Offline tDCS modulates prefrontal-cortical–subcortical-cerebellar fear pathways in delayed fear extinction

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Declarations of interest: none

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Abstract

Transcranial direct current stimulation (tDCS) has been studied as an add-on enhancer of extinction-based treatments for anxiety disorders. However, previous studies have failed to address one issue of translational significance: the to-be extinguished fear memory must be consolidated. Additionally, extant literature shows conflicting results about the anxiolytic effect of tDCS delivered to the right dorsolateral prefrontal cortex. To address these issues, we recruited thirty-four female participants to participate in a two-day fear conditioning procedure. On Day 1, laboratory fear acquisition was confirmed by self-reported contingency ratings. On Day 2, participants were randomly assigned to either the control group (n=18; completed the extinction session) or the tDCS group (n=16; whom received 20min tDCS session of 1mA [cathode – F4; anode – contralateral deltoid immediately before extinction).

Functional magnetic resonance imagining data showed that tDCS modulates the late phase of the delayed extinction process. In particular, neural activity of the frontal middle cortex, the left frontal superior cortex, and the left and right paracentral and postcentral cortices is increased during the processing of the CS-, supporting the processing of uncertainty. Furthermore, during the processing of threat, tDCS leads to an increased information flow from the contralateral prefrontal cortex along the cortical–amygdalo–hippocampal–cerebellar pathways. Thus, the boosted processing of uncertainty and the stronger coupling during threat processing might well be the mechanisms by which tDCS boosts stimuli discrimination, leading to decreased symptoms and enhanced clinical gains.

Keywords

Fear; Conditioning; partial conditioning; Delayed extinction; fMRI; tDCS; PPI; cortical–amygdalo–hippocampal–cerebellar pathways; Anxiety

Introduction

Anxiety disorders represent the most prevalent psychiatric conditions worldwide (1,2), negatively impacting individual lives, and impairing functioning in professional and social contexts (3). Notwithstanding the diversity and accessibility of pharmacological treatments targeting anxiety disorders, those currently available show limited efficacy (4) and present constraints with respect to tolerability and safety profiles (5). As for psychological treatments, despite their increased tolerability, their efficacy rates are no better than pharma (6,7) and are time-consuming. Hence, albeit the combination of medication with psychological treatments seems to be an option, it still presents limited therapeutic gains (e.g. . (8,9)). Aiming at exploring tDCS effect as an alternative medication-free treatment to decrease anxiety related symptoms, in this translatiolnal study we mapped its impact during experimental fear extinction.

Non-invasive brain stimulation (NIBS) strategies such as tDCS have exhibited promising effects in decreasing different psychiatric symptoms, either as a stand-alone or combined with pharmacotherapy and psychological interventions (10). Among NIBS, tDCS is the most portable and user-friendly technique, displaying a high safety and comfort profile (11). tDCS consists of a direct current delivered through electrodes aimed at changing cortical excitability by increasing or decreasing neuronal membrane polarity (12) or by altering the oscillatory activity of a particular network (13).

The effectiveness of tDCS in managing symptoms of anxiety has been anecdotally suggested in pre-clinical and clinical studies (14–16). More extensively, several laboratorial studies have used tDCS to study classical conditioning of the fear response - the translational model for the development, maintenance and elimination

of anxiety symptoms. According to this model, the fear response is elicited by environmental threats through an associative learning whereby the neutral stimulus (conditioned stimulus, CS+) is paired with an aversive stimulus (unconditioned stimulus, US) and a danger predicting value is assigned to it, eliciting the conditioned fear response (CR; 14). A deregulated fear response may lead to incorrect detection of danger and sustained anxiety-related symptoms (e.g. hyper-vigilance and excessive worrying). Thus, to eliminate the consolidated CR, extinction (the translational equivalent to exposure-based therapies) is implemented, consisting of consecutive presentations of the CS+ alone, leading to a new learning that inhibits the CS+/US association (18).

By delivering tDCS to the right or left DLPFC to enhance fear extinction, researchers aimed to artificially modulate brain activity and decrease the fear response (19–22). Counterintuitively, whereas previous studies have shown that tDCS using the anode electrode over the DLPFC leads to decreased fear, this gain was also associated with the adverse effect of decreasing stimuli discrimination (21,23). Differently, using the cathodal electrode over the DLPFC led to no effect in decreasing fear but seemed to result in increased stimuli discrimination (22,24). Such perplexing findings suggest the need to better understand the mechanism by which tDCS interferes with the fear network.

During fear extinction, participating regions compose the prefrontal cortical–amygdalo–hippocampal–cerebellar pathways of which the key structure is the amygdala (25). In healthy subjects, an increased activation of the amygdala is expected after fear conditioning and before extinction. Conversely, decreased activation is expected after extinction, setting the neural correlate of successful elimination /reduction of the fear response (26). The decreased activity of the

amygdala found in healthy subjects (during and after extinction) is due to excitatory as well as inhibitory projections from the anterior cingulate cortex (ACC) and the ventromedial prefrontal cortex (vmPFC; 24,25). Anxious individuals though, show aberrant downregulation of the amygdala, with its activity persisting after extinction (29).

The hippocampus is responsible for processing context features during the acquisition of the fear response (30,31). Additionally, the hippocampus participates in the consolidation of the fear memory along with the basolateral amygdala, the entorhinal cortex, the parietal and cingulate cortex (32). Furthermore, the hippocampus involvement is found in fear retrieval and extinction working in tandem with the vmPFC and the amygdala (33–36).

fMRI studies have also suggested the cerebellum participates in the extended fear network, associated with the regulation of the autonomic response during the early (37) and the late phases (38) of fear extinction, and in the processing of time, valence and prediction of stimuli contingency (for a review see 36).

Besides fMRI studies, electrophysiological studies have also shown the fear pathway. For example, the artificial induction of synchronized theta frequencies lead by the prefrontal cortex produces a coherent corresponding spike firing in the hippocampus and the amygdala (40,41). These coherent theta firing seems to correspond to processes of appraisal and experience of fear (42), fear extinction learning (40,41) or fear extinction reconsolidation (43). Also, the reduction of gamma power in the ACC and in the vmPFC is correlated with fear extinction (44).

The translational value of extinction for anxiety disorders relies on the specificities of delayed extinction, which allows for the temporal gap between learnings (the fear response acquisition and the fear response extinction) and the fear

response consolidation (45), mimicking real-life symptom onset and treatment. However, previous studies have used within-session acquisition and extinction learning procedures not allowing for a consolidation period (for a recent meta-analysis see 43). Consequently, what has been claimed the laboratory analogue for exposure-based therapies has, in fact, targeted recent memories which are known to present distinctive neural patterns and to be qualitatively more susceptible to interference (47).

Because long-term consolidated memories are a typical feature of treatments in anxiety disorders, to understand how tDCS interacts with the network when processing delayed extinction is of the utmost importance; otherwise, it compromises translational findings. To our knowledge, there is only anecdotal fMRI data that directly explore the effect of tDCS delivered to the right dorsolateral prefrontal cortex (rDLPFC) in delayed extinction. As such, we aimed to fill the gap by implementing a two-day fear conditioning procedure and observing how a single 20-min tDCS session over the right dorsolateral prefrontal cortex (rDLPFC) interferes with the neural activity and connectivity patterns of the prefrontal cortical–amygdalo–hippocampal–cerebellar pathway during delayed extinction. We hypothesized that off-line tDCS over the rDLPFC would lead to activity and connectivity changes in the entire pathway critically involved in the pathogenesis of anxiety disorders, from the frontal regions up to the cerebellum.

Materials and Methods

Participants

Forty-eight female participants were recruited to take part in the experiment, having given their informed consent according to the local ethical committee and

compliant with the Declaration of Helsinki recommendations. Three participants withdrew from the study after feeling discomfort during the experimental task. Data from eight participants were excluded because they did not acquire the fear response, and data from three participants were excluded due to technical problems. As such, 34 women completed the experiment, and were assigned to either the control (N=18), or tDCS group (N = 16). Due to technical problems, we lost data from one patient concerning US intensity, and ASI-3-PT, and from two patients in BSI; see Table 1 for details).

Exclusion criteria for participation included: (1) history of psychiatric disorders; (2) use of psychoactive medication; (3) pregnancy; (4) caffeine and/or alcohol intake 24 hours before sessions; (5) physical exercise two hours before sessions; (6) having had a meal two hours before sessions (48); (7) auditory or visual (non-corrected) deficits; and (8) the typical exclusion criteria for MRI scanners. Additionally, participants had to show fear acquisition on Day 1, to allow for observations on fear extinction processes on Day 2. Based on previous literature, the sample size was established at a minimum of 15 subjects per group (26,49,50).

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TABLE 1

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Procedure

Participants completed a partial-reinforcement auditory fear conditioning procedure for two consecutive days, allowing for the fear memory to consolidate (Day 1 – psychological assessment, habituation to stimuli, fear response acquisition; Day 2 – [tDCS session for the tDCS group, followed by] fear extinction). Before starting, participants underwent a brief assessment to cover for the exclusion criteria.

Also, the volume intensity of the US was individually set. The fear acquisition procedure occurred in the laboratory and the delayed fear extinction procedure inside the MRI scanner. For the first day, we collected skin conductance responses (SCRs) to measure the autonomic fear response, as well as self-report ratings on valence, arousal contingency and expectancy. For the second day we collected structural and functional MRI data.

Stimulus. Three stimuli were presented – the unconditioned stimulus (US), the conditioned stimulus (CS+), and the control stimulus (CS-; never paired with the US). As for the US, we used a woman's scream selected from the International Affective Digitized Sound System (IAPS, item 277; 48). The US volume was individually set in the first session between 90–96dB (according to the participants' comfort level), testing step-wise with a dummy aversive sound (IAPS, item 276; 48) added by a Visual Analog Scale for pain to measure discomfort (52) while the sound was delivered through noise-cancelling headphones. In the session proper, the US was presented for 2s. The CS+ and CS- were 12x12cm squares either blue or yellow (colours were counterbalanced across participants), with a white background, presented in a DELL P2012H monitor for 4s across sessions. Across sessions, we pseudo-randomized the CS+ and CS- trials such that no more than two consecutive presentations of the same category were allowed.

We used E-Prime (2.0.10.353 Standard SP1, Psychology Software Tools, Pittsburgh, PA) scripts to design, collect and analyse data. US triggers and onset markers were sent to a second computer where we collected the electrodermal activity across habituation and acquisition.

Day 1 – Habituation and fear acquisition. Participants were randomly assigned to the experimental groups (tDCS or control). The habituation phase

consisted of 8 non-reinforced CS+ and 8 CS- presentations. The acquisition phase consisted of a partial reinforcement procedure at 75% (i.e. 12 out of 16 CS+ presentations were paired with the aversive sound). US presentations overlapped with the last 2s of the CS+. In total, there were 16 CS+ and 16 CS- trials, each followed by a jittered interstimulus interval (ISI; a black fixation cross presented in the centre of the screen for 10 to 12s). The acquisition of the fear response was considered successful whenever in the late phase of acquisition (last 5 trials) the mean SCRs to the CS+ was higher than the mean SCRs to the CS-. For those showing faster habituation to the US in the late phase, we alternatively considered the middle phase (from the 6th to 10th trial inclusively). We assumed .01 μS as the minimum difference (53). Participants that showed fear response on Day 1 were invited for the second day and were instructed not to use hair products or have wet hair for the next session to avoid interference with tDCS stimulation.

Day 2 – tDCS session and delayed fear extinction procedure. Participants assigned to the tDCS group started Day 1 by completing the 20-minute tDCS session (including 30s of ramp up and 30s of ramp down), with a current intensity of 1mA, delivered through a tDCS 1-channel stimulator (TCT Research Limited, Hong Kong). The cathode was placed over the right DLPFC (F4, 10/20 international system) and the anode over the contralateral deltoid muscle (extra-cephalic montage). The sponges covering the electrodes were 5x5cm in size and were saturated with 10 mL of 0.9% saline solution immediately before the stimulation session. tDCS adverse effects were assessed immediately following stimulation (Table S3 in supplementary materials) and participants were then led to the extinction procedure inside the MRI scanner. Participants assigned to the control group went directly to the extinction session inside the MRI scanner. Before starting

extinction, participants were asked to recall the colour of the square paired with the US on Day 1, and no further information was offered about the auditory stimuli. The extinction session consisted of 10 CS+ and 10 CS- trials. Each trial consisted of a fixation cross presentation for 16s followed by the CS+ or CS- presentation for 4s. During extinction, the US was never presented. The stimuli were presented using an Avotec projector, controlled by "A Simple Framework" (54) under MATLAB R2014a (The MathWorks Inc., Natick, MA, USA). The presentation was then back-presented through a mirror attached to the head coil. Participants viewed the images passively in a block design for 7 minutes.

Data Acquisition, pre-processing and analysis

Psychological questionnaires. Data on baseline psychological status according to the Anxiety Sensitivity Scale –3-PT (55), the Behavioural Symptoms Inventory (56), the State Anxiety Inventory (STAI-1) and Trait Anxiety Inventory (STAI-2; 54) were collected using the experiment computer, screen and keyboard.

Skin conductance responses. We collected electrodermal activity on Day 1, using a pair of Powerlab 26T finger electrodes (MLT116F; ADInstruments, Ltd., Dunedin, New Zealand) attached to the medial phalanges of the index and middle fingers of the left hand. These electrodes were connected to a galvanic skin signal Amplifier (FE116; ADInstruments, Ltd., Dunedin, New Zealand) that collected data every 200 ms, filtering out frequencies above 50 Hz. The signal was pre-processed in MATLAB (2013, The MathWorks, Inc, Natick, Massachusetts, United States) using in-house scripts. We calculated the SCRs on a trial-by-trial basis using the first 2s after the onset of each stimulus. The 500 ms pre stimulus onset were used as baseline. For the SCRs, the first peak minus baseline values per trial were square

rooted (53). Responses starting before stimulus presentation were considered artefacts and were manually deleted.

Self-report measures. At the end of habituation and acquisition sessions, we collected offline valence and arousal self-reports per stimuli using Lang's (1980) nine-point Self-Assessment Manikin (SAM) scales (58). For valence, we used a 1-9 scale (1 = *highly unpleasant*; 9 = *highly pleasant*); for arousal we used a 1-9 scale (1 = *highly calm*; 9 = *highly excited*), for the expectancy of US presentations paired with the CSs presentation we used a 0-9 scale (0 = *I was sure the sound was not coming;* 9 = *I was sure the sound was coming*; finally for the contingency awareness concerning the CSs/US association we used a 0%-100% scale, in increments of 25% (0% = *the CS was never paired with the US,;* 100% = *the CS was always paired with the US* (22). Participants were asked to register their answers with the use of the keyboard.

MRI data. MRI data was collected on a 3T Siemens Magnetom Trio Tim scanner with a 12-channel head coil. We collected high-resolution structural T1 weighted images, using a MPRAGE pulse sequence (repetition time [TR] = 2530 ms; echo time [TE] = 3.29 ms; flip angle: 7 degrees; field of view [FOV] = 256 × 256 mm; matrix size: 256 × 256; voxel size: 1×1×1 mm; slice thickness = 1 mm; number of slices = 192 ascending interleaved). An EPI pulse sequence was used for T2* contrast (TR = 2000 ms; TE = 30 ms; flip angle: 90º; FOV = 256 × 256 mm; matrix size: 64×64; voxel size = 4×4×4 mm; slice thickness = 4 mm; number of slices = 30 ascending interleaved even-odd slices). We discarded the first two volumes of each run allowing for signal equilibration. For pre-processing proposes, we used SPM12 software version 7219 and in-house MATLAB scripts.

Definition of regions of interest (ROIs). To understand the activity and connectivity of the fear network, and how this is modulated by tDCS, we defined a set of cortical regions of interest - the bilateral DLPFC, and the vmPFC using neurosynth.org data (http://neurosynth.org/). Bilateral DLPFC were defined from automated meta-analysis out of 362 records, using the term "DLPFC" and the reverse inference method. We created a spherical ROI of 8mm radius using SPM toolbox Marsbar (http://marsbar.sourceforge.net/), centred over the significant cluster (Montreal Neurological Institute [MNI] coordinates; xyz = +-42,34,32) and checked its overlap with our stimulation site (F4) using *tool mni2tal* (Yale Bioimage suite; http://sprout022.sprout.yale.edu/mni2tal/mni2tal.html). To define the vmPFC we followed the same pipeline, using the term "vmPFC" and the reverse inference method. The vmPFC was defined from automated meta-analysis out of 199 records, creating a spherical ROI with 8 mm radius centred over the significant cluster (MNI coordinates; xyz=0, 30, -16).

A grey matter mask was used to reduce the number of multiple comparisons in each analysis (MNI grey matter mask template downloaded from Brain Imaging Centre, Montreal Neurological Institute, McGill University - https://digital.lib.washington.edu/researchworks/handle/1773/33312).

All masks were normalized into MNI space, binarized using SPM12 and finally checked for correct alignment.

Functional magnetic resonance imaging. SPM12 was used to analyse fMRI data (version 7219; https://www.fil.ion.ucl.ac.uk/spm/) in Matlab environment (9.3.0.713579 R2017b; https://www.mathworks.com/). Before pre-processing, we manually set the coordinates of the anterior commissure of the anatomical scan to zero and applied the same transformations to the functional scans of every subject.

The pre-processing of the functional data included slice-timing correction (sync interpolation), motion correction with respect to the first volume of the first functional run with a 7th degree B-spline interpolation, coregistration of the anatomical scan to the first volume of the first functional run on an individual basis. Functional data were smoothed using a Gaussian kernel (FWHM of 8 mm) and interpolated to 3 × 3 × 3 mm voxels.

The general linear model (GLM) was used to fit beta estimates to the events of interest. The default high-pass filter cut-off of 128 seconds was included. The first derivatives of the six motion parameters describing volume-to-volume motion were added (not convolved) as predictors of no interest to attract variance attributable to head movement. Experimental events were convolved with a canonical hemodynamic response function.

Based on the literature (59,60) we defined four regressors of interest: CS^{+}early, CS^{+} late, CS^{-}early and CS^{-}late, where the early phase included the first five and the late phase the last five trials. Model parameters were estimated using the classical Restricted Maximum Likelihood (REML) method, with an autoregressive AR(1) to account for serial correlations. Group-level significance testing was performed using Monte Carlo simulations (with the CoSMo Multivariate Pattern Analysis toolbox [CoSMoMVPA] in MATLAB) with 10,000 iterations, corrected for multiple comparisons using Threshold-Free Cluster Enhancement (TFCE; 55).

Whole brain contrast analysis. To understand how the neural activity during fear extinction is modeled by tDCS, we performed whole brain between group contrast analysis. Phase-dependent contrasts (CS+ early *vs.* CS+ late; CS- early *vs.* CS- late) and condition-dependent contrasts (CS+ early *vs.* CS- early; CS+ late *vs.* CS- late; early phase = first five trials; late phase = last five trials) were performed.

The *a priori* hypothesis defined that there would be distinctive condition-dependent (CS+ *vs.* CS-; increased or decreased stimuli discrimination) and phase-dependent (early *vs.* late) neural activity between groups (tDCS and control).

Contrast psychophysiological interaction (PPI) analysis. To understand the CSs-specific connectivity patterns between the cortical ROIs (DLPFC, and the vmPFC) and the whole brain grey matter mask during the early *vs.* late phase of extinction, we estimated the neural activity differences of time correlations between conditions using contrast PPI analysis (62–65) in SPM12. We extracted the first eigenvariate time-series data per seed ROI, controlling for all conditions of interest (F contrast). The resulting deconvolved seed ROI time course was multiplied by the task time course (convolved with the haemodynamic response function) to form the PPI predictor (63). We then computed one GLM with three regressors: the interaction term, the seed region's extracted signal and the experimental condition (PPI.ppi, PPI.Y and PPI.P, respectively). After model estimation, a contrast map was created with the PPI interaction as the regressor of interest. Between-groups analyses (two sample *t-tests*; two-tailed) were performed with the toolbox CoSMoMVPA and the resulting group level statistical Z maps were thresholded at $|z| > 1.96$ ($p < 0.05$, TFCE corrected). Clusters were defined as significant contiguous groups of voxels regardless of its extension. The *a priori* hypothesis that off-line tDCS over the rDLPFC interferes with the fear network during delayed extinction, defined that distinctive connectivity patterns would be found in the prefrontal cortical–amygdalo–hippocampal–cerebellar pathways during extinction for the tDCS group when compared with the control group. No direction was predicted.

Results

We sought to understand how tDCS modulates the fear pathway during the extinction of a consolidated fear response. For such, we implemented a 2-day experimental procedure. On Day 1, participants were split in two groups (the tDCS and the control group), and learned to fear the conditioned stimulus in the laboratory. To confirm fear acquisition, SCRs and self-reported arousal, valence, contingency and expectancy were collected. On Day 2, participants went through extinction inside the MRI scanner, where the neural activity and connectivity of the fear network was observed.

Psychophysiological data

SCRs collected during the habituation and acquisition phases from Day 1 confirmed the successful acquisition of the fear response. As expected, we found no differences in psychophysiological arousal between the CS+ and the CS- during the habituation phase, both for the control group (t (17) = -.38, p = .710, 95% IC [-.084, .058] and for the tDCS group (t (14) = -1.35, p = .198, 95% IC [-.116, .026], suggesting that the CSs were equated at baseline in terms of the arousal elicited. After fear acquisition, the CS+ elicited more increased responses than the CS-, both for the control group (t (17) = 2.84, p = .011, 95% IC [.030, .205]) and for the tDCS group (t (14) = 2.54, p = .023, 95% IC [.011, .130]; (Figure 1).

Behavioural data

Self-ratings data on valence and arousal of the CS+ and the CS- validate the psychophysiological data. After habituation, results showed no significant differences between the CSs for the control group for the arousal (t (17) = .13, p = .897, 95% IC [-.838, .950]) and valence ratings: t (16) = -.112, p = .912, 95% IC [-1.172, 1.053]) and the same was true for the tDCS group for arousal (t (15) = .37, p =

.718, 95% IC [-.600, .850]) and valence: t (15) =-1.14, p = 2.71, 95% IC [-1.433, .433]).

After acquisition, both groups evaluated the CS+ as evoking more arousal than the CS- (control group: t (16) = 7.90, p < .000, 95% CI [2.625, 4.551]; tDCS group: t (15) = 5.37, p < .000, 95% CI [1.923, 4.452]). Similarly, both groups evaluated the CS+ as being more negative than the CS- (control group: t (15) = -4.28, p = .001, 95% CI [-4.680, -1.570]; tDCS group: t (15) = -4.99, p < .001, 95% CI [-4.995, -2.005]).

Confirming that participants learned the association between the CS+ and the US, self-reports on the CS+/US and the CS-/US contingency after acquisition showed significant differences between stimuli, both in the control group (t (16) = 5.01, p < .000, 95% CI [1.42, 3.52]) and the tDCS group (t (15) = 13.76, p < .000, 95% CI [2.48, 3.39]).

fMRI results

Whole brain contrast analysis showed two clusters where activity patterns were distinctive between groups during late extinction for the contrast CS+ > CS- . Cluster 1 (peak voxel MNI coordinates: -18, 15, 45; mean z = 2.07) included, among other areas, the frontal middle and frontal superior left cortices. Cluster 2 (peak voxel MNI coordinates: -9, -33, 57; mean z = 2.00) included the left and right paracentral region and the right post central area (TFCE corrected at p < .05). Distinctive brain responses to the CSs per group are depicted in Figure 2. Whereas the tDCS group showed increased activity in both Cluster 1 and Cluster 2 during the processing of the CS-, the control group showed increased activity during the processing of the CS+ in Cluster 2 (for detailed information regarding the clusters' activity patterns per

region according to Automated Anatomical Labeling [AAL], see supplementary material Table S1).

PPI analysis per seed ROI for the whole brain grey matter (Friston et al, 1997; Di et al., 2018), by behavioural task contrast (contrast 1: CS+early > CS+late, contrast 2: CS+early > CS-early, contrast 3: CS+late > CS-late, contrast 4: CS-early > CS-late) was used to inspect phase-dependent (early *vs.* late) and condition-dependent (CS+ *vs.* CS-) functional connectivity patterns of the fear network. The seed ROIs were the lDLPFC, the VMPFC and the rDLPFC (the stimulation site, according to our original hypothesis). To observe the impact of tDCS, we estimated two-sample t-tests for each analysis.

The contrast CS+late > CS-late was the only one showing significant differences, and these were found specifically in the connectivity between the lDLPFC and four clusters. Cluster 1 peak voxel was located in the left insula (9354 voxels; xyz=-33, 15, -12, mean z = -2.72), Cluster 2 in the right inferior temporal region (66 voxels, xyz=54, -24, -21; mean z = -2.02), Cluster 3 in the right middle occipital (89 voxels; xyz = 42, -63, 24; mean z = -2.04) and Cluster 4 in the right supramarginal gyrus (67 voxels; xyz=54, -48, 27; mean z = -2.01; TFCE corrected at $p < .05$). Figure 3 shows the location and extension of each of the four clusters, and detailed information is depicted in Table S2.

Cluster 1 involved a broad set of brain regions (68 AAL regions) known to have distinctive roles in fear extinction. To better distinguish the contribution of each within the fear network and to disentangle the pattern of correlations between the activity of the lDLPFC and each of the 68 AAL regions, we observed the interaction graphs. Results showed that during the processing of the CS+ in late extinction, participants in the tDCS group presented a cross-cutting pattern of positive couplings

with stronger correlations between the neural activity of the lDLPFC and the neural activity of several cortical and subcortical regions, when compared with the control group. Namely, the stronger correlations during the processing of the CS+ in late extinction, were found between the lDLPFC and the bilateral hippocampus, the left amygdala, bilateral insula, bilateral caudate, bilateral putamen, bilateral pallidum, bilateral thalamus, bilateral cingulate cortex (anterior, middle and posterior), several regions of the inferior frontal cortex (left frontal triangular and orbital, left and right frontal medial orbital), and regions of the superior frontal cortex (including the right and left medial parts, and the right and left frontal middle, including the middle orbital frontal), and the precuneus. Furthermore, the steepest correlations were found between the lDLPFC and the cerebellum, including the vermis. Inversely, correlations between the lDLPFC and the regions of the right paracentral and right supplementary motor cortex seemed to be weaker.

Cluster 2 included the right inferior and middle temporal lobe. Again, a pattern of stronger correlations after tDCS stimulation was found between these regions and the lDLPFC. The same was true for the contiguous Cluster 3 (which included the right angular gyrus and the right middle occipital region) and Cluster 4 (which included the angular gyrus and the supramarginal gyrus). Detailed results are depicted in Table S2, and Figure S1, S2, S3 and S4 in the supplementary materials.

Besides the lDLPFC, no other phase or condition-dependent two-sample *t-tests* were statistically significant for the other seeds.

Discussion

Understanding how conditioned fear is extinguished in experimental situations that mimic the therapeutic changes may contribute to the development of

targeted clinical strategies for anxiety disorders. In our study, we examined how tDCS modulates neural responses and the connectivity within the fear pathway during a delayed extinction procedure. Due to known gender differences in what concerns anxiety responses (66) we tested only females who enrolled in two experimental sessions on two consecutive days (Day 1 in the laboratory room, and Day 2 inside the fMRI scanner).

On Day 1, the SCRs as well as self-ratings confirmed that the fear conditioning procedure was effective in building an associative fear memory and consequently in producing a conditioned fear response. On Day 2, we interfered with the fear pathway by delivering off-line tDCS immediately before delayed extinction.

We applied the cathode over the rDLPFC and the anode over the contralateral deltoid. We chose F4-contralateral deltoid montage, informed by previous preclinical studies showing its effectiveness in reducing anxiety related symptoms (14,15). The DLPFC is a hub region associated with the appraisal of fear experiences (42) and is also involved in fear extinction learning (46). Hence, the rDLPFC is interconnected with the vmPFC to downregulate the amygdala activity through emotion regulation processes and value detection (50). Moreover, current modelling literature shows that increased current intensity does not occur under the electrode but rather between electrodes (67), thus supporting our hypothesis according to which this montage allows for modulating the entire pathway that relies between electrodes and beyond. That is, we would interfere not only with cortical structures (such as the rDLPFC, and other frontal, motor and parietal areas) but also with subcortical structures and the cerebellum.

Importantly, our fMRI contrast analysis results confirmed the study hypothesis, showing that tDCS over the rDLPFC interfered with delayed extinction

learning by impacting the neural activity of two clusters during the processing of the CSs: Cluster 1 encompassed the frontal middle and frontal superior left regions, and Cluster 2 encompassed the paracentral and postcentral areas.

In the tDCS group, Cluster 1 frontal regions showed a steeped decrease during the processing of the CS+ compared to controls, but an increased activity during the processing of the CS-. Such distinctive stimuli-dependent response may be the neural correlate of the increased stimulus discrimination reported in previous studies (21,22). This is further supported by the decreased BOLD signal percentage change during the processing of the CS+ (supporting a consolidated learning about its value) and the simultaneous increase activity during the CS-. This increased activity for the CS- processing in late extinction though, may also support processes of reappraisal and detection value associated with uncertainty and the somewhat resistant extinction process that is typical of partial reinforcement schedules (68,69). That is, whereas on Day 1, the CS+ was learned to signal threat and the CS- was learned to be the safe cue, on Day 2, the absence of information about the CS- (due to no US presentations) induced a state of uncertainty (for further discussion, see 63).

In Cluster 2 regions, the tDCS group showed an inverted pattern when compared to controls. For the tDCS group, the central and paracentral regions showed negative signal percentage changes during the processing of the CS+ (supporting a stronger extinction of the conditioned response), while simultaneously showing an increased activity during the processing of the CS-, similar to the Cluster 1 pattern. This increased activation of the right central and post-central (somatosensory cortex) areas during the CS- is consistently reported during extinction learning studies (46), suggesting that tDCS may have enhanced extinction processing. In fact, the somatosensory (SI) region is involved in the fear network

through direct and indirect projections to the amygdala via insula for salient stimuli. In particular, the SI participates in processing emotional significance, emotion regulation and in the generation of the emotion of fear (71).

Interestingly, there were no differences found between groups with respect to the activity of core regions, such as the amygdalae and the hippocampi. This finding may be explained by one of two arguments: either the neural activity in both groups is equivalent (regardless of tDCS) or the lack of activity is due to methodological decisions that impact signal processing detection. In fact, an absent signal in the amygdala is frequently found due to the typically fast information processing of the ventral stream structures which is not compatible with the parameters adopted to model its neural response. When analysing whole brain neural activations, modelling for shorter durations will likely capture subcortical processing, whereas modelling for longer durations will lead to loss of subcortical activations (and the lack of differences detected in the BOLD signal in those regions) while gaining in cortical processes, such as memory and emotion regulation (72).

The impact of tDCS on the fear pathway was definitely confirmed with contrast PPI analysis results. We observed that cathodal stimulation of the rDLPFC increases the functional coupling between the lDLPFC and four clusters, during threat-specific processing in late extinction. This pattern of increased coupling and activation in the contralateral regions to the stimulation site has been previously reported in the literature. For example, Hanaoka and colleagues (2007) found a significant decrease in oxy-Hb during rTMS, followed by a significant increase in oxy-Hb in lDLPFC after low frequency rTMS (LF-rTMS) stimulation over the rDLPFC (73). Both LF-rTMS and tDCS using cathodal stimulation over the rDLPFC

inhibit neural excitability, and tDCS effects can last up to one hour (74), which explains the contralateral effect we have found during late extinction.

Cluster 1 was the broader cluster detected in between group analysis, and included 68 AAL regions, all of which participating in the prefrontal cortical–amygdalo–hippocampal–cerebellar pathway: the hippocampi, left amygdalae, insulae, basal ganglia, thalamus and the anterior to posterior cingulate cortex, along with the inferior, middle and superior frontal cortex, up to the precuneus and finally reaching the anterior and medial regions of the cerebellum. This stronger coupling found in the tDCS group between those regions and the lDLPFC, during the processing of the CS+, suggests that tDCS effectively impacts the fear pathway synchrony. While it does not unequivocally inform in terms of direction of the information flow, it provides important clues. On the one hand, the synchrony of neural activity across the fear pathway during late extinction might be the neural correlate of an absence of inhibitory control towards subcortical structures. Thus, such synchrony would additionally lead to an increased plasticity in subcortical regions during the processing of threat. On the other hand, such lack of inhibitory control may explain the preservative long-term fear response towards the CS+, which we reported in our previous study (22).

Also, because no group differences were found with respect to the processing of the CS-, we may argue that tDCS using cathodal electrode over the rDLPFC does not produce the generalization effect found after stimulation with the anode electrode (see 18), which supports the clinical benefits described in case reports (75) .

PPI contrast analysis also showed stronger time-course correlations between the lDLPFC and Cluster 2 regions during the processing of the CS+ in late extinction. Structures in the right inferior and middle temporal cortices such as the

entorhinal cortex are crucial for memory consolidation of fear extinction (32). As temporal regions serve as interface nodules between the amygdala, the hippocampus and the prefrontal cortex, our results show the increased coordination between frontal and temporal structures and support the use of delayed extinction when modulating consolidated fear memories.

The same stronger coupling was found between the lDLPFC and Clusters 3 and 4 that included the contiguous angular gyrus, the supramarginal and the right occipital region. The lateral occipital region is expected to work in pace with the prefrontal regions in visual fear signalling (76). Similarly, the lateral occipital activity is known to be functionally connected with the angular gyrus involved with encoding and retrieval processes (77) and the supramarginal gyrus involved in early and late extinction learning (46,78). Hence, that increased correlation between Clusters 3 and 4 and the lDLPFC during the processing of the CS+ in late extinction supports an efficient extinction process.

Here we show the online pattern of activity and connectivity for the prefrontal-cortical–subcortical-cerebellar pathway during the extinction processing of a previously consolidated fear memory. Additionally, we show that the relationship across the prefrontal-cortical–subcortical-cerebellar pathway is not inhibitory or anti-coupled in nature.

However, a number of limitations should be noted. Study results must not be generalized, as the sample involved only women given the gender differences across anxiety measures and fear conditioning responses (79). Future replication studies should consider homogeneous samples of only men or heterogeneous samples to inform the field on whether and how gender interferes with the fear pathways. Second, we used a control group instead of a sham group, not blinding participants to

the stimulation and not controlling for potential placebo effects. The use of a control group was intentional as a strategy to allow for increasing the recruitment rate by including participants not willing to do tDCS, and participants not complying with tDCS safety criteria. Third, although our hypothesis did not demand measures of successful extinction, we cannot state that the patterns of neural activity observed match successful extinction learning. However, our focus is on the ongoing delayed extinction and will advance the field by offering the information about the impact of tDCS using the cathode electrode over the rDLPFC and its use as a complementary strategy to exposure-based treatments. In future studies, however, simultaneous measures of extinction (e.g. SCRs and self-reports of contingency and expectancy) should be collected.

To date, most studies in fear extinction have failed to appreciate the importance of memory consolidation during the extinction-learning phase. Our results offer a first understanding of the effect of tDCS with the cathode electrode over the rDLPFC and extracephalic anode in extinction, supporting its clinical use. Here, we compared the mechanisms at play during extinction *vs*. extinction combined with tDCS where stimulation was used to inhibit the rDLPFC, a crucial hub to emotion regulation processes. Our data suggests that tDCS increased the processing of uncertainty associated with the safety cue, by frontal regions. Additionally, after tDCS the correlation between regions of the fear pathway was stronger during the processing of threat.

More importantly, as this experimental setting mimics real-life context changes, by allowing consolidation mechanisms to take place, we can state that targeting this regulatory system is a promising venue to non-invasive brain stimulation approaches for the treatment of anxiety disorders. The question of

whether the tDCS combined with exposure-based strategies is more successful via the stimulation of the DLPFC using the cathode electrode or the anode electrode should be targeted in future studies.

ACKNOWLEDGEMENTS

We thank Soares, M.J, Gerardo, B. for data collection and pre-processing. We Thank Professor Karl Friston for his wise advise in methodological choices and analysis.

Funding: This work was supported by a Foundation for Science and Technology of Portugal and Programa COMPETE grant to AGA and JA [grant numbers PTDC/MHC-PCN/3575/2012, SFRH/BD/80945/2011, PTDC/MHC-PAP/5618/2014 (POCI-01-0145-FEDER-016836)]. The Psychology Research Centre of Minho University is supported by the Portuguese Foundation for Science and Technology and the Portuguese Ministry of Education and Science through national funds and co-financed by FEDER through COMPETE2020 under the PT2020 Partnership Agreement [POCI-01-0145-FEDER-007653].

The Cognitive and Behavioural Centre for Research and Intervention
Of the Faculty of Psychology and Educational Sciences of the University of Coimbra is supported by the Portuguese Foundation for Science and Technology and the Portuguese Ministry of Education and Science through national funds and co-financed by FEDER through COMPETE2020 under the PT2020 Partnership Agreement [UID/PSI/01662/2013].

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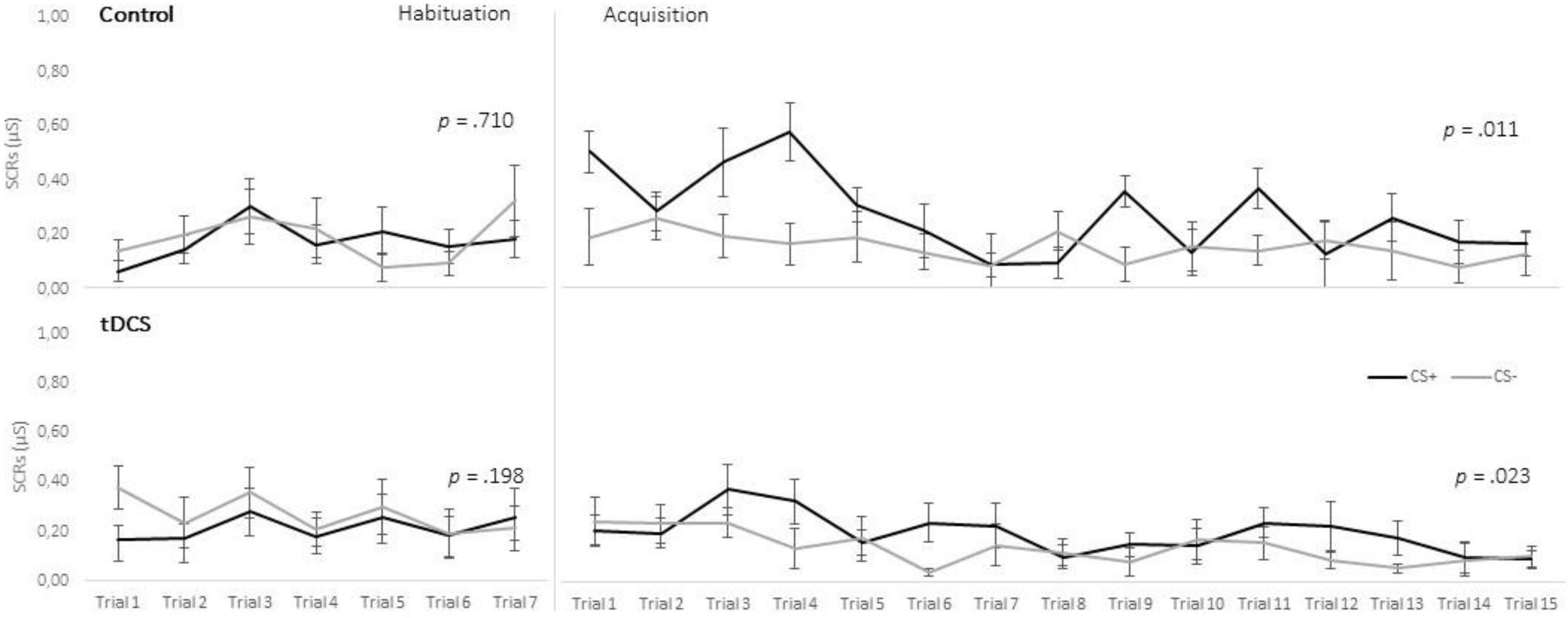
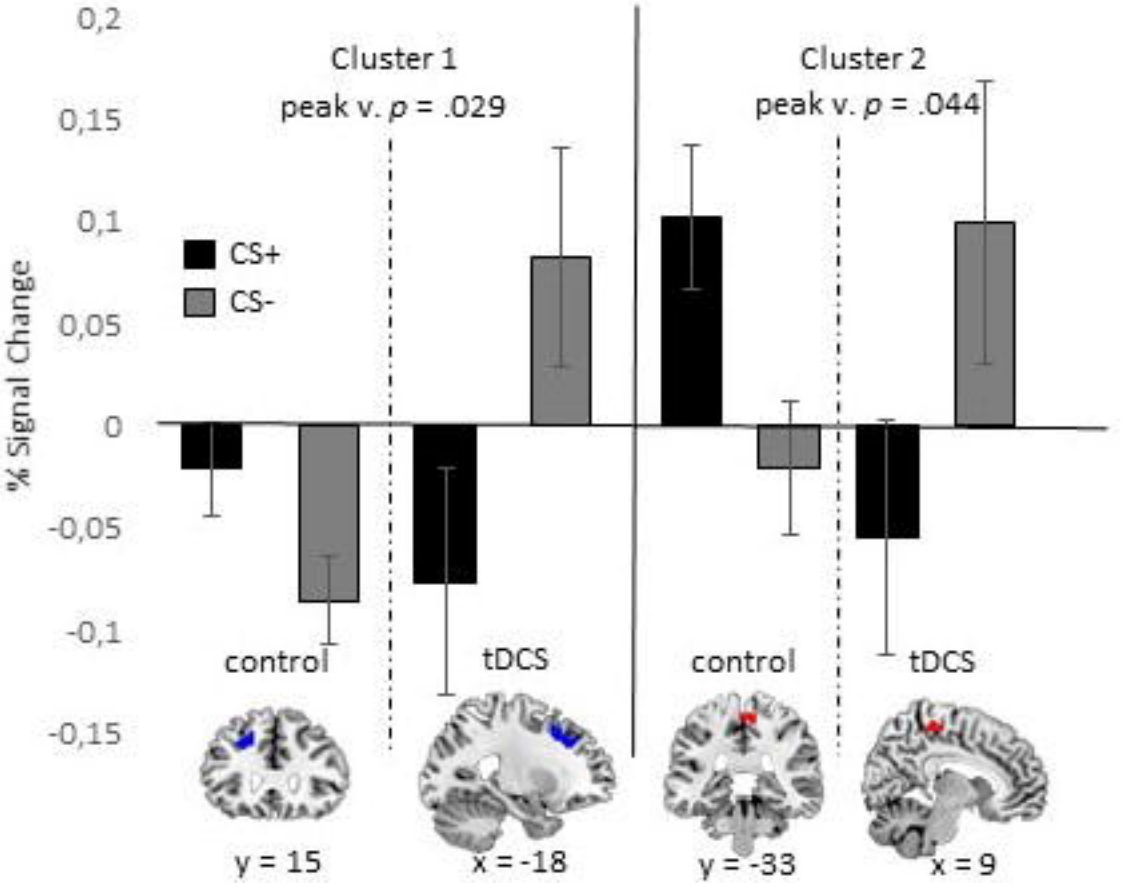
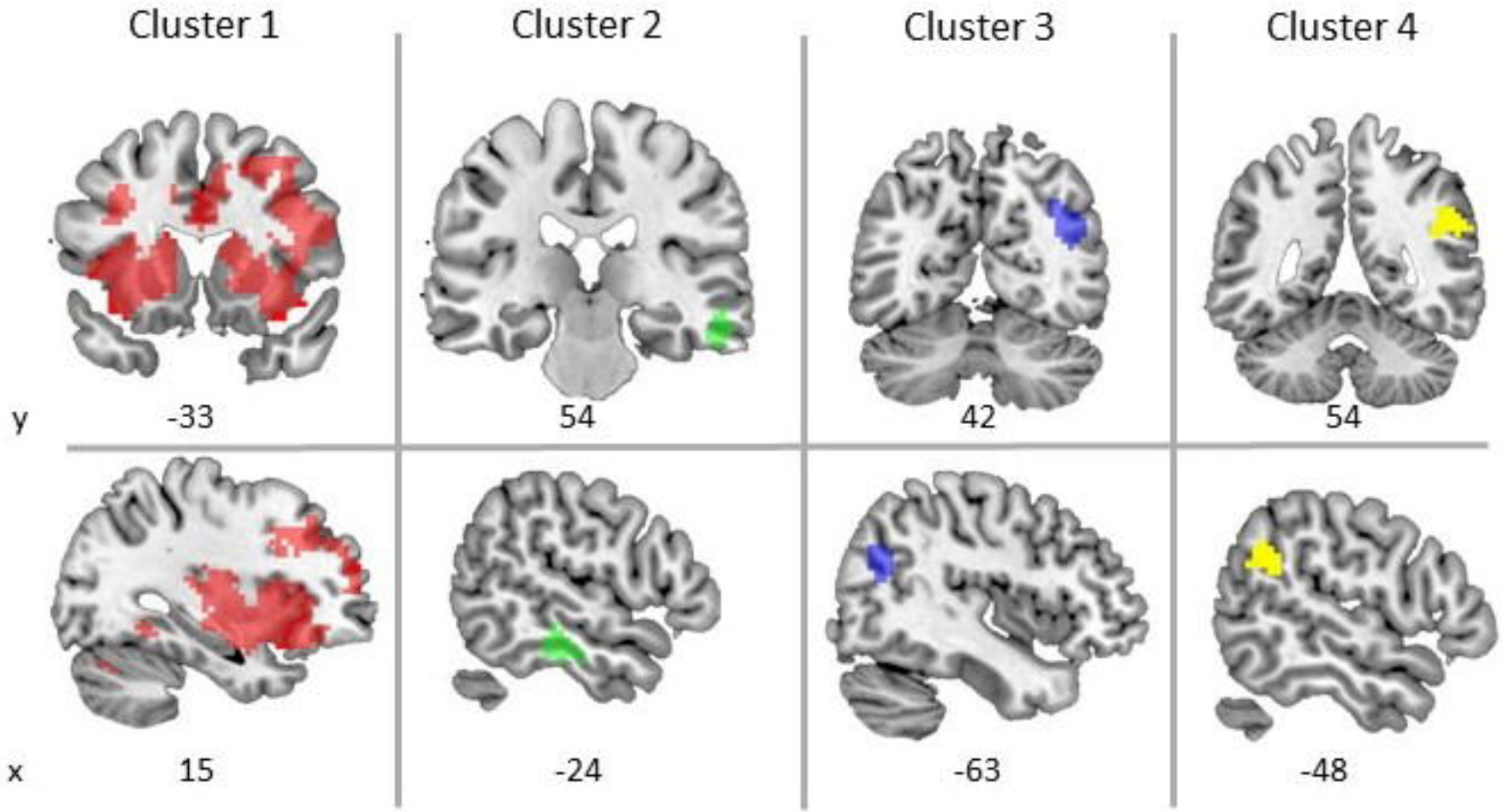

Control
Habituation
Acquisition
tDCS
SCRs (µS)
1,00
0,80
0,60
0,40
0,20
0,00
p = .710
p = .011
p = .198
p = .023
CS+
CS-
Trial 1
Trial 2
Trial 3
Trial 4
Trial 5
Trial 6
Trial 7
Trial 8
Trial 9
Trial 10
Trial 11
Trial 12
Trial 13
Trial 14
Trial 15


0,2
0,15
0,1
0,05
0
-0,05
-0,1
-0,15
% Signal Change
Cluster 1
peak v. p = .029
Cluster 2
peak v. p = .044
CS+
CS-
control
tDCS
control
tDCS
y = 15
x = -18
y = -33
x = 9


Cluster 1
Cluster 2
Cluster 3
Cluster 4
y
-33
54
42
54
x
15
-24
-63
-48

Figures captions

Figure 1. **Day 1 SCRs.** Day 1 SCRs for the habituation and acquisition procedures for the CS+ and the CS- per trial. CS+: conditioned stimuli; CS-: non-reinforced or control stimuli; tDCS: transcranial Direct Current Stimulation group; Control: control group. Error bars represent standard errors of the mean (SEM).

Figure 2. Mean Percentage signal change during late extinction per stimuli in control and tDCS group for cluster 1 (left; blue) and cluster 2 (right; red). Control: control group; tDCS: transcranial Direct Current Stimulation group. peak v. *p: p*-value in the voxel with the maximum activity. CS+: conditioned stimuli; CS-: non-reinforced or control stimuli. Error bars represent standard error of the mean (SEM). Images displayed according to the neurological convention (left-right).

Figure 3. Two-sample t-tests for group differences on lDLPFC-whole brain PPI analysis, during the processing of the CS+ in late extinction. Cluster 1 (red), cluster 2 (green), cluster 3 (blue), cluster 4 (yellow) were statistically significant at $p < .05$ (TFCE corrected). Top row: coronal view; bottom row: sagittal view; x, y = MNI coordinates. Images displayed according to the neurological convention (left-right).

Table 1. *Sample descriptive statistics.*

	Cathodal group n = 16	**Control group** n = 18	***p***	***df***
Age, Years	24.19 (5.94)	22.56 (5.47)	.410	32
Education, Years	15.25 (2.96)	15.00 (3.14)	.813	32
US Intensity, %	90.80 (3.13)	93.44 (4.50)	.061	31
ASI-3-PT	19.13 (12.10)	22.24 (15.02)	.519	31
BSI (GSI)	1.46 (.37)	1.80 (.42)	.859	29
STAI 1	37.13 (13.08)	36.72 (11.54)	.925	32
STAI 2	40.06 (11.94)	39.33 (12.06)	.861	32

Note. Mean values are depicted. The standard deviations (SD) are within parentheses. US: Unconditioned stimuli; ASI-3-PT: Anxiety Sensitivity Scale Portuguese version; BSI (GSI): Global index for symptoms intensity of the Behavioural Symptoms Inventory; STAI 1: State Anxiety Inventory; STAI 2: Trace Anxiety Inventory.