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Treatment with direct-current stimulation against cingulate seizure-like activity induced by 4-aminopyridine and bicuculline in an in vitro mouse model

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ABSTRACT

Clinical studies have shown that cathodal transcranial direct-current stimulation (tDCS) application can produce long-term suppressive effects on drug-resistant seizures. Whether this long-term effect produced by cathodal tDCS can counterbalance the enhancement of synaptic transmission during seizures requires further investigation. Our hypothesis was that the long-term effects of DCS on seizure suppression by the application of cathodal DCS occur through a long-term depression (LTD)-like mechanism. We used a thalamocingulate brain slice preparation combined with a multielectrode array and patch recording to investigate the underlying mechanism of the suppressive effect of DCS on anterior cingulate cortex (ACC) seizures. Patch-clamp recordings showed that cathodal DCS significantly decreased spontaneous excitatory postsynaptic currents (EPSCs) and epileptic EPSCs caused by the 4-aminopyridine. Fifteen minutes of DCS application reliably induced LTD, and the synaptic activation frequency was an important factor in LTD formation. The application of DCS alone without continuous synaptic activation did not induce LTD. Direct-current stimulation-induced LTD appeared to be *N*-methyl-D-aspartate (NMDA)-dependent, in which the application of the NMDA receptor antagonist D-1-2-amino-5-phosphonopentanoic acid (APV) abolished DCS-induced LTD, and the immediate effect remained. Direct-current stimulation-induced LTD and the long-term effects of DCS on seizure-like activities were also abolished by okadaic acid, a protein phosphatase 1 inhibitor. The long-term effects of DCS on seizures were not influenced by the depotentiation blocker FK-506. Therefore, we conclude that the long-term effects of DCS on seizure-like activities in brain slice occur through an LTD-like mechanism.

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Introduction

Epilepsy is a common neurological disorder. Approximately 1% of the population worldwide suffers from this disease. Thirty percent of patients with epilepsy suffer from drug-resistant seizures (Schiller and Najjar, 2008). Alternative treatment strategies, such as transcranial magnetic stimulation (TMS) and transcranial direct-current stimulation (tDCS), provide non-invasive approaches for controlling pharmacoresistant seizures. Previous studies showed that tDCS is effective for treating Alzheimer's disease related symptoms (Hansen, 2012), for Parkinson disease therapy (Benninger et al., 2010), for increasing motor learning ability (Karok and Witney, 2013), for lessening poststroke motor deficits (Ayache et al., 2012) and for treating intractable seizures (Yook et al., 2011). Clinical studies reveal the complex nature of the effects of tDCS, in which it can produce both short- and long-term suppressive effects on seizures (Ghai et al., 2000; Warren and Durand, 1998; Auvichayapat et al., 2013). However, the underlying mechanism of the long-term effect of tDCS has remained elusive. Additionally, the stimulation parameters, including orientation, field strength, and stimulation duration, must be tested in animal models to achieve its optimal effects.

The immediate effects of DCS on seizure-like activity have mostly been evaluated in the hippocampus and motor cortex (Bikson et al., 2001; Ghai et al., 2000; Gluckman et al., 2001). The immediate effect of DCS involves the passing of currents from the extracellular space (ECS), resulting in the polarization of cell membranes and modulation

Abbreviations: 4-AP, 4-aminopyridine; ACC, anterior cingulate cortex; aCSF, artificial cerebral spinal fluid; ANOVA, analysis of variance; APV, D-1-2-amino-5-phosphonopentanoic acid; ATP, adenosine triphosphate; BDNF, brain-derived neurotrophic factor; CSD, current-source density; DBS, deep brain stimulation; DCS, direct-current stimulation; DMSO, dimethyl sulfoxide; ECS, extracellular space; EEG, electroencephalogram; EGTA, ethylene glycol tetraacetic acid; FLE, frontal lobe epilepsy; fEPSP, field excitatory postsynaptic potential; GTP, guanosine-5′-triphosphate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; LTD, long-term depression; LTP, long-term potentiation; MEA, multielectrode array; MLA, methyllycaconitine; MT, medial thalamus; NKCC, Na–K–Cl cotransporter; NMDA, *N*-methyl-D-aspartate; PP1, protein phosphatase 1; sEPSC, spontaneous excitatory postsynaptic current; sEPSP, spontaneous excitatory postsynaptic potential; SPSS, Statistical Product and Service Solutions; tDCS, transcranial direct-current stimulation; Th, thalamus; TMS, transcranial magnetic stimulation.

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of neuronal activity (Andreasen and Nedergaard, 1996; Bikson et al., 2004; Jefferys, 1981). The orientation of pyramidal neurons is crucial for the effect of the electric field. The direction of the electric field must be parallel to the somatic–dendritic axis of pyramidal neurons to achieve a maximal effect (Gluckman et al., 2001).

Polarizing field can also exert long-lasting effect. It is known to influence long-term synaptic plasticity in the human motor cortex (Grundey et al., 2012; Kuo et al., 2007). These studies indicate that the long-term effect of tDCS likely occurs though a long-term potentiation (LTP)-like mechanism (Cheeran et al., 2008; Nitsche et al., 2003, 2004). A recent brain slice study confirmed that DCS-mediated potentiation is brain-derived neurotrophic factor (BDNF) — and *N*-methyl-D-aspartate (NMDA)-dependent (Fritsch et al., 2010). The LTP/long-term depression (LTD) mechanism is important in memory and learning, and the induction of LTD might be helpful in suppressing seizure. Previous studies used deep brain stimulation (DBS) and found that DBS could alter synaptic plasticity and change seizure thresholds (Gaito, 1980; Weiss et al., 1995). A recent study of DBS showed that an LTD stimulation protocol (0.1 Hz stimulation) could delay basolateral amygdala kindling (Velisek et al., 2002). Low-frequency stimulation-induced LTD also effectively lowers the frequency and amplitude of seizure-like activity in hippocampal slices (Albensi et al., 2004). These results indicate that DBS can induce LTD and might lead to the suppression of seizures. Although DCS-LTP has been characterized (Fritsch et al., 2010), unknown is whether DCS produces similar LTD-like effects. Moreover, whether changes in synaptic transmission mediated by DCS exert long-term effects on seizures needs further investigation.

Frontal lobe epilepsy (FLE) is the second most prevalent focal epilepsy syndrome. Epilepsy in the anterior cingulate cortex (ACC) is included as part of epileptic syndromes of frontal lobe origin, which often manifest with simple partial seizures (Nadkarni and Devinsky, 2009). Focal ACC epilepsies are often non-lesional with the cause unknown. Most focal ACC epilepsies are believed to be idiopathic and cryptogenic. Frontal lobe epilepsy and ACC seizures are often drug-resistant (Biraben et al., 2001; Zaatreh et al., 2002) and the evaluation of alternative treatments, such as tDCS, is needed. Seizures that arise from the ACC are difficult to study because this region lies deep within the brain, and the proximity between the right and left ACC increases the difficulty in identifying where seizures actually start (Geier et al., 1977; Mazars, 1970; Nadkarni and Devinsky, 2009). There are some reports of the application of the tDCS to the cingulate cortex (Nelson et al., 2014; Karim et al., 2010; Keeser et al., 2011). tDCS is shown to affect vigilance, decision making and emotion through alteration of ACC activities. tDCS usually affects large brain regions, and it is difficult to exclude non-specific effects. However, this tDCS feature may provide the potential to control or alter network activity across large brain areas. Whether tDCS modulates neuronal excitability and seizure activity in this brain region has not yet been characterized. Therefore, determining whether tDCS has suppressive effects on FLE or ACC seizures would be helpful for clinical treatment.

Previous studies showed that the orientation of the electric field must be parallel to axodendritic fibers of the cortical column to achieve a maximal effect, but obtaining such an orientation in an in vivo preparation is difficult. Additionally, the pharmacological manipulation is difficult in whole-animal preparations. In in vivo studies, the amounts of current passing through a particular brain region are difficult to measure, and also have some non-specific effects such as alteration on vascular activities. Therefore, in the present study, we used an in vitro brain slice preparation that was developed previously (Lee et al., 2007; Chang et al., 2011, 2013) to uncover the underlying mechanism of the effect of DCS on synaptic plasticity and seizure-like activity threshold. Thus, the present study investigated the long-term effects of polarizing field on ACC seizure-like activities. We tested the hypotheses that applied cathodal DCS is highly effective in controlling and modulating seizure-like activity and the long-term effects of cathodal DCS on the suppression of seizure-like activity occurs through an LTD-like mechanism.

Materials and methods

Slice preparation

Four to 8-week-old male C57BL/6J mice were used. The research protocol conformed to National Institutes of Health guidelines in accordance with the Institutional Animal Care and Utilization Committee, Academia Sinica (Taipei, Taiwan). After decapitation, the brain was quickly transferred to cooled oxygenated artificial cerebral spinal fluid (aCSF; 124 mM NaCl, 4.4 mM KCl, 1 mM NaH_2PO_3, 2 mM $MgSO_4$, 2 mM $CaCl_2$, 25 mM $NaHCO_3$, and 10 mM glucose, bubbled with 95% O_2 and 5% CO_2) for 3 min. Slices that contained the pathway from the medial thalamus (MT) to ACC were prepared according to a previously developed method (Lee et al., 2007). Slices (500 μm thickness) were made and then incubated in oxygenated aCSF at room temperature for 1 h. A single slice was then transferred to the recording chamber and kept at 32 °C under continuous perfusion (12 ml/min) with oxygenated aCSF.

Multielectrode array recording

Two types of multielectrode array (MEA) probes were used: 6 × 10 planar MEA (electrode diameter, 30 μm; electrode spacing, 500 μm; impedance, 50 kΩ at 200 Hz; Multi Channel Systems, Reutlingen, Germany) and 8 × 8 MEA (pyramidal-shaped electrode; diameter, 40 μm; tip height, 50 μm; electrode spacing, 200 μm; impedance, 1000 kΩ at 200 Hz; Ayanda Biosystems, Lausanne, Switzerland). A 60-channel amplifier was used with a band-pass filter set between 0.1 Hz and 3 kHz at 1200× amplification (MEA-1060-BC, Multi Channel Systems, Reutlingen, Germany). Data were acquired using MC Rack software at a 10 kHz sampling rate (Multi Channel Systems, Reutlingen, Germany).

Patch-clamp recording

Borosilicate glass pipettes (PC-10, 3–7 MΩ; Narishige, Japan) were used in whole-cell patch-clamp recordings. The pipette solution contained the following: 131 mM K-gluconate, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 2 mM ethylene glycol tetraacetic acid (EGTA), 20 mM KCl, 8 mM NaCl, and 2 mM Mg-adenosine triphosphate (ATP) and Na_3-guanosine-5′-triphosphate (GTP; Tocris Bioscience, Ellisville, MO, USA). The pipette solution also contained 6.7 mM biocytin. Recordings were made using an Axon Multiclamp 700B microelectrode amplifier (Molecular Devices, Sunnyvale, CA, USA) with 2× amplification. The amplified signals were digitized by a Power 1401 converter (CED, Cambridge, UK) using spike2 and Signal software.

Seizure-like activity induction and generation of electric fields

Seizure-like activity was induced by the application of 4-aminopyridine (4-AP; 250 μM) and bicuculline (5 μM). Our previous time-control studies showed that maximal and stable responses appeared 2–3 h after drug application (Chang and Shyu, 2013). All of the comparisons were made 2–3 h after 4-AP and bicuculline application. Uniform electric fields were generated by passing currents between two parallel Ag–Cl-coated silver wires placed inside the MEA chamber. Currents were generated by an isolated stimulator (A-M Systems, Carlsborg, WA, USA) under the control of a pulse generator (STG 1002, Multi Channel Systems). The definition of the electric field orientation was based on the direction of the axodendritic axis in the ACC. The orientations of dendrite and soma compartments were demonstrated using Golgi staining (Fig. 1A). The Ag–Cl electrode placed proximal to the ACC was defined as the anode, and the other electrode placed distal to the ACC was defined as the cathode. The field strength generated by the two field orientations (parallel and perpendicular to

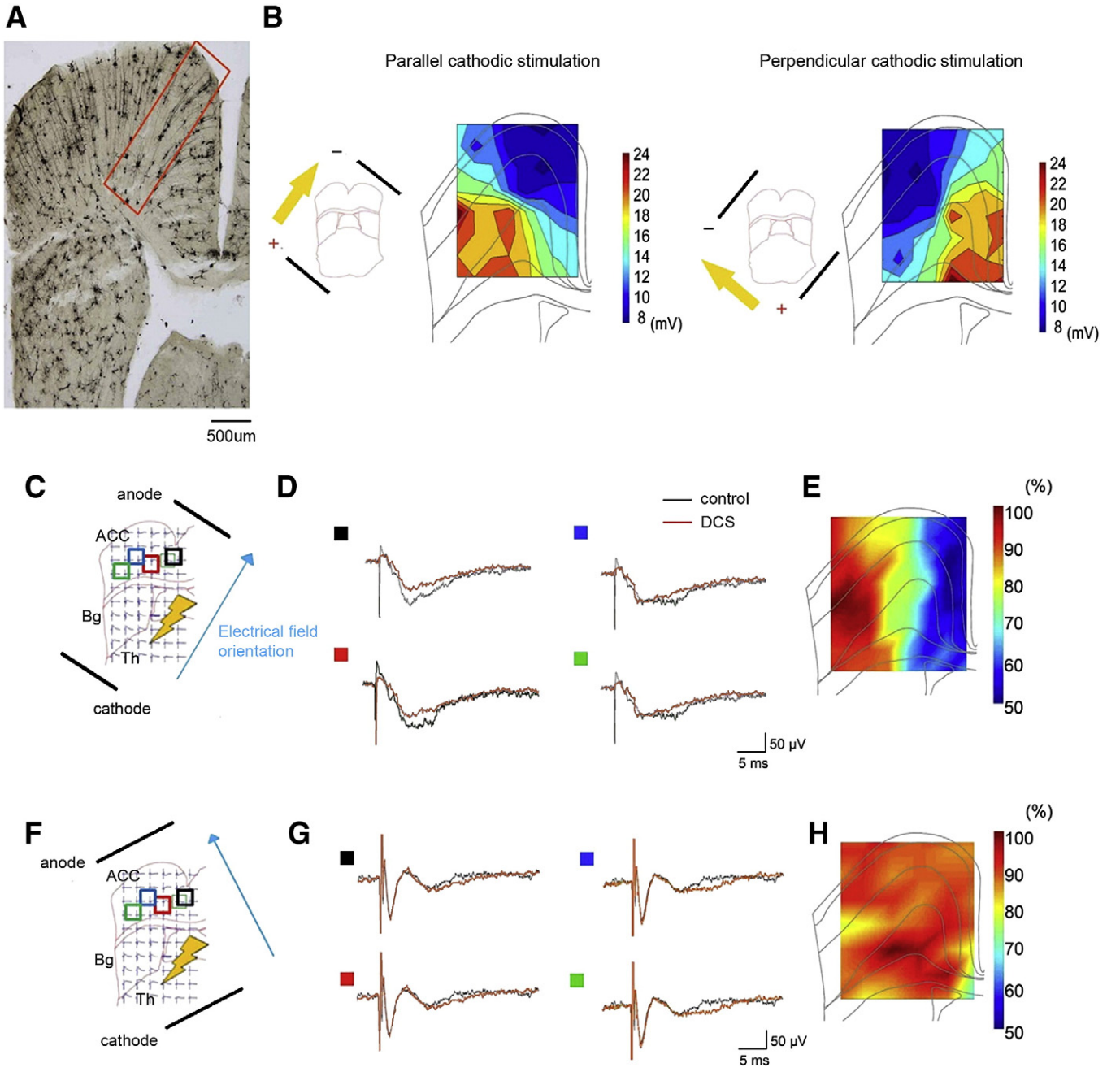

Fig. 1. Orientation of the electric field and typical effects of cathodal DCS on thalamic stimulation-evoked activity. (A) Golgi staining showed the orientation of axodendritic fibers in the ACC. (B) The orientation of the electric field was arranged according to the direction of axodendritic fibers in the ACC. Two orientations of the electric field were applied, and the field strength produced by the different orientations of the electric field was recorded by a MEA. The isopotential distribution of the electric field is plotted against the brain slice. (C) Effects of parallel cathodal DCS stimulation. The responses from four selected channels are enlarged in D. (D) Parallel cathodal DCS depressed thalamic stimulation-evoked activity in some channels. (E) Degree of depression on an isopotential color map. The effect of parallel cathodal DCS was the most prominent in the medial part of the ACC. (F) Effects of perpendicular cathodal DCS stimulation. The responses from four selected channels are enlarged in G. (G) Perpendicular DCS stimulation did not show any effect on thalamocingulate synaptic transmission. (H) Isopotential plot that shows that the evoked response recorded from all of the channels remained the same during perpendicular DCS.

the ACC axodendritic fibers) was recorded by the MEA. The isopotential color plot of field strength is shown in Fig. 1B. The tDCS strength in clinical studies is usually between 0.5 and 2 mA (Brunoni et al., 2012). The actual amount of current that reaches the targeted brain region will be decreased by the skull and vasculature. In order to mimic this situation, the maximum current used in this study is 400 μA. It is known that electrochemical damage to the targeted tissue could happen in in vivo preparation (Brummer and Turner, 1977). However, in the in vitro preparation, the tissue does not have to directly contact the electrode and therefore the electrochemical effects can be minimized (Durand and Bikson, 2001). We have systematically measured the pH values in aCSF in our recording chamber to investigate the possible alteration pH values caused by the electrochemical reaction during the DCS. We found that the pH values along the current axis between the electrodes were not significantly changed and the pH values were stable and maintained in constant level for 90 min. The intensity below 10 mV/mm which is in the range for inducing the long term effect, did not produce any significant effect on the pH value. However, the intensity of DCS higher than 10 mV/ mm did produce slight but significant change of pH value in aCSF (Supplementary information 1).

Thus any artifact in addition to the neuronal effect produced by the intensity used for testing the long term effect could be excluded in the present experiment. We have compared the effect of DCS with electrodes oriented in a different direction. Our results in Fig. 3 E showed that when the electrodes were oriented in 90° and 270° DCS did not have effect on evoked activities. Thus this result could be used as control experiment to exclude the possibility that there are side effects resulted from either junctional potentials or electrochemical reaction of the DCS that might damage the tissue in our experiment. Furthermore, submerged chamber for brain slice recording was used in this experiment. The main advantage of submerged chamber is the high diffusion rate of bath solution into and out of the slice (Croning and Haddad, 1998). In order to facilitate seizure-like activities, we keep relatively fast perfusion rate (8 ml/min). If there is any a reaction product or build-up of pH gradient, it is very unlikely that such effects could be accumulated in this high perfusion rate.

Drug application

The NMDA receptor antagonists D-1-2-amino-5-phosphonopentanoic acid (APV) and Na–K–Cl cotransporter (NKCC)-2 blocker furosemide were purchased from Sigma. The $GABA_A$ receptor antagonists bicuculline and 4-aminopyridine (4-AP) were purchased from Tocris Cookson (Ellisville, MO, USA). Stock solutions of 4-AP and APV in double-distilled water and bicuculline and furosemide in dimethyl sulfoxide (DMSO) were prepared, divided into small aliquots, and stored at −80 °C. The aliquots were thawed on the experimental days, and all of the drugs were applied to the bath solution according to the respective molar concentration.

Data analysis

The data were analyzed using MC Rack software (Multi Channel Systems) and subroutines in the MATLAB program (MathWorks, Natick, MA, USA). To detect oscillatory events, we set 3 standard deviations (SD) of the noise level as the threshold. The amplitudes of the peaks during an oscillation event that surpassed this threshold were automatically detected by MC Rack software. The time-point of each peak that exceeded this threshold was also detected, and the duration of an oscillation event was measured by subtracting the time point between the first and last peaks that surpassed the threshold. Two-dimensional current-source density (CSD) profiles were calculated from the field-potential profiles (Shimono et al., 2001) and color image plots generated to facilitate the visualization of CSD profiles. Blue represents current sinks, and red represents current sources.

Correlations between seizure-like activities recorded in the different channels were assessed by cross-correlation analysis. The maximal cross-correlation coefficients were calculated by selecting one channel as a reference.

The data in the text are expressed as mean ± SE. The statistical analyses were performed using Statistical Product and Service Solutions (SPSS) and Microsoft Excel software using Student's *t*-test. Measurements and one-way analysis of variance (ANOVA) in the text are expressed as mean ± SE, and *n* indicates the number of slices or neurons studied. The results were considered significant at $p \leq 0.05$.

Results

Cathodal DCS suppresses synaptic transmission in the thalamocingulate pathway

A method was developed to investigate the effects of DCS in the thalamocingulate pathway in a mouse brain slice preparation. The parallel orientation of the electric field was based on the direction of the axodendritic axis in the ACC. Previous clinical studies showed that a field strength that ranges from 0.22 to 7.7 mV is sufficiently strong to alter neuronal excitability in the cortical area (Miranda et al., 2006; Wagner et al., 2007). A field strength of 2–16 mV/mm was used in the present study. We used a 2 mV/mm DCS strength to induce long-term effects, and a higher field strength was used to induce an immediate effect on seizure-like activities.

The orientation of the electric field was arranged parallel to the orientation of dendrite and soma compartments in the ACC to achieve maximal efficiency (Fig. 1C). Field excitatory postsynaptic potentials (fEPSPs) in the ACC were elicited by stimulating the MT with a 0.1 Hz test pulse. Thalamic stimulation-evoked cortical responses from different cortical regions (numbers and colors boxes in upper panel of Fig. 1C) are magnified in Fig. 1D. The results showed that cathodal stimulation suppressed the thalamic stimulation-evoked responses in the medial part of the ACC (black and red marks in Figs. 1C and D), whereas it was less effective in suppressing thalamic stimulation-evoked responses in the lateral part of the ACC (green and blue marks in Figs. 1C and D). When the electric field was arranged perpendicular to the axodendritic fibers of the ACC (Fig. 1F), DCS did not show significant effects on thalamic stimulation-evoked activity in any of the cortical regions (Fig. 1G). The degree of depression was normalized and is shown as a color plot. Parallel cathodal DCS had a maximal effect in suppressing thalamic stimulation-evoked cortical activity in the medial part of the ACC (Fig. 1E). Perpendicular cathodal DCS slightly depressed cortical activity in the medial part of the ACC, but this depression was not significant (Fig. 1H). These results confirmed that the orientation of the electric field was important in regulating synaptic transmission in the thalamocingulate pathway.

Immediate effects of cathodal DCS on seizure-like activity

Clinical research has shown that FLE is often drug-resistant. Basic research studies have shown that the combination of 4-AP and bicuculline induced drug-resistant seizure-like activities (Bruckner et al., 1999). Therefore, epileptiform activity in the present study was induced by perfusing brain slices with 4-AP (250 μM) and bicuculline (5 μM), and the effects of cathodal DCS on seizure-like activities were tested. Typical traces of epileptiform activity in control slices and slices to which parallel and perpendicular cathodal DCS was applied are shown in Fig. 2. Only parallel cathodal stimulation (4–16 mV/mm) shortened the duration of seizure-like activity (Fig. 2A). Because perpendicular cathodal DCS is ineffective in altering synaptic transmission or seizure-like activity, the subsequent experiments focused on the effects of parallel cathodal DCS. A higher strength of cathodal DCS more effectively suppressed seizure-like activity. The suppressive effect of cathodal DCS exhibited a linear relationship with its strength. The duration of epilepsy was further decreased by a higher strength of DCS (Fig. 2B). Cathodal DCS also suppressed thalamic stimulation-evoked seizure-like activity immediately. The relative positions of the electrodes for stimulation and orientation of the electric field against the brain slice are shown in Fig. 2C. The application of cathodal DCS significantly shortened the duration of thalamic stimulation-evoked seizure-like activity (Figs. 2D, F; $n = 6$, $p < 0.05$). The application of cathodal DCS also restrained the propagation of thalamic stimulation-evoked seizure-like activity (Fig. 2E). The statistical analysis showed that the minimal threshold for generating seizure-like activity in response to stimulation in the MT was also significantly elevated by the application of cathodal DCS (Fig. 2G; $n = 6$, $p < 0.05$).

Field stimulation immediately altered cell firing patterns and enhanced sEPSC

Sixteen cells in 13 slices were recorded by patch clamp (Vm = −69 ± 1.6 mV). A step current was injected into cells (−60 pA to 190 pA) to induce action-potential firing. The firing patterns of control slices and slices to which cathodal DCS was applied were compared to determine the effects of DCS on the intrinsic properties

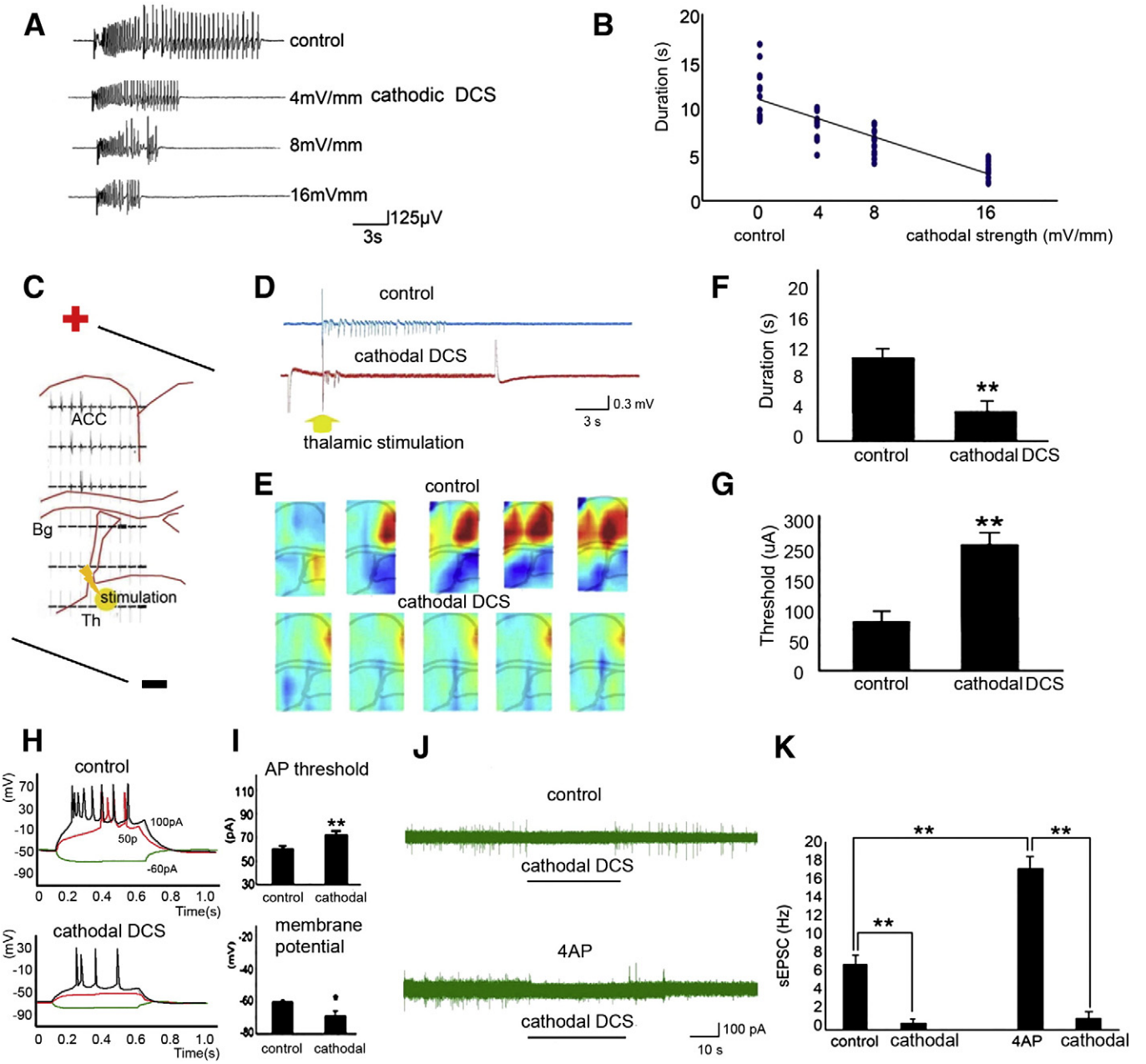

Fig. 2. Effects of different field strengths on seizure-like activity. (A) Effects of different strengths of cathodal DCS on seizure-like activity. (B) Cathodal strength had a linear relationship with seizure-like activity duration. Strong cathodal stimulation more effectively suppressed seizure-like activities. (C) Cartoon outline that indicates the relative position of the brain slice and electric field. (D) Typical example of thalamic stimulation-evoked seizure-like activity between the control and cathodal DCS groups. (E) Isopotential changes during the first 100 ms after thalamic stimulation. The application of cathodal DCS suppressed the propagation of seizure-like activity. (F) The duration of seizure-like activity was significantly decreased during cathodal DCS. (G) During cathodal DCS, the threshold of evoked seizure-like activities was significantly increased compared with the control group. (H) Typical example of a cell's response under control and cathodal DCS application conditions. (I) Cathodal DCS significantly hyperpolarized the transmembrane potential, and cathodal DCS significantly increased the step current to generate an action potential. (J) Effects of cathodal DCS on spontaneous postsynaptic currents. The application of cathodal DCS suppressed sEPSCs. The application of cathodal DCS also reduced EPSCs produced by 4-AP. (K) The application of cathodal DCS significantly decreased EPSCs and epileptic EPSCs.

of the neurons. The typical response is shown in Fig. 2H. The statistical analysis showed that the transmembrane potential was significantly hyperpolarized by cathodal DCS (Fig. 2I). The threshold for generating action-potential firing was also significantly increased by cathodal DCS (Fig. 2I; $n = 16$, $p < 0.05$). However, the action-potential amplitude (control: 60 ± 1.5 mV; cathodal DCS: 58 ± 2.2 mV) and firing frequency (control: 12 ± 1.1 Hz; cathodal DCS: 13 ± 2.2 Hz) were not significantly different between groups. To determine whether the application of DCS altered synaptic transmission, the patterns of spontaneous excitatory postsynaptic currents (sEPSCs) during control stimulation and cathodal DCS were analyzed. Cathodal DCS suppressed inward sEPSCs (upper sweep in Figs. 2J and K). The effects of tDCS were reversible, and EPSCs recovered after terminating the application of DCS. The amplitude of EPSCs was dramatically increased after the application of 250 μM 4-AP. The application of cathodal DCS significantly inhibited these large currents (lower sweep, Fig. 2J). The statistical results showed that cathodal DCS significantly decreased sEPSCs during the control period. The frequency of sEPSCs was significantly increased after the application of 4-AP, and cathodal DCS also inhibited enhanced sEPSCs caused by 4-AP (Fig. 2K; $n = 9$, $p < 0.05$).

Long-term depressive effects of cathodal DCS on thalamocingulate synapses

Direct-current stimulation produced long-term effects on synaptic transmission (Fritsch et al., 2010). We investigated whether cathodal DCS application could cause the LTD of thalamocingulate-synaptic transmission. The orientation of the electric field and brain slice is shown in Fig. 3A. Fifteen minutes of 4 mV/mm cathodal DCS was applied after 15 min of baseline recording. The electrode for stimulation was placed in the MT, and the test pulse was set to 0.1 Hz. The results showed that cathodal DCS depressed thalamocingulate synaptic transmission (11 of 13 slices, 84.62%; Fig. 3B). Synaptic depression lasted more than 1 h in the ACC after cathodal DCS treatment (Fig. 3C). Channels selected from different locations of the brain slice in Fig. 3A are shown in different colors in Fig. 3C. Thalamic stimulation itself did not cause synaptic depression (control-brown circles in Fig. 3C). Synaptic activation is important for generating LTD. The application of cathodal DCS alone without continuous synaptic activation did not cause LTD. Cathodal DCS also effectively generated LTD when the synaptic

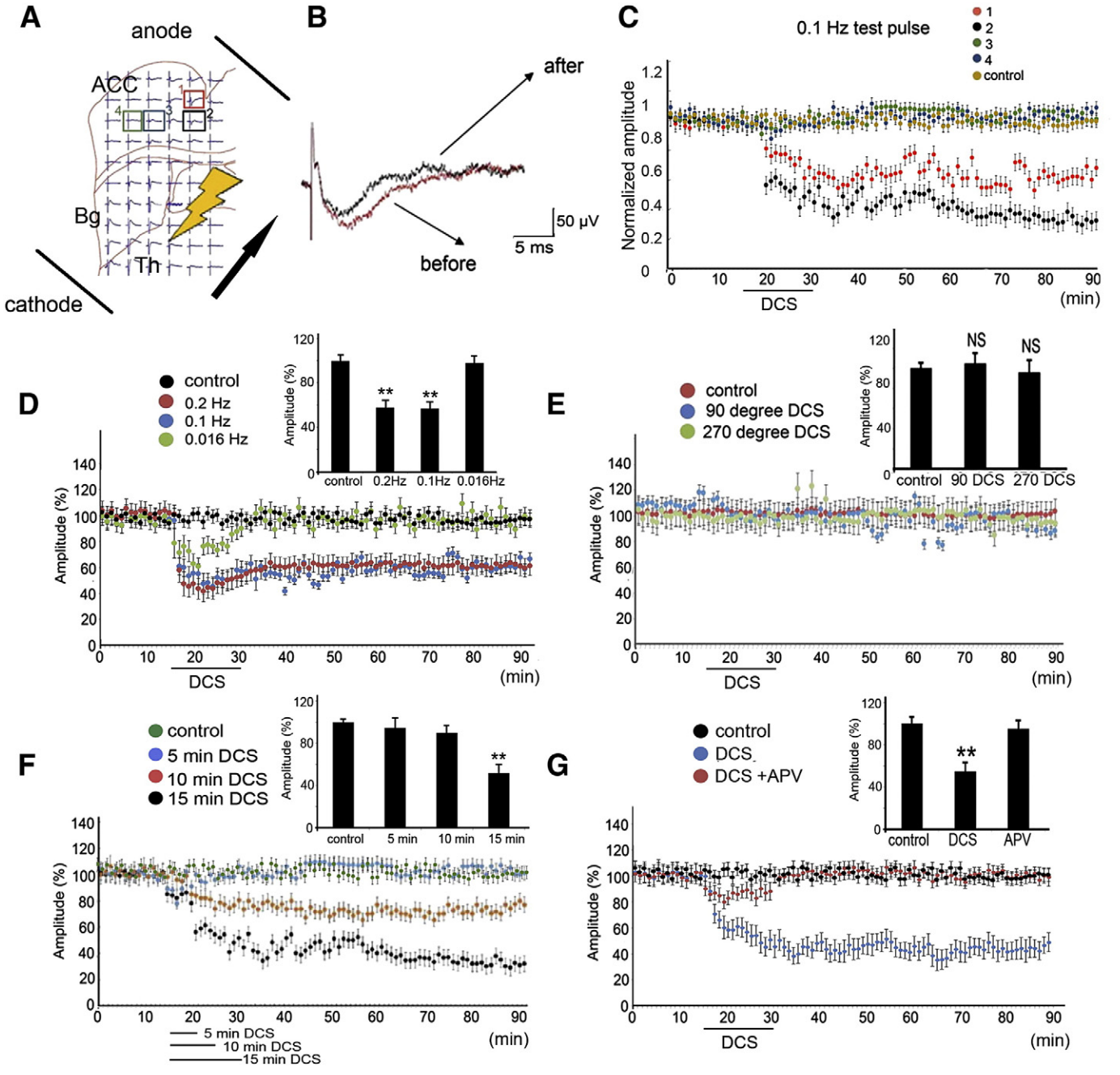

Fig. 3. Cathodal DCS caused long-term depression of the thalamocingulate pathway. (A) The cartoon outline indicates the orientation of the brain slice against the MEA and direction of the electric field. The responses of four selected channels are shown in C. (B) Typical example of evoked activity in the control and cathodal stimulation groups. (C) Amplitude of evoked activity against time in four channels in the control and cathodal stimulation groups. Cathodal DCS caused long-term depression of the thalamocingulate pathway under 0.1 Hz synaptic activation. (D) Cathodal DCS combined with 0.2 Hz synaptic activation also produced LTD, but cathodal DCS combined with 0.016 Hz synaptic activation only depressed synaptic transmission temporarily. The results showed that 0.1 and 0.2 Hz test pulses significantly lowered synaptic transmission in the thalamocingulate pathway. (E) The orientation of the electric field was important for DCS-induced LTD. Electric fields oriented at 90 and 270° were unable to generate DCS-induced LTD. No significant difference in thalamocingulate synaptic transmission was found after 90 and 270 degree electric field treatment. (F) Comparison of 5, 10, and 15 min cathodal DCS-induced LTD. Five minutes of DCS only transiently depressed evoked activity. Ten minutes of DCS also caused LTD but with lower efficiency. (G) The long-term depression caused by cathodal DCS was blocked by APV application.

activation frequency was set to 0.2 Hz or 0.1 Hz thalamic stimulation (Fig. 3D). However, DCS only temporarily depressed the synaptic response of 0.016 Hz thalamic stimulation without showing a long-term effect (Fig. 3D). The results showed that both 0.2 and 0.1 Hz stimulation significantly decreased thalamocingulate synaptic plasticity (Fig. 3D, bar graph). We also found that the orientation of DCS was important for the induction of LTD. The application of DCS at both a 90 and 270 degree orientation, which was perpendicular to the thalamocingulate pathway, did not have a significant effect on thalamic stimulation-evoked synaptic transmission (Fig. 3E). Fifteen minutes of DCS was the most effective in inducing LTD. A shorter duration (10 min) of cathodal DCS also caused long-term depression in the thalamocingulate pathway but with a lower success rate (1 of 6 slices, 16.7%) and less of an effect (Fig. 3F). Five minutes of cathodal DCS only transiently depressed thalamocingulate synaptic transmission. No LTD response was observed with 5 min of DCS application ($n = 5$; Fig. 3F). We applied an NMDA receptor antagonist to test whether NMDA receptors are involved in the formation of LTD-like responses after cathodal DCS application.

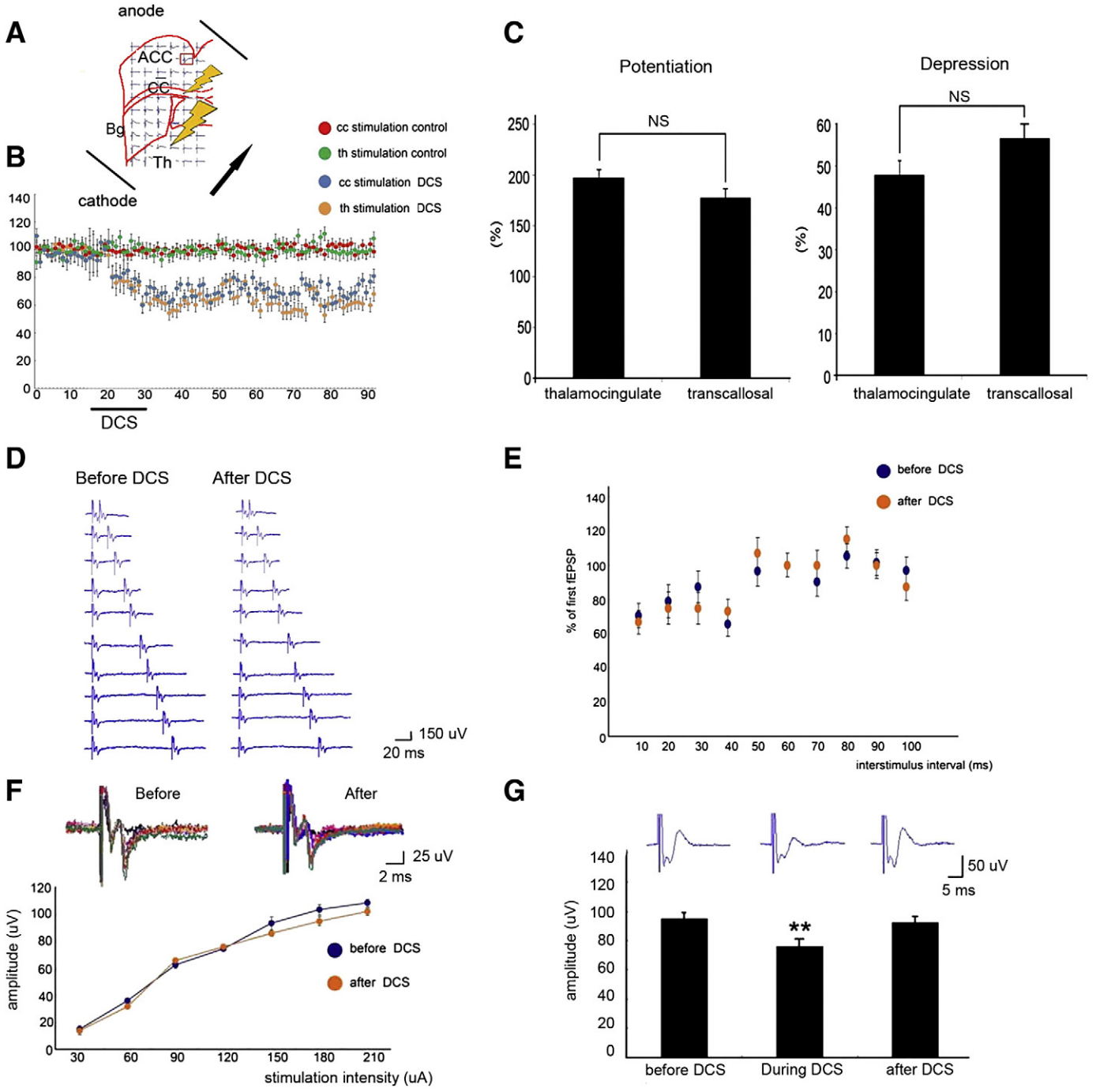

Fig. 4. Comparison of effects of cathodal DCS on thalamocingulate and transcallosal synaptic plasticities. (A) Cartoon outline of the orientation of the brain slice against the MEA and electrical stimulation site. (B) Effects of cathodal DCS on thalamocingulate and transcallosal synaptic transmissions. Cathodal DCS suppressed thalamocingulate and transcallosal synaptic transmissions. Thalamic activation alone (Th control) and transcallosal synaptic activation (cc control) did not modulate synaptic transmission. (C) No significant difference was found between the two groups. (D) Typical paired-pulse depression before and after cathodal DCS. (E) Inter-stimulation interval of 10–40 ms induced PPD both before and after cathodal DCS. The PPD effects were not significantly different between the before and after cathodal DCS groups. (F) Superimposed sweeps of the different stimulation intensities before and after the DCS (upper panel) and input–output relationship before and after cathodal DCS. (G) Typical evoked responses before, during, and after DCS. No test pulse was applied. During cathodal DCS, the evoked response was significantly decreased.

We found that the application of APV (25 μM) completely blocked the LTD-like response caused by DCS (Fig. 3G).

Cathodal DCS-induced LTD is not pathway-specific

To test whether the effect of cathodal DCS occurs locally or is pathway-specific, we also tested the effect of cathodal DCS on transcallosal synaptic activity. The relative position of the stimulating electrode and orientation of the electric field are shown in Fig. 4A. The effects of cathodal DCS on thalamocingulate and transcallosal synaptic transmissions are plotted against time. Both thalamic (th stimulation, green dots) and transcallosal (cc stimulation, red dots) stimulation did not significantly alter synaptic transmission during the test. Cathodal DCS caused LTD in both thalamic (orange dots) and transcallosal (blue dots) synaptic activation (Fig. 4B). The results showed that the degree of suppression by cathodal DCS was not significantly different between the two groups (Fig. 4C; $n = 5$, $p < 0.05$). These results indicate that cathodal DCS influenced synaptic transmission locally, regardless of the origin of the inputs.

Cathodal DCS did not influence presynaptic release mechanism

To test whether the effect of cathodal DCS influences synaptic transmission through a pre- or postsynaptic mechanism, we compared the paired-pulse response before and after cathodal DCS application. The interstimulus interval ranged from 10 to 100 ms. The typical paired-pulse responses elicited by thalamic stimulation before and after cathodal DCS are shown in Fig. 4D. We found that a 10–40 ms interstimulus interval produces paired-pulse depression (PPD) in both groups. The other stimulation interval did not have significant effects. The results showed that the PPD response was not significantly altered after DCS (Fig. 4E). The input–output relationship before and after pure cathodal DCS stimulation indicated that basal thalamocingulate synaptic transmission was unaltered (Fig. 4F). Thalamocingulate synaptic transmission was suppressed during the application of cathodal DCS without continuous synaptic activation. Pure cathodal DCS did not have after-effects on thalamocingulate synaptic transmission (Fig. 4G). These results indicate that DCS did not influence synaptic transmission by altering a presynaptic releasing mechanism.

Long-term depressive effects of cathodal DCS on seizure-like activities

The LTD effect induced by cathodal DCS was duration-dependent. To test whether the duration of cathodal DCS has a similar long-term effect on seizure-like activity, 0.1 Hz thalamic stimulation-evoked cingulate seizure-like activities were compared before, during, and after the application of 5, 10, and 15 min cathodal DCS. Thalamic stimulation evoked robust seizure-like activity in the cingulate cortex. 30 min after cathodal DCS application, the duration of thalamus evoked seizure-like activity was shortened (Fig. 5A). The duration of seizure-like activity induced by each thalamic stimulation is plotted against time (Fig. 5B). We found that 15 min of DCS application caused long-term suppressive effects on duration of seizure-like activities, but 5 and 10 min cathodal DCS application only transiently influenced seizure-like activities (Fig. 5B). The results showed that the seizure duration was significantly decreased after 15 min of cathodal DCS compared with 5 and 10 min of DCS application (Fig. 5C).

The long-term suppressive effect of cathodal DCS on seizure-like activities could be blocked by the application of APV. Cathodal DCS could still suppress seizure-like activity during its application (Fig. 5D). The results showed that the duration of seizure-like activity after cathodal DCS application significantly decreased compared with the control group. Under APV application, the duration of seizure-like activities after cathodal stimulation was not significantly different from the control group (Fig. 5E; $n = 6$, $p < 0.05$).

Suppression of seizure-like activities by cathodal DCS occurs through a PP1-dependent mechanism

Previous studies showed that NMDA receptor-dependent LTD occurs mainly through the activation of protein phosphatase 1 (PP1). PP1 dephosphorylates the GluA1 subunit of the AMPA receptor and causes LTD (Collingridge et al., 2010). To test whether DCS-LTD also occurs through the PP1 pathway, 200 nM of the PP1 inhibitor okadaic acid was applied in the patch pipette solution. Thalamic evoked response of single cell was recorded by patch clamp electrode. The results showed that the application of okadaic acid blocked DCS-induced LTD (Figs. 6A, B). The results also showed that the application of okadaic acid significantly blocked the depressive effect of cathodal DCS on the thalamic stimulation-evoked response but not the immediate effect during the application of DCS (Fig. 6C; $n = 5$, $p < 0.05$). We also tested whether pretreatment with okadaic acid can block the long-term suppressive effects of DCS of seizure-like activities. The results showed that pretreatment with okadaic acid blocked the long-term suppressive effects of cathodal DCS on seizure-like activities (Figs. 6D, E). The results showed that under the application of okadaic acid, the duration of seizure-like activities significantly decreased during the application of cathodal DCS but was restored to control levels after cathodal DCS (Fig. 6F; $n = 6$, $p < 0.05$).

Suppressive effect of cathodal DCS on seizure-like activities is not caused by depotentiation

Previous studies showed that the application of 4-AP enhances both glutamatergic and GABAergic transmission (Perreault and Avoli, 1991), a mechanism that is similar to potentiation. Previous studies also showed that the calcineurin pathway is important in mediating the depotentiation response (Lin et al., 2003). To test whether the suppressive effects of cathodal DCS on seizure-like activities occur through a depotentiation mechanism, we pretreated brain slices with the calcineurin inhibitor FK-506. Typical responses of seizure-like activity after FK-506 pretreatment and in the control group are shown in Fig. 7A. The results showed that pretreatment with FK-506 did not block the suppressive effect of cathodal DCS (Fig. 7B). The results showed that pretreatment with FK-506 did not have a significant effect on seizure-like activity duration in response to cathodal DCS application (Fig. 7C; $p < 0.05$). These results showed that the suppressive effect of cathodal DCS on seizure-like activities occurred through an LTD-like mechanism and not through depotentiation.

Discussion

We provided a method for investigating the effects of DCS on thalamocingulate and transcallosal synaptic plasticities and acute seizures. We found that cathodal DCS had both short- and long-term suppressive effects on seizure-like activities. We showed that the immediate effects of cathodal DCS on ACC seizure-like activities occurred through neuronal hyperpolarization. The long-term effect of DCS on seizure-like activities occurred through an LTD-like mechanism.

The position of the electric field relative to cortical structures is important (Bikson et al., 2004; Kabakov et al., 2012). Our results showed that DCS had immediate and long-term effects only when the orientation of the DCS field was parallel to axodendritic fibers. The duration of DCS application was also important. Our results showed that 5 min of cathodal DCS application did not induce LTD, which is consistent with a previous study that showed that 6 min of DCS did not cause LTP (Liebetanz et al., 2002). A previous study showed that 13 min of anodic DCS application was sufficient to cause a long-term effect on neuronal excitability (Nitsche and Paulus, 2001). Our results showed that 15 min of cathodal DCS was the optimal duration to induce LTD. Only 16.7% of the brain slices showed LTD after 10 min of cathodal

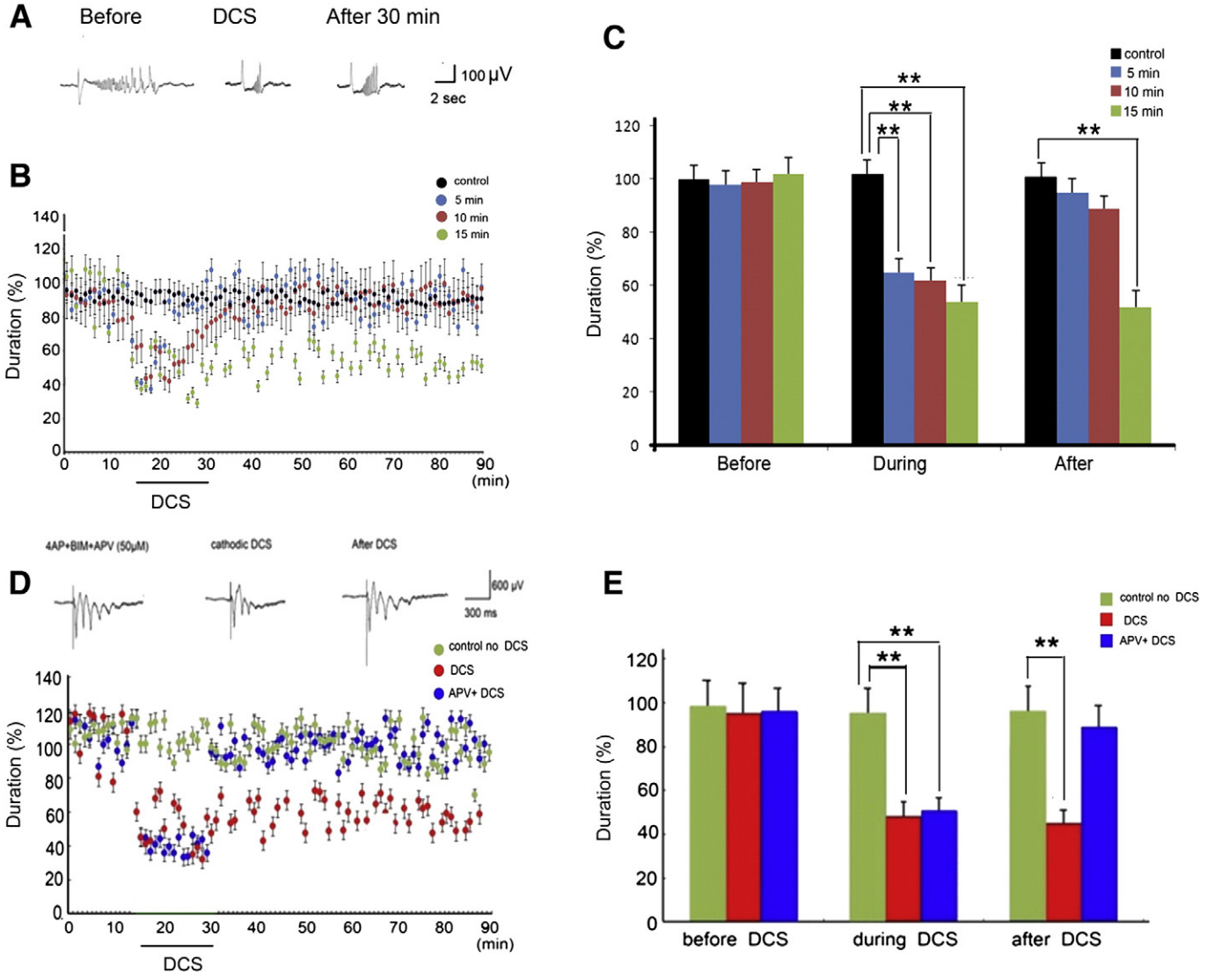

Fig. 5. Effects of cathodal DCS on thalamic stimulation-evoked seizure-like activity. (A) Typical example of thalamic stimulation-evoked seizure-like activities before, during, and 30 min after 15 min of cathodal DCS. (B) Time plot of seizure-like activity occurrence and duration. During cathodal stimulation, the occurrence of seizure-like activities decreased. The effects of 15 min of cathodal DCS on seizure-like activities suppression endured even when the application of DCS was terminated. However, 5 and 10 min durations did not have long-term effects on seizure-like activity duration. (C) The duration of seizure-like activity was significantly shortened after 15 min of DCS application. Five and 10 min durations of cathodal DCS only transiently suppressed seizure-like activities. (D) The long-term protective effect of cathodal DCS on seizure-like activities was blocked by APV. The application of cathodal DCS only temporarily suppressed seizure-like activities under the influence of APV. (E) The application of cathodal DCS, with and without APV treatment, significantly decreased the seizure-like activity duration compared with the control group. However, under the application of APV, the after-effects of cathodal DCS on seizure-like activity duration were blocked.

DCS. Therefore, 15 min of DCS more reliably induced long-term changes in synaptic transmission.

The underlying mechanism of the immediate anticonvulsant effects of cathodal DCS appears to involve the hyperpolarization of neuronal soma and desynchronization of neuronal activity. Previous studies showed that cathodal DCS application caused the hyperpolarization of neuronal soma (Lian et al., 2003; Nakagawa and Durand, 1991; Purpura and McMurtry, 1965). Our patch clamp technique also showed that during the application of cathodal DCS, the membrane potential (5.8 mV) was more hyperpolarized compared with the control group. Direct-current stimulation produces biphasic effects along the neural axis (Bikson et al., 2004). For example, anodal DCS depolarizes the soma and hyperpolarizes apical dendrites, and cathodal DCS has opposite effects. Because sodium channels are predominantly localized to the soma and initial segment (Vacher et al., 2008), the net effects of DCS depend on the polarization of the soma. Another possible mechanism is that the short duration DCS application could cause hypersynchronized neurons in epilepsy to fire out of phase (Durand and Warman, 1994). However, this possibility was unlikely in our experiment because we applied DCS for several seconds.

Previous studies indicated that tDCS directly affected the underlying cortex (Islam et al., 1995). Our results also showed that DCS alone did not alter PPD responses, indicating that presynaptic release was unaltered. The effects of DCS did not appear to be pathway-specific because synaptic activation from both the thalamus and contralateral ACC was able to induce LTD after cathodal DCS application.

Previous studies showed that the long-term effects of DCS occur through the modulation of synaptic transmission (Fritsch et al., 2010). In the present study, we showed that cathodal DCS was able to cause LTD in the thalamocingulate pathway. Deep brain stimulation, TMS, and tDCS are considered promising methods for the treatment of intractable seizures (Feindel, 1998; Polkey, 2004; Theodore and Fisher, 2004). Low-frequency TMS has been shown to effectively increase the latency of seizure onset in an in vivo model of pentylenetetrazol-induced seizures (Akamatsu et al., 2001), and low-frequency DBS has seizure-quenching effects (Velisek et al., 2002; Weiss et al., 1995). The LTD or

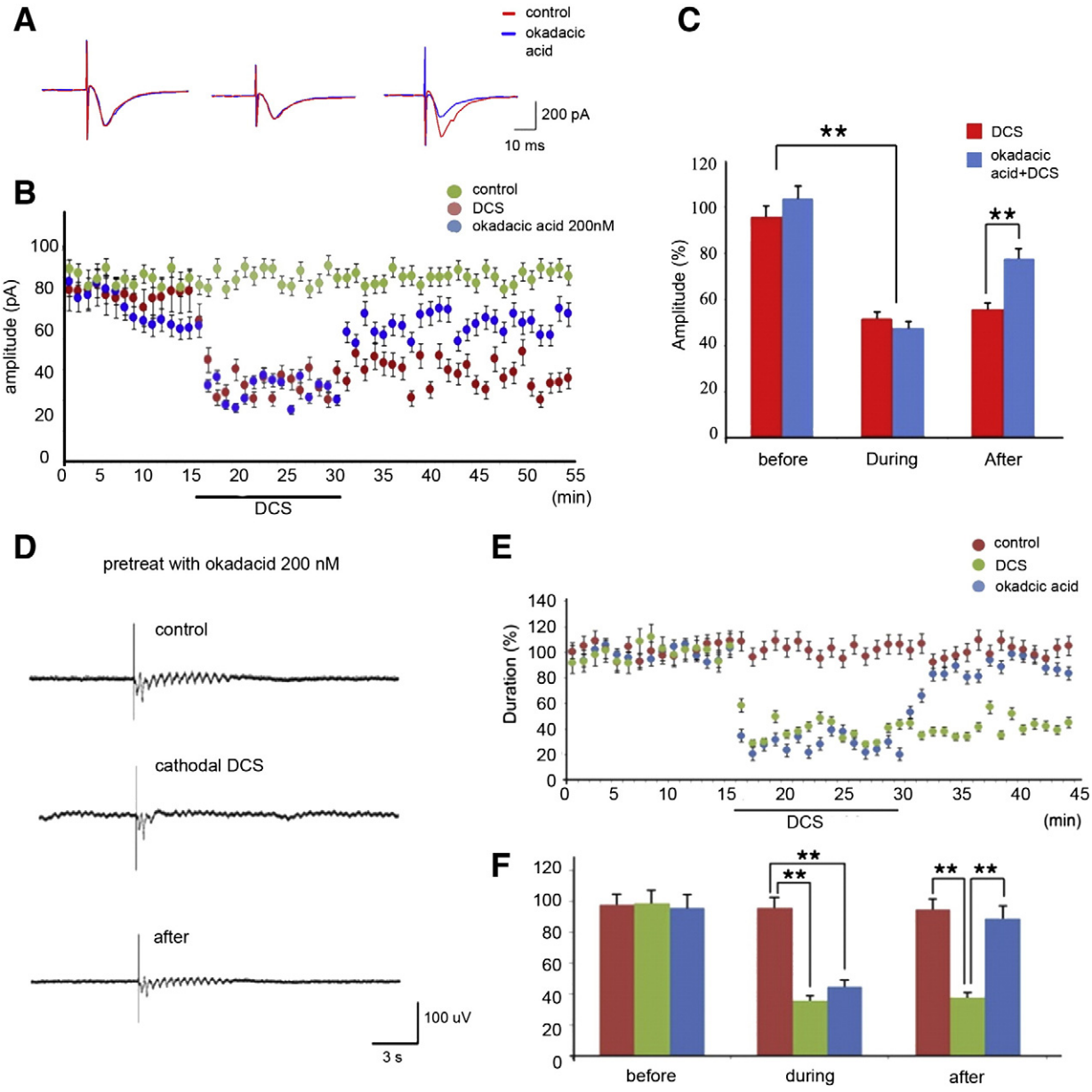

Fig. 6. Long-term suppressive effect of cathodal DCS on seizure-like activities occurs through an LTD-like mechanism. (A) Typical example of thalamic stimulation-evoked EPSCs with and without okadaic acid treatment. (B) The application of okadaic acid blocked the long-term effect of cathodal DCS on thalamocingulate synaptic transmission. (C) With okadaic acid treatment, synaptic transmission was significantly restored after cathodal DCS application. (D) Typical seizure-like activity response with application of okadaic acid. (D) The time plot of seizure-like activity duration showed that the long-term suppressive effects of cathodal DCS were blocked by pretreatment with okadaic acid. (E) With okadaic acid pretreatment, cathodal DCS only had a transient effect.

depotentiation of seizure-related potentiation is proposed to be part of the underlying mechanism of seizure suppression. The most common form of LTD requires the activation of postsynaptic NMDA receptors (Kemp and Bashir, 2001). In the present study, we showed that DCS alone without continuous synaptic activation was unable to generate LTD. We also found that the application of the NMDA-receptor antagonist APV blocked DCS-induced LTD. These results indicate the importance of activating postsynaptic NMDA receptors in DCS-induced LTD.

The induction of LTD involves the protein phosphatase cascade (Norman et al., 2000). Calcium entry via NMDA receptors triggers the calcium/calmodulin-sensitive calcineurin enzyme PP2B. This dephosphorylates inhibitor-1, which leads to the activation of PP1 (Mulkey et al., 1994). Increasing PP1 activity leads to dephosphorylation of the GluR1 subunit of the AMPA receptor and decreases AMPA receptor activity (Lee et al., 2000). To test whether DCS-induced LTD shares a similar mechanism with low-frequency stimulation-induced LTD, we preincubated brain slices with the PP1 inhibitor okadaic acid. Our results showed that the application of okadaic acid blocked DCS-induced LTD. The application of okadaic acid also reversed the suppressive effect of DCS on seizure-like activities. These results suggest that DCS-induced LTD and low-frequency stimulation-induced LTD might share a common mechanism. We also showed that the application of the NMDA receptor antagonist APV and protein phosphatase inhibitor okadaic acid blocked LTD and the seizure-like activity suppression effect of DCS. These results indicate that the long-term suppressive effect of DCS on seizure-like activities occurs through an LTD-like mechanism.

We observed that the amplitude of evoked responses dropped before DCS when okadaic acid is included in the patch pipette solution.

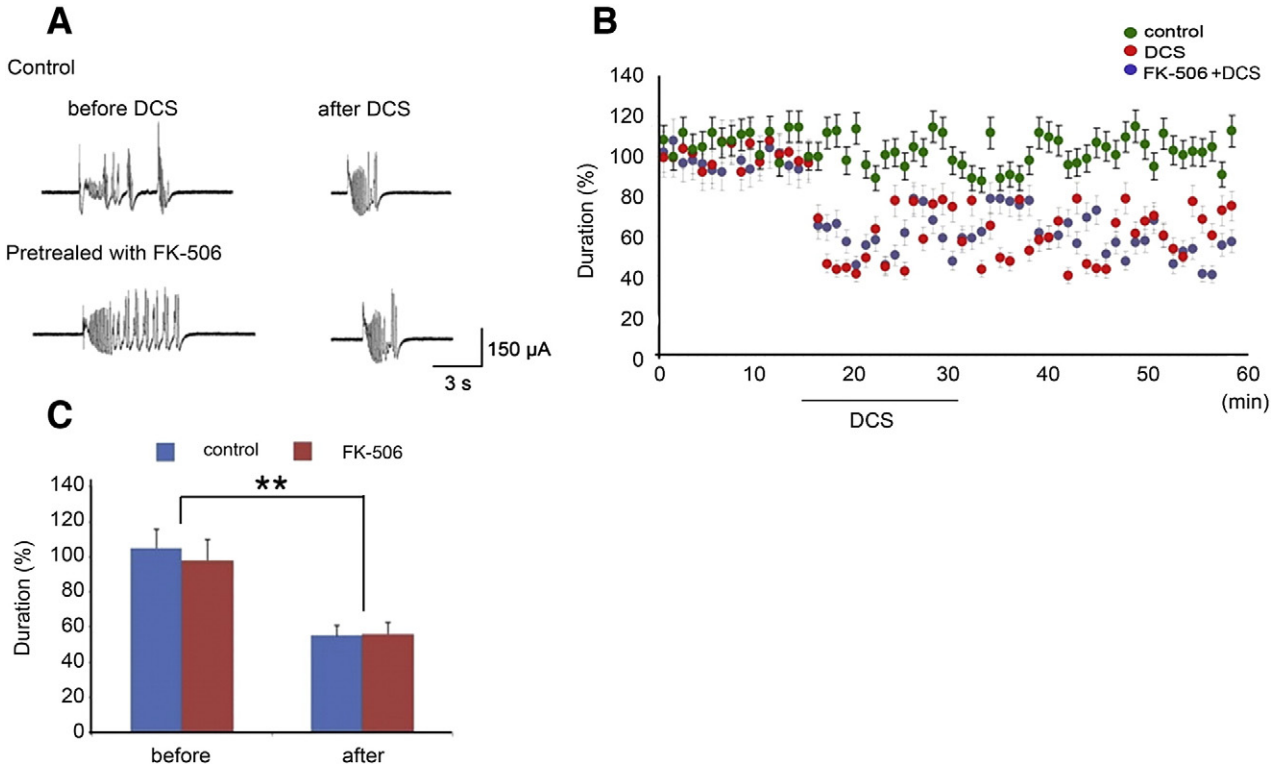

Fig. 7. The long-term suppressive effect of cathodal DCS does not occur through depotentiation. (A) With application of the calcineurin inhibitor FK-506, the seizure-like activity duration remained the same after cathodal DCS. (B) The time plot of seizure-like activity duration showed that FK-506 did not have a significant effect on seizure duration. (C) FK-506 did not alter seizure-like activity duration after cathodal DCS.

This could be because phosphatase inhibitors produce use-dependent depression of AMPA responses (Eto et al., 2002). When okadaic acid was applied internally, it produces a slowly developing attenuation in response to 0.05 Hz test pulse. When the test pulse is doubled to 0.1 Hz, the attenuation is faster and occludes subsequent LTD. Attenuation of evoked responses by okadaic acid was also shown in other studies (Kourrich, et al., 2008). Previous studies also showed that application of non-selective phosphatase inhibitors calyculin A or microcystin-LR attenuated Purkinje EPSC (Ajima and Ito, 1995). A possible mechanism underlying the attenuation caused by PP1-inhibitors is an increase in the phosphorylation state of a key substrate, such as synaptic GluR2 ser 880. Increased ser 880 phosphorylation induces internalization of the GluR2 subunit (Chung et al., 2000), and this would decrease the amplitudes of evoked responses.

High-frequency stimulation can induce LTP, and high-frequency kindling can induce seizure-like activities (Hoffman and Cavus, 2002). Previous studies showed that brief seizures could induce LTP and mossy fiber sprouting in the hippocampus (Ben-Ari and Represa, 1990). The mechanism of LTP formation might be similar to the mechanism of epileptogenesis. Previous studies showed that low-frequency stimulation reversed already-potentiated synaptic transmission, a phenomenon called depotentiation (Chen et al., 1996). Depotentiation is also a possible mechanism of the modulatory effects of tDCS or TMS in neurological disorders caused by hyperexcitability (Hoffman and Cavus, 2002). Calcineurin is known to be involved in the depotentiation process. The application of the calcineurin inhibitor FK-506 was shown to impair depotentiation (Jouvenceau et al., 2003). In the present study, we applied FK-506 and found that the potentiated response was not reversed. This result indicates that the suppressive effect of DCS on synaptic transmission or seizure-like activities does not occur through a depotentiation-like mechanism.

Enhancing the outcome of tDCS treatment may be possible if we better understand the underlying mechanism of tDCS. The common feature of tDCS is the long-lasting changes in regional cortical excitability. Treatment with DCS will likely be improved by combining it with pharmacological manipulations to enhance LTP or LTD. Our results showed that the long-term effect of DCS on seizure-like activities likely occurred through an LTD-dependent mechanism. Previous studies showed that methyllycaconitine (MLA) is able to enhance LTD (Yook et al., 2011). Previous studies also showed that MLA has a suppressive effect on seizures (Carroll et al., 2007). The combination of DCS and MLA may produce synergistic suppressive effects on seizures.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.expneurol.2015.02.002.

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