Long-term enhancement of visual responses by repeated transcranial electrical stimulation of the mouse visual cortex

Despoina Tsapa, Mehran Ahmadlou & J. Alexander Heimel[1]

Cortical Structure & Function group, Netherlands Institute for Neuroscience, an institute of the Royal Academy for Arts and Sciences, Amsterdam, The Netherlands

[1] To whom correspondence should be addressed. Meibergdreef 47, 1105 BA Amsterdam, The Netherlands, +31205664530, heimel@nin.knaw.nl

Abstract

Background: Transcranial electrical stimulation (tES) is a popular method to modulate brain activity by sending a weak electric current through the head. Despite its popularity, long-term effects are poorly understood.

Objective: We wanted to test if anodal tES immediately changes cerebral responses to visual stimuli, and if repeated sessions of tES produce plasticity in these responses.

Methods: We applied repeated anodal tES, like transcranial direct current stimulation (tDCS), but pulsed (8 s on, 10 s off), to the visual cortex of mice while visually presenting gratings. We measured the responses to these visual stimuli in the visual cortex using the genetically encoded calcium indicator GCaMP3.

Results: We found an increase in the visual response when concurrently applying tES on the bone without skin (epicranially). This increase was only transient when tES was applied through the skin (transcutaneous). There was no immediate after-effect of tES. However, repeated transcutaneous tES for four sessions at two-day intervals increased the visual response in the visual cortex. This increase was not specific to the grating stimulus coupled to tES and also occurred for an orthogonal grating presented in the same sessions but without concurrent tES. No increase was found in mice that received no tES.

Conclusion: Our study provides evidence that tES induces long-term changes in the mouse brain. Results in mice do not directly translate to humans, because of differences in stimulation protocols and the way current translates to electric field strength in vastly different heads.

Highlights

- Concurrent anodal tES increases calcium response in the mouse visual cortex
- Anodal tES over the visual cortex enhances visual responses over multiple sessions
- The increase in visual response is not specific to the visual stimulus coupled to the tES

Keywords: calcium imaging, potentiation, tES

Introduction

Transcranial electrical stimulation (tES) is the technique of running a weak electric current through the head. Particularly transcranial direct current stimulation (tDCS) has gained popularity in the last decade and by now it has been applied to more than 7000 subjects in over 17,000 sessions [1] in reported studies alone. It has been used in a research setting to treat stroke [2], depression [3], tinnitus [4], chronic pain [5] and a number of other neurological and psychiatric disorders [1]. In addition, tDCS has been reported to change a remarkably broad list of aspects of cognition, such as attention, learning, memory and decision making [6]; [7]; [8]; [9]; [10]. Concomitant with clinical work that evaluates whether tDCS is effective in treating the mentioned disorders [11]; [12]; [13], the beneficial effects of tDCS are also studied in rodent models of e.g. ADHD [14], pain [15] and food craving [16]. This rodent research can help to understand the mechanisms underlying changes induced by tES [17]. In human tDCS, typically 1–2 mA is applied through 5x5 cm plate electrodes wrapped in a saline-soaked sponge for ten minutes or more, though the used ranges vary widely [1]. There is doubt about how this weak current, of which less than 10% enters the brain, can influence neural processing [18]. First, it was shown in humans that tDCS can lead to changes in excitability directly after application [19]. Later animal work confirmed that weak DC currents can modulate cortical excitability and plasticity in vitro [20]; [21] and in vivo [22]; [23] and that these effects can last for minutes to hours after electrical stimulation [17]; [24].

In vivo work on the mechanism underlying these changes has focused on the motor cortex, which has the advantage of being close to the observable output of the brain. The visual cortex may be an equally interesting model to understand the effect of tDCS, because much is already known about plasticity in the visual cortex [25] and it is synaptically close to the brain's sensory input. Research in humans has shown that tDCS with one electrode placed over the occipital (visual) cortex modulates contrast sensitivity in both healthy [26]; [27]; [28] and amblyopic

individuals [29]. Anodal stimulation, i.e. the positive electrode placed over visual cortex, affects the strength of the visually evoked response in the occipital cortex with after-effects that can last for several minutes [30]; [31]; [32]; [33]. These findings have been replicated in mice, where anodal tDCS increases visual evoked potentials with after-effects persisting for ten minutes [34] or hours [24]. TDCS is often applied repeatedly over several days in therapeutic settings [35] or when used to enhance skill acquisition [7] or memory consolidation [6]. There are, however, no data yet from animal models about the effects on neural responses to sensory stimuli if tDCS is applied repeatedly. To understand the mechanisms underlying the effects of tDCS, it is useful to know what happens to neural activity during the electrical stimulation. We investigated this by measuring the effects of anodal direct current stimulation applied over short periods of time on the visual responses in the anesthetized mouse visual cortex by calcium imaging [36]; [37]. The advantage of calcium imaging is that there are no electrical artefacts in the measurement. Furthermore, we can image through the intact skin, and thus match the human situation more closely than is common in animal studies investigating neural responses. In addition, imaging through the intact skin prevents changes in sensory responses due exposure of the skull. We repeated the tES paradigm over several sessions spanning days to measure the chronic effect of anodal tES on visual responses.

Materials and Methods

Animals

We used 14 male and female, 2-7 months old offspring of a cross between cre-dependent GCaMP3 mice (Ai38 - B6;129S-Gt(ROSA)26Sortm38(CAG-GCaMP3)Hze/J, Jackson Laboratory; [36]) and the G35-Cre transgenic line. In G35-Cre mice, most cortical excitatory neurons express Cre-recombinase [38]. We also used 1 C57BL/6J wild type male for **Fig. 1B**.

Animals were on a 12h day/night cycle with ad libitum access to food and water. Experiments were carried out during the day cycle. For each animal, the imaging sessions were performed at two day intervals. Mice were randomly assigned to an experiment, but the experiment was not performed blind. The analysis of the calcium data was automated. Mice were housed solitary. All experiments were approved by the animal care and use committee of the Royal Netherlands Academy of Arts and Sciences.

Animal preparation for epicranial tES

We imaged transcutaneously (i.e. through the scalp), with or without electrical stimulation, except for the experiments presented in **Figures 2E-G**, where we removed the scalp and imaged and electrically stimulated epicranially, i.e. directly through the skull, to compare with previous animal work. For these experiments, we removed the scalp and attached the electrode tube and head fixation ring permanently to the skull. For this procedure, we anesthetized the animals with 5% isoflurane (induction) and 1.5-2.5% isoflurane (maintenance) in oxygen (0.8 L/min flow rate). To protect the mice's eyes, we used Cavasan ointment. During surgery, body temperature was maintained at 37°C with the use of a heating pad and a rectal probe. After induction of anesthesia, we prophylactically administered Metacam (1 mg/kg subcutaneous, for analgesia), dexamethasone (8 mg/kg subcutaneous, against cerebral edema/inflammation) and cefotaxime (25 mg/kg subcutaneous, against infection). Mice were head-fixed and the skull overlying the visual cortex was exposed. A metal ring (5 mm inner diameter) was fixed on the skull centered on the primary visual cortex (V1) with Loctite 454 and dental cement. A plastic tube [34] (3 mm inner diameter) was placed above left V1 (3 mm lateral and 0.4 mm anterior to the lambda cranial landmark [39]) and fixed on the skull with Loctite 454 and dental cement. The area between the plastic tube and the metal ring was covered with dental cement. The tube itself was filled with Kwik Cast silicone elastomer (World Precision Instruments) to protect the exposed skull when the animal was not being imaged. Animals recovered in their home cage

placed in a warm environment after the dental cement had dried. Animals were allowed to recover from the surgery for at least one week.

Calcium imaging

For each session of wide-field calcium imaging [37], anesthesia was induced with 5% isoflurane and maintained with 0.8-2% isoflurane. The isoflurane level was reduced if respiration became irregular or caused strong contraction of the animal's flanks, and increased upon spontaneous movement or movement in response to stretching of the leg. The temperature of the animal was maintained at 37°C by heating pad and rectal probe. For mice with a permanent electrode tube, the head ring was magnetically attached to a holder, and the silicone elastomer was removed. The other animals were head fixed with ear bars, and the hair on their head was cropped. The plastic electrode tube was placed above V1 and filled with a thin layer of EEG gel. A black cloth was used to prevent the light coming from the visual stimulus monitor to affect the recording. The cortex was illuminated through the skull with blue light (482 ± 25 nm). Fluorescent light passed through a 495 nm dichroic mirror and a green band-pass filter (525 ± 45 nm). Images were collected using a CCD (Teli, 11.04 µm per pixel) at 1.8 Hz acquisition rate through a 1x macroscope lens assembly using a VDAQ acquisition system (Optical Imaging Inc.). The camera was aligned parallel to the cranial surface, 3 mm lateral to the left and 0.4 mm anterior to the lambda landmark and focused 0.4 mm below the cranial surface. The blue excitation light was shuttered up to 2 s before onset of the visual stimulus, and directly after its end.

Visual stimulation

Visual stimuli were presented on a 42 inch gamma-corrected M4210 LG display screen, placed at a 30 cm distance at the front-right of the mouse, so that the horizontal view was from -30 to +70 degrees azimuth relative to the vertical midline and the vertical view from -45 to +45 degrees elevation (**Fig. 1A**). The visual stimuli were made with Matlab scripts using

Psychophysics Toolbox 3 [40], available at http://github.com/heimel/InVivoTools. First, we measured retinotopy to confirm that we were measuring from the visual cortex. For this, the screen was divided into four equal sized quadrants. Full contrast, 0.05 cycles per degree square-wave gratings filling one quadrant drifted in randomly changing directions (but using each quadrant exactly once per block of four stimuli), while the rest of the screen was a constant gray (**Fig. 1C**), following our previous imaging studies [38]. Next, we presented blocks of three visual stimuli (**Fig. 1D**). Two stimuli were full screen, black-and-white square-wave gratings of 0.05 cycles per degree and 100% contrast, moving for 8 s at 40 deg/s either in the upward left (135°) or upward right (45°) direction. The third stimulus was not an actual stimulus, but rather the absence of a visual stimulus and just the continued presentation of a blank equiluminant gray image, that was used to measure and subtract any slow late effects in calcium response to the two grating stimuli. We refer to this as the blank visual stimulus. All visual stimuli were separated by at least 10 s of presentation of an equiluminant gray image. The order of the three stimuli was randomly permutated for each block of the three stimuli. We first showed 25 blocks of both grating stimuli and the blank visual stimulus without tES. Next, we showed 50 blocks with one direction of motion (upward right, 45°) coupled to concurrent tES stimulation (described in next section), while the other direction (upward left, 135°) and blank visual stimuli were uncoupled to tES. Finally, we presented another 25 blocks of all three visual stimuli without tES.

Current stimulation

A current isolator (Isolator-19 stimulus, Axon Instruments) was connected through two electrodes to the mouse. The positive electrode was a platinum wire ending inside a plastic tube (3 mm inner diameter) filled with EEG gel centered above the left primary visual cortex of the mouse (3 mm lateral and 0.4 mm anterior to the lambda cranial landmark [39]), and the negative electrode was a sponge (4 cm^2) soaked in saline (0.9% NaCl) touching the ventral thorax of the

mouse (**Fig. 1A**). The current level was controlled by an Arduino device synchronized to the visual stimulus to give anodal DC pulses of 74 μA, corresponding to a current density of 0.010 mA/mm^2, or 25 μA and 0.0033 mA/mm^2 for **Fig 2G-H**. From the onset of the visual stimulus coupled with electrical stimulation, the current ramped up linearly in 1.5 s to the full strength. It remained at full strength for 5 s before linearly declining again in 1.5 s (**Fig. 1D**). We have also imaged with the same preparation, but without applying any current. We refer to these control experiments as 'unstimulated'. The animals had a variable number of imaging sessions, but all stimulated animals presented in this study had at least 4 sessions with tES during the imaging.

Analysis

For each pixel *i* of the CCD image, the fluorescence intensity at time *t* is given by $F_i(t)$. The last fluorescent image before the onset of the visual stimulus was used as the baseline fluorescence *F0*. For each pixel and frame after the onset of the visual stimulation, we calculated $\Delta F_i(t)/F0_i = (F_i(t) - F0_i)/F0_i$. The response of a pixel was defined as the mean $\Delta F_i(t)/F0_i$ during presentation of the visual stimulus. To compute the retinotopic map (**Fig. 1C**), each pixel was given a color that represented the monitor patch to which it showed the most response. This retinotopic map was used to define a region-of-interest (ROI) that was visually responsive on the hemisphere contralateral to the visual stimulation. The responses to the visual stimulus alone ($R_{uncoupled}$) and the visual stimulus paired with electrical stimulation ($R_{coupled}$) were computed as the mean $\Delta F_i(t)/F0_i$ over all pixels in the ROI during the 8 seconds of visual stimulation alone or during visual stimulation paired with electrical stimulation. We use 'visual response' for the response to a visual stimulus without simultaneous electrical stimulation. For averages over all blocks of visual stimuli within a session part (visual stimulation before, after or during electrical stimulation), the average response to the blank visual stimulus was subtracted from the responses to the visual stimulus. The concurrent tES effect size was measured as the $(R_{coupled} - R_{uncoupled}) / R_{uncoupled}$.

Statistics

Results are presented as mean ± SEM unless otherwise stated. For statistical testing, we used a Shapiro-Wilk test to determine if the values were consistent with a normal distribution. If this was the case, we used t-tests for comparison testing, otherwise the Mann-Whitney U test. For comparison within the same animal, we used paired testing. To determine if there was a significant increase over multiple sessions, we used the non-zero slope test. All tests used for significance calculations are mentioned in the text.

Results

We imaged neural activity through the scalp in anesthetized mice in response to visual stimulation and tES using the genetically encoded fluorescent calcium indicator GCaMP3 [36] (**Fig. 1A**). It is impossible to separate the GCaMP3 signal from endogenous flavoprotein fluorescence [41], but control measurements in wild type animals not expressing GCaMP3 showed that flavoprotein fluorescence was at least an order of magnitude smaller than the signal in GCaMP3 animals (**Fig. 1B**). After making a coarse retinotopic map to confirm the position of the visual cortex (**Fig. 1C**), we placed a small circular chamber on the skin above the visual cortex. The chamber was filled with transparent and conducting EEG-gel to image and apply current stimulation simultaneously. We first recorded visual responses inside the chamber to 25 blocks of 8 s periods of full screen gratings drifting to the top-right or top-left of the screen, alternated with periods of no visual stimulation of 10 s or more (see Methods) when an equiluminant grey background was shown (**Fig. 1D**). We then continued recording for 50 blocks, but now with one drifting direction paired with concurrent anodal tES. Finally, we quantified the response again for both directions but without electrical stimulation. The onset of each visual stimulus caused an increase in fluorescence, reflecting the neuronal response, but this

response and fluorescence reduced during the presentation of the stimulus by neuronal adaptation along the visual pathway (**Fig. 2A**). In the 50 blocks of the tES session, there was no significant difference in the response when the visual stimulus was coupled to tES compared to when the visual stimulus was not coupled to tES ($p = 0.6$, t-test, 4 animals x 4 sessions, 3 male, 1 female, **Fig. 2A**). Investigation of the individual blocks, however, showed that tES increased the responses (measured as average $\Delta F/F$ during the period of visual stimulation, see Methods) in the first blocks. The effect of tES diminished very quickly, while the visual (uncoupled) response stayed constant (**Fig. 2B**). The first three blocks showed a significant increase by tES measured as percentage of the visual response (block 1: $p = 0.0008$, block 2: $p = 0.0024$, block 3: $p = 0.0031$, Mann-Whitney U-test; average response of first three blocks shown in **Fig. 2B inset**), while the subsequent 47 did not ($p > 0.05$, Mann-Whitney U-test for each block, **Fig. 2C**). The time course of the response with concurrent tES was fitted with an exponential decay with a time constant of 5.63 blocks (explained variance $R^2 = 57\%$). We considered whether the diminishing tES effect was the result of a more global, habituating, brain reaction, like alertness, to the potentially noxious feeling of the skin stimulation. If this would be the case, we would expect that the effect of tES would not be specific to the brain region directly below the electrical stimulation area, but would occur throughout the brain. However, we found no calcium change in response to tES in the contralateral anterior brain area that was in our field of view the furthest away from the stimulated patch. There also was no difference in the effect in the first three versus the last three blocks in this area ($p = 0.28$, paired t-test, 4 mice x 4 sessions, **Fig. 2D**). We wondered if the reduction of the effect with time was a result of changing electrical properties of the skin during a tES-session. Changes in skin impedance, capacitance or ion distribution within the skin could change the induced electric field inside the brain and lead to changes in the effect evoked by the tES. For this reason, we decided to test the same visual and electrical stimulation protocol epicranially, i.e. after removing part of the scalp, in another set of animals. Not surprisingly, the response increase induced by epicranial tES was much

larger (2 male mice x 4 sessions, **Fig. 2E**), as now no current could flow through the skin directly under the anodal electrode and a larger electric field was present in the brain. Again, there was a decreasing effect during the first blocks of the session (**Fig. 2F**), but the tES-induced increase did not vanish, unlike what happened when we electrically stimulated transcutaneously. The response for concurrent epicranial tES was fitted by an exponential with the same time constant as for the transcutaneous tES plus a constant contribution (adjusted R^2 = 63%, **Fig. 2F**). To further check if the vanishing of the transcutaneous tES effect was because of a smaller current through the brain or because of changing electrical properties of the scalp, we then electrically stimulated in a new set of mice (3 male, 1 female) epicranially without skin at 25 µA, i.e. a third of the original current. Over all blocks, there was a clear effect of tES (**Fig. 2G**). Again, there was a decreasing effect in the first few blocks that was fitted with the same exponential decay plus a consistent increase in the response induced by tES (**Fig. 2H**). The epicranial tES-induced response increase in the late blocks was about a third of the increase of the larger epicranial current stimulation (0.16 % versus 0.54%), indicating that at this current range, the effect of epicranial tES scales linearly with the current. The initial effect of the low current transcutaneous tES, however, was not three times smaller than that of the larger current, suggesting that there is saturation in the initial effect of tES with increasing current.

Changes in visual response following tES

Before and immediately after fifty blocks of visual and electrical stimulation, we measured the calcium response to visual gratings drifting in both directions without simultaneous electrical stimulation. The size and shape of the calcium responses after the blocks with electrical stimulation were different from those before the blocks with electrical stimulation, in particular the late phase of the visual response (**Fig. 3A-B**). There was no difference, however, in the response to the direction that had been coupled to the tES compared to the direction that had

not been coupled (p = 0.6, paired t-test, 4 animals x 4 sessions, 3 male, 1 female, **Fig. 3C**). Furthermore, this change in visual response was not related to the tES as mice that had not received electrical stimulation showed exactly the same change (p = 0.74, Mann-Whitney U-test, 4 animals with each 4 tES sessions and 1 session without electrical stimulation, **Fig. 3D**). The observed changes were therefore related to brain adaptation due to anesthesia or repeated visual stimulation.

Long-term effects of tES

Surprisingly, long-term effects of tES became evident when we compared the average visual response in the first session to the average visual response in the last (fourth) session (**Fig. 4A**). Although there was no difference in the peak visual response between the grating direction that had been coupled to the tES and to the direction that had not been coupled, there was a clear increase in the peak visual response to both visual stimuli between the first and last session (p = 0.011, paired t-test, 4 animals, 3 males, 1 female). We also found a significant increase in the peak visual response when measured over all four sessions (p = 0.005, non-zero slope test, **Fig. 4B**). To make sure that the combination of imaging and visual stimulation by itself does not change the visual response, we measured visual responses in four other animals (2 males, 2 females) without tES in even more sessions. In these animals we did not find a change in the visual response in the first versus the last session (**Fig. 4C**) and the visual response was flat over 7 sessions (p = 0.8, non-zero slope test, **Fig. 4D**). A power analysis using the tES data showed that we had 92% chance to observe the effect at the 5% level in this unstimulated control group if it had existed. The tES thus increased the visual responses over sessions, but it did so regardless of the specific visual stimulus direction to which it had been coupled.

Discussion

Long-term effects of tES on human behavior can persist for days or even weeks after multiple sessions of tES [7]; [42]. This is a prerequisite for tES as a practical therapy. Long-term effects on cortical sensory responses had not been previously investigated in animals. We found that tES over the visual cortex in mice enhanced calcium responses to visual stimuli during electrical stimulation. More importantly, repeating tES over multiple days led to an enhanced visual response in the visual cortex.

A possible limitation of our study is that the age of the animals ranged from 2 to 7 months (i.e. ranging from young adult to mature adult). Age might be a factor in the effect of tES, but all chronic tES animals (male and female) showed the same trend of increased visual response. One important question is how comparable the electric fields that we induced in the mouse brain are to those induced in humans. In humans, usually, tDCS is applied continuously for 10-30 minutes. We have, however, alternated short periods with and without tES to also measure the direct effect of (weak) current stimulation on the visual response. Current was flowing for 50 times 8 seconds, thus about 7 minutes in total. This is shorter than what is commonly applied in humans. The repetition of many short periods of electrical stimulation may also induce effects that are different from one longer contiguous period of stimulation. Our protocol may also evoke effects similar to those caused by transcranial pulsed current stimulation (tPCS), but the pulse duration and interstimulus time used in tPCS are typically in the range of 1 to 500 ms [43] and thus much shorter than what we applied.

We measured lasting effects of transcutaneous tES at a current density of 10 A/m^2 directly below the electrode. This was not a relatively high current density. It is 5 times lower than in previous mouse visual cortex epicranial tDCS [24]; [34]. The current density was higher than the typical scalp surface current density of 0.8 A/m^2 applied in humans, but it would be better to

compare the electric fields that are induced in the brain, because these cause the changes in neural activity. In a typical human experiment, a 0.4 A/m^2 current density at the electrode produced a local field of maximally around 0.5 V/m [44]. The electric fields are very dependent on the stimulation parameters, but if we assume that the field strength scales linearly with the applied current, then a modeling study suggests that our stimulation induced a maximum field strength of about 2.6 V/m [45]. Our induced field is thus likely to be higher than in the typical human experiment, but less than an order of magnitude. It is therefore likely that our experiments evoked similar plasticity mechanisms as evoked in human tDCS.

The acute enhancement of cortical responses that we found during anodal transcranial stimulation had been reported before in animals [46] and is likely caused by a subthreshold depolarization of the soma of cortical pyramidal cells [47]. A similar direct enhancement of responses to visual stimuli, induced using stronger current densities, was previously seen in mouse visual cortex [24]; [34]. Anodal tDCS of human visual cortex also caused an increased visually evoked potential (VEP) [30]. Contrary to these and two other human visual cortex studies [31]; [32], we did not observe any after-effects directly following electrical stimulation. This difference is most likely due to differences in the temporal profiles and current strengths of the tES protocols.

Even within single sessions, we saw that the concurrent effect of electrical stimulation on calcium response to visual stimuli declined sharply after the first few trials. Others have not reported this, but have also not studied the development of the effect during repeated electrical stimulation over time during a session. Therefore, we do not know if this reduction is common. Because a decline with the same time-constant is also present when the scalp is removed, this reduction is not likely to be caused by changes in the skin. The decline could be caused by changes in the ion and charge distribution in the skull, brain or on the electrode interfaces

(saline-soaked sponge and EEG-gel filled tube) or by biochemical changes. Electrical and biochemical changes in the brain could be directly caused by tES or by neural adaptation in response to the tES-induced neural activity, either cell intrinsic or synaptic. Epicranial tES at a third of the transcutaneous stimulation current gives a larger increase than the transcutaneous tES. This suggests that the skin (and the fluid between the skull and skin) must be electrically shielding the brain by conducting more than two-thirds of the current around the skull. We also observed a change in the strength of the visual responses during a session, but this change was not related to the tES, because it was also present in animals that had not received electrical stimulation. Changes in brain state during prolonged anesthesia or repeated visual stimulation may have caused this effect.

Previously, it was shown that stronger supra-threshold electrical stimulation inside rat cortex leads to long-term potentiation (LTP), especially when repeated over multiple sessions [49]. A long-term enhancement of cortical sensory response strength by multiple tES sessions had not been shown before in animals. That anodal tDCS can have a lasting effect on behavior, however, was previously shown by improved skill learning in mice [21] and rats [50]. Cathodal tDCS had a lasting effect when applied during associative learning in rabbits [23]. In humans, behavioral effects of tDCS can last for days or weeks [7]; [42]; [6]. The long-term enhancement of visual responses that we observe may be a good model to investigate the mechanism behind these other long-term effects.

Mechanism

It is known that in awake mice repeated presentation of a visual stimulus by itself can lead to an NMDA-dependent visual response enhancement over subsequent sessions [51]; [52]. Under anesthesia, this enhancement does not occur, but the combined visual presentation and current stimulation may evoke the same potentiation mechanism. In our case, though, the response

enhancement was not visual stimulus specific, in contrast to potentiation in awake animals. It could be that the plasticity-inducing effects of the electrical stimulation have some persistence in time, carrying over from one visual stimulus to the next, or that neurons that responded well to both gratings have changed their response. The visually evoked response potentiation has been linked to thalamocortical LTP [52]. This suggests another explanation for the lack of specificity of the effect to the grating direction that we found. For gratings drifting in two different directions, over the course of one visual cycle many of the same dLGN neurons will be active. By increasing the activation of the postsynaptic V1 neurons regardless of their preferred direction, the synapses connecting the dLGN with V1 may all be strengthened. Plasticity of these thalamocortical synapses was previously suggested to be the site of action of cathodal tDCS in the rabbit [23]. Other pathways previously implicated in tES-induced plasticity and known to affect plasticity in the visual cortex are GABAergic [53], BDNF [21], cholinergic [54] and serotonergic [55] signaling.

Our findings are a confirmation that tES can have lasting effects on the brain, at induced electric fields that are around the same order of magnitude of those induced in humans. The molecular, genetic and measurement tools that are available in the mouse can thus be used to study the molecular changes induced by repeated tES and investigate which pathways underlie tES-induced plasticity. This will aid the understanding of the long-term effects of tES in a medical or non-medical use [56].

Acknowledgements

We thank Christiaan Levelt sharing experimental set-ups and mice, Emma Ruimschotel for genotyping, Fieke Dekkers and Jorrit Montijn for critical reading of the manuscript, and Hongkui Zeng and the Allen Institute for making the Ai38-line available. This work was supported by NWO VIDI grant 864.10.010.

Conflict of interest: There are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

Author contributions: D.T., M.A. and J.A.H. designed the experiment. D.T. performed the experiments, assisted by M.A. D.T. and J.A.H. analyzed the data. J.A.H. and D.T. wrote the manuscript.

References

[1] Bikson M, Grossman P, Thomas C, Zannou AL, Jiang J, Adnan T, et al. Safety of transcranial direct current stimulation: evidence based update 2016. Brain Stimul 2016;9:641-61.

[2] Hummel FC, Cohen LG. Non-invasive brain stimulation: a new strategy to improve neurorehabilitation after stroke? Lancet Neurol 2006;5:708-12.

[3] Nitsche MA, Boggio PS, Fregni F, Pascual-Leone A. Treatment of depression with transcranial direct current stimulation (tDCS): a review. Exp Neurol 2009;219:14-19.

[4] Vanneste S, Plazier M, Ost J, van der Loo E, Van de Heyning P, De Ridder D. Bilateral dorsolateral prefrontal cortex modulation for tinnitus by transcranial direct current stimulation: a preliminary clinical study. Exp Brain Res 2010:202;779-85.

[5] Lima MC, Fregni F. Motor cortex stimulation for chronic pain systematic review and meta-analysis of the literature. Neurology 2008;70:2329-37.

[6] Marshall L, Mölle M, Hallschmid M, Born J. Transcranial direct current stimulation during sleep improves declarative memory. J Neurosci 2004;24:9985-92.

[7] Reis J, Schambra HM, Cohen LG, Buch ER, Fritsch B, Zarahn E, et al. Noninvasive cortical stimulation enhances motor skill acquisition over multiple days through an effect on consolidation. P Natl Acad Sci USA 2009;106:1590-5.

[8] Coffman BA, Clark VP, Parasuraman R. Battery powered thought: enhancement of attention, learning, and memory in healthy adults using transcranial direct current stimulation. Neuroimage 2014;85:895-908.

[9] Reinhart RM, Woodman GF. Enhancing long-term memory with stimulation tunes visual attention in one trial. P Natl Acad Sci USA 2015;112:625-30.

[10] Bogdanov M, Ruff CC, Schwabe, L. Transcranial stimulation over the dorsolateral prefrontal cortex increases the impact of past expenses on decision-making. Cereb Cortex 2017;27:1094-102.

[11] Elsner B, Kugler J, Pohl M, Mehrholz J. Transcranial direct current stimulation (tDCS) for improving function and activities of daily living in patients after stroke. Cochrane Db Syst Rev 2013;11:CD009645.

[12] Shiozawa P, Fregni F, Benseñor IM, Lotufo PA, Berlim MT, Daskalakis JZ, et al. Transcranial direct current stimulation for major depression: an updated systematic review and meta-analysis. Int J Neuropsychop 2014;17:1443-52.

[13] Brunoni AR, Moffa AH, Fregni F, Palm U, Padberg F, Blumberger DM, et al. Transcranial direct current stimulation for acute major depressive episodes: meta-analysis of individual patient data. Brit J Psychiat 2016;208:522-31.

[14] Leffa DT, Bellaver B, Salvi AA, de Oliveira C, Caumo W, Grevet EH, Fregni F, Quincozes-Santos A, Rohde LA, Torres ILS. Transcranial direct current stimulation improves long-term memory deficits in an animal model of attention-deficit/hyperactivity disorder and modulates oxidative and inflammatory parameters. Brain Stimul. 2018;11:743-751.

[15] Cioato SG, Medeiros LF, Marques Filho PR, Vercelino R, de Souza A, Scarabelot VL, de Oliveira C, Adachi LN, Fregni F, Caumo W, Torres IL. Long-Lasting Effect of Transcranial Direct Current Stimulation in the Reversal of Hyperalgesia and Cytokine Alterations Induced by the Neuropathic Pain Model. Brain Stimul. 2016;9:209-17.

[16] Macedo IC, de Oliveira C, Vercelino R, Souza A, Laste G, Medeiros LF, Scarabelot VL, Nunes EA, Kuo J, Fregni F, Caumo W, Torres ILS. Repeated transcranial direct current stimulation reduces food craving in Wistar rats. Appetite. 2016;103:29-37.

[17] Brunoni AR, Fregni F, Pagano RL. Translational research in transcranial direct current stimulation (tDCS): a systematic review of studies in animals. Rev Neurosci 2011;22:471-81.

[18] Underwood E. Cadaver study challenges brain stimulation methods. Science 2016;352:397.

[19] Nitsche MA, Paulus W. Excitability changes induced in the human motor cortex by weak transcranial direct current stimulation. J Physiol 2000;527:633–9.

[20] Bikson M, Inoue M, Akiyama H, Deans JK, Fox JE, Miyakawa H, et al. Effects of uniform extracellular DC electric fields on excitability in rat hippocampal slices in vitro. J Physiol 2004;557:175-90.

[21] Fritsch B, Reis J, Martinowich K, Schambra HM, Ji Y, Cohen LG, et al. Direct current stimulation promotes BDNF-dependent synaptic plasticity: potential implications for motor learning. Neuron 2010;66:198-204.

[22] Cambiaghi M, Velikova S, Gonzalez-Rosa JJ, Cursi M, Comi G, & Leocani L. Brain transcranial direct current stimulation modulates motor excitability in mice. Eur J Neurosci 2010;31:704-9.

[23] Márquez-Ruiz J, Leal-Campanario R, Sánchez-Campusano R, Molaee-Ardekani B, Wendling F, Miranda PC, et al. Transcranial direct-current stimulation modulates synaptic mechanisms involved in associative learning in behaving rabbits. P Natl Acad Sci USA 2012;109:6710-5.

[24] Monai H, Ohkura M, Tanaka M, Oe Y, Konno A, Hirai H, et al. Calcium imaging reveals glial involvement in transcranial direct current stimulation-induced plasticity in mouse brain. Nat Commun 2016;7:11100.

[25] Levelt CN, Hübener M. Critical-period plasticity in the visual cortex. Ann Rev Neurosci 2012;35:309-30.

[26] Antal A, Nitsche MA, Paulus W. External modulation of visual perception in humans. Neuroreport 2001;12:3553-5.

[27] Kraft A, Roehmel J, Olma MC, Schmidt S, Irlbacher K, Brandt SA. Transcranial direct current stimulation affects visual perception measured by threshold perimetry. Exp Brain Res 2010;207:283-90.

[28] Antal A, Paulus W, Nitsche MA. Electrical stimulation and visual network plasticity. Res Neurol and Neuros 2011;29:365-74.

[29] Spiegel DP, Byblow WD, Hess RF, Thompson B. Anodal transcranial direct current stimulation transiently improves contrast sensitivity and normalizes visual cortex activation in individuals with amblyopia. Neurorehab Neural Re 2013;27:760-9.

[30] Antal A, Kincses TZ, Nitsche MA, Bartfai O, Paulus W. Excitability changes induced in the human primary visual cortex by transcranial direct current stimulation: direct electrophysiological evidence. Invest Ophth Vis Sci 2004;45:702-7.

[31] Accornero N, Voti PL, La Riccia M, Gregori B. Visual evoked potentials modulation during direct current cortical polarization. Exp Brain Res 2007;178:261-6.

[32] Strigaro G, Mayer I, Chen J, Cantello R, Rothwell JC. Transcranial direct current stimulation effects on single and paired flash visual evoked potentials. Clin EEG Neurosci 2015;46:208-13.

[33] Alekseichuk I, Diers K, Paulus W, Antal A. Transcranial electrical stimulation of the occipital cortex during visual perception modifies the magnitude of BOLD activity: a combined tES–fMRI approach. NeuroImage 2015;140:110-7.

[34] Cambiaghi M, Teneud L, Velikova S, Gonzalez-Rosa J, Cursi M, Comi G, et al. Flash visual evoked potentials in mice can be modulated by transcranial direct current stimulation. Neuroscience 2011;185:161-5.

[35] Rigonatti SP, Boggio PS, Myczkowski ML, Otta E, Fiquer JT, Ribeiro RB, et al. Transcranial direct stimulation and fluoxetine for the treatment of depression. Eur Psychiat 2008;23:74-6.

[36] Zariwala HA, Borghuis BG, Hoogland TM, Madisen L, Tian L, De Zeeuw CI, et al. A Cre-dependent GCaMP3 reporter mouse for neuronal imaging in vivo. J Neurosci 2012;32:3131-41.

[37] Andermann ML, Kerlin AM, Roumis DK, Glickfeld LL, Reid RC. Functional Specialization of Mouse Higher Visual Cortical Areas. Neuron. 2011;72:1025-1039.

[38] Heimel JA, Saiepour MH, Chakravarthy S, Hermans JM, Levelt CN. Contrast gain control and cortical TrkB signaling shape visual acuity. Nat Neurosci 2010;13:642-8.

[39] Heimel JA, Hartman RJ, Hermans JM, Levelt CN. Screening mouse vision with intrinsic signal optical imaging. Eur J Neurosci 2007;25:795-804.

[40] Kleiner M, Brainard D, Pelli D, Ingling A, Murray R, Broussard C. What's new in Psychtoolbox-3. Perception 2007;36:1.

[41] Tohmi M, Kitaura H, Komagata S, Kudoh M, Shibuki K. Enduring critical period plasticity visualized by transcranial flavoprotein imaging in mouse primary visual cortex. J Neurosci 2006;26:11775-85.

[42] Boggio PS, Nunes A, Rigonatti SP, Nitsche MA, Pascual-Leone A, Fregni, F. Repeated sessions of noninvasive brain DC stimulation is associated with motor function improvement in stroke patients. Rest Neurol Neuros 2007;25:123-9.

[43] Fitzgerald PB.Transcranial pulsed current stimulation: a new way forward? Clin Neurophysiol. 2014;125:217-9.

[44] Opitz A, Falchier A, Yan CG, Yeagle EM, Linn GS, Megevand P, et al. Spatiotemporal structure of intracranial electric fields induced by transcranial electric stimulation in humans and nonhuman primates. Sci Rep 2016;6:31236.

[45] Alekseichuk I, Mantell K, Shirinpour S, Opitz A. Comparative modeling of transcranial magnetic and electric stimulation in mouse, monkey, and human. Neuroimage. 2019;1;194:136-148

[46] Creutzfeldt OD, Fromm GH, Kapp H. Influence of transcortical dc currents on cortical neuronal activity. Exp Neurol 1962;5:436-52.

[47] Purpura DP, McMurtry JG. Intracellular activities and evoked potential changes during polarization of motor cortex. J Neurophysiol 1965;28:166-85.

[48] Haseneder R, Kratzer S, von Meyer L, Eder M, Kochs E, Rammes G. Isoflurane and sevoflurane dose-dependently impair hippocampal long-term potentiation. Eur J Pharmacol 2009;623:47-51

[49] Trepel C, Racine R. Long-term potentiation in the neocortex of the adult, freely moving rat. Cereb Cortex 1998;8:719-29.

[50] Dockery CA, Liebetanz D, Birbaumer N, Malinowska M, Wesierska MJ. Cumulative benefits of frontal transcranial direct current stimulation on visuospatial working memory training and skill learning in rats. Neurobiol Learn Mem 2011;96:452-60.

[51] Frenkel MY, Sawtell NB, Diogo ACM, Yoon B, Neve RL, Bear MF. Instructive effect of visual experience in mouse visual cortex. Neuron 2006;51:339-49.

[52] Cooke SF, Bear MF. Visual experience induces long-term potentiation in the primary visual cortex. J Neurosci 2010;30:16304-13.

[53] Bachtiar V, Near J, Johansen-Berg H, Stagg CJ. Modulation of GABA and resting state functional connectivity by transcranial direct current stimulation. Elife 2015;4,e08789.

[54] Kuo MF, Grosch J, Fregni F, Paulus W, Nitsche MA. Focusing effect of acetylcholine on neuroplasticity in the human motor cortex. J Neurosci. 2007;27:14442-7.

[55] Nitsche MA, Kuo MF, Karrasch R, Wachter B, Liebetanz D, Paulus W. Serotonin affects transcranial direct current-induced neuroplasticity in humans. Biol Psychiatry 2009;66:503–508.

[56] Dubljević V, Saigle V, Racine E. The rising tide of tDCS in the media and academic literature. Neuron 2014;82:731-6.

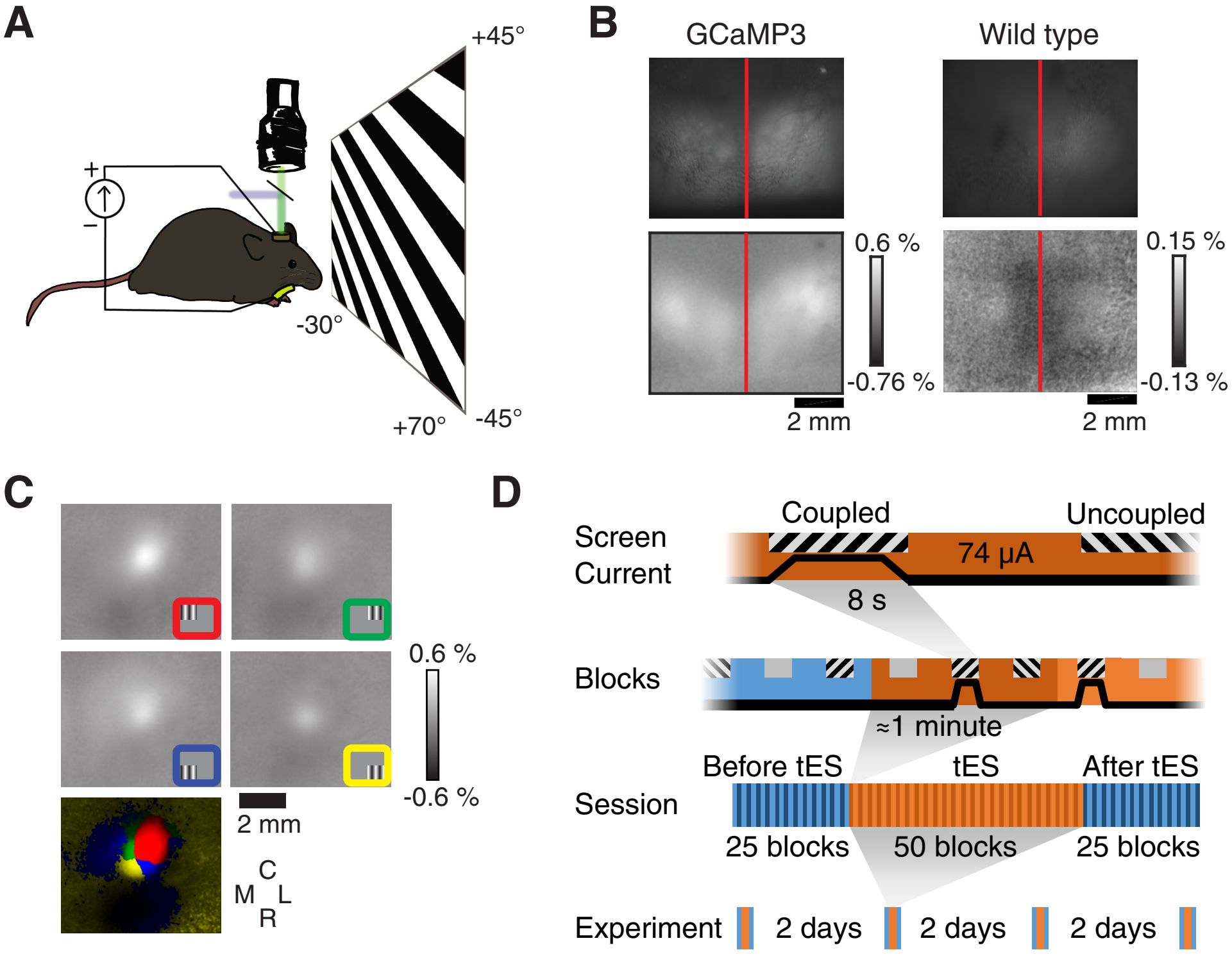
Figures

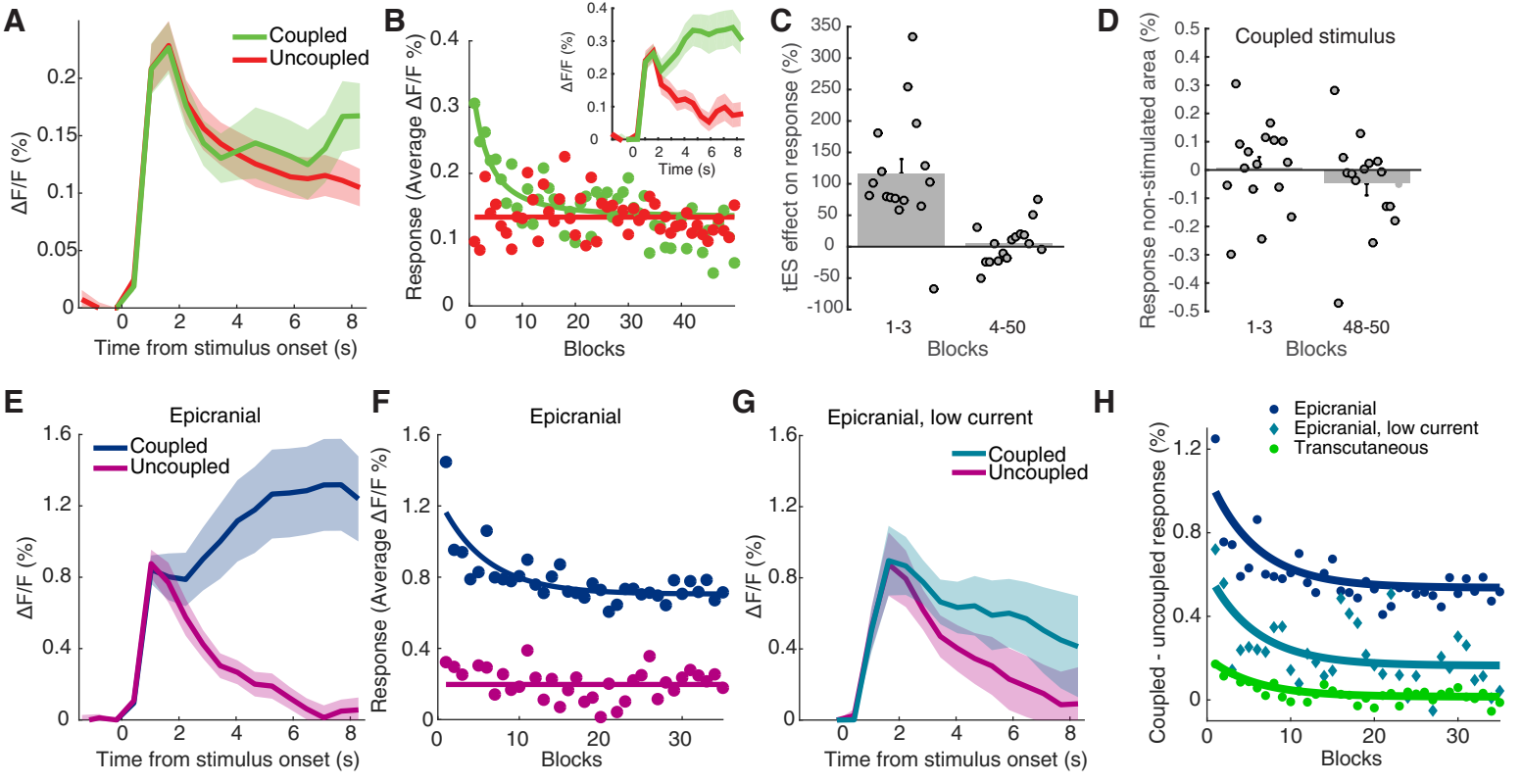
Figure 1. **Transcutaneous calcium imaging of visual and tES response. A**) Experimental setup for in vivo wide-field calcium imaging and transcranial current stimulation. **B**) Upper: Fluorescence images of the scalp of a GCaMP3 transgenic and a wild type mouse. Lower: ΔF/F during visual stimulation. Red line represents the midline. **C)** Example response to the visual stimulation in all monitor quadrants (grayscale panels). Bottom shows the retinotopic map where each pixel color represents the monitor quadrant to which the pixel showed the strongest visual response. CMRL refers to Caudal, Medial, Rostral and Lateral, respectively. **D**) Experimental paradigm. Top shows the visual stimulation for 8 s, which can be coupled to a ramping 8 s electrical stimulation. Each block of visual stimuli consists of the two grating stimuli and a blank visual stimulus, in random order. One session is composed of 25 blocks without tES, 50 blocks where the 45° grating is coupled with tES, and 25 blocks without tES. One experiment takes 7 days in which each mouse is imaged in 4 sessions. The sessions are 2 days apart.

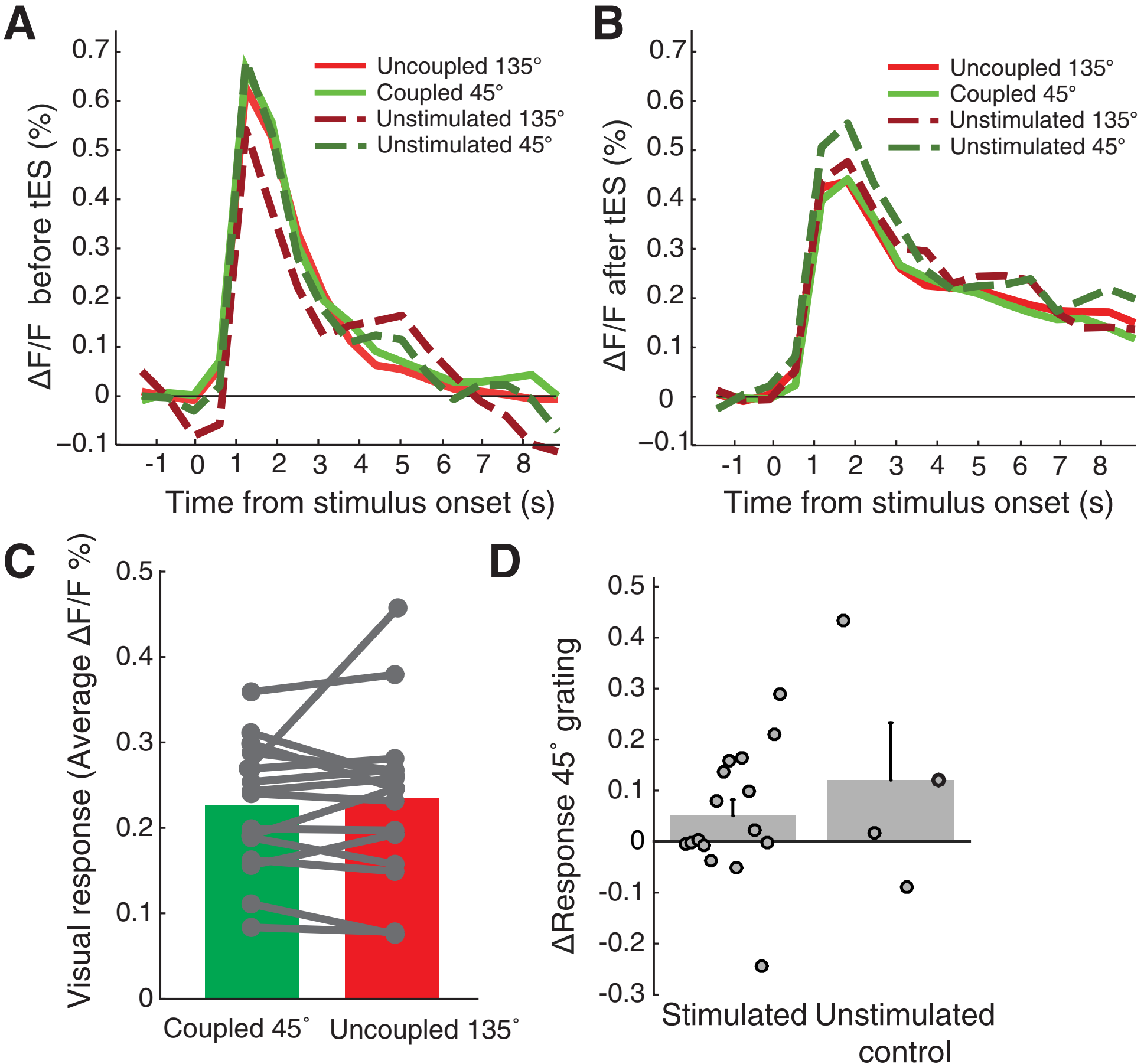
Figure 2. Anodal tES increases calcium responses to visual stimuli during electrical stimulation. **A**) Calcium response to visual stimulation coupled with simultaneous anodal transcutaneous tES (green) and uncoupled with tES (red) of 10 A/m^2 (4 animals x 4 imaging sessions), averaged over all visual stimulus blocks and all sessions. Time is relative to the onset of each visual stimulus. Band shows SEM. **B**) Response to visual stimulus alone (red) and concurrently coupled with transcutaneous tES (green) for all blocks within a tES session, averaged over all sessions. Each dot represents one block of a triplet of visual stimulation coupled and uncoupled with tES and blank visual stimulation. Curves are exponential fits. Block 1: $p = 0.0008$, block 2: $p = 0.0024$, block 3: $p = 0.0031$, Mann-Whitney U-test, all other blocks $p > 0.05$. Inset shows response like **A** for first 3 blocks. **C**) Effect of transcutaneous tES (difference between response with and without tES) relative to response averaged over the first three and later blocks for all sessions. Error bars denote mean ± SEM. **D)** There is no response to the visual stimulus coupled with transcutaneous tES in a non-visual, not electrically stimulated area at the corner of the imaged cortex, anterior and contralateral to the electrically stimulated visual cortex. There is no difference between the first and the last three blocks of stimulation ($p = 0.28$, paired t-test, 4 animals x 4 sessions). **E-F)** as **A-B** but with epicranial electrical stimulation of 10 A/m^2 (2 animals x 4 sessions). Curves are exponential fits with an offset and with a time constant fixed to the time constant found for transcutaneous stimulation. **G)** As **E** but with epicranial electrical stimulation of 3.3 A/m^2 (4 animals x 1 session). **H)** Mean difference between the coupled response and mean uncoupled response during a session plotted for the three conditions and fitted with exponentials with an offset and with a time constant fixed to the time constant found for transcutaneous stimulation.

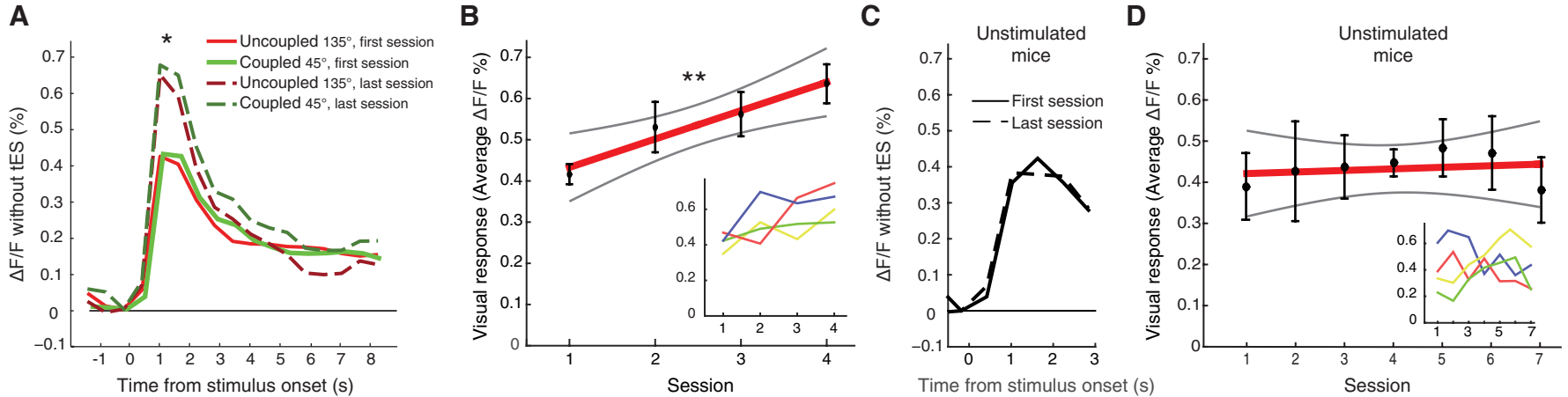
Figure 3. Visual response is not changed directly after tES. A) Visual response to two gratings drifting in orthogonal directions before transcutaneous tES and **B**) at the end of the imaging session after tES stimulation (4 animals x 4 sessions) or before and after sessions without tES (unstimulated, same 4 animals x 1 session), averaged over blocks and sessions. Solid lines represent data from sessions with tES and dashed lines are from later unstimulated sessions without tES. Coupled denotes the visual stimulus that was coupled to electrical stimulation during the tES blocks. Uncoupled denotes the visual stimulus that was not coupled to electrical stimulation, but was measured in a session with tES blocks. Average visual response is the relative change in fluorescence minus the average relative change for a blank visual stimulus. Time is relatively to the onset of each visual stimulus. **C**) Paired data show no difference of visual response between the coupled and uncoupled orientations ($p = 0.6$, paired t-test, 4 animals x 4 sessions). **D**) No difference in the change in visual response strength (without concurrent tES) to the 45° degree grating in sessions when it had been coupled with tES and in unstimulated control sessions without coupling to tES ($p = 0.74$, Mann-Whitney U-test). Error bars denote mean ± SEM.

Figure 4. tES unspecifically enhances visual response over sessions. A) Average changes in fluorescence in response to a visual stimulus, without concurrent tES, on the first imaging transcutaneous tES session compared to the last session after 7 days. Time is relatively to the onset of each visual stimulus. Difference at peak response: $p = 0.011$, paired t-test, 4 animals. **B)** Average visual response (cortical calcium response measured without tES) for each session for mice where visual stimulation was coupled with electrical stimulation ($p = 0.005$, non-zero slope test). Error bars denote mean ± SEM. Red line is linear regression line. Gray curves show 95% confidence interval. Inset shows individual mice. Blue line indicates the female mouse in this set. **C)** Average visual responses for different animals where visual stimulation had not been coupled with electrical stimulation (4 animals). **D)** Average visual responses over subsequent sessions in animals where visual stimulation was not coupled with electrical stimulation ($p = 0.8$, non-zero slope test). Inset shows individual mice.


A
+45°
-30°
+70°
-45°
B
GCaMP3
Wild type
0.6 %
-0.76 %
2 mm
0.15 %
-0.13 %
2 mm
C
0.6 %
-0.6 %
2 mm
C
M L
R
D
Coupled
Uncoupled
Screen
Current
74 µA
8 s
Blocks
≈1 minute
Before tES
tES
After tES
Session
25 blocks
50 blocks
25 blocks
Experiment
2 days
2 days
2 days


A
Coupled
Uncoupled
ΔF/F (%)
Time from stimulus onset (s)
B
Response (Average ΔF/F %)
Blocks
ΔF/F (%)
Time (s)
C
tES effect on response (%)
1-3
4-50
Blocks
D
Coupled stimulus
Response non-stimulated area (%)
1-3
48-50
Blocks
E
Epicranial
Coupled
Uncoupled
ΔF/F (%)
Time from stimulus onset (s)
F
Epicranial
Response (Average ΔF/F %)
Blocks
G
Epicranial, low current
Coupled
Uncoupled
ΔF/F (%)
Time from stimulus onset (s)
H
Epicranial
Epicranial, low current
Transcutaneous
Coupled - uncoupled response (%)
Blocks


A
B
C
D
Uncoupled 135°
Coupled 45°
Unstimulated 135°
Unstimulated 45°
ΔF/F before tES (%)
ΔF/F after tES (%)
Time from stimulus onset (s)
Visual response (Average ΔF/F %)
Coupled 45°
Uncoupled 135°
ΔResponse 45° grating
Stimulated
Unstimulated control


A
*
Uncoupled 135°, first session
Coupled 45°, first session
Uncoupled 135°, last session
Coupled 45°, last session
ΔF/F without tES (%)
Time from stimulus onset (s)
B
**
Visual response (Average ΔF/F %)
Session
C
Unstimulated mice
First session
Last session
ΔF/F without tES (%)
Time from stimulus onset (s)
D
Unstimulated mice
Visual response (Average ΔF/F %)
Session