1   **Arousal levels explain inter-subject variability of neuromodulation effects**

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Abstract

Over the past two decades, the postulated modulatory effects of transcranial direct current stimulation (tDCS) on the human brain have been extensively investigated, with attractive real-world applications. However, recent concerns on reliability of tDCS effects have been raised, principally due to reduced replicability and to the great interindividual variability in response to tDCS. These inconsistencies are likely due to the interplay between the level of induced cortical excitability and unaccounted individual state-dependent factors. On these grounds, we aimed to verify whether the behavioural effects induced by a common prefrontal tDCS montage were dependent on the participants' arousal levels. Pupillary dynamics were recorded during an auditory oddball task while applying either a sham or real tDCS. The tDCS effects on reaction times and pupil dilation were evaluated as a function of subjective and physiological arousal predictors. Both predictors significantly explained performance during real tDCS, namely reaction times improved only with moderate arousal levels; likewise, pupil dilation was affected according to the ongoing levels of arousal. These findings highlight the critical role of arousal in shaping the neuromodulatory outcome, and thus encourage a more careful interpretation of null or negative results.

Keywords

tDCS; arousal; pupil; interindividual variability; neuromodulation; state dependency; transcranial electrical stimulation; tES.

41   1. Introduction

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43   Founded on decades of experimentation, transcranial direct current stimulation (tDCS) is a research

44   tool capable of interacting with the central nervous system, that has been rediscovered at the

45   beginning of this century (1). Beside its value for basic research (2), tDCS has raised great interest

46   for real-world applications, like rehabilitative interventions for neurological and psychiatric diseases

47   (3) and cognitive enhancement (or detraction) in both young and older adults (4–7). However, the

48   development of more effective and generalizable stimulation protocols has been hindered by the gap

49   between our sparse knowledge of the physiological effects and the induced behavioral impact of tDCS

50   (8). What raises most concern is the lack of replicability among tDCS studies and the interindividual

51   variability in response to tDCS (9–15). In addition to non-optimal methodological practices, such as

52   inadequate control conditions and lack of statistical rigor, a complex interplay among biological

53   differences and the level of neuromodulatory effects might be crucial in explaining the reported

54   inconsistencies across studies (16–18). In particular, state-based factors, including the specific or

55   generalized levels of activation prior and during stimulation, the initial levels of performance,

56   wakefulness, task priming or novelty, might all play a decisive role. It appears conceivable to interpret

57   the final effects of tDCS as contingent on the level of network engagement (19,20). In line with this

58   prediction, several cognitive studies have reported a clear effect of baseline levels of different mental

59   capabilities on tDCS response (21–26). Most recently, individual differences in the behavioral effects

60   of prefrontal tDCS have been associated with the levels of excitability of the targeted cortex, indexed

61   by relative concentrations of GABA and glutamate (27).

62   Notably, tDCS affects large-scale brain systems extending well beyond the area under the stimulating

63   electrode (28–31). This approach translates into a lack of focality that closely resembles the spread

64   of the noradrenergic modulatory action exerted by the locus coeruleus (LC), which subtends arousal

65   functions. Several authors have highlighted the key adaptive role of this specific midbrain system in

66   shaping behavioral performance of primates (32–36). A large body of evidence suggests that the

67 exogenous direct currents and the endogenous noradrenergic modulatory action on target cells, share

68 the same central mechanism of neuronal gain control (34,37–39). Therefore, an interrelation between

69 the two stimulating activities seems reasonable to the extent that whenever the contrast between

70 activated and inhibited units becomes sufficiently increased or decreased any further added

71 neuromodulation can likely spoil the expected results. In this regard, a recent study has shown that

72 offline anodal tDCS may hinder the LC endogenous action during response inhibition processes due

73 to the induced alterations of pre-existent neural excitability levels (40). Given the above

74 considerations, it appears evident that great part of the tDCS behavioral variability reasonably stems

75 from the interdependency between the induced cortical excitability and the varying levels of arousal

76 experienced by participants before and during the experimental sessions.

77 The aim of this study was to verify whether the behavioural and physiological responses induced by

78 a common prefrontal tDCS montage were dependent on the participants' arousal levels. We selected

79 the tDCS montage used to stimulate prefrontal cortex in attentional and vigilance tasks (40–43),

80 which is also commonly used in a variety of other settings, such as language-related, executive

81 functions, episodic and visual working memory tasks (44–47). The tDCS was applied during an

82 auditory oddball task aimed to probe cognitive performance as a function of arousal levels (48–50).

83 Our task, indeed, was purposefully designed to keep participants alerted over uncertain intervals (i.e.,

84 variable inter stimulus interval) in a way that online tDCS effects would be necessarily subjected to

85 more frequent fluctuations of arousal (51,52).

86 We tracked pupillary changes as a proxy for the LC modulatory action (50,53–55). Accordingly, we

87 used reaction times (RT) and pupil dilation peaks (PD) as measures of LC phasic response to the

88 relevant stimuli (target), and pre-stimulus pupil diameter (PrePD) as a physiological marker of the

89 LC tonic discharge activity. Furthermore, because LC endogenous activity is closely related to the

90 perceived anxiety (56–58) subjective arousal levels were evaluated by means of State-Trait Anxiety

91 Inventory (STAI-Y) (59).

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93   2. Methods

94   Mindful that the mere sensory stimulation could mimic the expected arousal effects, prior to

95   conducting the study, we ran a control experiment to validate our blind-controlled tDCS protocol

96   with respect to the potential alteration of arousal due to subjective sensations. To this end, ten healthy

97   participants were recruited. Pupillary dynamics were recorded at rest using the exact same setting as

98   in our main experiment (see section 2.3 and 2.4). Statistical analyses revealed no difference in eye-

99   blink rate and subjective discomfort between sham and real stimulation, ruling out the possibility of

100   tDCS confounding effects on arousal (see supplementary material).

101

102   2.1 *Participants*

103   Fifteen right-handed healthy participants took part in the main experiment. Data of one subject were

104   rejected prior to analyses due to the excessive noise in her/his pupil signal (i.e., interpolation rate >

105   30% of the whole epoch; see 2.3). The remaining 14 participants (8 females) had a mean age of 22.4

106   (SD = 3.9) and a mean score to the STAI-Y trait of 44.9 (SD = 4.1). Participants had no history of

107   neurological or psychiatric illness and had normal or corrected-to-normal visual acuity. Ethical

108   approval was obtained by the Ethics Committee of the IRCCS Centro San Giovanni di Dio

109   Fatebenefratelli, Brescia, Italy. All participants were given written informed consent.

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111   2.2 *Study design and task procedure*

112   A single-blind within-subject design was implemented for this experiment. The testing sessions were

113   organized in two days separated by at least 48h in order to exclude any tDCS carryover effects. In

114   each session we collected behavioral and pupil data for the whole task duration (~18 min).

115   Participants completed the task twice: at *baseline* (T1) without any electrodes mounted on their scalp,

116   and subsequently either during *sham* or *real* stimulation (T2) (Figure 1b).

117   Participants were randomly assigned and counterbalanced across two session-orders of tDCS

118   protocol, so as to rule out any extra confounding variable. Importantly, they were kept blind to the

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119   ongoing experimental condition (i.e., sham or real). However, any proportion of variability possibly

120   due to either orders of stimulation was accounted for by including the order group as an independent

121   fixed factor (see section 2.5). As for the time of the day, the same participant was tested at around the

122   same hour to control for any arousal variation due to the daily metabolic cycle and circadian rhythms

123   (60).

124   Participants seated in a soundproof dark room at the distance of about 55 cm from a 17-in LCD

125   monitor and with the only source of light provided by a grey fixation cross. The auditory oddball task

126   was presented using E-Prime presentation software (61) by means of two constant-loudness speakers

127   (Figure 1a).

128   In every task condition there was a fixed total number of trials (420) of which 20% included targets

129   (84) and 80% standards stimuli (336). The stimuli order was then pseudorandomized in a way that

130   target tones (880Hz) occurred after at least three standard tones (800 Hz). The interstimulus interval

131   was set to a range of 2.1-2.9 s and both stimuli lasted for 70 ms including 5 ms of fade in-out edit. In

132   so doing, we ensured enough time (~8 s) for any pupil dilation to return to baseline before overlapping

133   to the next target trial (50,53). Along with a short training session, participants were instructed to

134   readily press a button with their right index finger whenever detecting a target tone, and to keep their

135   gaze on the fixation cross throughout the task. Speed of response and gaze fixation were emphasized

136   before each task execution.

137   At the end of each experimental session participants were given a questionnaire to rate the perceived

138   sensations or discomforts that influenced their performance (62,63).

139   Finally, a careful screening on the amount of sleep, caffeine intake, nicotine and alcohol consumption

140   was carried out next to the above questionnaire. None of these factors was found to be associated
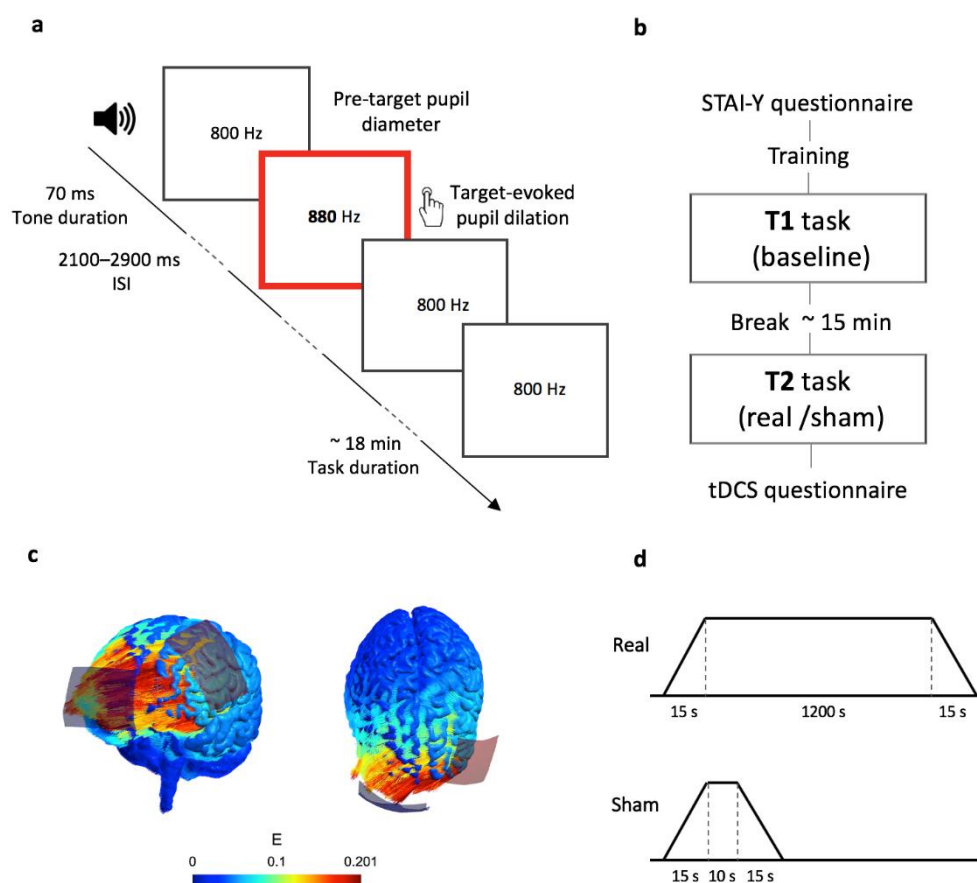
141   with either stimulation sessions.

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Fig. 1. Study design and task paradigm. a, Example of a trial sequence. **b**, Overview of the experimental timeline, showing two testing sessions each one with two task conditions: baseline and stimulation. **c**, Simulation results for the applied tDCS montage and parameters using SimNIBS toolbox (Saturnino et al., 2019). The colors denote the electric fields simulated in a default head model. **d**, Schematic representation of the stimulation protocol

2.3 *Pupil signal recording and pre-processing*

Participants seated on a chair with adjustable height allowing for the use of a fixed chinrest, and thus keeping variability in the eye-to-camera distance and visual angle as low as possible. For the pupil diameter recording, an EyeLink 1000 Plus system (SR Research, Osgood, ON, Canada) was set up at 500 Hz sampling rate with left-monocular and pupil-CR tracking mode. A 9-point calibration procedure was performed before each recording session. After the final session, participants were

171   asked to wear hand-crafted goggles whose left side incorporated an artificial eye with a 4 mm pupil,

172   carefully positioned over the subject's left eye. This allowed for a precise conversion of pupil

173   arbitrary units from the eye-tracker system output to millimeters. Pupil signal was processed offline.

174   Eye blink correction was implemented with a custom script in MATLAB (MathWorks, Inc, Natick,

175   MA, USA). A shape-preserving piecewise cubic interpolation method was chosen to interpolate

176   values ranging from 70 ms before blink onset to 300 after blink offset. Epoch segmentation (-1 s to

177   +2.5 s, relative to target onset), baseline correction (subtractive method, from -800 ms to +200 ms)

178   and visual inspection of pupil traces was carried out in the Brain Vision EEG analyzer software (Brain

179   Products GmbH, Munich, Germany). We extracted two variables of interest from pupil signal: (i)

180   pupil dilation (PD), as the peak value of the maximum dilation after targets presentation and (ii) Pre-

181   stimulus pupil diameter (PrePD) as the mean of 1 s data prior to tone presentation. All epochs with a

182   peak pupil diameter exceeding ±2 mm were rejected (50).

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184   2.4 *tDCS protocol*

185   A battery-driven current stimulator (Brain- STIM, EMS, Bologna, Italy) was used to deliver 1 mA

186   (0.028 mA/cm^2) direct current stimulation via two rubber electrodes (35 cm^2) which were inserted

187   inside two saline-soaked sponges. These were fixated with an elastic mesh stretching over the entire

188   head. In order to ensure a stable impedance level as well as keeping skin sensations at the minimum,

189   conductive electro-gel was also applied.

190   Similarly to previous studies (43), the electrodes montage consisted in placing the anode over the

191   area F3 of the EEG 10-20 system and the return (cathode) electrode over the right supra-orbital area

192   as reported in Figure 1c. The duration of the stimulation consisted of about 17 min (1040 s) with 15

193   s of currents fade-in and fade-out. Configuration of the sham condition included 15 s of fade-in, 10 s

194   of actual current delivery and 15 s of fade-out given at the beginning of the experiment only (see

195   Figure 1d).

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197   2.5 *Statistical analyses*

198   As expected, the nature of our oddball task caused ceiling effects in the correct responses for all

199   conditions (accuracy rate > 98%). All the trials that included either a false alarm or a missed response

200   were left out from subsequent analyses on RT, as well as trials corresponding to RT faster than 150

201   ms or exceeding 1.96 standard deviations from the mean (number rejected trials: $M = 3.14$, $SD =$

202   1.39). All valid RT were then log-transformed to the base e in order to ensure a normal distribution

203   of the data. We considered only trials having no missing values at the two main outcomes RT and

204   PD, resulting in 52 trials overall. Importantly, these data points were not collapsed across conditions;

205   hence *Trial* was included in the analyses as an independent fixed factor, and thus affording a greater

206   reliability and robustness of the findings.

207   In order to study the effect of tDCS on the behavioral and physiological responses, we performed two

208   linear mixed models (LMM) on RT and on PD as dependent variables. Individual (subject-specific)

209   variation was accounted for by considering *Subjects* as random effect. Fixed effects, repeated within

210   subjects, were specified for *Condition* (2 levels, *real* and *sham*), *Time* (2 levels, *T1* and *T2*) and *Trial*

211   (52 levels); whereas *Order* (2 subgroups, *sham-real* and r*eal-sham*) was considered as a between-

212   subject fixed effect. In addition, the interaction *Condition* x *Time* was assessed. Post hoc comparisons

213   were adjusted with Sidak correction for multiple comparisons.

214   The above LMM were subsequently adjusted for subjective arousal (measured by STAI-Y State

215   score) and for physiological arousal (evaluated by PrePD) in order to assess their effects on tDCS-

216   induced modulation. Akaike information criteria (AIC) was used to select the best fitted models (the

217   lower AIC the better model) and the corresponding predictors.

218   Finally, to control for any interdependence between the subjective and physiological measures of

219   arousal, we calculated Pearson's (r) two tailed correlations between PrePD and STAI-Y State score.

220   Correlations coefficients were all non-significant (p's > 0.05). All statistical analyses were conducted

221   on SPSS Statistics (IBM Corp, Armonk, NY, USA).

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223   3. Results

224   Despite the random assignment, the participants included in the two *Order* subgroups exhibited

225   different levels of physiological tonic arousal (PrePD) already at the baseline of the first experimental

226   session, that is before applying the tDCS electrodes (two tailed independent t-tests [t = -3.64, df =

227   11.82, *p* = .003]). No difference was found between the subgroups in the STAI-Y scores [t = -.41, df

228   = 11.57, *p* = .68].

229   As for the reported sensations, a Wilcoxon matched pair test revealed no significant difference

230   between sham and real stimulation [$Z = 1.34, p = 0.18$]. It was also ensured that their written responses

231   were consistent with their oral report. Therefore, it was safe to assume that participants were

232   completely unaware of the type of stimulation protocol.

233

234   3.1 Reaction times – RT

235   The unadjusted linear mixed model on RT [AIC = -2574] revealed no significant effects of the *Order*

236   [$F_{(1,11)} = .59, p = .477$] and a trend toward significance for *Condition* [$F_{(1,2419)} = 3.76, p = .053$] and

237   *Trial* [$F_{(51,98)} = 1.47, p = .05$], with slower RT occurring at the end of each tasks. A significant effect

238   of *Time* [$F_{(1,2399)} = 12.15, p < .001$] showed that performance significantly improved from *T1* [$M =$

239   5.98; *SE* = .05] to *T2* sessions [$M = 5.96; SE = .05$], indicating an overall practice effect. Importantly,

240   we found a significant *Condition* x *Time* interaction effect [$F_{(1,2403)} = 12.08, p = .001$], indicating a

241   different trend for real and sham conditions. The post-hoc comparison for *Time* revealed a significant

242   performance improvement during *sham* ($p < .001$), but not during *real* stimulation ($p = .99$). This

243   finding suggests that real tDCS hindered the practice effect that was present in the sham condition.

244   Next, LMM adjusted for STAI-Y and PrePD were separately performed (see Supplementary Table

245   1). We found an overall significant contribution of STAI-Y [$F_{(1,2312)} = 9.44, p = .002$] and more

246   importantly a significant 3-way interaction [*Condition* x *Time* x STAI-Y: $F = 19.1$, df = 3/1857, $p <$

247   .001], indicating that the subjective level of arousal affected the interaction *Condition* x *Time* on RT.

248   Specifically, STAI-Y state scores were predictive of the performance variations across tDCS

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249 conditions. During sham session a performance improvement was observed for all the continuum of

250 arousal, although it diminished as the level of STAY-Y increased. In the real tDCS condition, RT

251 proved to be faster only when the levels of arousal were low, whereas such pattern was abolished or

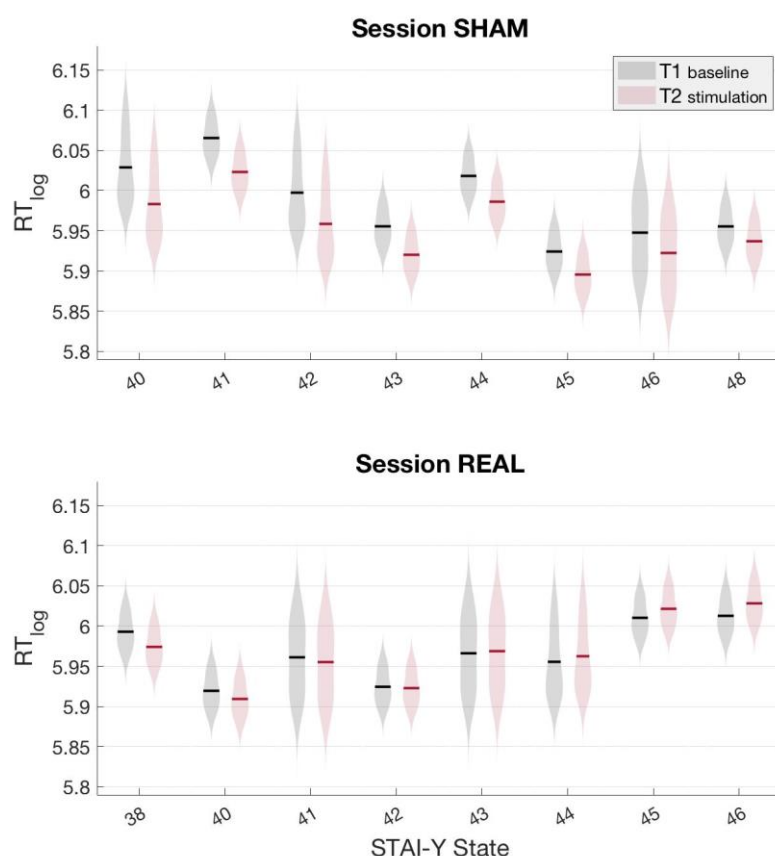252 even reversed with higher levels of arousal (i.e., higher STAI-Y scores, see Figure 2).

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Fig. 2. Reaction times by subjective arousal. Average log-based RT of model fitted values are plotted as a function of STAI-Y scores, with results from session sham (top panel) and real (bottom panel). Each mean value is marked over the corresponding distribution of the data. Colors grey and red represent the baseline (T1) and stimulation (T2) task, respectively.

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275 After adjusting for PrePD, *Condition* and *Time* remained significant [$F_{(1,2131)} = 6.88$, $p = .009$; $F_{(1,1967)}$

276 $= 9.44$, $p = .032$ respectively], although the physiological predictor did not reach statistical

277 significance [PrePD: $F_{(1,1834)} = 3.52$, $p = .061$]. Also in this case, the 3-way interaction [*Condition* x

278 *Time* x PrePD: $F_{(3,1307)} = 5.57$, $p = .001$] revealed that the interaction between *Condition* and *Time*

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279   was affected by participants' physiological level of arousal. Consistently with the aforementioned

280   effects of subjective levels of arousal, RT improvement across time was consistent in the sham

281   condition, but larger during trials with a reduced PrePD. During real tDCS, a trend toward lower or

282   no improvement was observed as physiological arousal increased (see Figure 3).
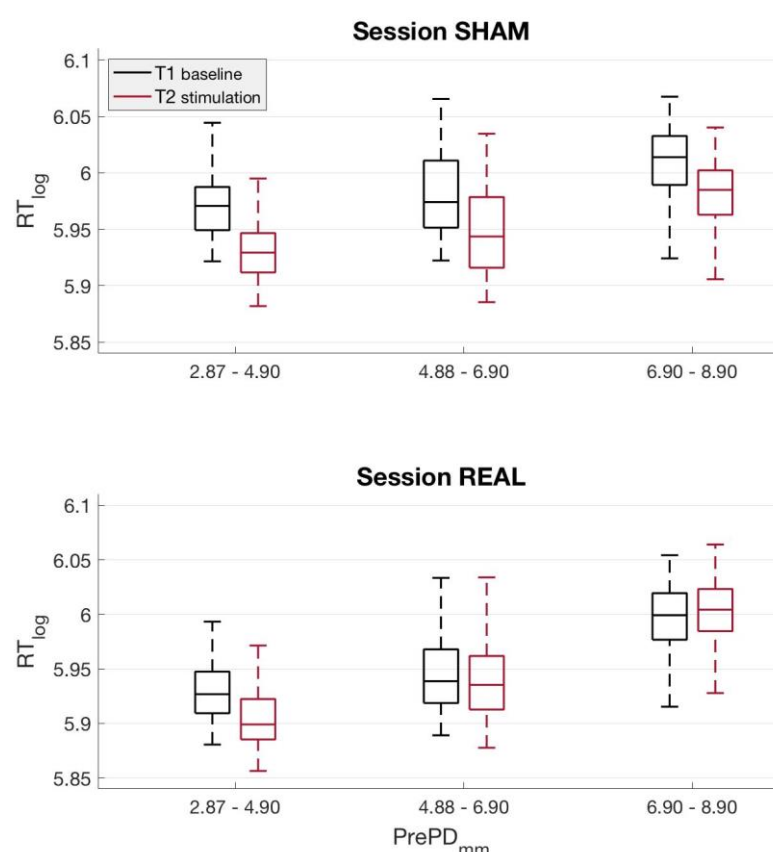
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Fig. 3. Reaction times by physiological arousal. On each box, the interquartile range, the whiskers and the median of predicted log-based RT are represented for three linearly interspaced bins of pre-target pupil diameter, with results from session sham (top panel) and real (bottom panel). Colors grey and red represent the baseline (T1) and stimulation (T2) task, respectively.

303   Based on the present results, a far more consistent trend emerged from the adjusted models as

304   compared to the same raw data (see Figure 4). This finding corroborates the importance of not

305   disregarding discrepancies rooted in interindividual differences, such as in physiological and

306   subjective arousal, but rather include them as predictors along with individual random effects.
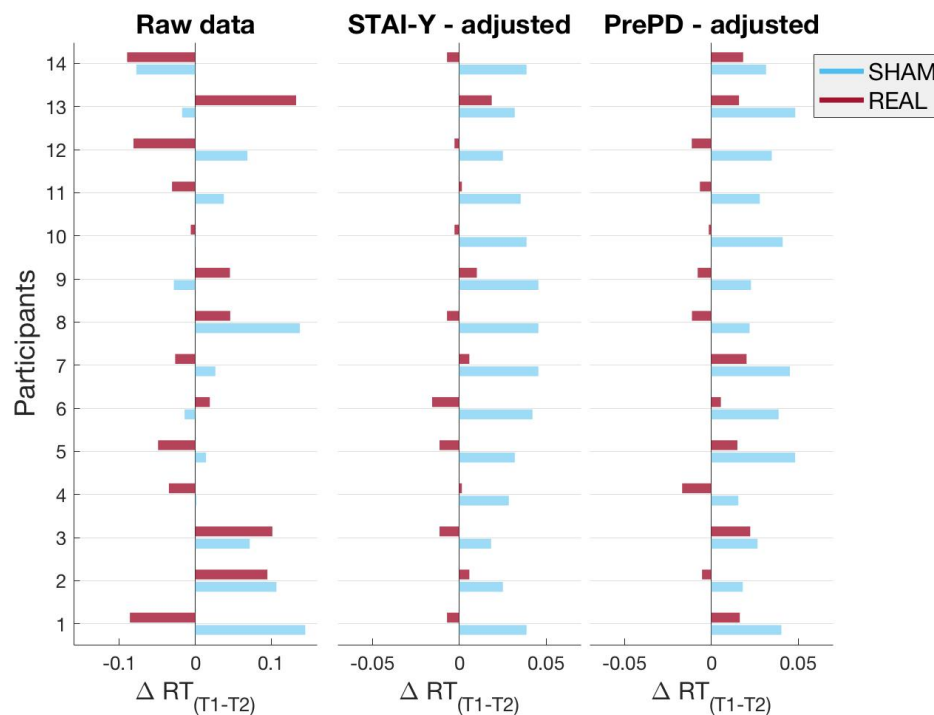
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318 **Fig. 4. Subject variability of reaction time change.** Average log-based RT differences between the baseline (T1) and

319 stimulation (T2) tasks are plotted on the vertical axis for each participant, separately for session sham (blue bars) and real

320 (red bars). A different bar plot is used to represent mean differences from raw (left panel) and fitted data from the adjusted

321 models using STAI-Y (middle panel) and PrePD (right panel) predictors. Negative and positive values on the horizontal

322 axis indicate slower and faster performance, respectively.

325 3.2 Pupil dilation – PD

326 In the unadjusted LMM on PD, [AIC = 964.66], all fixed effects were significant [*Condition*: $F_{(1,1340)}$

327 = 10.46, p = .001; *Time*: $F_{(1,1445)}$ = 15.83, p < .001; *Trial*: $F_{(51,88)}$ = 5.94, p < .001] except for the factor

328 *Order* [$F_{(1,11)}$ = 1.96, p = .18] and the interaction between *Condition* and *Time* [$F_{(1,1362)}$ = .75, p =

329 .38]. Importantly, pupil dilation decreased from *T1* [M = .375; SE = .023] to *T2* sessions [M = .34; SE

330 = .023], indicating a general habituation of the phasic pupillary responses. However, no specific effect

331 of tDCS on PD was revealed.

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332   The adjustment for STAI-Y got worse the model fitting [AIC = 986.38], making the interaction

333   *Condition* x *Time* x STAI-Y not significant [$F_{(1,1066)}$ = 1.08, p = .35] (see Supplementary Table 2).

334   On the contrary, adjusting for PrePD strongly improved the model fitting [AIC = -365.48], with

335   significant PrePD [$F_{(1,1794)}$ = 2231.23, $p < .001$] and interaction *Condition* x *Time* x PrePD effects

336   [$F_{(3,1172)}$ = 6.5, $p < .001$]. In detail, during the sham condition a decrease in pupil dilation consistently

337   occurred throughout the range of PrePD values, whereas during real tDCS the pupil dilation

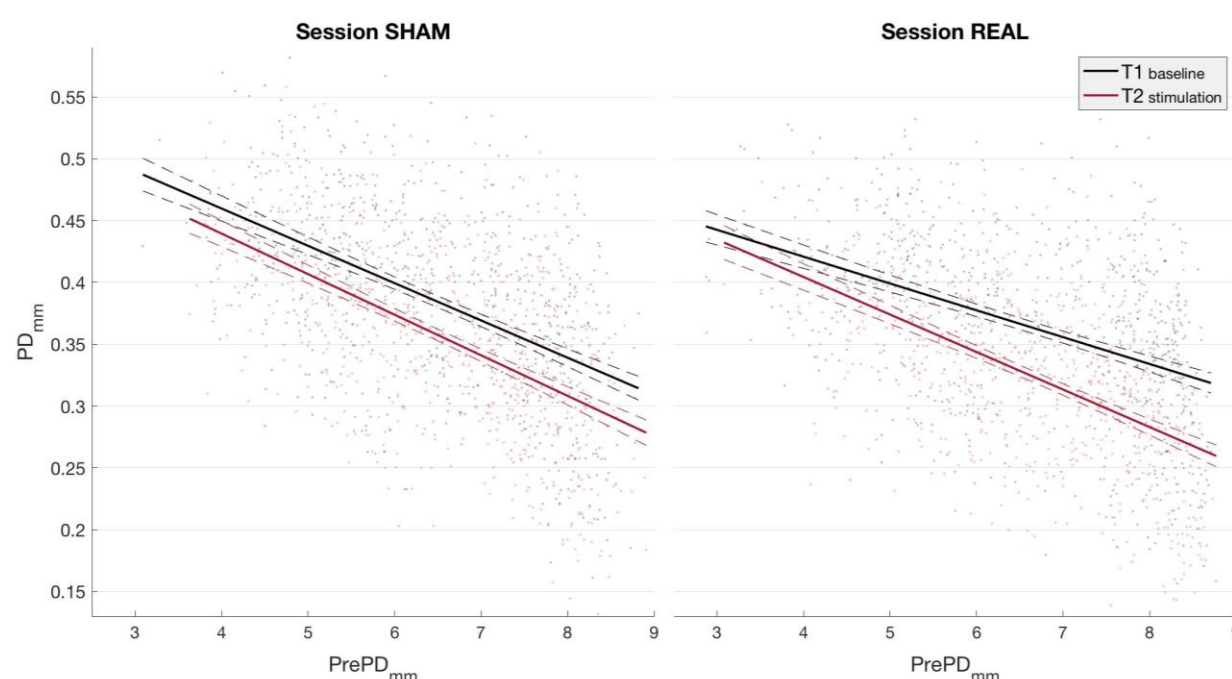338   progressively shifted toward a maximal suppression during trials with larger PrePD (Figure 5).

340   **Fig. 5. Pupil dilations explained by physiological arousal.** Model fitted PD values are plotted against pre-target pupil
341   diameter, with results from session sham (**left panel**) and real (**right panel**). Grey and red best-fitting lines describe the
342   trend of pupil dilation data points over pre-target pupil diameter respectively for the baseline (T1) and stimulation (T2)
343   task. Dashed lines represent prediction functional bounds, i.e. the uncertainty of predicting the fitted lines.

346   4. Discussion

347   In the present study, we addressed the question of whether variable effects of single session tDCS

348   could be dependent on the degree of arousal experienced before and during the experiment.

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349 Subjective and physiological levels of arousal significantly accounted for the variation of reaction

350 times across two experimental sessions. Real tDCS appeared to hinder the practice effect observed

351 during the sham condition, with a trend becoming especially evident at higher levels of arousal. As

352 for pupil dilation, its values were significantly tied to the corresponding physiological fluctuations of

353 arousal. In particular, a more reduced pupillary response emerged during real tDCS as arousal levels

354 increased.

355 These results shed light on one relevant factor, which seems to account for the paucity of consistency

356 across tDCS effects in some experiments. What effectively emerges is that arousal is predictive of

357 the modulations induced by tDCS on task performance. A number of studies, which reported a

358 considerable inter- and intra-individual variability in response to tDCS protocols, investigated the

359 impact of demographic characteristics (e.g., age, gender), cortical architecture variations or

360 physiological measures specific to the targeted areas (e.g., levels of excitability of the primary motor

361 cortex), yet without considering general measures of activation comparable to arousal (9,64–68).

362 Here, we collected ratings on the subjective level of anxiety (i.e., STAI-Y State) before each

363 experimental session, thus serving as a fixed measure of arousal. Pre-target pupil diameter was instead

364 used as a dynamic proxy of arousal, allowing us to track its ongoing fluctuations (50,52,69). We

365 confirmed that pupil dilation values were negatively related with pre-target pupil diameter across all

366 conditions, as frequently reported in the literature (50,70–72).

367 When our measures of arousal were accounted for by statistical analyses, a clear picture emerged,

368 indicating that the effects induced by tDCS on the behavioral responses were dependent on both

369 subjective and physiological levels of arousal levels. In the sham session, participants speeded up

370 their responses when they completed the task for the second time. This practice effect emerged

371 somewhat independently of both the subjective and physiological levels of arousal, although a slightly

372 more pronounced improvement appeared with lower levels in either measures. During the application

373 of real tDCS, however, performance ceased to improve with the exception of trials characterized by

374 smaller pre-target pupil diameter and participants with a lower score at the STAI-Y questionnaire. A

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375   negative or null behavioral outcome of anodal tDCS is not uncommon in the literature and learning

376   impairments have been reported in a host of different tDCS studies involving specific learning

377   outcomes, such as unimproved working memory for recognition or implicit categorization, blocked

378   consolidation of visual perception and inhibited motor learning (68,73–78).

379   We chose response speed as behavioural measure, given that its intrinsic low sensitivity heavily relies

380   on prior levels of fatigue and general activation (79–82). The interpretation of our behavioural results

381   is arguably consistent with an inverted U-shape curve between task performance and arousal.

382   According to this relationship, performance decline would occur when arousal levels are either too

383   high or too low (33,83). None of the participants reported sleep deprivation or otherwise drowsiness-

384   related conditions. Therefore, we can assume that the lower values of our predictors effectively

385   corresponded to moderate and not low levels of arousal. With this in mind, the finding that facilitatory

386   effects are principally associated with a moderate level of cortical excitation seems to support the

387   proposed cellular mechanism for a cortical excitation-inhibition balance (16,84). On these grounds,

388   tDCS exogenous modulation would negatively impact on the normal cortical functioning whenever

389   the levels of endogenous neural activity increase to the extent of a dysfunctional neuronal gain, with

390   spontaneous task disengagement causing slower responses. A direct consequence of this mechanism

391   would be the inhibition of task learning effects, unless the endogenous system is sufficiently inactive,

392   as in low arousal trials. The latter scenario provides an additional argument for when single session

393   tDCS is found to improve task performance in the face of variable but otherwise moderate and well-

394   balanced arousal levels. The understanding that an unbalanced combination of endogenous and

395   exogenous excitability-increase events can, in fact, lead to negative effects is also coherent with

396   frameworks on brain activity-dependent plasticity and on signal-to-noise ratio mechanisms (20,78).

397   Results on pupil dilation, which represents a physiological response to relevant stimuli, corroborate

398   the above interpretation. Only when the ongoing levels of arousal were considered in the analyses, a

399   specific effect of tDCS on pupil dilatation was revealed. An overall reduction of pupil dilation

400   occurred when participants completed the task for the second time (T2), consistently with a

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401 physiological habituation effect that paralleled the practice effect seen in the behavioral results

402 (85,86). In particular, pupil dilation evenly decreased for the entire range of arousal in the sham

403 session, but crucial variations emerged during the application of real tDCS: looking at the lower end

404 of the arousal range, pupil dilation values were not as much reduced as in sham session. Conversely,

405 a more pronounced reduction in pupil dilation was observed in trials associated with higher arousal.

406 These, in fact, corresponded to the trials of unimproved response times following real tDCS.

407 Therefore, habituation of a phasic response may not necessarily indicate the same outcome direction

408 as the better performance after a practice effect (85,87). Pupil dilations primarily reflect the timely

409 increase of neural gain control, which translates into a system's responsivity amplification, and as

410 such can be ascribed in the aforementioned inverted-U curve (34,50,71,88). The implication is that

411 the additive effect of an exogenous neuromodulation would, on the one hand, contrast the natural

412 habituation effect on pupil dilation occurring below the intermediate range of tonic arousal and, on

413 the other hand, accentuate task disengagement at higher levels of tonic arousal, hence a greater

414 reduction in phasic response. An analogous explanation was put forward in a recent tDCS work

415 showing a reduction of pupil dilation - but no behavioral effects - during a Go-NoGo task, whereby

416 it was argued that an offline tDCS enhancement of neuronal membrane potential could hinder or

417 replace the endogenous gain control mechanisms of locus coeruleus (40).

418 Furthermore, outside the tDCS literature, phasic pupillary responses were found to be reduced

419 whenever participants' attention was not directed to the task, such as during episodes of mind

420 wandering (72,89–91). Indeed, recent empirical and theoretical formulations of mind wandering have

421 proposed that the locus coeruleus–norepinephrine system is tightly linked to different internally-

422 driven cognitive states, i.e., on- and off-task states with various degrees of deliberate control (36,92).

423 In this respect, the possibility of a direct and focally targeted tDCS modulation of mind wandering

424 has been recently debated with uncertain conclusions (93). Based on this knowledge, it is not unlikely

425 that our tDCS effects would also be partly dependent on the arousal-mediated propensity of mind

426 wandering activity during the task. Although not covered by the aims of this study, the above

17

427   possibility justifies the argument for a selective alteration of arousal via exogenous neuromodulation.

428   For example, vigilance decrements and physiological sleep pressure were somewhat diminished after

429   prolonged frontal anodal tDCS (41,94) with a magnitude of effects greater than caffeine (95,96).

430   Whereas for the arousal modulation related to a specific event, stimulus-locked bursts of electric

431   random noise stimulation were used to enhance performance and LC phasic responses, as indexed by

432   skin conductance measurements (25). Despite this compelling evidence, it is still difficult to conclude

433   that certain tDCS montages can directly modulate the deep brain arousal structures (97–99). Note

434   though that other mechanisms could involve changes in the neocortical neurons, whose membrane

435   potential shifts are known to be coupled with alteration in pupil diameter (34,100,101).

436

437   In summary, our data collectively offer an explanation for the negative or null effects of a common

438   prefrontal tDCS application. We are aware that the interpretation of these particular results may not

439   apply to all tDCS studies. Nevertheless, the large variability of arousal levels that we found across

440   participants leads us to reflect more closely on what may mask the desired effects in the varied and

441   still growing landscape of stimulation studies, which often fail to incorporate, but simply

442   acknowledge, the crucial aspect of individual state-dependent variables (102–105).

443   The importance of brain state is not a novel idea in the literature on non-invasive brain stimulation.

444   The ongoing or basal levels of activation, included in the concept of "state-dependency", have been

445   extensively reported to impact the effects of transcranial magnetic stimulation (TMS) (106).

446   Nevertheless, considering the mechanisms of action of tDCS, which modulates excitability of neurons

447   by hyperpolarizing or depolarizing their membrane potential (107,108), tDCS effects might be more

448   sensitive to the arousal levels than TMS. In a similar vein, these considerations might be applicable

449   to any kind of current stimulation modality.

450

451   Taken together, the discussed findings should encourage a more careful interpretation of null or

452   negative effects of tDCS. This is far from saying that all replication failures are due to inherently

453 inefficacious tDCS protocols with exhausted future potential (15). Such observations should instead

454 help ascribing the outcome of those protocols to the interrelation of the locus coeruleus–

455 norepinephrine system and the spreading of induced currents in the brain (40,42). In this sense, future

456 tDCS studies might consider useful to have both dynamic and fixed measures of arousal as an accurate

457 way to monitor its impact on the final outcome. If successful, these achievements would be of great

458 help also in assessing the degree of effectiveness with which tDCS protocols are being utilized to

459 treat or ameliorate clinical conditions.

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463 **Disclosure statement**

464 This work has not been published and has not been submitted for publication elsewhere while under

465 consideration. The authors declare no potential conflict of interest.

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