *Cortisol level results of pairwise post-hoc testing for the active and placebo groups separately.*

Cortisol							
ACTIVE				**PLACEBO**			
Timepoints		**p-value**		**Timepoints**		**p-value**	
T1	**T3**	1		**T1**	**T3**	1	
T1	**T4**	0.104		**T1**	**T4**	1	
T1	**T5**	1		**T1**	**T5**	1	
T1	**T6**	1		**T1**	**T6**	1	
T3	**T4**	0.008	**	**T3**	**T4**	0.025	*
T3	**T5**	1		**T3**	**T5**	1	
T3	**T6**	0.09		**T3**	**T6**	1	
T4	**T5**	0.0000763	***	**T4**	**T5**	0.01	*
T4	**T6**	0.0000763	***	**T4**	**T6**	0.08	
T5	**T6**	0.002	**	**T5**	**T6**	1	

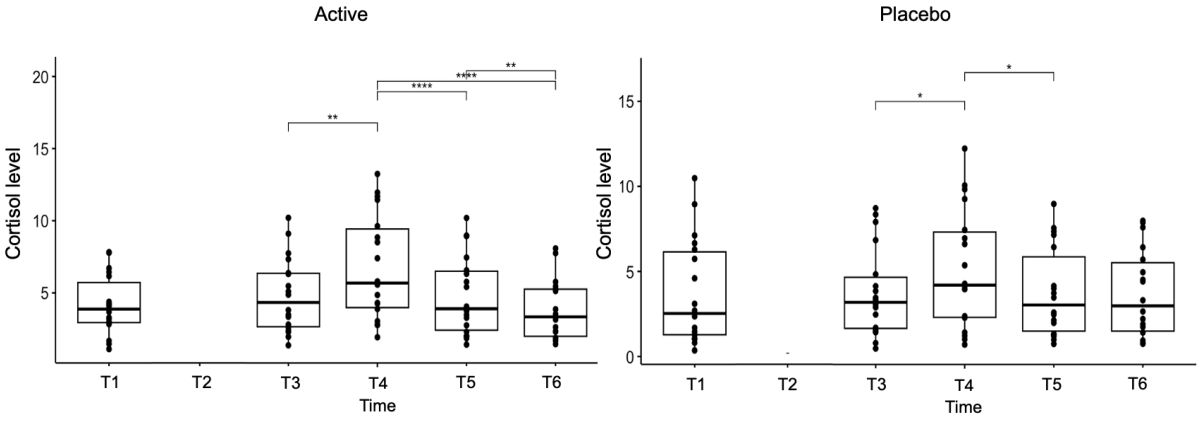

Figure 3.4. *Box-plot of cortisol levels in the active and placebo groups at different timepoints. The significant post-hoc tests are reported in the plot (* p<0.05, ** p<0.01, ***p<0.001).*

When the baseline correction was considered to explore the different effects on the stress reactivity as evaluated with the cortisol level, no significant differences between active and placebo groups were found (p = 0.548).

3.3. PANAS score

The descriptive statistics of PANAS scores in the two groups is reported in Table 3.7.

Table 3.7. *Descriptive statistics for PANAS Positive and Negative scores in active and placebo groups at the different timepoints.*

		Active						Placebo					
		T1	T2	T3	T4	T5	T6	T1	T2	T3	T4	T5	T6
PANAS positive	median	2.7	2.65	2.55	2.2	2	1.8	2.95	2.8	2.5	2.2	2.15	2
	iqr	1.15	0.975	1.27	1.12	0.525	0.7	1.15	0.925	1.05	0.8	1.32	1.48
	min	1.4	1.4	1.3	1.1	1.2	1.3	2	1.8	1.67	1.3	1	1
	max	4.2	4	4.2	3.9	3.7	3.6	4.1	4	4.4	4	4	3.7
PANAS negative	median	1.3	1.4	1.45	1.15	1	1	1.35	1.55	1.55	1.2	1	1
	iqr	0.4	0.4	0.475	0.3	0.175	0.1	0.4	0.55	0.85	0.475	0.1	0.175
	min	1	1	1	1	1	1	1	1.1	1	1	1	1
	max	3.2	3	2.8	2.3	2.3	2.1	1.7	2.9	4.4	3.2	2.3	1.7

No significant interaction between group and time ($F(5, 16) = 0.6387$, $p = 0.6734$) nor significant effect of group were observed ($F(1, 21) = 0.0340$, $p = 0.8554$), while a significant effect of time was found ($F(5, 16) = 9.8723$, $p = 0.0002$) (Figure 3.5).

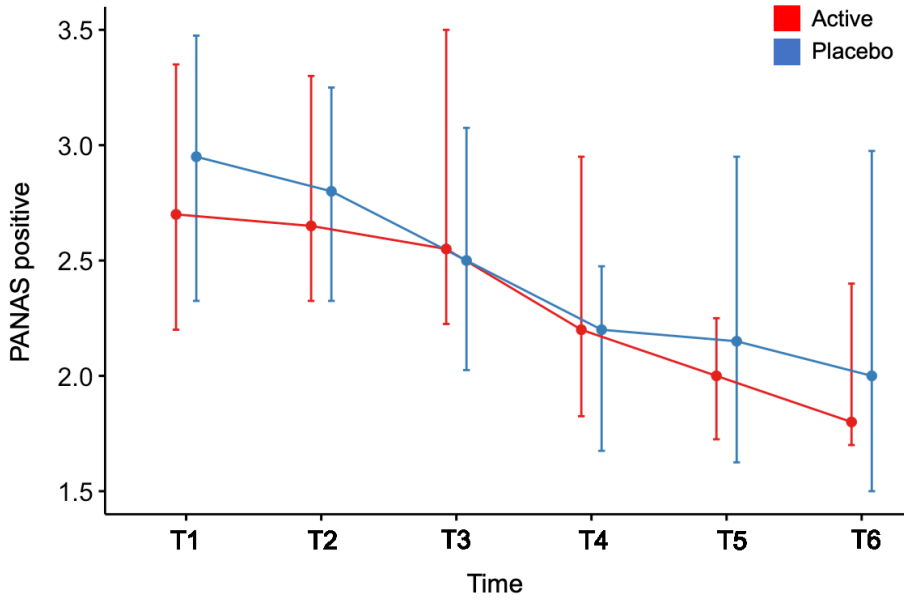

Figure 3.5. *Plot of PANAS positive scores in the active and placebo groups at the different timepoints (median and iqr are shown).*

Given the only significant main effect of time, pairwise tests for the time variable, ignoring group, were performed and showed statistically significant differences between various timepoint pairs (Table 3.8).

Table 3.8. *PANAS positive score results of pairwise post-hoc testing for the time variable.*

PANAS Positive			
Timepoints		p-value	
T1	T2	1	
T1	T3	1	
T1	T4	0.00095	***
T1	T5	0.00016	***
T1	T6	<0.0001	***
T2	T3	1	
T2	T4	0.00058	***
T2	T5	0.00016	***
T2	T6	<0.0001	***
T3	T4	0.001	**
T3	T5	0.001	**
T3	T6	0.001	**
T4	T5	1	
T4	T6	1	
T5	T6	1	

Since the group effects was no significant, no post-hoc comparisons between active and placebo were performed. However, to get a better idea of the phenomenon in the two groups, the Friedman test was performed within the active and the placebo groups separately.

Regarding the active group, the PANAS positive score was statistically significantly different at the various time points (Friedman test, X2(2) = 80.2, p = <0.0001, large effect size Kendall's W 0.786). Pairwise Wilcoxon signed rank test between groups revealed statistically significant differences between various timepoint pairs (Table 3.9, Figure 3.6).

The PANAS positive score for the placebo group was statistically significantly different at the various time points (Friedman test, X2(2) = 69.9, p = <0.0001, large effect size Kendall's W

0.685. Pairwise Wilcoxon signed rank test between groups revealed statistically significant differences between various timepoint pairs (Table 3.9, Figure 3.6).

Table 3.9. *PANAS positive score results of pairwise post-hoc testing for the active and placebo groups separately.*

PANAS Positive

ACTIVE				PLACEBO			
Timepoints		**p-value**		**Timepoints**		**p-value**	
T1	**T2**	1		**T1**	**T2**	1	
T1	**T3**	1		**T1**	**T3**	1	
T1	**T4**	0.372		**T1**	**T4**	0.015	*
T1	**T5**	0.049	*	**T1**	**T5**	0.012	*
T1	**T6**	0.005	**	**T1**	**T6**	0.005	**
T2	**T3**	1		**T2**	**T3**	1	
T2	**T4**	0.168		**T2**	**T4**	0.016	*
T2	**T5**	0.034	*	**T2**	**T5**	0.019	*
T2	**T6**	0.003	**	**T2**	**T6**	0.022	*
T3	**T4**	0.039	*	**T3**	**T4**	0.476	
T3	**T5**	0.005	**	**T3**	**T5**	1	
T3	**T6**	0.005	**	**T3**	**T6**	0.778	
T4	**T5**	1		**T4**	**T5**	1	
T4	**T6**	0.388		**T4**	**T6**	1	
T5	**T6**	1		**T5**	**T6**	1	

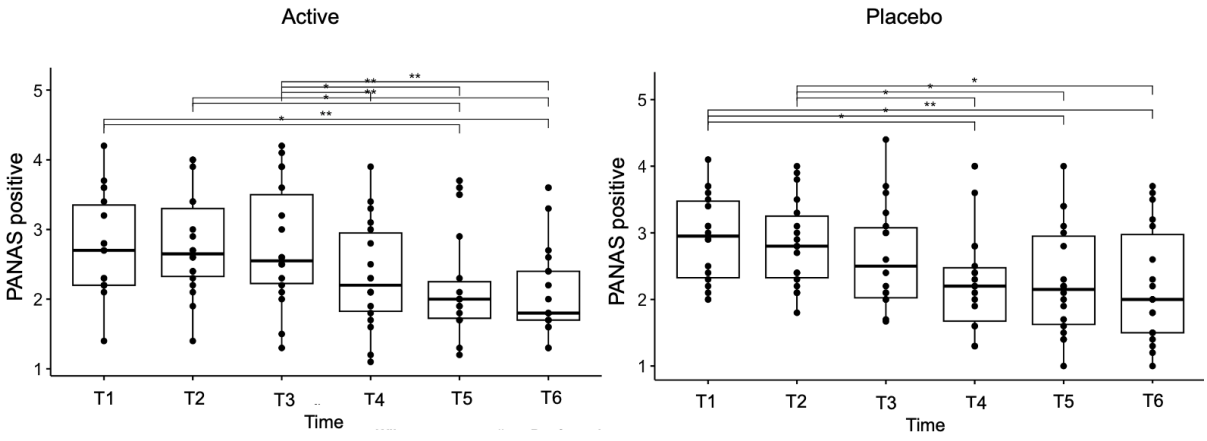

Figure 3.6. *Box-plot of PANAS positive score in the active and placebo groups at different timepoints. The significant post-hoc tests are reported in the plot (* p<0.05, ** p<0.01, ***p<0.001).*

When the baseline correction was considered, the stress reactivity as evaluated with the PANAS positive score was no significantly different between active and placebo groups (p = 0.133).

As for the PANAS negative score, no significant interaction between group and time (F(5, 15) = 0.2638, p = 0.9261) nor significant effect of group were observed (F(1, 20) = 0.5200, p = 0.4792), while a significant effect of time was found (F(5, 15) = 14.5947, p < 0.0001) (Figure 3.7).

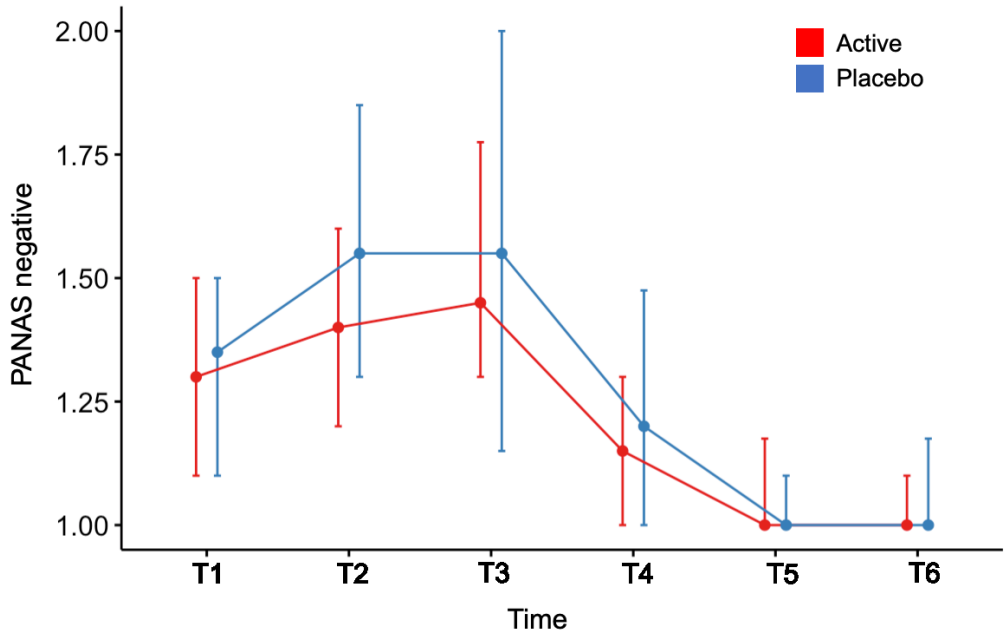

Figure 3.7. *Plot of PANAS negative scores in the active and placebo groups at the different timepoints (median and iqr are shown).*

Given the only significant main effect of time, pairwise tests for the time variable, ignoring group, were performed and showed statistically significant differences (Table 3.10).

Table 3.10. *PANAS negative score results of pairwise post-hoc testing for the time variable.*

PANAS Negative			
Timepoints		p-value	
T1	T2	0.531	
T1	T3	0.918	
T1	T4	0.054	
T1	T5	0.00099	***
T1	T6	0.001	**
T2	T3	1	
T2	T4	0.00075	***
T2	T5	<0.0001	***
T2	T6	<0.0001	***
T3	T4	<0.0001	***
T3	T5	<0.0001	***
T3	T6	<0.0001	***
T4	T5	0.027	*
T4	T6	0.046	*
T5	T6	1	

Since the group effects was no significant, no post-hoc comparisons between active and placebo were performed. However, to get a better idea of the phenomenon in the two groups, the Friedman test was performed within the active and the placebo groups separately.

As for the active group, the PANAS negative score was statistically significantly different at the various time points (Friedman test, $X2(2) = 70.8$, $p = <0.0001$, large effect size Kendall's W 0.694). Pairwise Wilcoxon signed rank test between groups revealed statistically significant differences between various timepoint pairs (Table 3.11, Figure 3.8).

The PANAS negative score for the placebo group was statistically significantly different at the various time points (Friedman test, $X2(2) = 51.2$, $p = <0.0001$, large effect size Kendall's W, 0.502). Pairwise Wilcoxon signed rank test between groups revealed statistically significant differences between T2-T5, T2-T6, T3-T4, T3-T5 and T3-T6 (Table 3.11, Figure 3.8).

Table 3.11. *PANAS negative score results of pairwise post-hoc testing for the active and placebo groups separately.*

PANAS Negative

ACTIVE				PLACEBO			
Timepoints		**p-value**		**Timepoints**		**p-value**	
T1	**T2**	1		**T1**	**T2**	0.196	
T1	**T3**	1		**T1**	**T3**	1	
T1	**T4**	0.03	*	**T1**	**T4**	1	
T1	**T5**	0.008	**	**T1**	**T5**	0.387	
T1	**T6**	0.01	*	**T1**	**T6**	0.414	
T2	**T3**	1		**T2**	**T3**	1	
T2	**T4**	0.012	*	**T2**	**T4**	0.232	
T2	**T5**	0.011	*	**T2**	**T5**	0.005	**
T2	**T6**	0.006	**	**T2**	**T6**	0.02	*
T3	**T4**	0.016	*	**T3**	**T4**	0.01	**
T3	**T5**	0.009	**	**T3**	**T5**	0.01	*
T3	**T6**	0.006	**	**T3**	**T6**	0.007	**
T4	**T5**	1		**T4**	**T5**	0.137	
T4	**T6**	1		**T4**	**T6**	0.212	
T5	**T6**	1		**T5**	**T6**	1	

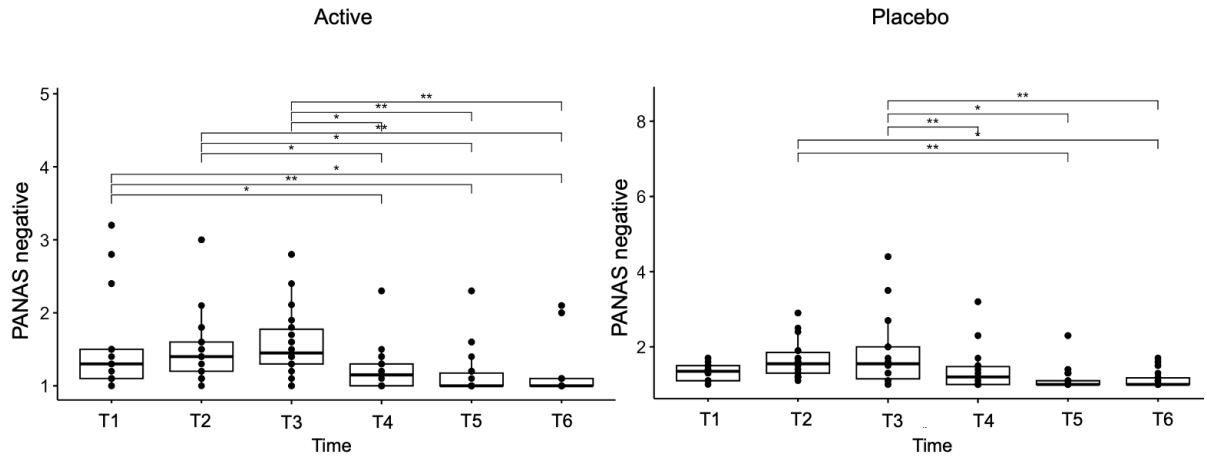

Figure 3.8. *Box-plot of PANAS negative scores in the active and placebo groups at different timepoints. The significant post-hoc tests are reported in the plot (* p<0.05, ** p<0.01, ***p<0.001).*

When the baseline correction was considered, the stress reactivity as evaluated with the PANAS negative score was no significantly different between active and placebo groups ($p = 0.668$).

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4. Discussion

The present study suggests that administering a single 20-minute session of anodal tDCS at 1mA to the left DLPFC immediately before and during exposure to psychosocial stress did not significantly affect the release of cortisol or positive and negative emotion regulation (PANAS) even if the analysis is done considering the measures acquired before the stressful event as baseline, to bring out the groups "stress reactivity". Instead in self-reported levels of state stress (VAS) if the difference before and after the stress event (baseline correction) is tested a significant dissimilarity emerges, indicating that subjects experience and judge the stress event differently, and this difference is due to the left DLPFC stimulation. Similar results were obtained in previous studies that used VAS to investigate the effects of non-invasive brain stimulation focused on the left DLPFC in postoperative spine pain (Hamed et al., 2022) and alcohol abuse (Yuan et al., 2020), but, unlike the present study, an experimental design involving multiple stimulation sessions was used in these cases.

The significant effects observed in the subjective and objective measures over time, in both stimulation groups, indicate that the MAST stress paradigm is effective, at least in healthy controls.

Since stimulation protocols may vary according to the purpose of the specific study it is useful to consider that a current strength of 1-2 milliamperes (mA) is usually used and the duration of the stimulation is typically around 20-30 minutes. Stimulation duration has been shown to modulate the length of time before cortical excitability returns to baseline levels post-stimulation (Nitsche et al., 2001). For example, receiving 9 min of tDCS created after-effects of up to 30 min, whereas stimulating for 13 min increased this time to 90 min. Morover tDCS stimulation can be administered in single sessions or repeated over multiple

days: interventions comprised of multiple sessions can induce enduring therapeutic effects and can ameliorate symptoms of several major psychiatric disorders, both acutely and in the long-term (Kekic et al., 2014).

Our results demonstrate a difference in VAS scores between the active and sham groups, a novelty compared to other studies available to date that have adopted a similar protocol to study the emotional processing by using anodal tDCS over the left DLPFC: the VAS on mood does not show changes stimulating with a single session of 1 mA current for 10 or 20 minutes (Peña-Gómez et al., 2011; Nitsche et al., 2012). Although tDCS has previously shown no influence on VAS scores it is important to note that differences in VAS scores have been recorded in studies stimulating left DLPFC with TMS (Pascual-Leone et., al 1996).

The results of the cortisol concentration and PANAS scores are in line with the previous literature where generally no influences related to this kind of stimulation were recorded (Peña-Gómez et al., 2011; Plazier et al., 2012; Morgan et al., 2014; Wolkenstein et al., 2014; Carnevali et al., 2019), but even here there are exceptions, especially for the PANAS (Plewnia et al., 2015).

It should be noted that if autonomic nervous system outcomes obtained with instrumental recordings, such as hearth rate variability (Carnevali et al., 2019) or galvanic skin response (Feeser et al., 2014) are evaluated, clear influences due to left DLPFC stimulation with tDCS emerges.

Transcranial direct current stimulation applied to the left DLPFC has been extensively studied as a possible tool to modulate the emotional, or psychosocial, stress response. However, many studies focused on both left DLPFC stimulation (Vierheilig et al., 2016; Baeken et al., 2018; Deldar et al., 2018; Voss et al., 2019) and right DLPFC stimulation (Brunoni et al., 2013; Bogdanov and Schwabe, 2016) did not find tDCS effects on emotional stress reactivity. Conversely, other studies targeting left DLPFC (Boggio et al., 2009; Peña-Gómez et al., 2011;

Maeoka et al., 2012; Rêgo et al., 2015; Carnevali et al., 2019) or right DLPFC have found a significant decrease in emotional stress reactivity after tDCS or at least in a subset of emotional outcomes (Plewnia et al., 2015).

Therefore, in some studies targeting DLPFC, anodal tDCS lowered emotional stress reactivity, while in other the studies did not show significant effects on similar outcomes.

The data from our study show that the effects of a single session of DLPFC tDCS stimulation may not be strong and stable enough to induce objectively relevant effects on psychosocial stress reactivity in young healthy individuals, but, at the same time, indicate that there is a subjective effect that is promoted.

The present results should therefore be considered as work in progress and indicative for the dependence of tDCS effects on various technical, contextual and task-related factors.

Despite the abundance of studies in literature and the heterogeneous results, the current study demonstrates a limited impact of a single tDCS session on the reactivity to acute psychosocial stress. These findings call for further exploration on how prefrontal tDCS can be enhanced to produce objectively significant effects on stress reactivity, measurable by laboratory markers.

One potential strategy to improve effectiveness is to administer a series of multiple stimulation sessions rather than relying on a single session. In addition, it has been suggested that combining tDCS sessions with a task that activates or trains the specific neural process targeted by the stimulation can enhance its effectiveness, several studies have proposed that the largest effects of tDCS are observed when neural networks and cognitive functions are activated or trained during the stimulation process (Martin et al., 2014; Gill et al., 2015; Mancuso et al., 2016; Pisoni et al., 2018; Simonsmeier et al., 2018), possibly due to the fact that synaptic activity may be a prerequisite for tDCS effects to occur (Kronberg et al., 2017).

Similarly, combining prefrontal tDCS with cognitive behavioral therapy (Bajbouj and Padberg, 2014) can enhance treatment response in depression, PTSD, and anxiety disorders

(Segrave et al., 2014; Li et al., 2016; Kozel et al., 2018; Chalah and Ayache, 2019). However, it is worth noting that prefrontal non-invasive stimulation (eg. tDCS, TMS) by itself, without any cognitive practice or therapy, does not appear to produce lasting improvements in cognitive performance in neuropsychiatric patients (Martin et al., 2016, 2017).

This highlights the potential benefits of combining tDCS with cognitive practice or cognitive therapy for the augmentation of its effects on emotion regulation processes with consequences on the individual psychosocial stress response.

Conclusion

This study provides a comprehensive analysis of the immediate effects of single-session left prefrontal tDCS on psychosocial stress reactivity.

The findings suggest that there is not an objective significant effect of stimulation on stress reactivity, measured with salivary cortisol concentration, but there is a subjective difference in judging the level of stress induced by the controlled stress event, indicating that it is possible to influence how the subject feels the psychosocial stress through stimulation.

However, it is important to note that the effects of tDCS on stress responses may be influenced by a range of technical, experimental, neurobiological, and mental state factors that vary across subjects. Therefore, it is premature to draw conclusive results on the overall direct effects of prefrontal tDCS on stress responses.

These initial findings imply that further research is necessary to better understand the potential of prefrontal tDCS as a tool to modulate the stress response. Additionally, it will be essential to develop and integrate this technique with other interventions to optimize its effectiveness.

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