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Effects of repeated anodal transcranial direct current stimulation on auditory fear extinction in C57BL/6J mice

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Abstract

Background: Trauma-based psychotherapy is a first line treatment for post-traumatic stress disorder (PTSD) but not all patients achieve long-term remission. Transcranial direct current stimulation (tDCS) received considerable attention as a neuromodulation method that may improve trauma-based psychotherapy.

Objective: We explored the effects of repeated anodal tDCS over the prefrontal cortex (PFC) on fear extinction in mice as a preclinical model for trauma-based psychotherapy.

Methods: We performed auditory fear conditioning with moderate or high shock intensity on C57BL6/J mice. Next, mice received anodal tDCS (0.2 mA, 20 min) or sham stimulation over the PFC twice daily for five consecutive days. Extinction training was performed by repeatedly exposing mice to the auditory cue the day after the last stimulation session. Early and late retention of extinction were evaluated one day and three weeks after extinction training respectively.

Results: We observed no significant effect of tDCS on the acquisition or retention of fear extinction in mice subjected to fear conditioning with moderate intensity. However, when the intensity of fear conditioning was high, tDCS significantly lowered freezing during the acquisition of extinction, regardless of the extinction protocol. Moreover, when tDCS was combined with a strong extinction protocol, we also observed a significant improvement of early extinction recall. Finally, we found that tDCS reduced generalized fear induced by contextual cues when the intensity of conditioning is high and extinction training limited.

Conclusions: Our data provide a rationale to further explore anodal tDCS over the PFC as potential support for trauma-based psychotherapy for PTSD.

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Introduction

Post-traumatic stress disorder (PTSD) is a debilitating neuropsychiatric disorder that can develop after a person has experienced or witnessed a traumatic event [1,2]. While up to 70% of people experience trauma, approximately 4% of those exposed to trauma develop PTSD [3] yielding a lifetime prevalence of 2–3.5% [4–6]. Risk factors for PTSD include trauma type and prior trauma exposure [3]. Treatment guidelines for PTSD recommend trauma-based psychotherapy, such as prolonged exposure (PE), and pharmacotherapy, notably with selective serotonin reuptake inhibitors [7–13]. However, trauma-based psychotherapy may be more effective as first-line intervention compared to pharmacotherapy [14] and it is presently unclear how pharmacotherapy influences the effectiveness of concomitant psychotherapy [15]. While roughly

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one third of PTSD cases fully recover within one year, approximately one third of patients fail to achieve full remission despite treatment [16–18].

Trauma-based psychotherapy conceptualizes PTSD as a failure to adequately process the trauma due to avoidance of trauma reminders [19]. In vivo exposure exercises are designed to activate the trauma cognitive structure, through presentation of trauma reminders in a safe context, and to disconfirm the expected disastrous outcome [19,20]. The fear conditioning paradigm is widely used to study the formation of traumatic memories with associated fear responses [21,22] and fear extinction procedures are commonly used to model PE [23,24]. In fear conditioning, subjects acquire a fear response through association of a specific cue with an aversive stimulus (typically an electrical shock) and repeated exposure to the fear-provoking cue in the absence of the unconditioned stimulus results in a gradual lowering of fear expression in a process termed fear extinction [22,25,26]. When the aversive stimulus is stronger or more frequently associated with the cue during conditioning, the resulting fear memory becomes more resistant to extinction [27–32]. Importantly, patients with PTSD show slow rates of extinction learning and impaired extinction recall, implying aberrant activity in the involved brain circuits (for review see Ref. [33]). The process of fear extinction is driven by the interplay between the amygdala, the hippocampus (HPC) and the ventromedial prefrontal cortex (vmPFC) [34–36]. The ventral HPC and vmPFC (equivalent of rodent infralimbic cortex (IL)) are crucial for extinction memory formation [36–38] and facilitation of activity and plasticity in these brain regions is suggested to result in enhanced extinction in rodents [36,38–42]. The extinction paradigm has been used extensively in rodents and humans to identify interventions that may enhance fear extinction (see review [9,17,43]), and in this context, transcranial direct current stimulation (tDCS) has received considerable interest [44–46].

tDCS is a non-invasive neuromodulation method based on delivering a weak direct current into the brain through electrodes placed over the scalp [47,48]. The induced intracerebral current flow alters cortical excitability and spontaneous activity [47–49]. Depending on the stimulation procedure, tDCS is able to induce neuroplastic after-effects and is thought capable of modulating cognitive functions such as learning and memory [47,49,50]. Numerous studies explored the effects of tDCS on cognition and emotional processing in neuropsychiatric disorders [44,50–53] and, thus far, clinically relevant effects have been obtained with repeated stimulations in the treatment of depression [50,54–56]. Nonetheless, it remains to be established whether similar results can be achieved in the treatment of PTSD.

In humans, tDCS above the prefrontal cortex (PFC) is reported to affect fear expression [57,58] and previous reports described overgeneralization of fear to non-reinforced stimuli after anodal tDCS [59,60]. In studies investigating the effects of tDCS on fear extinction in healthy volunteers, anodal tDCS over the vmPFC before and during extinction training was found to accelerate the acquisition of extinction, but no significant effect on the retention of extinction was reported [60,61]. In veterans with PTSD, a moderately better retention of extinction was observed in veterans who received anodal tDCS over the vmPFC following extinction training compared to those stimulated during extinction training [62]. Recently, beneficial effects of repeated anodal tDCS over the vmPFC, paired with combat-related virtual reality training, were demonstrated on psychophysiological arousal and symptom-severity in veterans with PTSD [63]. Taken together, these studies suggest the potential of tDCS targeting the vmPFC to augment the efficacy of fear extinction. Nevertheless, pertinent questions regarding the long-term effects of tDCS on fear extinction, the timing of stimulation relative to extinction training and the optimal stimulation parameters remain to be determined.

In preclinical studies conducted in mice [54–66], tDCS over the PFC was shown to reverse drug-induced (β-adrenergic or CB1 receptor antagonist) impairments in acquisition and consolidation of fear memory, but to the best of our knowledge, no previous studies explored the effect of tDCS on fear extinction in mice. We hypothesized that repeated anodal tDCS over the PFC may have the potential to facilitate fear extinction, given the supporting evidence that tDCS over the PFC may alter neural activity and plasticity in the medial PFC (mPFC) and HPC [67–72], and the pivotal role of plasticity in these limbic brain regions in the extinction of conditioned fear [36,73,74]. The aim of this study was to investigate the effect of repeated anodal tDCS over the PFC on auditory fear extinction and generalization in mice. In our experimental set-up, the stimulation electrode was surgically placed over the left PFC, similar to previous work in mice [65,67,75,76]. While the lateralization of brain function is not established in rodents, these previous studies found that anodal tDCS over the left PFC affect fear memory [63], depression-like behavior [67,75], and bilateral changes in c-Fos expression in cortical regions (IL) and subcortical regions (HPC) [67]. Furthermore, we applied a repeated anodal tDCS procedure which was previously demonstrated to be effective in preclinical studies [67,75] and clinical studies for depression [54–56]. We used several protocols for auditory fear conditioning and fear extinction to investigate the boundary conditions under which facilitation of fear extinction could be observed [77].

Materials and method

Animals and housing

Male C57BL6/J mice (Janvier, France), aged 8–10 weeks at the start of the experiments were used. The animals were group
housed (4–5 per cage, 1290D Eurostandard type III cages) under standard laboratory conditions of temperature (19–25 °C) and humidity (30–70%) in a 12h light/dark cycle with food and water available ad libitum. One week before surgery, mice were habituated to handling. After surgery, mice were single housed (1264C Eurostandard type II cages) for the remainder of the experiment. All procedures met local guidelines for animal experiments (Royal Decision 2013-05-29/12, directive 2010/63/EU), complied the ARRIVE guidelines [78] and were approved by the Ethical Committee for Animal Experiments of the Vrije Universiteit Brussel (ECD 16-213-2).

**Experimental design**

The stimulation electrode was surgically placed over the left PFC one week before the start of the fear conditioning experiments. Three days following auditory fear conditioning, anodal tDCS (0.2 mA, 20 min) or sham stimulation was performed over the left PFC 2x/day for 5 consecutive days. One day after the completion of ten tDCS sessions, mice were subjected to fear extinction training. Extinction recall was assessed in the extinction context 1- and 21-days post extinction training, whereas fear renewal was determined in the conditioning context 1.5 h after late extinction recall (Fig. 1). In addition, locomotor activity and anxiety-like behavior were assessed in the open field test (OFT) and elevated plus maze (EPM) test in the week following extinction training (described in Appendix - Supplementary A.1). In experiments 1–3, mice were conditioned with 0.6 mA shocks and subjected to fear extinction training with respectively 40 (experiment 1), 8 (experiment 2) or 4 (experiment 3) CS⁻ presentations. In experiments 4–5, mice were conditioned with 1 mA shocks and subjected to fear extinction training with 40 (experiment 4) or 8 (experiment 5) CS⁻ presentations respectively (Fig. 1).

**Surgery**

A plastic electrode holder base (2.1 mm internal diameter) was stereotaxically placed above the left PFC, 1 mm anterior and 1 mm left to bregma [79], under general isoflurane anesthesia, essentially as previously described [67,75,76]. The full method is described in Appendix (Supplementary A.1).

**Transcranial direct current stimulation**

Before the start of the tDCS procedure, mice were briefly (1–2 min) anesthetized with isoflurane to fill the electrode holder base with saline solution (0.9% NaCl) and to screw the stimulation electrode (anode, 3.5 mm² contact area, DIXI Medical, France) into the holder base. A larger rubber-plate reference electrode (cathode, 4.5 cm², MedCaT, The Netherlands) was placed onto the ventral thorax and was fixed using painters’ tape. During stimulation, mice were awake and able to freely move in their home cage due to a commutator system (Bilaney Consultants GmbH, Germany). Mice were stimulated 2 × 20 min/day (anodal tDCS, 0.2 mA, 4 h inter-stimulation interval) for five consecutive days over the left PFC using a direct current stimulator (custom-made constant current supply powered by a 9 V battery [80]) with a linear fade in/fade out of 10 s. Sham stimulated mice were subjected to the same procedure, but no current was delivered (Fig. 1).

**Auditory fear conditioning**

Auditory fear conditioning and fear extinction were carried out in a fear conditioning apparatus containing a test box (17 cm width x 17 cm length x 24 cm height) placed in a soundproof chamber (Isolation Cubicle 46000-550, Ugo Basile). Two context configurations were used (A: checkered walls, white rubber ground floor, washed with hospital antiseptic concentrate (1–3%), 15 lux light intensity; B: grey walls, metal grid, washed with 1% acetic acid, 125 lux light intensity, plexiglass plate on top). When mice were placed in the test box, there was always an acclimation period of 2 min to the box (HAB) prior to tone presentation. Two different tones were used (2.5 kHz or 7.5 kHz, 80 dB sound pressure level, 30 s duration) that were randomly allocated as generalization cue (CS⁺) or conditioning cue (CS⁻). Tone frequency was counterbalanced within the experimental groups.

On day 1 of all experiments, mice were habituated to context A and exposed to five presentations of CS⁻. The interval between tone presentations was randomized between 20 and 120 s. On day 2, mice were placed in context B and exposed to five presentations of CS⁻ and CS⁺. CS⁻ and CS⁺ were presented alternatingly and the last 2 s of each CS⁺ presentation coincided with an unconditioned stimulus (US, 0.6 or 1 mA electric foot shock, 2 s). The interval between CS⁻ and CS⁺ presentations was randomized between 20 and 120 s. On day 4, a fear retrieval test was carried out in context A.
during which mice were exposed to subsequent blocks of four CS− and CS+ presentations with a 20–120 s interval. During fear extinction training in context A on day 10, mice were exposed to four CS− presentations, followed by 40, 8 or 4 CS+ presentations with 5 s interval. On day 11, early retention of extinction was tested in context A using blocks of four CS− and CS+ presentations (early extinction recall). On day 31, late retention of extinction was tested in context A (late extinction recall) followed 1.5 h later by a fear renewal test in context B, similarly using blocks of four CS− and CS+ presentations (Fig. 1).

Freezing behavior was analyzed using an automated video monitoring system (Ethovision software, Noldus, The Netherlands). Freezing was defined as the difference of pixels (max. 0.3%) between two consecutive frames during 1 s or more. Additionally, integrated data were manually corrected by a blinded observer for false positives. Time frames considered by the software erroneously as freezing, were subtracted manually from the total freezing time.

Statistical analysis

Statistical analysis was performed using Graphpad Prism software 7.0. Values are expressed as mean ± 95% confidence interval (CI) or as individual datapoints and alpha was set at \( p < 0.05 \), two-tailed. Statistical analysis was performed by repeated measures (RM) two-way ANOVA followed by Tukey’s or Bonferroni’s multiple comparisons test for post-hoc analysis (see Appendix – Supplementary A.1 for more information on the applied statistics).

The descriptive statistics and additional results from statistical analysis can be found in Appendix (Supplementary A.2 and C).

Results

Experiments 1–3: modulation of auditory fear extinction and generalization by repeated anodal tDCS following moderate intensity fear conditioning (0.6 mA shocks)

We observed no significant interaction or differences between experimental groups during the fear retrieval test but a significant cue effect, indicating that the randomization was effective in all experiments (Fig. 2A–C, Table 1). Post-hoc analysis showed that mice were capable of discriminating between cues, given that freezing was significantly higher during presentation of CS+ compared to CS− and significantly higher during CS− presentation compared to HAB (Supplementary Table C1.1).

We observed no significant interaction or treatment effect during the early extinction recall, late extinction recall or fear renewal tests in all experiments (Fig. 2A–C, Table 1). This indicates that tDCS did not significantly affect the long-term efficacy of auditory fear extinction in the used experimental conditions, regardless of the number of CS+ presentations during fear extinction training. In experiment 1, the lack of significant cue effect during early extinction recall reflects the notion that across experimental groups freezing levels were low regardless of cue type as a consequence of strong extinction training (Fig. 2A, Supplementary Table C1.1). At later time points of experiment 1, a

Fig. 2. Modulation of auditory fear extinction and generalization with repeated anodal tDCS in experiments with a shock intensity of 0.6 mA during fear conditioning. The graphs illustrate the freezing responses of sham and tDCS mice during the acclimation period (HAB) or evoked by cue presentation (CS−, CS+) in the fear retrieval, extinction training, early extinction recall, late extinction recall and the fear renewal test. (A) Overview of experiment 1 during which mice were exposed to 40 CS+ presentations during extinction training. (B) Overview of experiment 2 during which mice were exposed to 8 CS+ presentations during extinction training. (C) Overview of experiment 3 during which mice were exposed to 4 CS+ presentations during extinction training. Data are presented as means with 95% CI. Freezing scores during CS− and CS+ presentation are presented as the average of four tone presentations during the fear retrieval, early extinction recall, late extinction recall and the fear renewal test (presented as CS− BLOCK and CS+ BLOCK).
indicating that acquisition of extinction could be demonstrated, in
Table 2). This suggests that tDCS may facilitate the rate of acquisi-
tion or extinction when the conditions for extinction training are
between fear retrieval, early extinction recall and late extinction
test, in all experiments ( Supplementary Table C1.2).

During early extinction recall we found no signiﬁcant interaction or treatment effect
for both experiments 1–5 are presented in


cant cue effect, indicating that the


cant interaction or treatment effect


cant overall effect on auditory fear extinction.


cant cue effect, indicating that the
during presentation of CS+ compared to CS−. We observed no significant interaction or treat-
We further explored the effects of repeated anodal tDCS on fear
To HAB, while freezing was not statistically different between CS− and CS+ in experiment 2 (Supplementary Table C1.1). In experiment 3, freezing was not significantly different between CS− presentation and HAB but was significantly higher during presentation of CS+ compared to CS− (Supplementary Table C1.1). At later time points of experiment 2–3, freezing was significantly higher during presentation of CS+ compared to CS− and signiﬁcantly higher during CS− presentation compared to HAB (Supplementary Table C1.1).

We carried out additional analyses to establish the efficacy of the used extinction procedures focusing on the evolution of freezing during CS− presentations over time. During acquisition of extinction, we observed no overall treatment effect for all experiments (Fig. 2A–C, Table 2). We observed a signiﬁcant time effect, indicating that acquisition of extinction could be demonstrated, in experiment 1 and 2, but not in experiment 3 (Table 2). Interestingly, we observed a signiﬁcant interaction in experiment 2 (Fig. 2B, Table 2). This suggests that tDCS may facilitate the rate of acquisition of extinction when the conditions for extinction training are suboptimal, but post-hoc analysis did not reveal signiﬁcant treatment differences during individual CS− presentation (Fig. 2B, Supplementary Table C2.2).

When comparing freezing levels during CS+ presentation be-
tween fear retrieval, early extinction recall and late extinction
cue effect but a signiﬁcant interaction or treatment effect
but a signiﬁcant time effect (Table 2). This indicates that tDCS did not have a signiﬁcant overall effect on auditory fear extinction. Moreover, post-hoc analysis showed that freezing during CS+ presentation lowered signiﬁcantly over time, compared to the fear retrieval test, in all experiments (Supplementary Table C1.2). Similarly, we found no signiﬁcant interaction or treatment effect but a signiﬁcant time effect when comparing freezing levels for CS+ during late extinction recall and fear renewal, demonstrating that fear renewal occurred in all experiments but remained unaffected by tDCS (Table 2).

The results from statistical analysis of the OFT and the EPM are described in Appendix (Supplementary A.2, B.1, B.2 and C.3).

Experiments 4–5: modulation of auditory fear extinction and
generalization by repeated anodal tDCS following high intensity fear conditioning (1 mA shocks)

We further explored the effects of repeated anodal tDCS on fear extinction and generalization in mice subjected to a high intensity fear conditioning procedure. We observed no signiﬁcant interaction or differences between experimental groups during the fear retrieval test but a signiﬁcant cue effect, indicating that the randomization was effective in all experiments (Fig. 3A–B, Table 1). Post-hoc analysis showed that mice were able to discriminate between cues, given that freezing was signiﬁcantly higher during presentation of CS+ compared to CS− and signiﬁcantly higher during CS− presentation compared to HAB (Supplementary Table C1.1).

During early extinction recall we found no signiﬁcant treatment effect but a signiﬁcant interaction and cue effect for both experi-
Figure 1

Table 1

The results of statistical analysis of the fear retrieval, early extinction recall, late extinction recall and fear renewal test. The F and p values for experiment 1–5 are presented in the table. Statistical analysis: Repeated measures two-way ANOVA. Signiﬁcance (p < 0.05) is shown in bold. tDCS: transcranial direct current stimulation, n: number of mice.

<table>
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<tr>
<th>Figure</th>
<th>Experiment Groups</th>
<th>Fear retrieval</th>
<th>Early extinction recall</th>
<th>Late extinction recall</th>
<th>Fear renewal</th>
</tr>
</thead>
<tbody>
<tr>
<td>2A 1</td>
<td>Sham (n = 7) tDCS (n = 7)</td>
<td>Interaction: F (2,24) = 0.06398, p = 0.9382</td>
<td>Interaction: F (2,24) = 0.4086, p = 0.6691</td>
<td>Interaction: F (2,24) = 0.09577, p = 0.9090</td>
<td>Interaction: F (2,24) = 0.03648, p = 0.9642</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cue effect: F (2,24) = 22.26, p &lt; 0.0001</td>
<td>Cue effect: F (2,24) = 2.981, p = 0.0698</td>
<td>Cue effect: F (2,24) = 13.92, p &lt; 0.0001</td>
<td>Cue effect: F (2,24) = 16.81, p &lt; 0.0001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Treatment: F (1,12) = 0.04380, p = 0.8377</td>
<td>Treatment: F (1,12) = 0.8295, p = 0.3804</td>
<td>Treatment: F (1,12) = 0.2512, p = 0.6253</td>
<td>Treatment: F (1,12) = 0.3788, p = 0.5497</td>
</tr>
<tr>
<td>2B 2</td>
<td>Sham (n = 11) tDCS (n = 12)</td>
<td>Interaction: F (2,42) = 0.4648, p &lt; 0.0001</td>
<td>Interaction: F (2,42) = 0.3766, p = 0.6885</td>
<td>Interaction: F (2,42) = 0.2984, p = 0.7435</td>
<td>Interaction: F (2,42) = 0.3840, p = 0.6835</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cue effect: F (2,42) = 72.26, p &lt; 0.0001</td>
<td>Cue effect: F (2,42) = 18.33, p &lt; 0.0001</td>
<td>Cue effect: F (2,42) = 37.64, p &lt; 0.0001</td>
<td>Cue effect: F (2,42) = 33.86, p &lt; 0.0001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Treatment: F (1,21) = 0.2054, p = 0.6550</td>
<td>Treatment: F (1,21) = 0.7072, p = 0.4099</td>
<td>Treatment: F (1,21) = 0.9125, p = 0.3503</td>
<td>Treatment: F (1,21) = 0.1103, p = 0.7431</td>
</tr>
<tr>
<td>3C 3</td>
<td>Sham (n = 12) tDCS (n = 13)</td>
<td>Interaction: F (2,46) = 1.404, p = 0.2559</td>
<td>Interaction: F (2,46) = 1.047, p = 0.9008</td>
<td>Interaction: F (2,46) = 0.8034, p = 0.4540</td>
<td>Interaction: F (2,46) = 1.392, p = 0.2588</td>
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<tr>
<td></td>
<td></td>
<td>Cue effect: F (2,46) = 48.55, p &lt; 0.0001</td>
<td>Cue effect: F (2,46) = 19.79, p &lt; 0.0001</td>
<td>Cue effect: F (2,46) = 30.97, p &lt; 0.0001</td>
<td>Cue effect: F (2,46) = 22.46, p &lt; 0.0001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Treatment: F (1,23) = 0.2320, p = 0.6346</td>
<td>Treatment: F (1,23) = 1.605, p = 0.2179</td>
<td>Treatment: F (1,23) = 3.321, p = 0.0814</td>
<td>Treatment: F (1,23) = 1.057, p = 0.3145</td>
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<tr>
<td>3A 4</td>
<td>Sham (n = 14) tDCS (n = 15)</td>
<td>Interaction: F (2,54) = 0.1126, p = 0.9308</td>
<td>Interaction: F (2,54) = 0.4099, p &lt; 0.0001</td>
<td>Interaction: F (2,54) = 3.170, p &lt; 0.0001</td>
<td>Interaction: F (2,54) = 6.317, p = 0.0355</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cue effect: F (2,54) = 173.4, p &lt; 0.0001</td>
<td>Cue effect: F (2,54) = 30.16, p &lt; 0.0001</td>
<td>Cue effect: F (2,54) = 41.60, p &lt; 0.0001</td>
<td>Cue effect: F (2,54) = 36.63, p &lt; 0.0001</td>
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<tr>
<td></td>
<td></td>
<td>Treatment: F (1,27) = 0.002551, p = 0.9601</td>
<td>Treatment: F (1,27) = 0.0116, p = 0.9308</td>
<td>Treatment: F (1,27) = 1.454, p = 0.2384</td>
<td>Treatment: F (1,27) = 5.376, p = 0.0282</td>
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<tr>
<td>3B 5</td>
<td>Sham (n = 15) tDCS (n = 16)</td>
<td>Interaction: F (2,58) = 0.1549, p = 0.8569</td>
<td>Interaction: F (2,58) = 0.0272, p &lt; 0.0001</td>
<td>Interaction: F (2,58) = 3.836, p = 0.0272</td>
<td>Interaction: F (2,58) = 9.306, p = 0.0256</td>
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<td></td>
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<td>Cue effect: F (2,58) = 81.44, p &lt; 0.0001</td>
<td>Cue effect: F (2,58) = 0.0001, p = 0.9714</td>
<td>Cue effect: F (2,58) = 37.47, p &lt; 0.0001</td>
<td>Cue effect: F (2,58) = 7.701, p = 0.0006</td>
</tr>
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<td></td>
<td>Treatment: F (1,29) = 0.7069, p = 0.4074</td>
<td>Treatment: F (1,29) = 1.897, p = 0.1790</td>
<td>Treatment: F (1,29) = 2.627, p = 0.1159</td>
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</tbody>
</table>
Table 2
The results of statistical analysis of the acquisition of extinction for CS\(^+\) (freezing levels of CS\(^-\) specifically during fear extinction training), extinction for CS\(^-\) over time (freezing during CS\(^-\) presentation in the fear renewal to the extinction recall tests (early and late)) and renewal of the fear response for CS\(^+\) (freezing during CS\(^-\) presentation in the fear renewal to the late extinction recall test). The F and p values for experiment 1–5 are presented in the table. Statistical analysis: Repeated measures two-way ANOVA. Significance (p < 0.05) is shown in bold. tDCS: transcranial direct current stimulation, n: number of mice.

<table>
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<th>Experiment</th>
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<tr>
<td></td>
<td></td>
<td>Acquisition of extinction for CS(^+)</td>
</tr>
<tr>
<td>1</td>
<td>Sham (n = 7), tDCS (n = 7)</td>
<td>Interaction: F (39,468) = 0.5383, p = 0.9904</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Interaction: F (2,24) = 0.1020, p = 0.9034</td>
</tr>
<tr>
<td>2</td>
<td>Sham (n = 11), tDCS (n = 12)</td>
<td>Interaction: F (7,147) = 2.226, p = 0.0352</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Time effect: F (7,147) = 6.771, p &lt; 0.0001</td>
</tr>
<tr>
<td>3</td>
<td>Sham (n = 12), tDCS (n = 13)</td>
<td>Interaction: F (3,69) = 0.5808, p = 0.6296</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Time effect: F (3,69) = 0.050, p = 0.1149</td>
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<tr>
<td>4</td>
<td>Sham (n = 14), tDCS (n = 15)</td>
<td>Interaction: F (39,1053) = 1.003, p = 0.0461</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Time effect: F (39,1053) = 5.016, p = 0.0335</td>
</tr>
<tr>
<td>5</td>
<td>Sham (n = 15), tDCS (n = 16)</td>
<td>Interaction: F (7,203) = 3.172, p = 0.2190</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Time effect: F (7,203) = 4.417, p = 0.0444</td>
</tr>
</tbody>
</table>

Group effects showed a significant difference in freezing during CS\(^+\) presentation for experiment 4 (p = 0.0317, d = 0.8) but not for experiment 5 (p = 0.0945, d = 0.649) (Supplementary Table C2.1). This suggests that tDCS can improve the short-term efficacy of extinction training, particularly in combination with a strong extinction procedure. During late extinction recall of experiment 4–5, we did not observe a significant interaction or treatment effect, indicating that tDCS had no long-lasting effects on auditory fear extinction, but observed a significant cue effect (Fig. 3A–B, Table 1). Freezing was significantly higher during presentation of CS\(^+\) compared to CS\(^-\) and significantly higher during CS\(^+\) presentation compared to HAB (Supplementary Table C1.1). Interestingly, during the fear renewal test, we observed a significant cue effect and treatment effect but no significant interaction for both experiment 4 and 5 (Fig. 3A–B, Table 1). In both experiments, freezing was significantly higher during presentation of CS\(^+\) compared to CS\(^-\), but not different between HAB and CS\(^-\) presentation (Supplementary Table C1.1). Post-hoc analysis (Supplementary Table C2.1) found no significant treatment differences during CS\(^-\) presentation in experiment 4 (p = 0.0551) but significantly lower freezing during CS\(^-\) presentation in experiment 5 (p = 0.0043). Together these data suggest that tDCS may prevent a generalized fear response during fear renewal following a strong conditioning procedure.

When analyzing freezing levels during CS\(^+\) presentations specifically, we observed a significant time effect and treatment effect but no significant interaction during acquisition of extinction for both experiments (Fig. 3A–B, Table 2).

When comparing freezing levels during CS\(^+\) presentation between fear retrieval, early extinction recall and late extinction recall, we observed a significant time effect but no significant interaction or treatment effect (Fig. 3A–B, Table 2). Post-hoc
analysis showed that freezing during CS\textsuperscript{+} presentation significantly lowered over time, compared to the fear retrieval test, in all experiments (Supplementary Table C1.2). Similarly, we found a significant time effect but no significant treatment effect or interaction when comparing freezing levels during CS\textsuperscript{+} presentation in late extinction recall and fear renewal, demonstrating that fear renewal occurred in all experiments across experimental groups (Fig. 3A–B, Table 2).

The results from statistical analysis of the OFT and the EPM are described in Appendix (Supplementary A.2, B.1, B.2 and C.3).

**Discussion**

In this series of experiments, we investigated the effects of repeated anodal tDCS over the PFC on auditory fear extinction and generalization in mice under different experimental conditions for fear conditioning and fear extinction.

In experiments 1–3, we found no significant effect of repeated anodal tDCS on the acquisition or retention of fear extinction. Given that a significant reduction in freezing responses was observed in all experiments with moderate fear conditioning intensity (0.6 mA shocks), across treatment groups, we reasoned that potential effects of tDCS on the efficacy of fear extinction might have been obscured because there was little room for further improvement of extinction learning. To eliminate floor effects on freezing and to ensure that we would be able to detect suppressed fear responses [28], we further explored the effects of repeated anodal tDCS under experimental conditions that were more challenging. In humans, persistence of PTSD symptoms depends on previous exposure to trauma and the type of trauma [3]. In rodents, the persistence of fear memory depends on the number of cue-shock pairings and the shock intensity [27,32,81]. Increasing the intensity of delivered foot shocks during fear conditioning may be thus an etiologically relevant model to study fear memories that are more resistant to extinction [28–30,82]. When subjected to a high intensity fear conditioning procedure (1 mA shocks), we consistently found a significant effect of repeated anodal tDCS on the acquisition of extinction (experiment 4–5). When looking at the evolution of freezing behavior during extinction training, our data suggest an effect of tDCS on the acquisition rate of extinction rather than having an overall effect on fear expression. In the early extinction recall, we observed an improved retention of extinction for CS\textsuperscript{+} in tDCS treated mice. The observed effect was relatively small and only significant in combination with a strong extinction procedure. Moreover, the effect of tDCS on freezing behavior for CS\textsuperscript{+} was lost during the late extinction recall. Nonetheless, the observations that repeated anodal tDCS may facilitate the acquisition rate and short-term retention of fear extinction are in line with prior studies in healthy volunteers and PTSD patients targeting the vmPFC [60–63,83]. In contrast to some studies, we did not observe an increase in the response to the generalization cue (CS\textsuperscript{–}) following application of tDCS [59,60]. However, we observed an interesting treatment effect during the fear renewal sessions with a trend towards lower freezing during habituation (HAB) in the renewal context (the context in which mice were initially conditioned) and during CS\textsuperscript{–} presentation in the tDCS groups of experiments 4–5. Post-hoc analysis showed a significant difference for freezing during CS\textsuperscript{–} presentation only in experiment 5, indicating that mice in the tDCS group correctly identified the CS\textsuperscript{–} as a safe signal, while sham mice showed a similar fear response during habituation in the renewal context, CS\textsuperscript{–} presentation or CS\textsuperscript{+} presentation. This suggests that tDCS may have long-lasting effects on fear extinction for contextual cues and may suppress a generalized fear response towards CS\textsuperscript{+} when extinction training is suboptimal. Renewal of the fear response for CS\textsuperscript{+} occurred in all experiments without significant differences between the experimental groups. Placing mice in the renewal context is expected to reactiviate the initial fear memory, since extinction training does not erase this initial CS\textsuperscript{–}–US association but depends on the formation of a new context-dependent extinction memory [35,84,85]. Indeed, in extinction [86,87], reconsolidation [88] and reactivation-extinction [89] based procedures, recovery of fear has been observed, illustrating that
preventing the return of fear in the renewal context is not very robust.

In the OFT and EPM test, we observed a significant effect of repeated anodal tDCS above the PFC on locomotor activity in the OFT but no significant effect on open arm exploration in the EPM test (Supplementary Figures B1, B2 and Supplementary Tables C3). This indicates that tDCS may have long-lasting effects on (exploratory) activity but not on anxiety-like behavior, which is consistent with previous literature findings [75]. However, it seems unlikely that this effect would have a major impact on freezing levels in our experiments since we did not observe significant treatment differences for freezing during the acclimation period (HAB) or during CS+ presentation. We therefore maintain that the observed effects of repeated anodal tDCS on freezing during cue presentation reflect fear expression and were dependent on the intensity of fear conditioning and extinction training, rather than non-specific effects on locomotor activity or anxiety-like behavior.

To the best of our knowledge, our study is the first to explore the effect of repeated anodal tDCS on auditory fear extinction in mice. A single tDCS session, using stimulation parameters identical to those applied in our study, was previously shown to elicit a significant increase in the expression of the immediate-early gene c-Fos in the mouse limbic system [67]. This was observed in brain areas adjacent to the stimulation site, such as the mPFC (including the IL), but also more distal brain regions, notably the HPC. However, after ten tDCS sessions, this significant increase in c-Fos expression was no longer observed. This suggests long-lasting changes in neuronal responsiveness following application of repeated anodal tDCS [67]. Indeed, long-term potentiation (LTP) is enhanced in hippocampal and PFC slices obtained from rodents subjected to anodal tDCS [70–72,90]. Interestingly, these effects of anodal tDCS on LTP are dependent on Brain-Derived Neurotrophic Factor (BDNF) [70,72,91]. Given that BDNF plays a critical role in fear extinction [92–98] through modulation of plasticity in neuronal projections from the ventral HPC to the infralimbic PFC [38,41,42,99,100] we proposed that repeated anodal tDCS may facilitate fear extinction. We partially confirmed our hypothesis using a repeated tDCS protocol that was previously shown to induce long-lasting effects in tests for depression-related behaviors in mice [67,75] and that is similar to protocols that improve mood symptoms in major depressive disorder [55,56,101–103].

In our study, mice were stimulated repeatedly during five consecutive days prior to extinction training [67,75], instead of receiving a single stimulation immediately before, during or after extinction training [60–62,83]. Therefore, the observed behavioral effects in this study may be the result of cumulative effects on plasticity [70,72,104]. Indeed, previous studies demonstrated that tDCS may elicit changes in the expression of plasticity-associated genes, such as c-Fos, Arc, CAMKII, CREB and BDNF in cortical and hippocampal regions [70,105–108]. Moreover, a single session of tDCS using stimulation parameters similar to our study was shown to increase long-term plasticity, long-term memory formation and the expression of BDNF for up to one week following stimulation [72]. Similarly, single and repeated application of tDCS using stimulation parameters identical to our study were shown to elicit antidepressant-like effects in mice that lasted for at least three weeks after stimulation [67]. It is likely that changes in expression of plasticity-related genes also underlie the long-term antidepressant-like properties of tDCS [50,67,109–111]. Indeed, tDCS increased c-Fos expression in several limbic regions including several subregions of the PFC and HPC [67]. We hypothesize that tDCS improves extinction learning by increasing the expression of these plasticity-related genes in targeted brain regions such as the PFC. While it is unlikely that tDCS would specifically target the IL to improve extinction learning, we propose that extinction learning will specifically recruit the IL, which was primed for improved plasticity by tDCS. In line with this notion, chronic rTMS treatment over the PFC was found to enhance extinction in mice 10 days following the last stimulation [112]. This enhancement was associated with increased c-Fos expression in the IL, basolateral amygdala and ventral HPC following extinction training [112]. Nevertheless, further research is necessary to elucidate the mechanism through which repeated anodal tDCS may affect fear extinction and to identify the optimal stimulation parameters that may yield long-term effects on fear extinction. Timing of electrical stimulation relative to extinction training may be critical [39]. According to the activity-selectivity hypothesis, pairing of tDCS with a learning task may induce long-lasting neuroplastic effects more effectively [113–116], since the effects of brain stimulation may rely on the neural activation state at the time of stimulation [115–117]. In this context, promising long-term effects of tDCS may be found on PTSD symptoms in patients [63].

A limitation in our study is that all experiments were conducted in male mice. Therefore, additional studies are necessary to determine whether our findings can be extrapolated to female mice. Another important consideration is the equivalence of our stimulation protocol to those applied in humans [51,75]. While the duration, the amount and the repetition of stimulation are similar to protocols used in humans [55,56,101–103], the current density applied to the skull in this study is higher than that applied in humans due to the relative size of the stimulation electrode [51,75]. Consequently, this may have resulted into different voltage distributions and diffused stimulation of other brain areas [51]. Moreover, the specificity with which tDCS is able to target specific areas of the brain of rodents or humans is limited, given the diffused spatial resolution of tDCS and the size of the electrodes relative to specific brain areas [51,118]. In our study for example, beneficial effects of stimulation of the IL may have been partly obscured by concomitant stimulation of the PL. Moreover, given the placement of the reference electrode over the ventral thorax, the possible effect of transcutaneous stimulation of peripheral nerves should be further scrutinized in future studies [119]. Finally, while the PFC is typically targeted to augment fear extinction [62,63,83,112], stimulation of the HPC merits consideration, given the role of HPC projections to the IL in fear extinction [41,42].

Conclusion

Taken together, this is the first preclinical study to show that repeated anodal tDCS over the PFC may augment the rate of acquisition and short-term retention of auditory fear extinction in mice. Moreover, tDCS may have long-term effects on generalized fear responses induced by contextual cues. While the observed effect sizes were small, our results are promising and provide a framework for future research, to further optimize stimulation parameters, investigate underlying mechanisms, and explore whether similar results can be obtained in models of impaired extinction [120].

CRediT authorship contribution statement

Andries Van Schuerbeek: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing - original draft, Visualization, Project administration. Marie-Anne Vanderhasselt: Conceptualization, Methodology, Writing - review & editing, Supervision, Funding acquisition. Chris Baeken: Conceptualization, Methodology, Writing - review & editing, Supervision. Anouk Pierre: Conceptualization, Validation, Investigation, Writing - review & editing. Ilse Smolders: Conceptualization, Resources, Writing - review & editing, Supervision. Vincent Van Waes:
Methodology, Validation, Resources, Writing - review & editing, Supervision. **Dimitri De Bundel**: Conceptualization, Methodology, Validation, Formal analysis, Resources, Writing - original draft, Visualization, Supervision, Funding acquisition.

**Declaration of competing interest**

None.

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**Appendix A. Supplementary data**

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