MODULATION OF LTP AT RAT HIPPOCAMPAL CA3-CA1 SYNAPSES BY DIRECT CURRENT STIMULATION

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ABSTRACT

Transcranial direct current stimulation (tDCS) can produce a lasting polarity-specific modulation of cortical excitability in the brain and it is increasingly used in experimental and clinical settings. Recent studies suggest that the after-effects of tDCS are related to molecular mechanisms of activity-dependent synaptic plasticity.

Here we investigated the effect of DCS on the induction of one of the most studied N-methyl-D-aspartate (NMDA) receptor-dependent forms of long-term potentiation (LTP) of synaptic activity at CA3-CA1 synapses in the hippocampus. We show that DCS applied to rat brain slices determines a modulation of LTP, that is increased by anodal and reduced by cathodal DCS.

Immediate early genes, such as c-fos and zif268 (egr1/NGFI-A/krox24), are rapidly induced following neuronal activation and a specific role of zif268 in the induction and maintenance of LTP has been demonstrated. We found that both anodal and cathodal DCS produce a marked subregion-specific increase in the expression of zif268 protein in the cornus ammonis (CA) region, while the same protocols of stimulation produce a less pronounced increase in c-fos protein expression in the CA and in dentate gyrus (DG) regions of the hippocampus. Brain derived neurotrophic factor (BDNF) expression was also investigated and it was found to be reduced in cathodal stimulated slices.

The present data demonstrate that it is possible to modulate LTP by using DCS and provide the rationale for the use of DCS in neurological diseases in order to promote the adaptive and suppress the maladaptive forms of brain plasticity.

Keywords: direct current stimulation; synaptic plasticity; long-term potentiation; hippocampus.
**INTRODUCTION**

Transcranial direct current stimulation (tDCS) has recently been introduced as a tool to stimulate non-invasively the intact human brain (Priori et al. 1998; Nitsche and Paulus 2000; Nitsche et al. 2008) and several preliminary studies evaluated the therapeutic potential of tDCS in neurological diseases, including stroke (Schlaug et al. 2008), epilepsy (Nitsche and Paulus 2009) and movement disorders (Wu et al. 2008). However, the neurobiological mechanisms underlying the after-effects of tDCS are still poorly defined.

Early studies on animal preparations show that the application of weak polarizing currents, at an intensity lower than that needed for triggering action potentials, induces lasting polarity-specific changes in spontaneous and evoked neuronal activity: anodal polarization increases neuronal activity, while cathodal polarization decreases it (Bindman et al. 1962, 1964; Creutzfeld et al. 1962; Purpura and McMurtry 1965). Similarly to these experimental results, tDCS has been shown to induce relatively long-lasting and polarity-specific changes in the excitability of human motor cortex (Nitsche and Paulus 2001; Lang et al. 2004a; Lang et al. 2011).

It has been suggested that the after-effects of tDCS might originate from persistent modifications of synaptic efficacy similar to those underlying long-term potentiation (LTP) and long-term depression (LTD) of synaptic activity, both in experimental (Islam et al. 1995) and in human studies (Liebentanz et al. 2002). The synaptic effects of DCS have been investigated recently by Fritsch et al. (2010) in slices of mouse primary motor cortex (M1). These authors showed that: 1) anodal DCS applied to M1, in the absence of simultaneous synaptic activation, elicits only a short-lasting synaptic potentiation; 2) by coupling DCS with low frequency stimulation (at 0.1 Hz) a long-lasting LTP is obtained; 3) LTP is polarity-specific (no effects using cathodal DCS) and also related to the frequency of synaptic co-activation (lower and higher frequencies do not produce LTP); 4) LTP induction is dependent on N-methyl-D-aspartate (NMDA) receptor activation and requires activity-dependent brain-derived neurotrophic factor (BDNF) secretion. These data confirm that DCS may induce LTP, however, human studies suggest that tDCS not only may induce LTP but can also modulate LTP induced by different techniques of stimulation (Siebner et al. 2004; Lang et al. 2004b; Nitsche et al. 2007; Antal et al. 2008) and interfere with learning and memory (Reis et al. 2008, 2009; Dockery et al. 2009), two functions that appear strongly related to LTP (Kim and Linden 2007). Cumulatively these studies reveal the complex nature of tDCS effects, characterized by the capability of inducing LTP/LTD and of modulating the induction of the
same phenomena. While the first aspect has been extensively studied and characterized by Fritsch et al. (2010), who revealed the physiological and molecular basis of DCS-induced LTP, limited information is available about the basis of the effects of DCS on LTP induction. Understanding the basis of the modulatory effect of DCS would be particularly relevant for its clinical application in humans, where it could be used to shape the plastic properties of the brain.

The aim of present study was to investigate the effects of DCS on LTP from an experimental point of view. To this end, we evaluated the effects of anodal and cathodal DCS of rat brain slices on LTP using one of the most studied and compelling models of synaptic plasticity: the synapse between the axons of the pyramidal cells of the CA3 region and the dendrites of the neurons of the CA1 region of the hippocampus (Shaffer collateral pathway).

A second part of the study was aimed at investigating the mechanisms underlying the observed effects of DCS on synaptic plasticity. Protein synthesis is crucial for the expression and maintenance of activity-dependent modifications of synaptic efficacy. In order to confirm the interaction of DCS with these mechanisms, we explored, in the same experimental preparation, the effect of DCS on the expression of c-fos and zif268, two immediate early genes whose activation is protein synthesis-independent and that are supposed to act as regulators of downstream target genes in coupling short-term events with long-term functional modifications (Cole et al. 1989; Abraham et al. 1991, 1994; Jones et al. 2001). Because brain derived neurotrophic factor (BDNF) is implicated in synaptic modifications related to the maintenance of LTP (Lu 2003), we also investigated the effects of DCS on BDNF expression in our experimental preparation.

METHODS

Ethical approval

All animal procedures were approved by the Ethical Committee of the Catholic University and were fully compliant with Italian (Ministry of Health guidelines, Legislative Decree No. 116/1992) and European Union (Directive No. 86/609/EEC) legislation on animal research.

Experimental preparation
Male Wistar rats (weight: 150-200 g, 3-4 week old) were used for the experiments. Coronal hippocampal slices were prepared following standard procedures (Podda et al. 2010). Slices 400 µm thick were used for the electrophysiological recordings and LTP induction protocol and for the determination of BDNF levels by ELISA, while slices 150 µm thick were destined to immunohistochemistry. Sections ranged from -2.6 to -4.2 mm from bregma (Paxinos and Watson 2005). The slices destined to BDNF determination and immunohistochemistry were obtained from separate groups of animals. The animals were anesthetized by inhalation of halothane (Sigma, Milan, Italy) and decapitated. The brain was rapidly removed and put in ice-cold cutting solution containing in mM: 124 NaCl, 3.2 KCl, 1 NaH2PO4, 26 NaHCO3, 2 MgCl2, 1 CaCl2, 10 glucose, 2 Na-pyruvate and 0.6 ascorbic acid (pH 7.4, 95% O2 - 5% CO2). The brain was cut with a vibratome (VT1000S, Leica Microsystems, Nussloch, Germany) and the obtained sections in the region of the hippocampus were incubated in the cutting solution at 32°C for 60 min and then at room temperature (RT, 20-25 °C) until use.

Direct current stimulation (DCS)

DCS was applied using a battery driven constant current stimulator (Eldith - NeuroConn GmbH, Ilmenau, Germany). This stimulator is the same commonly used in human studies. The brain slices obtained after cutting received anodal or cathodal DCS or were used as a control. On both stimulated and control slices an LTP induction protocol was applied at the CA3-CA1 hippocampal synapse (see below) to determine if DCS modulates LTP at that level. DCS was applied to the brain slice while it was kept submerged in artificial cerebrospinal fluid (aCSF) solution bubbled with 95% O2 - 5% CO2 (pH 7.4). The aCSF contained (in mM): 124 NaCl, 3.2 KCl, 1 NaH2PO4, 1 MgSO4, 2 CaCl2, 26 NaHCO3, 10 glucose. The stimulation was applied through two Ag-AgCl round electrodes, with a diameter of 9 mm, submerged in the solution and connected with the two poles of the DC stimulator; for this purpose a homemade glass container of 8×2×6 cm (W×H×D) was used: the slice was placed in the middle and the electrodes laterally on the left and right side at a distance of 5 cm one from the other. Control slices were placed inside the glass container for the same amount of time as their stimulated counterparts, but the stimulator was not turned on. For anodal and cathodal stimulation the slice was oriented to obtain a current flow approximately parallel to the soma-
dendritic axis of CA1 pyramidal cells (Figure 1A), as this orientation was shown to be effective in inducing somatic polarization of these cells (Bikson et al. 2004). A current of 200-250 µA, corresponding to 100 µA at the level of the brain tissue, was delivered for 20 min. The intensity of the current near the slice was checked with an amperemeter before the stimulation and was kept constant by adapting the current delivered by the stimulator in every experimental session. The intensity of 100 µA was chosen based on previous studies on the same animal model (Liebetanz et al. 2006, 2009).

Electrophysiological recordings and hippocampal LTP induction

For each animal, an LTP induction protocol was applied at the Shaffer collateral-CA1 hippocampal synapse on control and DC-stimulated (anodal or cathodal DCS) brain slices. The stimulated and the corresponding control slice were always collected from the same animal. In stimulated slices, the electrophysiological recording procedure was started immediately after DCS. The stimulated and control slices were studied in a random order in each animal, so that the average time interval from the cutting procedure to the electrophysiological recordings was the same for anodal-stimulated, cathodal-stimulated and control slices.

For electrophysiological recordings slices were transferred to a submerged recording chamber and continuously perfused with aCSF (bubbled with 95% O₂-5% CO₂). The flow rate was kept at 1.5 ml/min by means of a peristaltic pump (Minipuls 3, Gilson, Villiers, France) and bath temperature was maintained at 30-32 °C by an in-line solution heater and temperature controller (TC-344B, Warner Instruments, Hamden, CT, USA). Ionotropic GABA-A receptors were blocked by adding 100 µM picrotoxin to the perfusing solution. A cut between CA1 and CA3 areas prevented the spread of epileptic activity into area CA1.

Field excitatory postsynaptic potentials (fEPSP) were recorded in the hippocampal CA1 region using a glass capillary microelectrode filled with aCSF (tip resistance 2-5 MΩ) positioned in the middle third of the stratum radiatum of the CA1 region and connected to a MultiClamp 700A amplifier (Molecular Devices, Sunnyvale, CA, USA). Presynaptic axons were stimulated using a bipolar tungsten electrode (Warner Instruments, Hamden, CT, USA) positioned along the Shaffer collateral fibers pathway and connected to a Grass S11 stimulator (Grass Technologies, West Warwik, RI, USA) (Figure 1B).

Data acquisition and stimulation protocols were performed by Digidata 1440 Series interface
and pClamp 10 software (Molecular Devices). Data were filtered at 1 kHz, digitized at 10 kHz and analyzed both online and offline.

Identification of hippocampal subfields and electrode positioning were done with 4× and 40× water immersion objectives on an upright microscope equipped with differential interface contrast optics under infrared illumination (BX5WI, Olympus, Tokyo, Japan) and video observation (C3077-71 CCD camera, Hamamatsu Photonics, Japan).

The stimulation intensity that produced one-third of the maximal response was used for the test pulses and LTP induction protocol. After 20 min of stable baseline response to test stimulation delivered once every 20 s, LTP was induced with a standard high frequency stimulation (HFS) paradigm consisting of four trains of 50 stimuli at 100 Hz (500 ms each) repeated every 20 s (Snyder et al. 2001). This LTP-induction protocol was used for the majority of experiments in this study. Responses to test pulse were recorded thereafter every 20 s for 60 min to measure LTP. The amplitude and slope of fEPSPs were measured from 55 to 60 min after HFS and averaged. The magnitude of LTP was expressed as percentage changes of the mean fEPSP peak amplitude and slope normalized to baseline values (i.e., the mean values of the last 10 min of recordings before tetanus). The amplitude of fEPSP was measured from baseline to peak; the slope was calculated between 20% and 80% of the maximal amplitude. Data included in the study refer to brain slices exhibiting stable fEPSP amplitude and slope increases of at least 15% at the end of LTP recording.

Before the LTP induction protocol, in order to check for a possible effect of DCS on the basic synaptic properties, input-output (I/O) curves were obtained by recording fEPSPs induced by presynaptic stimulation at intensities ranging from 10 to 55 V (in steps of 5 V).

In a set of experiments LTP was induced by theta burst stimulation (TBS), comprised of 10 bursts, at an interburst frequency of 5 Hz and an intraburst frequency of 100 Hz; each burst consisted of 5 pulses (Jo et al. 2011).

For population spike recordings a microelectrode filled with aCSF was placed extracellularly in the stratum pyramidale of area CA1 and the stimulation site was the same as the Shaffer collateral site used for recordings of fEPSPs in the stratum radiatum. Stimulation and recording sites are shown in figure 5A. Population spike I/O curves were constructed by plotting population spike amplitude versus stimulus intensities (1-50 V). Population spike amplitude was calculated as the difference between the average of the two peak positivities and the peak negativity (Marder and Buonomano 2003).
**Immunohistochemistry**

Immunohistochemical analysis was performed for c-fos and zif268 proteins, as well as for the neuronal marker NeuN. For each animal, one of the two slices obtained at a single section level (see above) was stimulated (anodal or cathodal DCS) and the other one was used as control. All brain slices were also immunostained for NeuN. After DCS, slices were kept in the aCSF solution for 6 hours before being immunoprocessed; control slices were treated in the same way, except for these were not stimulated. Slices were first fixed in 4% paraformaldehyde and washed with PBS three times, permeabilized with 1% Triton X-100 for 30 min, and blocked with PBS containing 5% BSA for 1 hour at RT to prevent nonspecific binding of antibodies. Slices were incubated for 1 hour at RT with specific primary antibody diluted in PBS, 1% BSA, 0.1% Tween 20, or in the same buffer in the absence of any antibody (no primary antibody control). Antibodies anti c-fos protein (rabbit IgG polyclonal antibody, 1:250) or anti zif268 protein (rabbit anti-Egr-1, 1:200) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and antibody anti NeuN (mouse IgG monoclonal antibody, 1:250) were purchased from Chemicon International Inc. (Millipore Corporation, Billerica, MA). Slices were then washed five times with PBS and incubated for 1 hour at room temperature with 1:150 dilution of Texas Red-conjugated goat anti-mouse IgG and FITC-conjugated goat anti-rabbit IgG (Jackson Immunoresearch Laboratories Inc., Suffold, UK), diluted in the same buffer used for the primary antibodies. The slices were washed five times with PBS and mounted with PBS/glycerol in a 1:1 dilution.

**BDNF determination**

For each animal, one of the two slices obtained at a single section level (see above) was stimulated (anodal or cathodal DCS) and the other one was used as control. After DCS, slices were kept in the aCSF solution for 6 hours before being processed; control slices were treated in the same way, except for these were not stimulated. Hippocampus was then isolated from the collected brain slices in order to be processed for the quantification of BDNF protein by enzyme-linked immunosorbent assay (ELISA). Briefly, tissues were sonicated in lysis buffer (10 μl/mg tissue), containing: 137 mM NaCl, 20 mM Tris, 1% Nonidet P-40, 10% glycerol, 1 mM PMSF, 10 μg/ml aprotinin, 1 μg/ml leupeptin. Sonicates were then diluted two-fold with Dulbecco's Phosphate Buffered Saline (DPBS), containing in mM: 2.7 KCl, 137 NaCl, 1.47
KH₂PO₄, 8.1 Na₂HPO₄, 0.5 MgCl₂, 0.9 CaCl₂ (pH 7.35). Samples were then stored at -80°C until assayed for BDNF.

BDNF was measured by ELISA using commercial kits (R&D Systems, MN, USA) following manufacturer’s instructions. All assays were performed on F-bottom 96-well plates (Nunc, Wiesbaden, Germany). Tertiary antibodies were conjugated to horseradish peroxidase. Wells were developed with tetramethylbenzidine and measured at 450 nm.

**Data analysis**

**Electrophysiological recordings.**

In each animal the amount of LTP was measured in at least one DC-stimulated (anodal or cathodal DCS) and one control slice. Operators performed recordings and analysis unaware of the group of origin of the slices being processed.

FEPSP amplitude and slope were normalized to the average of baseline values obtained in the last 10 min of recording before application of LTP induction protocol (HFS or TBS) and expressed as percentage values. In each slice (DC-stimulated or control) the average of normalized values between 55 and 60 min after LTP induction protocol was calculated for both amplitude and slope. LTP was expressed as variation from the baseline and mean values were then obtained for anodal-stimulated, cathodal-stimulated and each of the two groups of control slices. FEPSP amplitude and slope increases in anodal- and cathodal-stimulated slices were compared with those of the respective control slices by mean of two-tail unpaired Student’s *t*-test. Within each brain slice, amplitude and slope after LTP induction were also compared with those of baseline conditions by mean of two-tail paired Student’s *t*-test. *P*<0.05 was taken as significant.

I/O curves for fEPSP amplitude and slope were analyzed with 2-way ANOVA, with stimulus intensity and treatment as main factors.

Data relating to CA1 population spike amplitude at different stimulus intensities were analyzed by nonlinear dose response curve fitting. The individual curves describing the relationship between output spike amplitude (y) and stimulus intensity (x) were fitted to a sigmoidal four-parameter logistic function:

\[
y = A1 + \frac{A2 - A1}{1 + 10^{(\log x_0 - x)p}}
\]
where $A_1$ and $A_2$ are the expected minimum and maximum responses, respectively, $p$ is the slope, and $\log x_0$ is the stimulus intensity expected to generate a half-maximal response ($ES_{50}$). Because CA1 population spikes were never observed at stimulus intensities below 1 V, the expected minimum response was zero in all cases. Constrained curve fitting and two-tail unpaired Student’s $t$-test were used to assess the significance of differences between the calculated $ES_{50}$ values on DC-stimulated and control slices.

**Immunohistochemistry.**
Quantitative analysis of c-fos, zif268 and NeuN labeled cells was performed using analysis^B image software (Olympus, Hamburg, Germany) with the aid of Olympus XM10 Digital Camera attached to an Olympus BX51M microscope. Sections were viewed at 20× magnification and counts of immunolabeled cells were made by an observer (G.F.) blind to the treatment within square grids of defined size (100×100 µm) that were placed over each area. The same magnification and exposure time were used for all samples. In each animal, cells in the CA1, CA3 and DG regions of the hippocampus were counted in at least one DC-stimulated (anodal or cathodal DCS) and one control slice and expressed as mean number of cells/mm$^2$. The number of positive cells in anodal- and cathodal-stimulated slices was compared with that of the respective control slices by mean of two-tail unpaired Student’s $t$-test. $P<0.05$ was taken as significant.

**BDNF determination.**
BDNF concentrations were determined from the regression line for a standard curve generated by using highly purified recombinant human BDNF at various concentrations performed contemporaneously with each assay. The standard curve also served as an internal control over the sensitivity and range of each assay. All samples were assayed in duplicate, and quality control pools at low, normal and high concentrations were present in each assay. Concentrations were expressed as pg/mg of tissue. BDNF levels in anodal- and cathodal-stimulated slices were compared with that of the respective control slices by mean of two-tail unpaired Student’s $t$-test. $P<0.05$ was taken as significant.

**RESULTS**
HFS and TBS stimulations elicited reliable and stable LTP in both control and DCS-exposed slices: measured fEPSP were significantly increased at 60 min after stimulus, in terms of amplitude and slope (P<0.001 for both parameters; Figures 2 and 4). DCS did not influence the success rate of LTP induction that was 100% in all experimental conditions.

Anodal stimulation

LTP elicited by HFS was significantly greater in anodal-stimulated than in control slices, both for amplitude and slope (control: n=18 and stimulated: n=18, from 18 rats). Mean fEPSP amplitude increase was 47.8±6.7% (mean±SEM) in control slices and 134.0±13.3% in stimulated slices (P<0.0001) (Figure 2A,B). Mean fEPSP slope increase was 57.3±9.1% in control slices and 145.8±11.0% in stimulated slices (P<0.0001) (Figure 2B).

Useful information about synaptic properties can be obtained by constructing I/O curves. They were examined, in baseline conditions, in control and stimulated slices, to verify if there was any variation of the basal synaptic function induced by DCS. Plots of fEPSP amplitude and slope versus stimulus intensity showed that the I/O function did not differ significantly between stimulated and control slices (2-way ANOVA; Amplitude: F_{1,9}=0.069, P=1.000; Slope: F_{1,9}=0.190, P=0.995) (Figure 3).

It is known that different LTP induction protocols may have different effect on LTP magnitude and activate heterogeneous signaling pathways (Hernandez et al. 2005; Smith et al. 2009). We therefore performed additional experiments in order to test whether the effects of DCS were influenced by induction protocol used.

LTP at Shaffer-collateral-CA1 synapse was induced by TBS, a widely used protocol that it is intended to mimic endogenous theta frequency EEG activity (4–8 Hz) recorded in the rat hippocampus during behavioral activities (Buzsaki 2002; Jo et al. 2011). As shown in Figure 4, similarly to what we observed following HFS, the magnitude of LTP induced by TBS was higher in slices stimulated with anodal DCS compared to that of control slices. Mean fEPSP amplitude increase was 110.9±9.4% in control slices and 160.2±5.0% in stimulated slices (control: n=6 and stimulated: n=6, from 6 rats; P<0.001, Figure 4A,B). Mean fEPSP slope increase was 137.6±8.1% in control slices and 154.7±11.7% in DC-stimulated slices (P<0.001; Figure 4C,D).

These data indicate that, in our experimental conditions, the direction of the change produced
by anodal DCS is not influenced by the mode of LTP induction. However, it should be pointed out that in control slices the TBS-induced LTP was greater than HFS-induced LTP, whereas the percent increase of LTP magnitude produced by anodal DCS is lower in TBS-stimulated slices compared to HFS-stimulated slices. To test whether the same DCS that enhanced LTP also produces after-effects on postsynaptic cell excitability, that would account for its facilitation of subsequent LTP, we recorded extracellular responses in stratum pyramidale from area CA1 (population spikes) in control and stimulated slices while delivering stimuli at Shaffer-collateral over a series of intensities (see Methods). The extracellularly recorded population spike reflects the number of synchronously firing cells (Andersen et al. 1971) and the I/O function of a population of cells provides a measure of post-synaptic excitability. As shown in figure 5C the shape of the I/O relationship was similar in anodal-stimulated and control slices. Statistical analysis revealed no significant difference between the stimulus intensity required to elicit a half maximal response (ES$_{50}$) (P>0.5); in both control and stimulated slices (control: \( n=16 \) and stimulated: \( n=16 \), from 16 rats) this value was \( \sim 28 \) V (29.6±2.3 V and 27.0±2.8 V in control and stimulated slices, respectively). Slope values were 0.06±0.01 and 0.07±0.01 mV/V in control and stimulated slices, respectively (P>0.5). However, in DC-stimulated slices mean responses to all stimuli were slightly smaller than in controls, with a non significant (P=0.23) decrease of \( \sim 15\% \) observed in anodal-stimulated slices at the maximal stimulus intensity (Figure 5C). Taken together our electrophysiological data indicate that anodal DCS exerted a facilitatory effect on LTP at Shaffer-collateral-CA1 hippocampal synapses, which is not associated to either changes in basal synaptic transmission or increases in post-synaptic excitability. For both zif268 and c-fos proteins, immunohistochemical analysis was conducted on 6 anodal-stimulated and 6 corresponding control slices, from 6 animals. Zif268 analysis in the CA subregions showed an increased number of positive cells in all anodal-stimulated slices compared to the respective controls: in CA1 the mean number of cells/mm$^2$ was 155±31 (mean±SEM) in control slices and 621±111 in stimulated slices (P<0.005); in CA3 it was 123±47 in control slices and 523±89 in stimulated slices (P<0.005). The number of zif268 immunolabeled cells in the DG region was lower than in CA and showed no significant variation in the stimulated group (controls: 64±8 cells/mm$^2$, stimulated: 65±8 cells/mm$^2$; P=0.95) (Table 1; Figure 6). C-fos analysis in the CA subregions showed an increased number of positive cells in anodal-stimulated slices compared to the respective controls: in CA1 the mean number of cells/mm$^2$
was 52±5 in control slices and 71±7 in stimulated slices (P=0.07); in CA3 it was 43±10 in control slices and 90±20 in stimulated slices (P=0.06). The number of c-fos immunolabeled cells was also increased in the DG region of the stimulated group (controls: 31±2 cells/mm², stimulated: 41±3 cells/mm²; P<0.05) (Table 1; Figure 7).

Counting of NeuN labeled cells in CA and DG revealed no significant difference between control and stimulated slices (CA: 2128±40 and 2214±27 cells/mm² in control and stimulated slices respectively, P>0.05; DG: 1890±40 and 1822±54 cells/mm² in control and stimulated slices respectively, P>0.05), indicating no loss of vital neurons following anodal DCS.

BDNF concentration was determined in the hippocampal tissue obtained from 8 anodal-stimulated and 8 corresponding control slices (from 5 rats). Mean BDNF concentration was not significantly modified in anodal stimulated slices: it was 18.01±2.61 pg/mg of tissue (mean±SEM) in control slices and 30.45±15.26 pg/mg in stimulated slices (P>0.05).

Cathodal stimulation

HFS-induced LTP was significantly lower in cathodal stimulated than in control slices, both for amplitude and slope (control: n=16 and stimulated: n=16, from 16 rats). Mean fEPSP amplitude increase was 53.7±8.8% (mean±SEM) in control slices and 32.1±4.0% in stimulated slices (P<0.0001) (Figure 2C,D). Mean fEPSP slope increase was 55.4±8.5% in control slices and 25.3±10.8% in stimulated slices (P<0.0001) (Figure 2D).

As for anodal stimulation, I/O curves were obtained before HFS in control and cathodal stimulated slices. Plots of fEPSP amplitude and slope versus stimulus intensity showed that the I/O function did not differ significantly between stimulated and control slices (2-way ANOVA; Amplitude: F_{1,9}=0.057, P=1.000; Slope: F_{1,9}=0.107, P=0.999) (Figure 3).

The effect of cathodal stimulation was also tested on LTP induced by TBS and the picture that emerged was similar to that observed following HFS. Indeed, a reduction of LTP magnitude was observed in cathodal-stimulated compared to control slices (control n=6 and stimulated n=6, from 6 rats; Figure 4C,D). Mean fEPSP amplitude increase was 102.1±13.3% in control and 41.6±4.1% in stimulated slices (Figure 4C,D). Mean fEPSP slope increase was 120.4±7.3% in control and 71.3±12.0% in stimulated slices (Figure 4D). Comparison of effects produced by cathodal DCS on TBS-LTP versus HFS-LTP evidenced a difference in term of magnitude, being the DCS more effective in reducing LTP elicited by TBS.

Analysis of population spike I/O function did not reveal significant differences between
control and stimulated slices (control: $n=16$ and stimulated: $n=16$, from 16 rats) in term of shape of the I/O curve, ES$_{50}$ value (24.4±2.2 V in control slices and 25.7±1.3 V in stimulated slices; $P>0.5$) and slope value (0.07±0.01 mV/V for both control and stimulated slices). Mean responses at all stimulus intensities were smaller in cathodal-stimulated slices compared to control slices with a decrease of ~40% in the maximal response (Figure 5D, $P<0.05$).

For both zif268 and c-fos proteins, immunohistochemical analysis was conducted on 6 cathodal-stimulated and 6 corresponding control slices, from 6 animals.

Zif268 analysis in the CA subregions showed an increased number of positive cells in all cathodal-stimulated slices compared to the respective controls: in CA1 the mean number of cells/mm$^2$ was 147±33 (mean±SEM) in control slices and 611±117 in stimulated slices ($P<0.005$); in CA3 it was 161±44 in control slices and 592±88 in stimulated slices ($P<0.005$). The number of zif268 immunolabeled cells was lower in the DG region and showed no significant variation in the stimulated group (controls: 56±4 cells/mm$^2$, stimulated: 49±1 cells/mm$^2$; $P=0.13$) (Table 1; Figure 6).

C-fos analysis in the CA subregions showed an increased number of positive cells in the CA1 subregion of cathodal-stimulated slices compared to the respective controls: in CA1 the mean number of cells/mm$^2$ was 42±6 in control slices and 76±13 in stimulated slices ($P<0.05$); in CA3 it was 50±19 in control slices and 48±17 in stimulated slices ($P=0.95$). The number of c-fos immunolabeled cells was not significantly modified in the DG region of the stimulated group (controls: 35±2 cells/mm$^2$, stimulated: 43±5 cells/mm$^2$; $P=0.19$) (Table 1; Figure 7).

Counting of NeuN labeled cells in CA and DG revealed no significant difference between control and stimulated slices (CA: 2167±25 and 2235±18 cells/mm$^2$ in control and stimulated slices respectively, $P>0.05$; DG: 1855±3 and 2027±74 cells/mm$^2$ in control and stimulated slices respectively, $P>0.05$).

BDNF concentration was determined in the hippocampal tissue obtained from 8 cathodal-stimulated and 8 corresponding control slices (from 5 rats). Mean BDNF concentration was significantly reduced in cathodal stimulated slices: it was 36.40±8.98 pg/mg of tissue (mean±SEM) in control slices and 15.45±3.75 pg/mg in stimulated slices ($P<0.05$).

DISCUSSION

The results of our study show that DCS applied to rat brain slices determines a modulation of LTP at CA3-CA1 hippocampal synapses, in a polarity-specific manner: anodal DCS markedly
increased LTP while cathodal DCS reduced it. This supports the hypothesis of a link between the after-effects of DCS and the mechanisms of activity-dependent synaptic plasticity. Input-output curves before LTP-induction, both for anodal and cathodal stimulation, showed that there were no changes in the basal synaptic transmission after DCS and that this stimulation likely affects synaptic plasticity. Supporting this view our analysis of input-output function of CA1 population spikes, as a measure of post-synaptic excitability, showed no significant differences among control and stimulated slices as for the ES$_{50}$ and the slope values. However, it should be pointed out that population spike amplitude was decreased in both anodal and, more markedly, in cathodal stimulated slices. These changes, that may reflect a certain degree of reduction in pyramidal cell excitability, cannot account for the enhancement of LTP observed following anodal stimulation, but might contribute to LTP reduction observed following cathodal stimulation. Our conclusion of a major effect of DCS on synaptic plasticity mechanisms is also supported by the study of Fritsch et al. (2010) who showed that LTP is not induced by DCS alone, but only in the presence of simultaneous synaptic activation. Although the data obtained in one region can not be immediately extended to another, it might be that at multiple levels of the central nervous system the modulatory effect of DCS is not sufficient to modify synaptic activity, but it can change the response of the synapse to some kind of repeated activation (low frequency stimulation in the cited study on the primary motor cortex and LTP-induction protocol in our study on the hippocampus). A similar phenomenon was described in humans by Ziemann et al. (1998), who reported that cortical transitory deafferentation, in analogy with our DCS conditioning protocol, modulates plasticity making the cortex modifiable by inputs (repeated stimulation at 0.1 Hz) that are normally subthreshold for inducing changes in excitability. The effects of tDCS on LTP using several transcranial magnetic stimulation (TMS) based protocols capable of inducing LTP in the intact human brain have been evaluated: interestingly, in analogy with our experimental data, it has been shown that anodal tDCS may enhance the level of LTP induced by a facilitatory TMS based protocol (Nitsche et al. 2007), while cathodal tDCS can have the opposite effect (Lang et al. 2004b; Nitsche et al. 2007; Antal et al. 2008).

In vitro different LTP-induction protocols are known to differentially affect LTP magnitude and to activate heterogeneous signaling pathways (Hernandez et al., 2005; Smith et al., 2009). Our study demonstrates that DCS modulation of LTP occurs independently on the induction protocol we used. Indeed, the effects produced by anodal and cathodal stimulation on LTP
induced by high frequency stimulation (HFS) and by theta burst stimulation (TBS) were similar in terms of direction of the change (i.e., anodal DCS increased LTP while cathodal DCS reduced it). However, our data show that the magnitude of LTP in control condition and, more interestingly, the entity of DCS effects, differ when using HFS and TBS. In control condition mean fEPSP amplitude increase was >100% following TBS and ~50% following HFS. The entity of anodal DCS effects on TBS-stimulated slices were smaller compared to those elicited in HFS-stimulated slices, which could be explained by the fact that in control slices synaptic strength following TBS was already close to maximum, therefore additional potentiation by anodal DCS was limited. On the contrary the inhibitory effects elicited by cathodal DCS on TBS-LTP were slightly higher than those observed on HFS-LTP.

Different molecular pathways can underlie the observed effects of DCS and it is only possible to speculate about them (Zaghi et al. 2010). Since DCS does not initiate action potentials, its effects are likely to be initially mediated by membrane potential changes that could influence molecular targets sensitive to polarizing electric fields. Based on current knowledge about the mechanisms of LTP (Malenka and Nicoll 1999; Malenka and Bear 2004; Kim and Linden 2007), it can be hypothesized that DCS influences some molecules specifically involved in LTP induction, possibly including Ca\(^{2+}\) channels or the NMDA receptor. The NMDA receptor in particular accounts for the major component of LTP induction at hippocampal Shaffer collateral pathway (Collingridge 1983, 1987; Grover and Teyler 1990) and has a voltage-dependent mechanism that might make it susceptible to the polarity-specific effects of DCS (Nowak et al. 1984; Mayer et al. 1984). Based on the 'tagging and capture' hypothesis (Frey and Morris 1997; Barco et al. 2008) another possible explanation of the effect of anodal stimulation is that it determines the formation of molecular tags for some “proteins of plasticity” coded by genes induced by the successive tetanic stimulation. The formation of these molecular tags might be of different origin: modifications of existing proteins related to DC field exposure or to changes in spontaneous neuronal spiking and/or miniature synaptic potentials, or even the expression of new proteins by early gene induction. These hypotheses would be coherent with our findings, since the experimental design of the present study actually evaluates a phenomenon of metaplasticity, where DCS modulates, in a polarity-specific manner, the response to a successive protocol of synaptic potentiation. Nonetheless the possibility of DCS of influencing cortical plasticity has been demonstrated in humans (Siebner et al. 2004; Lang et al. 2004b). A similar mechanism of action would give strong support to the clinical use of tDCS, where it could be used to increase the response of the brain to interventions to promote plasticity, such as the neurorehabilitation techniques
commonly used for the recovery of motor function or language after stroke (Talelli and Rothwell 2006; Schlaug et al. 2008). The role of protein synthesis in the sustained effects of DCS in the rat has been demonstrated from the beginning (Gartside et al. 1968). BDNF is attributed a main role as an effector in the consolidation of the late phase of LTP (Lu 2003). Interestingly Barco et al. (2005) demonstrated that BDNF is not only critical for the maintenance of LTP at CA3-CA1 synapses, but also for synaptic capturing. Indeed, while BDNF mutant mice showed normal LTP induction, they exhibited a dramatic reduction of synaptic potentiation induced by a following tetanic stimulation of second pathway to the same population of CA1 neurons, suggesting that BDNF might contribute to the processes of tagging and capture at that level. This suggests that this neurotrophin might be involved in the mechanisms mediating the observed effects of DCS in the hippocampus. The role of the BDNF in DCS-induced LTP has recently been confirmed in the motor cortex (Fritsch et al. 2010) and is coherent with the finding in humans that BDNF polymorphysms modulate the effect of preconditioning with cathodal tDCS in a protocol of homeostatic plasticity (Cheeran et al. 2008). We found no significant variation of BDNF level in anodal stimulated slices and a significant reduction in cathodal stimulated slices. We can hypothesize that inhibition of ongoing BDNF synthesis by cathodal DCS contributes to the observed reduction in LTP produced by this polarity of stimulation. However, the interpretation of our data on BDNF expression suffer from a limitation due to the small sample and a high variability related to the little amount of tissue that is possible to collect from the isolation of the hippocampus. Also, the amount of BDNF that is available for activity-dependent release might differ from the total protein level obtained from homogenized slices. For this reasons, the present data should be confirmed in future specifically designed studies. Nonetheless our results do not contrast with the role of BDNF in synaptic plasticity described in the literature and are coherent with the direction of the modulation of LTP determined by anodal and cathodal DCS. To this respect, BDNF-TrkB signaling plays a crucial role in the regulation of LTP (Minichiello et al. 2009) and has already been demonstrated to be a key mediator of the LTP produced by anodal DCS in rat motor cortex (Fritsch et al. 2010) and to be influenced by repetitive TMS protocols (Wang et al. 2011). Moreover, a better knowledge of the influence of DC exposure on the pathways of CREB-dependent BDNF expression would shed light on the mechanisms of modulation of this neurotrophin by DCS. Further studies are needed to specifically investigate the above molecular patterns. Protein synthesis associated with some kind of cell perturbation depends on the early
activation of cascade signaling pathways that can involve several downstream genes. We focused our attention on the expression of two immediate early genes (IEGs), c-fos and zif268, that are rapidly induced following neuronal activation (Saffen et al. 1988) and that are thought to be involved in the mechanisms of synaptic plasticity (Tischmeyer and Grimm 1999; Pláteník et al. 2000). Of these, zif268 is likely to be more specifically related to LTP, since it is expressed under virtually all LTP-inducing situations and shows a remarkably high correlation with the duration of LTP (Cole et al. 1989; Wisden et al. 1990; Abraham et al. 1991; Davis et al. 2003). In a recent study Aydin-Abidin et al. (2008) found that repetitive transcranial magnetic stimulation, another technique of non-invasive brain stimulation capable of inducing plastic changes in the cerebral cortex, applied in vivo to the rat brain modulates the expression of c-fos and zif268 in the neurons of different areas. This suggests that these proteins might have a role also in the modulation of synaptic plasticity induced by DCS.

Here we studied the products of these genes by immunohistochemistry in the same region and following the same stimulation procedure used in the electrophysiological investigation. As previously reported, in basal conditions zif268 is most highly expressed in the CA than in the DG region of the hippocampus (Schlingensiepen et al. 1991). In CA1 and CA3 areas, the number of zif-positive cells was much higher (about four times) in DC-stimulated slices than in controls, whilst no difference was observed in the DG region. C-fos expression was more diffusely enhanced by both anodal and cathodal DCS, however this increase was less pronounced and less region-specific than that observed in the case of zif268. Since both DC polarities produced similar effects on zif268 expression in CA1 and CA3 areas, despite the different prevalent orientation of the neurons in these regions, while no effect was observed in DG, the most likely explanation for these findings seems to reside in the intrinsic properties of the different cell populations. However a contribution of the specific cell polarization related to the non homogeneous spatial orientation of the neurons along CA and DG regions can not be completely ruled out (Bikson et al. 2004).

At first sight it might be surprising that DCS produced a polarity-specific modulation of LTP at CA3-CA1 synapses, while IEG expression was increased by either anodal or cathodal stimulation in that region. On the basis of present findings, we can only speculate about the possible mechanisms responsible for the dissociated effect of DCS on LTP and on IEGs. It should be considered that the induction of IEG expression is very fast during neuronal activation and that they in turn activate downstream target genes: thus, IEGs might be involved in the maintenance of already induced LTP (Jones et al. 2001) or in the modulation
of the response of the post-synaptic cell to a following activation. Previous studies have shown that both *zif268* and, less consistently, *c-fos* can be modulated by tetanic stimulation inducing LTP (Cole et al. 1989); here we show that also DCS alone influences the levels of these proteins. Based on the assumption that IEGs are not likely to act as final effectors, we can hypothesize that the enhanced expression of IEGs in CA contributes to the modulation of LTP induction at that level in the presence of other factors shaping its direction, such as specific electrical or conformational modifications of other molecular targets determined by anodal or cathodal DCS. Moreover, it might also be that different molecular changes take place after DCS and these are responsible for the direction of LTP change.

In conclusion, our data confirm the effect of DCS on synaptic plasticity showing that it may modulate LTP induction in a polarity specific manner and affect gene expression. Although these data were obtained in rat CA3-CA1 hippocampal synapses with an *in vitro* model of brain stimulation and it is not possible to determine to which extent they can be extrapolated to the stimulation *in vivo* and to other brain regions, they may suggest that similar effects could be involved in the electrophysiological or behavioral modifications determined by tDCS in humans.

It is suggested that tDCS could be used to modulate LTP induction in the intact human brain and, in pathological conditions, it might serve as an experimental tool to enhance the adaptive and suppress the maladaptive forms of brain plasticity.

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**Disclosures.** No conflict of interest
REFERENCES


Bindman LJ, Lippold OC, Redfearn JW. The action of brief polarizing currents on the cerebral cortex of the rat (1) during current flow and (2) in the production of long-lasting after-effects. *J


**Table 1**

Mean number of immunolabeled cells in the cornus ammonis and dentate gyrus regions of the hippocampus.

<table>
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<th>Animal</th>
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<th>CA3 (cells/mm²)</th>
<th>DG (cells/mm²)</th>
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<td>stim</td>
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</table>

Mean number of immunolabeled cells in the cornus ammonis and dentate gyrus regions of the hippocampus.
Figure 1
A) Schematic representation of continuous electric field application. The slice was oriented to obtain an electric field approximately parallel to the soma-dendritic axis of CA1 pyramidal cells. Anodal stimulation is represented in the figure.
B) Schematic representation of electrophysiological recordings. SC: Shaffer collaterals; CA: cornus ammonis; DG: dentate gyrus.

Figure 2
Anodal DCS increases while cathodal DCS decreases LTP induced by high frequency stimulation (HFS). FEPSP amplitude in anodal-stimulated (A), cathodal-stimulated (C) and control slices is expressed as percentage of the mean of the baseline values in the last ten minutes before tetanus (t from -10 to 0 min). Arrow indicates HFS application. B,D) Bar graphs comparing HFS-LTP, in terms of fEPSP amplitude and slope, in control and DCS-exposed slices during the last 5 min of recording. * P<0.0001.

Figure 3
Input-output relationship of synaptic function. FEPSP amplitude and slope in anodal- and cathodal-stimulated slices (filled diamonds), compared to the respective control slices (empty diamonds), by stimulating the presynaptic axon at increasing intensities from 10 to 55 V.

Figure 4
Anodal DCS increases while cathodal DCS decreases LTP induced by theta burst stimulation (TBS) protocol. FEPSP amplitude in anodal-stimulated (A), cathodal-stimulated (C) and control slices is expressed as percentage of the mean of the baseline values in the last ten minutes before tetanus (t from -10 to 0 min). Arrow indicates TBS application. B,D) Bar graphs comparing TBS-LTP, in terms of fEPSP amplitude and slope, in control and DCS-exposed slices during the last 5 min of recording. * P<0.001.

Figure 5
Changes in the evoked responses of area CA1 pyramidal cells following DCS stimulation. A) Schematic representation of the Shaffer collaterals (SC) stimulating site and recording site in the CA1 stratum pyramidale for population spike recordings. B)
Representative evoked responses at 30 V in control and DC-stimulated slices. C,D) Population spike amplitude is plotted as a function of stimulus intensity. Data represent mean±SEM of the results for each stimulus intensity. The lines drawn through the points represent best-fit curves (see Methods).

**Figure 6**

**Immunohistochemistry for zif268.** A) NeuN (first column), and zif268 (second column) immunolabeled cells in the CA region, in representative slices. The number of neurons expressing zif268 (third column: merge of NeuN and zif positive cells) in CA subregions is markedly higher in both anodal- and cathodal-stimulated slices compared to controls. The CA1 subregion is represented in the figure. B) Bar graphs representing the mean relative variation in the expression of zif268 in the different hippocampal subregions after both anodal and cathodal DCS compared to the respective controls (control condition = 100%, horizontal dashed line) (see Table 1 for analytical values). Error bars represent standard error.

**Figure 7**

**Immunohistochemistry for c-fos268.** A) NeuN (first column), and c-fos (second column) immunolabeled cells in the CA region, in representative slices. The CA1 subregion is represented in the figure. B) Bar graphs representing the mean relative variation in the expression of c-fos268 in the different hippocampal subregions after both anodal and cathodal DCS compared to the respective controls (control condition = 100%, horizontal dashed line) (see Table 1 for analytical values). Error bars represent standard error.

**Table 1**

Mean number of zif268 and c-fos immunolabeled cells in the cornus ammonis (CA) and dentate gyrus (DG) regions of the hippocampus of control, anodal- and cathodal-stimulated slices. * P<0.05 compared to controls; ** P<0.005 compared to controls.