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Transcranial direct current stimulation does not improve memory deficits or alter pathological hallmarks in a rodent model of Alzheimer's disease

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ABSTRACT

Alzheimer's disease (AD) is a progressive and debilitating degenerative disorder for which there are currently no effective therapeutic options. Non-invasive neuromodulation, including transcranial direct current stimulation (tDCS), has been investigated for the treatment of cognitive symptoms in AD. Results from clinical and preclinical studies, however, have been somewhat controversial. We investigate whether tDCS delivered to triple transgenic (3xTg) AD mice improves memory deficits and mitigates the development of AD-type neuropathology.

3xTg AD mice and controls were implanted with paddle electrodes over the skull. The cathode was anterior to bregma and the anode anterior to lamda. tDCS was delivered for 20 min/day, 5 days/week over three weeks at 50 μA. Though this amplitude was lower than the one used in the preclinical literature, it generated a high current density compared to the clinical scenario. Memory testing was conducted during treatment weeks 2 and 3. Post-mortem pathological AD markers were studied. Our results show that performance of 3xTg mice in the novel object recognition and Morris water maze tests was significantly impaired compared to that of controls. In addition, AD transgenics had an increased expression of tau, phosphorylated-tau and amyloid precursor protein in the hippocampus. tDCS did not improve behavioural deficits or mitigated the development of AD neuropathology in 3xTg animals. In summary, we found that tDCS at the settings selected in our study was largely ineffective in improving memory performance or altering the expression of AD pathological hallmarks in a validated mouse model.

1. Introduction

Memory deficits are a hallmark of numerous neuropsychiatric disorders, including dementias. Alzheimer's disease is the most common form of dementia accounting for 50–60% of cases. With the anticipated increase in life expectancy, it is estimated that around 40 million people worldwide will be diagnosed with AD by 2030 (World Health Organization, 2012). In addition to human suffering, the socioeconomic burden of the disease is enormous with more than US$ 400 billion spent per year in global health-care costs (World Health Organization, 2012). The most commonly used medications to treat AD are acetylcholinesterase inhibitors (Ballard et al., 2011; Birks, 2006; Lockhart et al., 2009). The benefits of these drugs on memory, however, are somewhat limited (Ballard et al., 2011; Birks, 2006; Birks and Harvey, 2006).

Over the last decade several neuromodulation techniques have been proposed to treat different cognitive aspects of AD. Transcranial direct current stimulation (tDCS) has been considered a promising non-invasive modality, particularly due to its safety profile. To date, a number of clinical trials have been conducted using anodal, but also cathodal dorsolateral prefrontal cortex or temporal tDCS (Boggio et al. 2009, 2012; Bystad et al., 2016; Chang et al., 2018; Cotelli et al., 2014;

List of abbreviations: AD, Alzheimer's disease; CTL, control; LTM, long-term memory; NOR, novel objet recognition; STM, short-term memory; tDCS, transcranial direct current stimulation; 3xTg, Triple transgenic mice

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Ferrucci et al., 2008; Khedr et al., 2014; Penolazzi et al., 2015). Overall, results have been somewhat inconsistent (Chang et al., 2018) with trials showing cognitive improvement (Boggio et al. 2009, 2012; Cotelli et al., 2014; Ferrucci et al., 2008) or no significant differences between stimulated patients and sham-treated controls (Bystad et al., 2016).

Preclinical studies to test the effects of tDCS have largely shown improvement in different forms of memory and cognitive function (Dockery et al., 2011; Kamida et al., 2011; Leffa et al., 2016; Manteghi et al., 2017; Nasehi et al. 2017a, 2017b; Pedron et al., 2014; Podda et al., 2016; Wu et al., 2017; Yoon et al., 2016; Yu et al., 2015). These, however, have not been conducted in AD models.

Triple transgenic (3xTg) mice are suited to mimic some aspects of AD, inasmuch as animals present age-related cognitive deficits and relevant neuropathological substrates, including the accumulation of amyloid precursor protein and tau (Gimenez-Llort et al., 2007; Oddo et al. 2003a, 2003b).

We test whether tDCS delivered to 3xTg AD mice improves hippocampal memory performance and mitigates the development of neuropathology.

2. Materials and methods

2.1. Animals

This study was approved by the Toronto Western Research Institute Animal Care Committee and is in accordance with the guidelines of the Canadian Council on Animal Care. Breeding pairs for homozygous 3xTg AD mice (stock #34830) and WT 129SV/C57BL6 founder strains (stock #101043) were purchased from Jackson's laboratory, bred and housed at the Toronto Western Hospital. Tail clippings of parents were used to confirm genotype at The Hospital for Sick Children Genetics Analysis Facility (Toronto). All mice had *ad libitum* access to food and water and were housed in a room maintained at a constant temperature (20–22 °C) on a 12 h: 12 h light–dark cycle.

2.2. Surgery and stimulation

Based on our previous behavioural and neuropathology work with 3xTg AD mice (Mann et al., 2018), six-months male animals were anesthetized with isoflurane and fixed to a stereotactic instrument (Model 900, David Kopf Instruments). Following skull exposure, two paddle electrodes were implanted on top of the bone (E303/76; 1 mm width, 3.25 mm length, 0.66 mm thickness; Plastics One; Roanoke, VA, USA). The one to be used as cathode was placed in a region 2 mm anterior to bregma (i.e. over the anterior cingulum and part of the secondary motor cortex) perpendicular to the interfrontal suture, with 1.6 mm extending to each hemisphere. The electrode to be used as anode was positioned 1 mm anterior to lambda, over the retrosplenial and secondary visual cortices, above the dorsal temporal hippocampus (e.g. CA1 region and dentate gyrus). Once in place, electrodes were fixed with dental acrylic cement. To test whether the system was working, one day after the implants stimulation was delivered to the animals at incremental levels for a few seconds. When currents in the 150–200 μA range were delivered, mice tended to scratch the region of the surgical cap, which suggests that these amplitudes were not confortable and potentially associated with the development sensory phenomena (Bregman et al., 2014).

Following one week of postoperative recovery, mice were given daily constant current stimulation for 20 min/day over 3 weeks (5 day/week with weekends off-total of 15 sessions). This timeframe was selected to mimic the one commonly used in the clinic, where patients are stimulated for 20min/day over 3–4 weeks. The selection of current amplitude was based on current density, as we were not able to measure electric fields associated with the clinically relevant value of 0.5–1V/m. In humans receiving 2 mA through conventional tDCS electrodes (35 cm^2), current density would be in the order of 1–2 μA/mm^2. Bearing in mind that our electrodes had 3.25 mm^2, we have sought to administer 5 μA. Because this would comprise a relatively low dose even for stimulation delivered through implanted cerebral electrodes, we have decided to use 50 μA instead. Control mice (CTL) had electrodes implanted but received no stimulation.

2.3. Behavioural testing

Novel place/object recognition and Morris Water Maze were carried out on stimulation weeks 2 and 3, respectively. tDCS was delivered in the morning and testing conducted after stimulation offset. One transgenic control animal died during the study.

2.3.1. Novel object recognition (NOR)

Mice were habituated for 15 min over three days to a rectangular arena. On day 4, they underwent a 5-min acquisition phase for novel object recognition with identical objects positioned in opposite corners. Forty-five minutes later, animals were tested for short-term memory (STM NOR). During this phase, one familiar (F) object was replaced by a novel one (N) and animals allowed to explore the apparatus for 5 min (Magen et al., 2012). Twenty-four hours later (day 5), mice were tested for long-term memory (LTM NOR). The former novel object was replaced by a newer one and animals were given 5 min to explore. The location of novel and familiar objects was counterbalanced in all experiments (Davis et al., 2013; Leger et al., 2013). Recorded videos were manually scored by a blinded-experimenter. Exploration of the object was considered when the animal's nose was within 2 cm of the object. Percent time exploring the novel object was calculated using the following formula: $\%T = (TN/(TN + TF)) \times 100\%$. The number of mice per group was as follows: WT-CTL (n = 8), WT-tDCS (n = 8), 3xTg AD-CTL (n = 7) and 3xTg AD-tDCS (n = 8).

2.3.2. Morris Water Maze (MWM)

The MWM test was conducted as previously described (Mann et al., 2018). Briefly, a 120 cm diameter circular water tank was filled with 26 ± 1 °C water made opaque by white non-toxic paint. The escape platform was submerged 1 cm below the surface. Five visual cues were equally spaced on a white curtain surrounding the tank. Mice were trained for three days with three trials per day (inter-trial interval of ~15 s). A trial was considered complete either when the mouse reached the platform or when 60 s have elapsed. If the mouse failed to find the platform, it was guided to it and allowed to spend 5 s prior to being returned to the home cage. One hour after the last training session, spatial memory was assessed in a single probe trial. During the probe, the platform was removed from the pool and the mouse was given 60 s to search for it. Behavioural data was acquired and summarized using the automated tracking system EthoVision XT (Noldus Inc., Leesburg, VA). Four outliers with swimming impairment (WT-CTL n = 2; WT-tDCS n = 1; AD-tDCS n = 1) were identified and removed from the analysis. The number of animals per group was as follows: WT-CTL (n = 6), WT-tDCS (n = 7), 3xTg AD-CTL (n = 7) and 3xTg AD-tDCS (n = 7).

2.4. Tissue collection

Twenty-four hours after the MWM probe trial mice were deeply anesthetized with isoflurane and decapitated. Brains were removed and divided in hemispheres. Hippocampi and prefrontal cortical regions (prelimbic cortex, anterior cingulum and a small portion of the secondary motor cortex) were dissected, collected, immediately flash frozen in liquid nitrogen and stored at −80 °C (Gondard et al., 2015). Western blotting was performed as previously described (Gondard et al., 2015; Mann et al., 2018). Samples were homogenized in RIPA lysis buffer (50 mM Tris-HCl, pH 7.4, 2 mM EDTA, pH 8, 150 mM NaCl, 1% Triton X-100, protease inhibitor cocktail; Roche) on ice for 30 min and then centrifuged (13,000 × *g*, 15 min, at 4 °C). A detergent

compatible (DC) protein assay (Biorad) was performed. 70 μg of protein per sample were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes (Biorad). After blocking in a solution of 0.1 M Tris-buffered saline with 0.1% Tween-20 (TBST) supplemented with 5% non-fat milk for 30min, membranes were incubated at 4 °C overnight with rabbit monoclonal Phospho-tau (1:1000; Cell Signaling), mouse monoclonal tau antibody (1:1000; Abcam) and mouse monoclonal Aβ-6E10 antibody (1:200; Biolegend). On the following day, membranes were washed three times with TBST, and incubated with horseradish-peroxidase- conjugated anti-mouse IgG or horseradish-peroxidase- conjugated anti-rabbit IgG (1:5000; GE Healthcare) for 1 h at room temperature. Membranes were then washed three times for 10 min and protein expression was visualized using enhanced chemiluminescence kits (ECL: GE Healthcare or ECL Plus: Thermo Fisher Scientific), followed by exposure to x-ray film for detection. Loading of total protein was confirmed using anti-GAPDH antibodies (1:1000; Cell Signaling).

2.5. Data analysis and statistics

Behavioural data was quantified using EthoVision XT (automated tracking; Noldus Inc.) and imported into GraphPad Prism for statistical analysis. Western blot bands were quantified using ImageJ (National Institutes of Health). Pixel densities were analyzed using rectangular areas of uniform size for each band. A semi-quantitative analysis was performed by densitometry and samples normalized to the respective GAPDH bands. Results were calculated and graphically shown as ratio relative to GAPDH bands.

Two-way repeated measures ANOVA (Fisher post hoc) was used to analyze training results in the MWM (group and time used as factors). Factorial ANOVA was used to compare the remainder data with genotype (WT, 3xTg AD) and treatment (CTL, tDCS) considered as factors. Results are shown as means ± standard error of the mean (SEM).

3. Results

3.1. NOR

No significant interaction ($F_{1,27} = 1.38$, $P = 0.25$), genotype ($F_{1,27} = 2.88$, $P = 0.10$) or treatment effects ($F_{1,27} = 0.25$, $P = 0.61$) were recorded when the percentage of time exploring the novel vs. the old object was measured after training (Fig. 1A). In contrast, animals tested one day later (LTM) presented a significant genotype effect ($F_{1,27} = 6.17$, $P = 0.02$) but no treatment effect ($F_{1,27} = 0.06$, $P = 0.81$) or treatment vs. genotype interaction ($F_{1,27} = 0.50$, $P = 0.49$; Fig. 1B). LTM results were due to a significantly worse performance of AD mice compared to WTs controls (p = 0.03) and a trend towards a worse performance of AD-CTLs compared to the WT tDCS group (p = 0.06).

3.2. Morris Water Maze

During training in the MWM, animals had a reduction in the latency to find the platform over time ($F_{2,46} = 12.7$, $P < 0.01$; Fig. 2A). On the third day of training, we found that AD-CTLs took significantly longer to find the platform than WT-CTLs ($P = 0.005$) and WT-tDCS animals ($P = 0.02$).

On the probe testing to assess spatial memory 1 h after the platform was removed from the pool, a significant genotype effect ($F_{1,23} = 6.42$, $P = 0.02$) but no treatment effect ($F_{1,23} = 1.00$, $P = 0.34$) or interaction genotype x treatment were recorded ($F_{1,23} = 0.16$, $P = 0.69$; Fig. 2B). AD-tDCS mice crossed the platform zone fewer times than WT-CTLs ($P = 0.02$) or WT-tDCS animals ($P = 0.045$).

As for the time spent in the target zone (TZ), no significant interaction ($F_{1,23} = 0.38$, $P = 0.54$), genotype ($F_{1,23} = 2.34$, $P = 0.14$), or treatment effects were noticed ($F_{1,23} = 0.50$, $P = 0.49$; Fig. 2C).

3.3. Western blot

To test whether tDCS affected AD pathological hallmarks we measured total-tau, Ser416 residue phosphorylated tau and APP levels in the hippocampus and cortex. In the hippocampus, a genotype effect was observed for total-tau ($F_{1,27} = 17.6$, $P = 0.0003$), phosphorylated-tau ($F_{1,27} = 97.83$, $P < 0.0001$), and APP ($F_{1,27} = 47.34$, $P < 0.0001$). Expression of these proteins was largely noted in AD transgenics (Fig. 3A). In contrast, no significant interaction or treatment effect were observed when hippocampal levels of total-tau ($F_{1,27} = 2.17$, $P = 0.152$; $F_{1,27} = 0.028$, $P = 0.868$, respectively), phosphorylated-tau ($F_{1,27} = 0.318$, $P = 0.577$; $F_{1,27} = 0.039$, $P = 0.845$, respectively) or APP were considered ($F_{1,27} = 1.371$, $P = 0.252$; $F_{1,27} = 1.35$, $P = 0.256$, respectively).

In the cortex, no significant interaction, genotype or treatment effects were noticed when the expression of total-tau ($F_{1,27} = 0.013$, $P = 0.909$; $F_{1,27} = 0.237$, $P = 0.63$; $F_{1,27} = 0.02$, $P = 0.887$, respectively) and Ser416 residue phosphorylated-tau were considered ($F_{1,27} = 2.509$, $P = 0.125$; $F_{1,27} = 0.456$, $P = 0.5$; $F_{1,27} = 0.029$, $P = 0.865$, respectively; Fig. 3B). However, AD mice had a higher expression of cortical APP compared to WT controls ($F_{1,27} = 328.0$, $P < 0.0001$). tDCS had no effect on cortical pathology regardless of genotype ($F_{1,27} = 1.31$, $P = 0.263$).

4. Discussion

We found that tDCS delivered at the settings selected in our study did not alter memory performance in either AD mice or healthy controls. tDCS was also ineffective in mitigating AD pathology in 3xTg

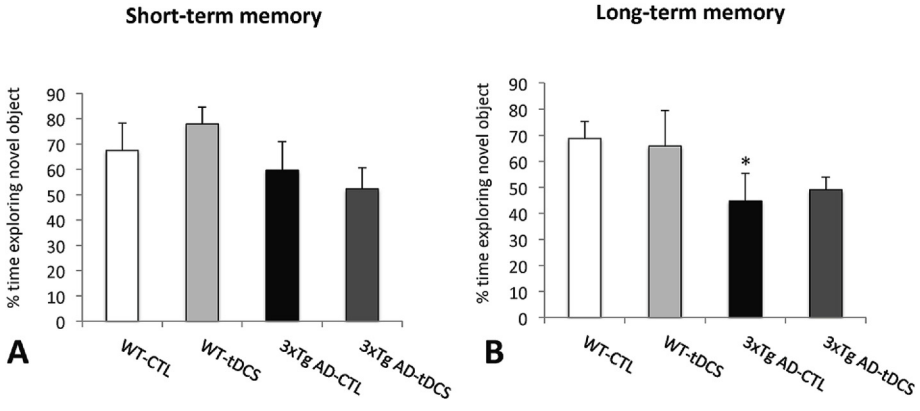

Fig. 1. Novel Object Recognition testing. An initial acquisition phase was conducted with identical objects positioned in opposite corners of an arena. Animals underwent testing 45 min (short-term memory) or 24 h later (long-term memory) with one of the familiar objects being replaced by a novel one. The percentage of time spent exploring the novel object was calculated. While no differences across groups were noted at short-term (A), a significant genotype effect was recorded at long term (B). This was due to a significantly worse performance of Alzheimer's disease (AD) mice compared to wild-type (WT) controls and a trend towards a worse performance of AD mice compared to the WT tDCS group. WT-CTL (n = 8), WT-tDCS (n = 8), 3xTg AD-CTL (n = 7) and 3xTg AD-tDCS (n = 8). *P < 0.05 when WT-CTL and 3xTg AD transgenics were compared. Data represent mean ± standard error.

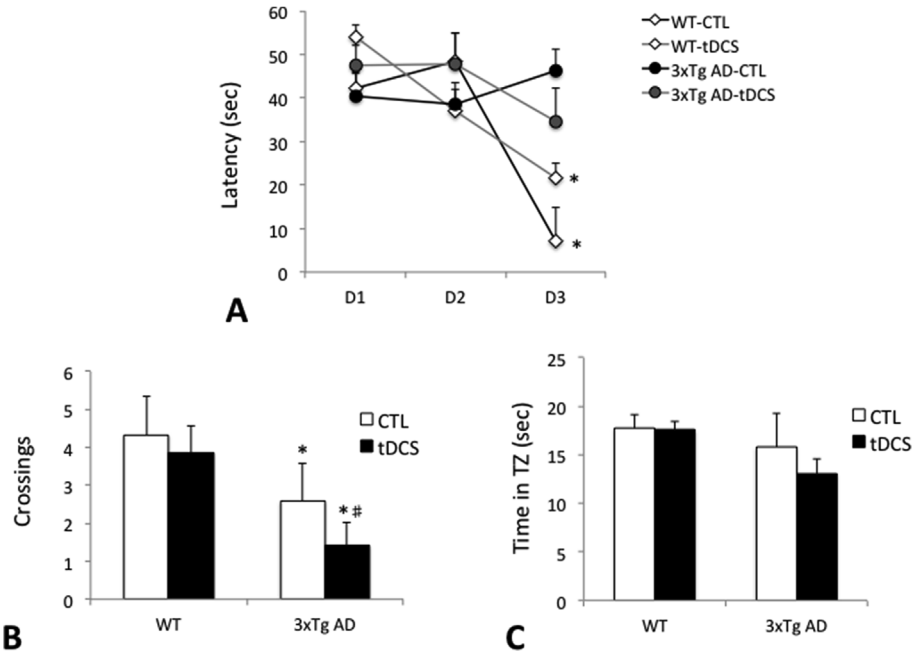

Fig. 2. Morris Water Maze. Mice were trained for 3 days followed by probe testing without the platform 1 h after training. (A) During training, animals had an improvement in performance over time. On the third day, however, Alzheimer's disease controls (AD-CTL) took significantly longer to find the platform than wild-type (WT)-CTLs and WT-tDCS animals. (B) AD-tDCS mice crossed the platform zone fewer times than WT-CTLs or WT-tDCS animals during probe testing. (C) No significant differences across groups were observed when the time spent in the target zone (TZ) was considered. WT-CTL (n = 6), WT-TDCS (n = 7), AD-CTL (n = 7), AD-TDCS (n = 7). *P < 0.05 when WT-CTL and 3xTg AD transgenics were compared; #P < 0.05 when WT-tDCS and 3xTg AD transgenics were compared. Data represent mean ± standard error.

animals.

In the clinic, a number of studies have been conducted to test the safety and efficacy of tDCS in AD (Boggio et al. 2009, 2012; Bystad et al., 2016; Chang et al., 2018; Cotelli et al., 2014; Ferrucci et al., 2008; Khedr et al., 2014; Penolazzi et al., 2015). While most have shown some degree of cognitive improvement (Boggio et al. 2009, 2012; Cotelli et al., 2014; Ferrucci et al., 2008), some found no significant differences between stimulated and sham-treated patients (Bystad et al., 2016).

Preclinical work using tDCS in rodents has largely shown memory improvement in different paradigms and conditions, including post-traumatic brain injury (Yoon et al., 2016), diabetic rats (Wu et al., 2017), epileptic animals (Kamida et al., 2011), tests to measure short-term memory (Leffa et al., 2016), working memory (Pedron et al., 2014), spatial memory (Dockery et al., 2011; Podda et al., 2016), and fear memory (Manteghi et al., 2017; Nasehi et al. 2017a, 2017b). In most of these studies, tDCS was applied transcranially or through the skull for 10–20 min/day over several days with the anode placed in front of bregma. Current, however, was often much higher than the one used in our study. As described in the methods, our choice of amplitude was based on density. We note that current density following the selected value of 50 μA would already be higher than the one used in the clinic. In humans, adverse events such as itching, tingling and redness in the stimulated regions often preclude the use of higher amplitudes. Corroborating the notion that current may affect memory performance

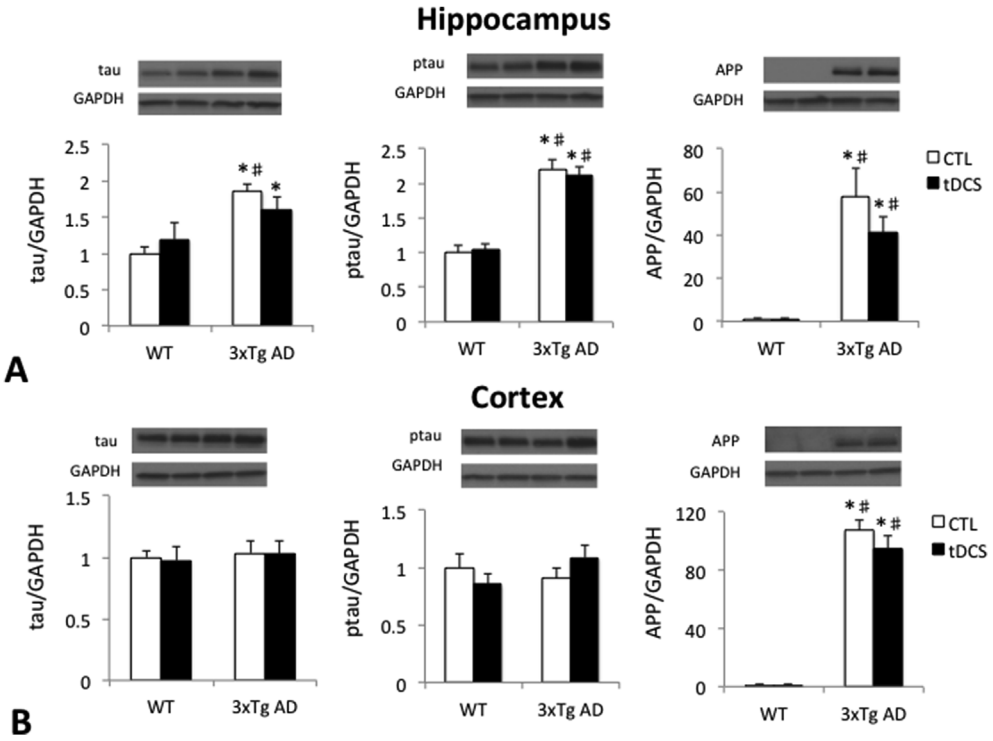

Fig. 3. Chronic tDCS had no effect on Alzheimer's disease (AD) pathological markers. (A) Hippocampal and (B) cortical levels of tau, Ser416 residue phosphorylated-tau (ptau) and amyloid precursor protein (APP) measured with Western blot. While these three markers were increased in the hippocampus, only APP was increased in the cortex of AD transgenics. tDCS exerted no effect in the expression of these proteins in either region. *P < 0.05 when wild-type controls (WT-CTL) and 3xTg Alzheimer's disease transgenics were compared; #P < 0.05 when WT-tDCS and 3xTg AD transgenics were compared. Data represent mean ± standard error.

following tDCS, Yu and colleagues conducted dose curve response experiments showing that stimulation below 100 μA was somewhat ineffective when given to rats injected with hippocampal Aβ1-40 (Yu et al., 2015). In our study, when current amplitude was increased above 150–200 μA mice tended to scratch the region of the electrode implants (Bregman et al., 2014). This validates the integrity of our system, while suggesting that animals might have been disturbed by the experienced sensation.

Another important difference between our study and others was the use of transgenic AD mice. Numerous rodent models have been proposed for the study of AD, including inducible and genetic preparations (Gotz et al., 2018). Transgenic animal models are often obtained through the integration of gene variants associated with the expression of proteins involved in the pathophysiology of the disease (Gotz et al., 2018). In this study we chose to work with triple transgenic animals because they present age-related cognitive deficits and AD-related neuropathological changes (Gimenez-Llort et al., 2007; Oddo et al. 2003a, 2003b). In previous work, we have shown that animals with 6–7 months of age presented some degree of memory impairment, APP and tau accumulation (Mann et al., 2018). Based on DBS-induced improvements observed in that study we have decided to deliver tDCS to 3xTg mice between the 6th and 7th months of age. While memory deficits and AD-type pathology were noticed, tDCS was largely ineffective in mitigating these abnormalities. As treatments are more effective when delivered during early stages of AD, we find it unlikely that tDCS would have been more effective in older animals.

As with other electrical stimulation techniques, during tDCS electrons flow from the cathode to the anode (Brunoni et al., 2019). Current intensities used in the clinic do not generate action potentials, but modulate synaptic transmission in a non-linear manner (Batsikadze et al., 2013; Brunoni et al. 2012, 2019; Monte-Silva et al., 2013). Excitatory or inhibitory responses have been attributed to the effects of stimulation on intracellular concentrations of Ca^{+2} and the subsequent development of different forms of plasticity (Brunoni et al., 2019; Yavari et al., 2018).

In several human trials, anodes were placed over the dorsolateral prefrontal cortex or temporal regions (Boggio et al. 2009, 2012; Bystad et al., 2016; Chang et al., 2018; Cotelli et al., 2014; Ferrucci et al., 2008; Khedr et al., 2014; Penolazzi et al., 2015). As we were largely testing hippocampal memory function, we have decided to implant the anode over cortical areas covering the hippocampus. As for prefrontal cortical regions, homology between rodents and humans is fairly controversial (Hamani et al. 2010, 2011; Hamani and Nobrega, 2012; Hamani and Temel, 2012). However, the proposed rodent region with the highest degree of functional, connectivity and anatomical homology with the human dorsolateral prefrontal cortex seems to be the dorsal prelimbic region (Hamani et al. 2010, 2011; Hamani and Nobrega, 2012; Hamani and Temel, 2012). This structure is buried underneath associative motor and cingulate cortical regions.

Finally, we note that studies conducted in animal models are not without limitations. It is possible that more subtle memory changes reported by humans receiving tDCS might not have been captured or observed in rodents undergoing well-validated but somewhat limited memory tests. It is also plausible that humans may require less current than rodents for an effect.

In summary, we found that tDCS delivered to transgenic animals at relatively low currents did not counter memory deficits or the development of AD-type neuropathology. Though stimulation amplitude in our experiments was lower than in other studies, current density was much higher than the one used in humans (i.e. when the dimensions of the electrodes are taken into account). By following previous work and enhancing stimulation intensity, we would have likely reduced the translational nature of our study.

As a final remark, we stress that our data is not suited to refute the use of tDCS in patients with memory deficits. It just suggests that stimulation at amplitudes closer to the ones used in humans were ineffective in AD transgenics. Instead of discrediting the technique, we believe that technological advancements are required so that higher amplitudes may be delivered to patients.

Conflicts of interest

The authors report no conflict of interest related to this work.

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