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14. ABSTRACT
An increasing number of studies using human subjects substantiate the use of transcranial direct current stimulation (tDCS) as a noninvasive approach to treat various neurological symptoms. tDCS has been tested in conditions from motor to cognition dysfunctions. Performance enhancement of healthy subjects using tDCS has also been explored. The underlying physiological mechanism for tDCS effects is hypothesized to be through changes in neuroplasticity and we have previously demonstrated that in vivo anodal tDCS can enhance neuroplasticity of hippocampal CA1 neurons. The purpose of this study was to determine whether the underlying electrophysiological changes that occur following in vivo tDCS are polarity specific. We also examined both the CA1 and CA3 regions of the hippocampus to determine whether the tDCS effects were subfield specific. We conducted in vivo tests of cathodal tDCS versus anodal tDCS on synaptic plasticity of CA1 and CA3 neurons of male rats. In each region we assessed long term potentiation (LTP), paired pulse facilitation (PPF) and long term depression (LTD). In the CA1 region, we found anodal tDCS significantly enhanced not only LTP and PPF, but also LTD. There was no statistical difference in LTP, PPF or LTD of hippocampal CA1 neurons resulting from cathodal tDCS. Neither anodal nor cathodal tDCS induced significant changes in neuroplasticity of hippocampal CA3 neurons. Results indicate that the effects of tDCS are subfield specific and polarity dependent with anodal tDCS having greater impact on synaptic activity in the rat hippocampus than cathodal tDCS.

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Polarity and subfield specific effects of transcranial direct current stimulation on hippocampal plasticity

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ABSTRACT

An increasing number of studies using human subjects substantiate the use of transcranial direct current stimulation (tDCS) as a noninvasive approach to treat various neurological symptoms. tDCS has been tested in conditions from motor to cognition dysfunctions. Performance enhancement of healthy subjects using tDCS has also been explored. The underlying physiological mechanism for tDCS effects is hypothesized to be through changes in neuroplasticity and we have previously demonstrated that *in vivo* anodal tDCS can enhance neuroplasticity of hippocampal CA1 neurons. The purpose of this study was to determine whether the underlying electrophysiological changes that occur following *in vivo* tDCS are polarity specific. We also examined both the CA1 and CA3 regions of the hippocampus to determine whether the tDCS effects were subfield specific. We conducted *in vivo* tests of cathodal tDCS versus anodal tDCS on synaptic plasticity of CA1 and CA3 neurons of male rats. In each region we assessed long term potentiation (LTP), paired pulse facilitation (PPF) and long term depression (LTD). In the CA1 region, we found anodal tDCS significantly enhanced not only LTP and PPF, but also LTD. There was no statistical difference in LTP, PPF or LTD of hippocampal CA1 neurons resulting from cathodal tDCS. Neither anodal nor cathodal tDCS induced significant changes in neuroplasticity of hippocampal CA3 neurons. Results indicate that the effects of tDCS are subfield specific and polarity dependent with anodal tDCS having greater impact on synaptic activity in the rat hippocampus than cathodal tDCS.

1. Introduction

Transcranial direct current stimulation (tDCS) is a non-invasive brain stimulation technique that has gained popularity in many clinical settings as a potential therapeutic approach in alleviating symptoms associated with neurological disorders. Clinical studies have explored the beneficial effects of tDCS as treatment for various neurological diseases including Alzheimer's disease (Boggio et al., 2009; Ferrucci et al., 2008), Parkinson's disease (Benninger et al., 2010; Boggio et al., 2006), multiple sclerosis (Ferrucci et al., 2014), and stroke (Monti et al., 2008), as well as psychiatric disorders, such as depression (Dell'Osso et al., 2012; Fregni, Boggio, Nitsche, Rigonatti, & Pascual-Leone, 2006) and schizophrenia (Andrade, 2013). In healthy humans, tDCS also has been shown to enhance cognition through language learning (Flöel, Rösser, Michka, Knecht, & Breitenstein, 2008), working memory (Fregni et al., 2005), verbal fluency (Iyer et al., 2005), probabilistic classification learning (Kincses, Antal, Nitsche, Bártfai, & Paulus, 2004), image recall (Penolazzi et al., 2010), and picture naming (Hansen, 2012; Sparing, Dafotakis, Meister, Thirugnanasambandam, & Fink, 2008). Increased understanding of the neurobiological effects of direct current is critical for optimizing clinical applications of tDCS.

Earlier studies attribute the effects of tDCS on the nervous system to its ability to modulate the excitability of neurons in a polarity-dependent manner (Bindman, Lippold, & Redfearn, 1962). Prevailing evidence indicates that anodal tDCS increases and cathodal tDCS reduces neuronal excitability with sustained after-effects in the motor cortex of healthy human subjects (Liebetanz, Nitsche, Tergau, & Paulus, 2002; Nitsche & Paulus, 2000; Nitsche et al., 2003, 2005). However, the relationship between stimulation polarity and neuronal excitability is more complex, as neurons are not modulated homogenously; neuronal subtype, orientation relative to the electric field and depth in the cortical layers influence the response to stimulation (Radman, Ramos,

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Brumberg, & Bikson, 2009; Stagg & Nitsche, 2011). Furthermore, the after-effects of polarizing currents are dependent on the intensity and duration of stimulation (Stagg & Nitsche, 2011). Thus, more investigation is needed to understand the multifaceted effects of anodal and cathodal tDCS on neuronal excitability and plasticity.

Recent work has examined the effect of *in vitro* direct current stimulation (DCS) and *in vivo* tDCS on synaptic plasticity. Anodal tDCS was demonstrated to increase LTP whereas cathodal stimulation decreased LTP in mouse motor cortex (Fritsch et al., 2010). Similar results were obtained with anodal DCS in rat hippocampal slices (Ranieri et al., 2012). Further studies determined that the polarity dependent effects of DCS on synaptic plasticity vary based on axonal orientation (Kabakov, Muller, Pascual-Leone, Jensen, & Rotenberg, 2012) and dendritic location (Kronberg, Bridi, Abel, Bikson, & Parra, 2017). We previously demonstrated that anodal tDCS increased LTP and PPF in the Schaffer collateral-CA1 synapse of the rat hippocampus (Rohan, Carhuatanta, McInturf, Miklasevich, & Jankord, 2015). Likewise, anodal tDCS was observed to increase LTP in mouse hippocampal slices at CA3-CA1 synapses (Podda et al., 2016). However, only cathodal tDCS was reported to induce PPF in the somatosensory cortex of behaving rabbits (Márquez-Ruiz et al., 2012). Further study is warranted to elucidate the mechanisms underlying the effects of anodal and cathodal tDCS on synaptic plasticity.

The polarity dependent effects of *in vivo* tDCS on cellular LTP and LTD in the hippocampus remain largely unexplored. Here, we examined the effects of both anodal and cathodal tDCS in the rat CA1 and CA3 hippocampal regions and evaluated LTP, LTD, and PPF. We found that anodal tDCS, but not cathodal tDCS, enhanced LTP at the Schaffer collateral-CA1 synapses of rat hippocampus. Anodal tDCS enhanced PPF consistent with previous data (Rohan et al., 2015), while cathodal tDCS did not induce a significant change compared to control groups. In the mossy fiber-CA3 synapses neither anodal nor cathodal tDCS produced a significant LTP response.

2. Materials and methods

2.1. Animal handling

All rats were maintained according to National Institutes of Health and Wright-Patterson Air Force Base (WPAFB) Institutional Animal Care and Use Committee guidelines. The study protocol was reviewed and approved in compliance with the Animal Welfare Act and with all applicable federal regulations governing the protection of animals in research.

Forty animals (7–8-week-old male Sprague Dawley rats weighing approximately 400–550 g) were used for this study and were purchased from Charles River. Upon arrival to WPAFB facilities, animals received a 10 day acclimation period before surgical electrode placement.

2.2. Surgical implantation of cranial electrode

Data from our previously published work (Rohan et al., 2015) were obtained from larger electrodes that required direct implantation of the electrode onto the scalp. Here, we have used a circular 2.5 mm radius electrode that is better adapted to our specialized tDCS system such that only the electrode casing is implanted onto the scalp in which it can then be connected to the tDCS electrode.

Animals were anesthetized with isoflurane (Med-Vet International, Mettawa, IL) using 5% induction, followed by 2–3% isoflurane to maintain anesthetic depth. A 5 mm diameter, circular, head electrode casing (Tangible Solutions, Fairborn, OH) was attached to the skull from 0 mm to −5 mm bregma. Luting dental cement (GC Fuji I, GC America Inc., Alsip, IL) was applied to base of the head electrode casing and to the skull, followed by an acrylic dental cement (Sigma-Aldrich, St. Louis, MO) to secure the electrode. Animals were given a minimum of 7 days recovery period before tDCS treatment. Rats were randomly selected for control, anodal tDCS, or cathodal tDCS treatment

2.3. tDCS treatment

On the same day, prior to stimulation, animals were acclimated to the testing room for 10 min. A conducting medium (SignaGel, Parker Laboratories, Fairfield, NJ) was placed into the head casing prior to connecting the head electrode. The reference electrode (12 mm diameter, Tangible Solutions, Fairborn, OH) was placed on the rats shaved chest with SignaGel as the conducting medium. Once the electrodes were in place, the animal was wrapped with a flexible cohesive bandage (PetFlex, Med-Vet, Mettawa, IL.) and placed into an open arena. tDCS was then applied at 0.25 mA using a constant-current stimulator (Magstim DCstimulator; Neuroconn, Ilmenau, Germany) for 30 min. The control group was prepared the same way as the stimulation groups but did not receive any current. The head electrode was either connected to the stimulator's negative terminal or positive terminal to deliver anodal or cathodal tDCS, respectively.

2.4. Brain slice preparation

Animals were euthanized immediately after completion of tDCS administration. Brain slices were prepared as previously described (Rohan et al., 2015). Brains were rapidly removed following euthanasia and were kept viable with ice cold artificial cerebrospinal fluid (ACSF) that was continuously oxygenated (95%/5% O_2/CO_2). ACSF was prepared fresh daily and consisted of (in mM): 124 NaCl, 3 KCl, 1.25 KH_2PO_4, 10 D-Glucose, 1 $MgSO_4$, 36 $NaHCO_3$, and 2 $CaCl_2$ (pH ~ 7.4). Cerebellum and approximately 1 cm of frontal cortex were removed and the remaining brain was sectioned at 350 μm thickness using a vibratome (VT1000S Leica Microsystems, Buffalo Grove, IL) in the transverse plane, at 20–30°, lateral from the horizontal axis. Brain slices were maintained in warmed oxygenated ACSF and allowed to recover for at least 60 min prior to recording. Recordings were done using a microelectrode array system (Quad II, Automate, Berkeley, CA). One hippocampal slice was placed onto the pre-coated microelectrode array probe, using small weights to anchor the slices down. For recordings of the Schaffer collateral – CA1 synapses, the slice was positioned such that neurons from the CA1 and surrounding region were recorded (Fig. 1A). For recordings of the mossy fiber – CA3 synapses, the slice was positioned such that neurons from the CA3 and surrounding region were recorded (Fig. 1B). The probe containing the brain slice was then assembled with the Quad II system, per the manufacturer's instructions. A perfusion cap was used to circulate fresh oxygenated ACSF into the probe and prevent the slices from drying. The ACSF solution and oxygen entering the probe chamber were maintained at 32–34 °C. Sections were perfused with humidified oxygen to prevent drying and maintain viability.

2.5. Electrophysiology recording

All electrophysiology data were obtained using AlphaMed's Quad II systems (Automate, Berkeley, CA), as previously described (Rohan et al., 2015). Probes were initially pretreated with 70% ethanol for 5 minutes followed by 0.1% polyethylenimine (PEI) in 25 mM borate buffer for at least 24 h prior to use. Data acquisition and stimulation protocols were performed using Mobius software (Automate, Berkeley, CA). A biphasic stimulating current of 10–100 μA was applied to the Schaffer collateral or the mossy fiber region of the hippocampus to obtain an input/output relationship curve. Evoked field potentials mainly consisting of inward deflections were recorded from neurons within the CA1 (for Schaffer collateral – CA1 recordings) or CA3 (for mossy fiber-CA3 recordings) region of the hippocampus at a sampling frequency of 20 kHz using the Quad II multichannel amplifier, digitized and graphically displayed using Mobius software. Input/output data were obtained by delivering multiple biphasic stimuli at various

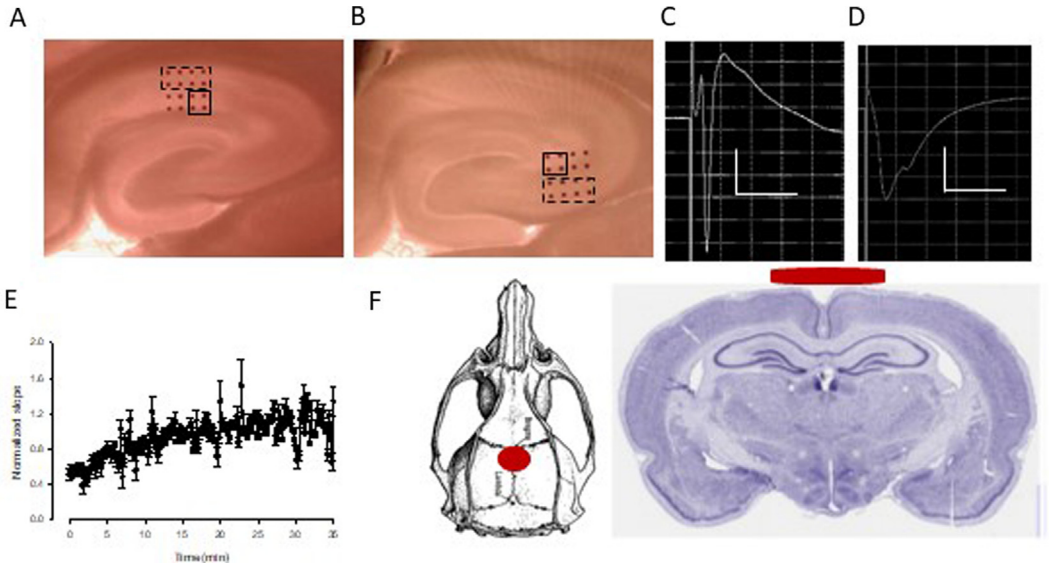

Fig. 1. Positioning of hippocampal slices onto the 4 × 4 microelectrode array dish. (A, B) Typical positioning of our hippocampal slice in the microelectrode array dish when Schaffer collateral-CA1 region (A) or mossy fiber-CA3 (B) is being analyzed. Dotted rectangular box indicates the hippocampal area that is being recorded, whereas the solid rectangular box indicates the typical position where stimulation occurs within the microelectrode array. (C–D) A mixture of population spikes (C) and field potentials (D) were observed. Both amplitudes and slopes were used to calculate LTP and LTD. Scale bar: 0.2 mV, 10 ms. (E) Graph showing the average, normalized baseline recording from all animals. Baseline recording for at least 30 min were obtained prior to initiation of LTP or LTD recording. (F) Position and approximate size of the electrode on the rat skull. The image on the left depicts a rat skull modified to demonstrate the positioning of the tDCS electrode (red circle) in our experimental setup. The image on the right depicts a Nissl-stained image (Mikula, Trotts, Stone, & Jones, 2007) modified to show a coronal tissue section close to the center of the tDCS electrode (red bar). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

intensities, ranging from 10 to 100 μA. Baseline field potentials are set between 50 and 60% of maximum amplitude for each slice. PPF and paired pulse depression (PPD) were obtained by delivering sets of two consecutive stimuli of equal intensity that were 40 ms apart. Degrees of PPF and PPD were quantified by calculating the paired pulse (PP) ratio in which the response amplitude from the second stimulating pulse was divided by the response amplitude from the first pulse. Thus, a PP ratio of $>$ 1 indicates PPF whereas a PP ratio of less than 1 indicates PPD. Spontaneous spiking activity of neurons within the CA1 or CA3 region were recorded for 10 min and frequency of firing was calculated using the Mobius software. Amplitudes of spontaneous spikes were calculated using Mobius as well as Excel and Sigmaplot v13.0 software. A threshold value of 15 μV was used to calculate spike frequency and amplitude.

For LTP and LTD experiments we use the input/output relationship curve to determine the size of the stimulating current that resulted in half of the maximal output response. Typically, a stimulating current size of 30–50 μA induced a half-maximal response and thus was used for our LTP and LTD experiments. Baseline recording was obtained for each slice for at least 30 min prior to LTP or LTD measurements. LTP was induced by delivering 3 trains of theta burst stimulation (TBS), consisting of 10 repeats of 4 high frequency stimulations (100 Hz) every 200 ms to the Schaffer collateral regions. LTD was induced by delivering low frequency (1 Hz) paired pulse stimulations (interpulse duration of 50 ms) for 15 min (Kemp, McQueen, Faulkes, & Bashir, 2000). Evoked responses or field potentials (fEPSPs and population spikes) were monitored at 12 s intervals for at least 30 min following LTP induction. Percent potentiation was calculated by computing the percent difference in population spike amplitude or fEPSP slope at either 30 min following LTP induction by TBS from baseline. Averages of 10 data points were calculated to obtain baseline and LTP values.

2.6. Data analysis

Field potentials were calculated by fitting a line to the inward deflecting curve and calculating the slope. Amplitudes of population spikes were also calculated. Slope and amplitude calculations were performed using Mobius software (Automate, Berkeley, CA). Quantitation of LTP was obtained by averaging 10 data points at the indicated times (just prior to LTP induction by TBS and at 30 min following TBS). Percent LTP or percent potentiation refers to the slope or amplitude of fEPSP at 30 min after TBS minus the slope, or amplitude of baseline fEPSP prior to TBS, divided by the slope or amplitude of baseline fEPSP values. Normalized fEPSP data refers to the slope or amplitude of fEPSP divided by the average slope or amplitude of all fEPSP points prior to TBS. Quantitation of PPF/PPD was obtained by dividing the slope or amplitude of the fEPSP response due to the second stimulus, divided by the slope or amplitude of the fEPSP response due to the first stimulus to obtain the PP ratio. Data from multiple microelectrodes within the CA1 region of a hippocampal slice were averaged together to obtain the response from that particular slice. Typically, 1–4 hippocampal slices per rat were used and n values are indicated as the number of rats followed by the number of slices used. For our PPF/PPD data analysis, multiple stimulations at distinct locations in one slice were performed and various microelectrodes within the CA1 region were recorded and counted as the sample size in the statistical analysis of this data.

Data are represented as means with the standard error of the mean (SEM) and were statistically compared using an unpaired, two-tailed *t*-test. A calculated P of less than 0.05 is considered significantly different. All quantitation and statistical analysis as well as graphs were generated using Microsoft Excel and Sigmaplot v.12.5 and v.13.

3. Results

3.1. Anodal versus cathodal tDCS and long term potentiation

At some of the channels recorded, evoked responses were in the form of population spikes whereas at other channels, evoked responses were in the form of field potentials (Fig. 1C). Therefore, we calculated both the amplitude of all evoked responses from all recorded channels and the slope of field potentials. Percent LTP was calculated as the

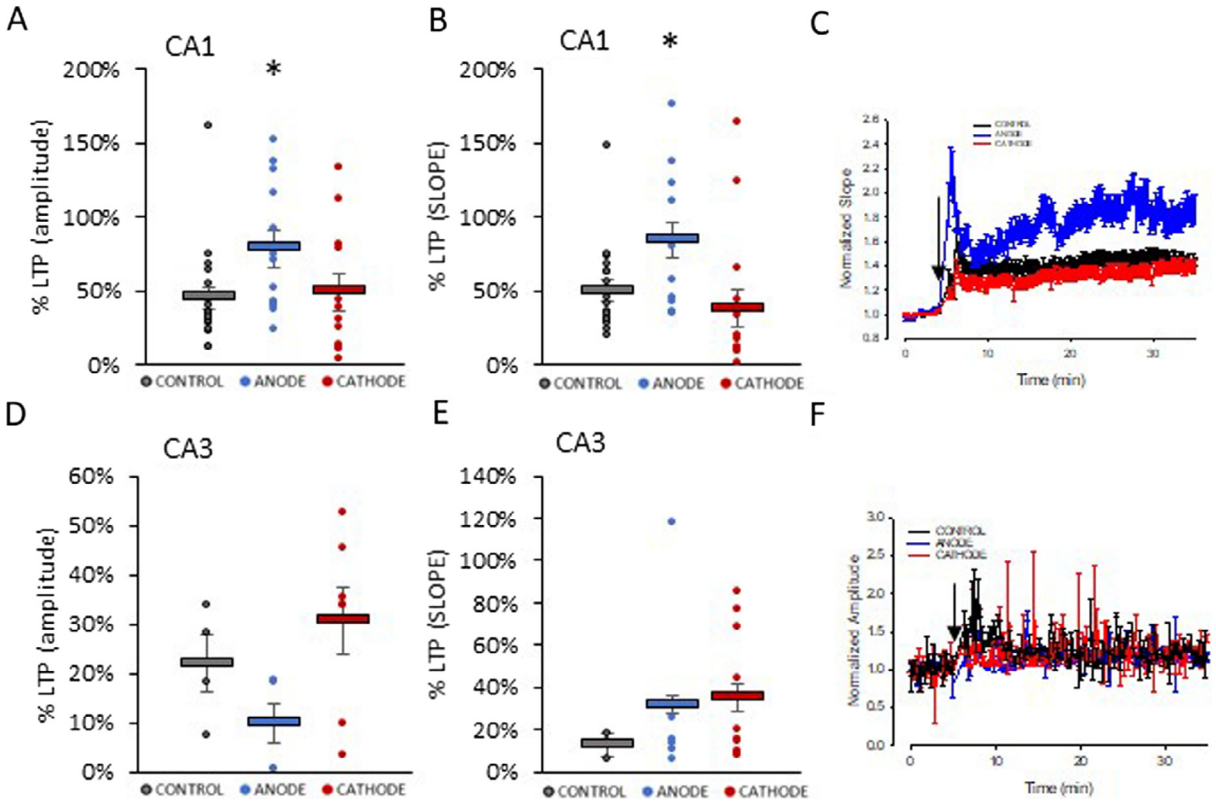

Fig. 2. Differential effects of anodal and cathodal tDCS on synaptic plasticity. (A–C) Anodal but not cathodal tDCS enhances LTP in rat Schaffer collateral – CA1 synapses. (A) Amplitudes of field responses were used to calculate CA1 LTP. Control or sham-treated animals experienced an average of 47 ± 7% LTP, whereas animals treated with anodal tDCS experienced an average of 78 ± 12% LTP. Animals treated with cathodal tDCS has an average LTP of 49 ± 12%. Data failed the normality test. Kruskal Wallis ANOVA on ranks analysis yielded significant difference among the 3 groups (n = 8 rats, 12–20 slices, P = 0.043). Non-parametric Mann-Whitney rank sum test showed significant difference between the control (sham) group and the anodal tDCS group (P = 0.014). (B) Slopes of field potentials were used to calculate CA1 LTP. Control or sham-treated animals experienced an average of 49 ± 7% LTP, whereas animals treated with anodal tDCS experienced an average of 84 ± 16% LTP. Animals treated with cathodal tDCS has an average LTP of 38. Animals treated with cathodal tDCS has an average LTP of 13%. Data failed the normality test. Kruskal Wallis ANOVA on ranks analysis yielded significant difference among the 3 groups (n = 8 rats, 10–18 slices, P = 0.005). Non-parametric Mann-Whitney rank sum test showed significant difference between the control (sham) group and the anode tDCS group (P = 0.033). (C) Averaged recording trace from animals exposed to control (black), anodal tDCS (blue) or cathodal tDCS (red). Arrow denotes LTP induction by TBS. (D–E) Anodal or cathodal tDCS had no statistically significant effect on LTP in mossy fiber – CA3 synapses (n = 8, P > 0.05). (D) Amplitudes of field responses were used to calculate CA3 LTP. Control animals experienced an average of 22 ± 6% LTP. Animals treated with anodal tDCS experienced an average of 16 ± 3% LTP. Animals treated with cathodal tDCS experienced an average of 31 ± 7%LTP. Data passed the normality test. ANOVA analysis did not yield significant differences among the 3 groups (n = 3–6 rats, 4–7 slices, P = 0.421). (E) Slopes of field potentials were used to calculate CA3 LTP. Control animals experienced an average of 13 ± 4% LTP. Animals treated with anodal tDCS experienced an average of 32 ± 18% LTP. Animals treated with cathodal tDCS experienced an average of 32 ± 10% LTP. Data did not pass the normality test. Kruskal Wallis ANOVA on ranks analysis did not yield significant differences among the 3 groups (n = 3–6 rats, 3–10 slices, P = 0.448). (F) Averaged normalized recording trace from animals exposed to control (black), anodal tDCS (blue) or cathodal tDCS (red). Arrow denotes LTP induction by TBS. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

change in amplitude or slope of evoked response 30 min following high frequency stimulation divided by the baseline amplitude or slope prior to LTP induction. Our data did not pass the normality test and consequently, the non-parametric Kruskal Wallis ANOVA on ranks was used for statistical analysis. CA1 LTP was reliably induced using TBS and LTP data from 2 to 4 slices were obtained per animal.

We have previously demonstrated that we can achieve over two-fold enhancement of LTP at the hippocampal Schaffer collateral-CA1 synapses by administration of 0.25 mA anodal tDCS for 30 min (Rohan et al., 2015). Here, we report that 0.25 mA anodal tDCS for 30 min delivered through a smaller electrode with more localized current positioned at the same scalp location can also elicit nearly 2-fold LTP enhancement at the Schaffer collateral-CA1 synapse of rat hippocampus (Fig. 2A and B). We found significant differences in the 3 experimental groups (control, anodal tDCS and cathodal tDCS) when either amplitude (P = 0.043, n = 8 rats per group, 1–4 slices per rat) or slope (P = 0.014, n = 8 rats per group) was used for analysis. The non-parametric Mann Whitney rank sum test yielded a significant difference between the control and the anodal tDCS group (P = 0.014 for amplitude and P = 0.033 for slope). Averaged slope recordings from rats treated with either anodal (blue) or cathodal (red) tDCS or control (black) are shown in Fig. 2C. However, cathodal stimulation at the same stimulation intensity and duration (0.25 mA, 30 min) did not elicit hippocampal CA1 LTP enhancement (Fig. 2A–C).

We also measured LTP at the mossy fiber-CA3 synapses (Fig. 2D and E). Whereas Schaffer collateral-CA1 synapses undergo LTP that are primarily NMDA-mediated, the mossy fiber-CA3 synapses undergo LTP that are mostly non-NMDA-mediated. Unlike the Schaffer collateral-CA1 synapse in which LTP induction can be reliably induced with TBS, mossy fiber-CA3 synapses could not be reliably induced by TBS using our microelectrode array system. However, we did achieve small levels of LTP in at least one slice per animal (3–6 rats per group). With this limitation in mind, our data did not result in any significant difference between degrees of LTP following anodal or cathodal tDCS (0.25 mA, 30 min, Fig. 2D and E). Furthermore, no significant differences were observed in the input-output function of CA1 (Fig. 2F) or CA3 (not shown) neurons.

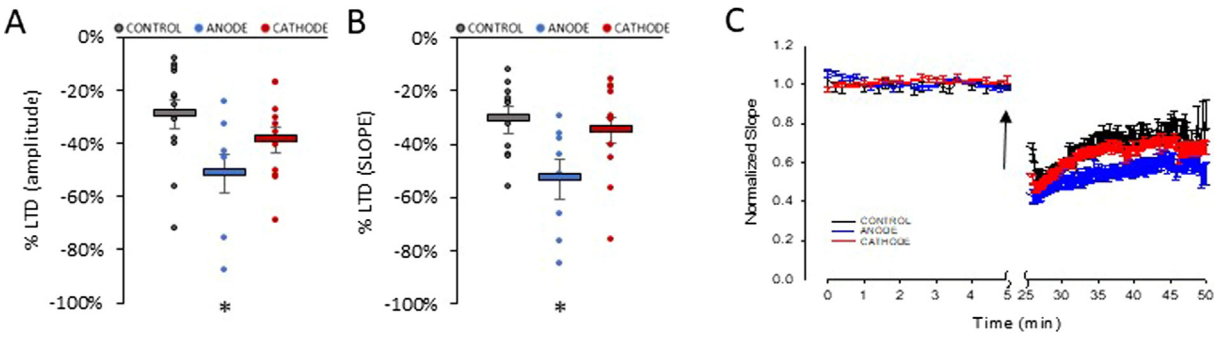

Fig. 3. Differential effects of anodal and cathodal tDCS on long term depression (LTD). (A,B) Anodal but not cathodal tDCS enhances LTD in rat Schaffer collateral – CA1 synapses, obtained by measuring response amplitude (A) or slope (B). (A) Control? animals experienced an average of 26 ± 5% LTD, whereas animals treated with anodal tDCS experienced an average of 47 ± 7% LTD. Animals treated with cathodal tDCS had an average LTD of 36 ± 5%. Data did not pass the normality test. Kruskal Walis ANOVA on ranks analysis yielded significant difference among the 3 experimental groups (n = 8 rats, 8–13 slices, P = 0.030). The non-parametric Mann Whitney rank sum test yielded significant difference between the control and anodal tDCS groups (P = 0.010). (B) Control ? animals experienced an average of 31 ± 4% LTD, whereas animals treated with anodal tDCS experienced an average of 53 ± 7% LTD. Animals treated with cathodal tDCS had an average LTD of 35 ± 6%. Data did not pass the normality test. Kruskal Walis ANOVA on ranks analysis yielded significant difference among the 3 experimental groups (n = 8 rats, 8–11 slices, P = 0.031). The non-parametric Mann Whitney rank sum test yielded significant difference between the control and anodal tDCS groups (P = 0.017). (C) Averaged current trace demonstrating a more pronounced LTD arising from animals subjected to anodal tDCS (blue), compared to control animals (black) or animals subjected to cathodal tDCS (red). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.2. Anodal versus cathodal tDCS and long term depression

In addition to LTP, we evaluated the effect of tDCS on LTD, another form of synaptic plasticity in which synaptic strength gets weakened (rather than strengthened as is the case with LTP) by repeated prolonged low frequency stimulation (Fig. 3). Previous studies by others indicate that low frequency paired pulse stimulation is more effective in inducing LTD in hippocampus from adult rats (Kemp et al., 2000). Therefore, we induced LTD at the Schaffer collateral-CA1 synapses of the hippocampus with 15 min of low frequency paired pulse stimulation at 1 Hz. Percent LTD (%LTD) was calculated as the change in amplitude or slope of evoked response 15 min following slow frequency stimulation, divided by the baseline amplitude or slope prior to LTD induction. Interestingly, we found that 30 min of 0.25 mA anodal tDCS induced a significantly greater LTD compared to the control group (Fig. 3A and B). Our data did not pass the normality test and consequently, Kruskal Wallis ANOVA on ranks was used as our statistical analysis. There was a significant difference among the 3 experimental groups when either amplitudes (P = 0.030, n = 8 rats per group) or slopes (P = 0.031, n = 8 rats per group) were used to calculate %LTD. The non-parametric Mann Whitney rank sum test yielded significant difference between the control and the anodal tDCS group (P = 0.010 for amplitude and P = 0.017 for slope). However, cathodal tDCS did not induce significant changes in LTD levels (Fig. 3A–C).

3.3. Anodal versus cathodal tDCS and paired pulse facilitation

PPF is another form of synaptic plasticity. PPF at both the Schaffer collateral-CA1 and mossy fiber-CA3 synapses was induced by 2 consecutive stimulating pulses that are 40 ms apart (Fig. 4A), at multiple intensities ranging from 10 to 100 μA. Unlike LTP or LTD which are examples of long term synaptic plasticity, PPF is a form of short term synaptic plasticity that is believed to arise from an increase in intracellular calcium concentration at the presynaptic terminal from closely paired stimulation, which then triggers a greater amount of synaptic vesicle fusion to the presynaptic membrane and increased neurotransmitter release in the synaptic cleft. We found that anodal tDCS enhanced PPF at the Schaffer collateral-CA1 synapse, consistent with our previous data (Fig. 4B). Two-way ANOVA revealed significant differences among the 3 experimental groups (n = 8, P = 0.001). Post-hoc comparison analysis using the Holm-Sidak method revealed significant difference between the control and anodal tDCS groups (P = 0.002) and also between the anodal tDCS and cathodal tDCS groups (P = 0.003). One-way ANOVA revealed statistical difference among the groups during the 40 μA stimulation, between the control and anodal tDCS groups (P = 0.041). However, cathodal tDCS did not significantly alter PP responses (Fig. 4B). Neither anodal nor cathodal tDCS induced significant changes in PPF at the mossy fiber-CA3 synapse (Fig. 4C).

3.4. Spontaneous activity

Our previous work showed no observable change with frequency or amplitude of spontaneous activity of neurons within the CA1 region resulting from anodal tDCS (Rohan et al., 2015). Here, we looked at spontaneous activity level of neurons within the CA3 region of the hippocampus. A spike threshold of 15 μV was used to calculate spike frequency and amplitude. There was no statistically significant effect of anodal or cathodal tDCS on the averaged frequency of spontaneous firing activity of neurons within the CA3 region (Fig. 5A). However, a trending increase in spontaneous spiking activity was observed in rats that have undergone cathodal tDCS. Averaged frequency of non-evoked, spontaneous spiking activity were 5.5 ± 1.5, 8.6 ± 2.3, and 12.1 ± 2.8 Hz for the control, anodal tDCS and cathodal tDCS groups, respectively. However this increase in frequency was not found to be statistically significant.

The averaged amplitudes of the spikes from each experimental group were not statistically different (Fig. 5B). A vast majority of spikes (> 98%) have amplitudes that were less than 50 μV. Averaged amplitude of spontaneous spikes are 15.7 ± 4, 17.2 ± 3.9, and 15.4 ± 4.7 μV for the control, anodal tDCS and cathodal tDCS groups, respectively. However, a closer analysis of the spike amplitude histogram as well as the cumulative histogram revealed increased incidence of spikes with greater amplitude ranging from 50 to 150 μV in rats that had been subjected to anodal or cathodal tDCS (Fig. 5C,D). In a 10-minute recording period, only 0.61% of spontaneous spikes had amplitudes of ≥50 μV, whereas 1.5% and 0.9% of spontaneous spikes had amplitudes ≥50 μV in rats treated with anodal and cathodal tDCS, respectively.

4. Discussion

The general consensus is that tDCS modulates neuronal excitability in a polarity specific manner, with anodal stimulation increasing and cathodal stimulation reducing neuronal excitability (Bikson et al., 2004; Liebetanz et al., 2002; Medeiros et al., 2012; Nitsche & Paulus, 2000;

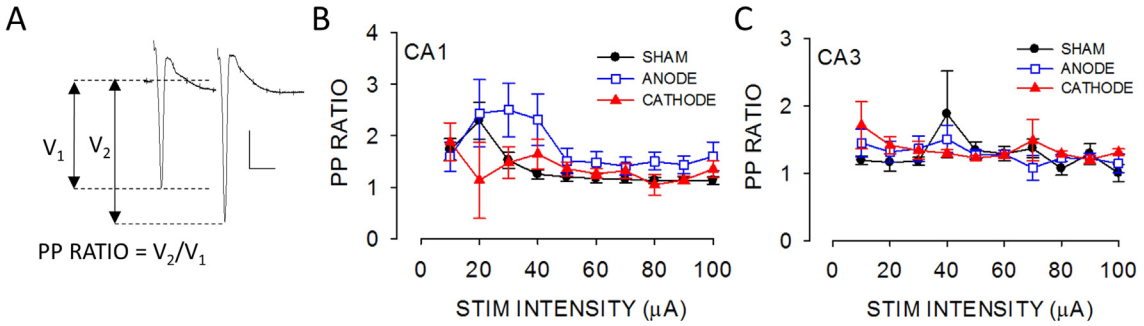

Fig. 4. Differential effects of anodal and cathodal tDCS on PP stimulations. (A) PPF was induced by delivering 2 consecutive stimuli that were 40 ms apart. PP Ratio was calculated as the ratio between the response amplitude arising from the 2nd stimulus (V_2) and the response amplitude arising from the 1st stimulus (V_1). Scale bar: 0.2 mV, 20 ms. (B) Anodal but not cathodal tDCS enhances PPF in rat Schaffer collateral – CA1 synapses. Two-way ANOVA analysis revealed a statistically significant difference among groups (n = 8 rats, 29–46 slices, P = 0.001). Post-hoc comparison analysis using the Holm-Sidak method revealed significant difference between anodal tDCs and control groups (P = 0.002) and also between the anodal tDCS and cathodal tDCS groups (P = 0.003). One way ANOVA revealed statistical difference among the groups during the 40 μA stimulation, between the control and anodal tDCS groups (P = 0.041). (C) Anodal or cathodal tDCS had no statistically significant effect on PPF in mossy fiber – CA3 synapses (n = 8 rats, 9–24 slices, P > 0.05, two-way ANOVA).

Nitsche et al., 2003). Based on previous studies suggesting that anodal and cathodal tDCS have opposite effects on neuronal excitability, we anticipated that anodal tDCS would increase LTP and PPF and decrease LTD, whereas cathodal tDCS would induce opposing effects on plasticity of rat hippocampal neurons. However, we did not observe any statistically significant changes in neuroplasticity of neurons in CA1 or CA3 region of rats that had been subjected to cathodal tDCS (Figs. 2 and 3).

4.1. Polarity matters – anodal versus cathodal tDCS

We know from past studies that direct current electric field can indeed alter the morphological shape of neurons and modify protein expression (Pelletier & Cicchetti, 2015). Previous *in vitro* experiments

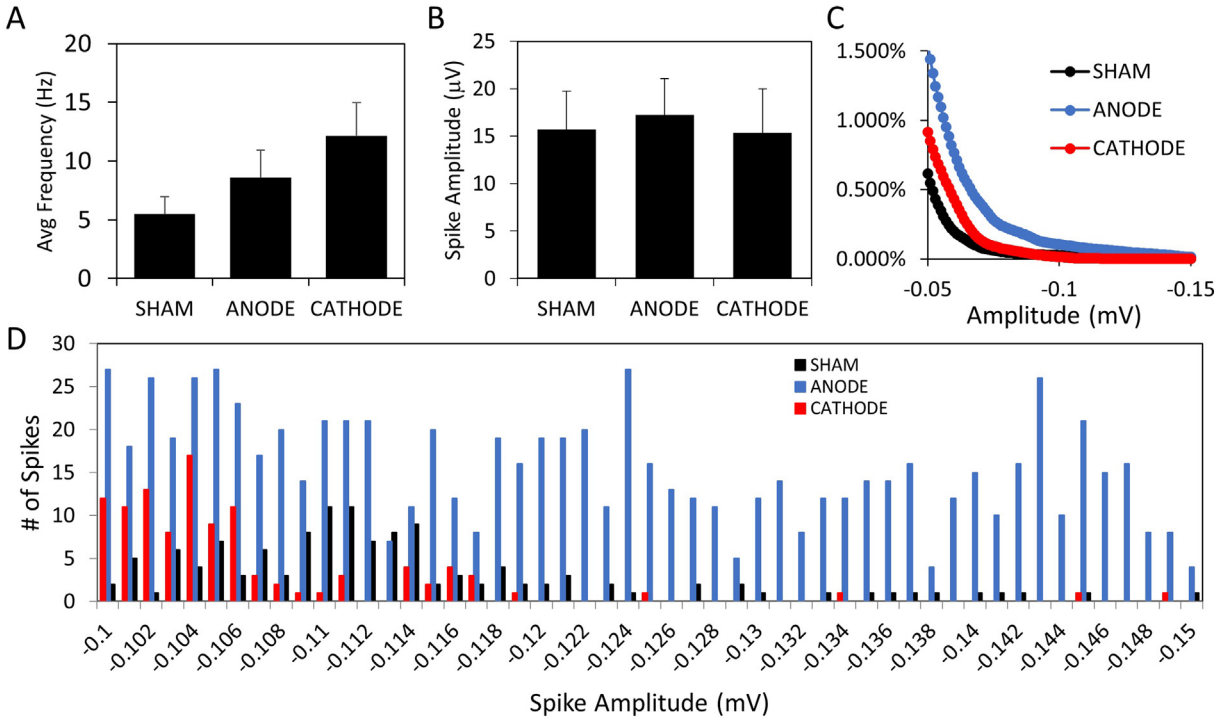

Fig. 5. Effects of anodal and cathodal tDCS on spontaneous spiking activity of CA3 neurons. (A) Non-statistically significant increases in frequency of spontaneous spiking activity of CA3 neurons were observed from rats subjected to cathodal tDCS. Control or sham-treated animals have an averaged spontaneous activity frequency of 5.5 ± 1.5 Hz, whereas animals treated with anodal tDCS have an averaged frequency of 8.6 ± 2.3 Hz. Animals treated with cathodal tDCS have an averaged frequency of 12.1 ± 2.8 Hz. Data passed the normality test. ANOVA analysis yielded a no significant change in the frequency spontaneous spiking activity (n = 8 rats, 11–16 slices, P = 0.091). (B) Anodal or cathodal tDCS had no statistically significant effect on the overall averaged amplitude of spontaneous spiking activity of CA3 neurons. Control animals had an averaged spontaneous spike amplitude of 15.7 ± 4.0 μV. Animals treated with anodal tDCS had an averaged spontaneous spike amplitude of 17.2 ± 3.9 μV. Animals treated with cathodal tDCS had an averaged spontaneous spike amplitude of 15.4 ± 4.7 μV. (C) A partial cumulative amplitude histogram of spontaneous spiking activity with amplitudes from between 50 and 150 μV from CA3 neurons. Inward spike amplitudes are plotted in reverse order. Rats subjected to anode (blue) and cathode (red) tDCS appeared to have greater occurrence of spikes with larger amplitudes (50–150 μV) compared to control rats (black). (D) A partial amplitude histogram of large spontaneous spikes (100–150 μV amplitude) from animals treated with anodal tDCS (blue), cathodal tDCS (red) or control (black), showing greater incidence of larger spikes in anodal as well as cathodal groups, compared to control (sham). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

revealed that anodal and cathodal DCS of rat hippocampal slices induced an enhancement and reduction of LTP, respectively (Ranieri et al., 2012). However, a more recent *in vitro* study reported that both anodal and cathodal DCS induced an enhancement of LTP and a reduction in LTD (Kronberg et al., 2017). Differences between our data and previously reported data from *in vitro* DCS on hippocampal slices are somewhat expected, given that the electric field generated by *in vivo* tDCS, as experienced by hippocampal neurons, is likely to be different than the electric field generated by *in vitro* DCS. In addition, many *in vitro* studies involved acute or concurrent DCS, in which measurements were obtained during or immediately after stimulation. In contrast, although brain harvest was performed within minutes after tDCS administration, viable brain slices were allowed to recover for at least 1 h before recordings were obtained. Thus, the findings we observed are persistent effects of tDCS, perhaps resulting from translocation and phosphorylation of synaptic receptors such as the ionotropic glutamate AMPA receptor (Stafford, Brownlow, Qualley, & Jankord, 2018).

Previous work performed in the motor cortex demonstrated a significant effect of cathodal tDCS on behavior while our results from the hippocampus did not show a significant effect. This difference may be attributed to differences in proximity and orientation of motor versus hippocampal neurons relative to the tDCS electrode. Hippocampal neurons span a greater depth within the brain, have varied morphology as well as orientation relative to the electrical field compared to cortical motor neurons. GABAergic interneurons are arranged heterogeneously in the hippocampal network and they regulate the activity of the more homogenously layered pyramidal cells (Ishizuka, Cowan, & Amaral, 1995; Jinno, 2009; Pelkey et al., 2017), thus adding several degrees of complexity to the interpretation of the polarity experienced by individual interneurons and how that subsequently modulates the activities of CA1 pyramidal neurons.

4.2. Region specific tDCS effects – CA1 versus CA3

Another finding of this study is the specificity with which tDCS affects the CA1 but not CA3 region of the hippocampus. No statistically significant changes were observed at the mossy fiber-CA3 synapse following tDCS administration. However, unlike the consistently induced LTP in the CA1 region, LTP was not consistently induced in the CA3 region using our microelectrode array system. Most of the previous studies conducted on mossy fiber-CA3 LTP have used glass electrodes and either extracellular or whole cell patch clamp recording rather than microelectrode array. Possible reasons for our inconsistent LTP induction in this region can be that the distance among electrodes in our array was not ideal and that the mossy fiber-CA3 synaptic organization is not as uniform as the Schaffer-collateral-CA1 synapses such that a slight difference in the positioning of the slice onto the array can potentially activate greater proportion of non-glutamatergic synapses (i.e., GABA-ergic synapses) such that LTP could not be consistently generated. Our data on mossy fiber-CA3 LTP will need to be verified using either extracellular recording or whole cell patch clamp recording with glass electrodes. With this limitation in mind, we did not see augmentation of CA3 LTP with tDCS in our hippocampal slices as measured using a microelectrode array system. One possible explanation is the potential involvement of NMDA receptors in tDCS-mediated effects, since Schaffer collateral-CA1 LTP is mainly NMDA-dependent (Harris, Ganong, & Cotman, 1984) whereas mossy fiber-CA3 LTP is mainly NMDA-independent (Katsuki, Kaneko, Tajima, & Satoh, 1991). Multiple previous studies support the hypothesis that tDCS effects on synaptic plasticity may be dependent on NMDA receptors (Impey, de la Salle, Baddeley, & Knott, 2017; Liebetanz et al., 2002; Nitsche et al., 2003, 2004; Rohan et al., 2015).

We also observed a trend towards an increase, although not statistically significant, in the frequency of spontaneous spiking activity of CA3 neurons following anodal and cathodal tDCS during 10-minute recordings (Fig. 5A). An increase in frequency of spontaneous activity of CA3 neurons has been previously reported 10–30 min following *in vitro* electrical stimulation of the entorhinal cortex (Deadwyler, Gribkoff, Cotman, & Lynch, 1976). A possible mechanism for the observed increase in the frequency of spontaneous firing is that *in vivo* tDCS resulted in increased neuronal excitability in the entorhinal cortex potentially leading to increased spontaneous spiking activity of CA3 neurons. Here, we did not assess the effects of tDCS on oscillatory patterns of hippocampal neurons. Further work is needed to verify whether anodal or cathodal tDCS can also alter the hippocampal network oscillations, as this can potentially provide another mechanistic explanation for the beneficial effects of tDCS in several cognitive processes.

4.3. Conclusions

In summary, current polarity and region play a critical role in tDCS effects on neuroplasticity of hippocampal neurons. Anodal, but not cathodal, tDCS modulates synaptic plasticity of the hippocampus through effects on LTP and LTD. Further, the effects of anodal tDCS were observed in the NMDA dependent region of CA1 while no effect was observed in the NMDA-independent CA3 region. Thus, our data support the hypothesis that the effects of tDCS on the hippocampus are specific to current polarity and unique to the sub-region examined.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. Work was funded by Air Force Office of Scientific Research, award #16RHCOR362.

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