Task-specific elevation of motor learning by conjunctive transcranial direct current stimulation

Short title: Conjunctive tDCS enhanced motor learning

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Transcranial direct current stimulation (tDCS) is used for modulating brain functions, but the optimal protocol remains unclear. We found that mouse learning to run on a rotarod was enhanced by anodal tDCS during ("online") but not before or after ("offline") the task performance. The enhancement was task-specific, since online tDCS during rotarod learning did not affect learning of beam walking, and vice versa. For mice underwent middle cerebral artery occlusion (MCAO), online anodal tDCS restored motor learning capability in a task-specific manner. Transcranial calcium imaging showed that anodal and cathodal tDCS elevated and suppressed cortical neuronal activity, respectively, suggesting that elevated spiking in task-activated neural circuits underlie the learning enhancement. Thus, the efficacy of tDCS could be elevated by conjunctive activation of targeted neural circuits.

One sentence summary: Transcranial direct current stimulation applied in temporal conjunction with the motor task enhances motor learning in a task-specific manner.

Transcranial direct current stimulation (tDCS) is now widely used for non-invasive modulation of cognitive functions in healthy subjects and patients with brain disorders, ranging from neurological and psychiatric

diseases to stroke-induced dysfunction (*1-4*). The stimulation parameters and electrode configurations varied greatly, and the optimal protocol of tDCS remains unclear. The present study is based on the idea that modulation of neuronal spiking due to tDCS-induced membrane potential changes (*5-8*) could be more effective in modulating neural circuits that are activated at the time of tDCS (*2, 8*). Using a mouse rotarod learning paradigm, we examined this idea in normal wild-type mice as well as stroke model mice that were produced by MCAO. In both cases, we found that motor learning was markedly enhanced by applying anodal but not cathodal tDCS at the primary motor cortex (M1), and the learning enhancement could not be transferred to a different motor learning task. These results, together with *in vivo* calcium imaging of cortical neuronal activity in response to tDCS, support the notion that the efficacy of tDCS could be increased if its application is temporally conjunctive with the execution of targeted brain functions.

Mice were subjected to a rotarod running task that began each day with a 5-min familiarization period for rotarod running at a constant low rotation speed, followed by three 5-min trials with gradually increasing speed (4 to 40 rpm, day 1 & 2; 8-80 rpm, day 3 & 4) (*9*) that were interrupted by 5-min inter-trial intervals (ITIs) off the rotarod (Fig. 1A). Mice were subjected to tDCS at designated time with anodal ("+") or cathodal ("-")

currents, or without current (sham control "S") (Fig. 1B). The mice normally learned to run on the rotarod over the four training days, as shown by increasing duration of staying on the rotarod (Fig. 1C) and increasing terminal rotor speed when the mouse dropped off the rotarod (Fig. 1D). When tDCS was applied to the right primary motor cortex (M1) during the familiarization period and all three task trials each day ("online" stimulation), we found a significant increase in both the time on the rotarod and the terminal speed, beginning on the second day of training (Fig. 1, C and D, and movie S1 and S2). This enhancement of motor learning remained detectable at 14 but not 28 days after training (fig. S1, A and B). The results were further quantified by the rate of learning, as defined by the normalized difference of the terminal speed between the first and last training trials (Fig. 1E). Doubling the anodal tDCS current magnitude to 0.2 mA caused occasional convulsion in mice, whereas reducing current magnitude to 0.05 mA resulted in no learning enhancement (Fig. 1E, and fig. S2, A and B). We thus chose 0.1 mA for the standard anodal tDCS in this study. Furthermore, we found no enhancement of rotarod learning when the same online anodal tDCS was applied to the primary visual cortex (V1, Fig. 1E, and fig. S3, A and B), indicating site specificity for the tDCS effect. The rotarod learning was not affected by the procedure of surgery and electrode installation, as shown by comparing the learning of mice that were not subjected to the procedure (fig. S4, A and B).

In contrast to the learning enhancement described above, we found that anodal tDCS (at 0.1 mA) applied during all 5-min ITIs before or after rotarod running ("offline" stimulation) had no effect on rotarod learning (Fig. 1, F to H, and fig. S5, A and B). Furthermore, no effect was found when anodal tDCS was applied continuously for 20 min before the task onset (Fig. 1H, and fig. S5, C and D), a protocol often used in clinical research (*10*). In contrast to anodal tDCS, online *cathodal* tDCS (0.1 mA) at M1 also had no effect on rotarod learning (Fig. 1E, and fig. S6, A and B). However, when the cathodal current was increased to 0.2 mA, learning was impaired at the 3rd and 4th day of training (Fig. 1E, and fig. S6, C and D). Unlike that found for anodal tDCS, both online and offline cathodal stimulation at 0.2 mA resulted in similar impairment of learning (Fig. 1, E and H, and fig. S6, C and D). As shown later, this may be attributed to the long-lasting (>5 min) suppression of neuronal firing by cathodal tDCS. Taken together, these findings show that tDCS could bi-directionally modulate rotarod learning, and that the enhancement effect is significant only when anodal tDCS is applied in conjunction with the performance of the rotarod task.

The effect of online anodal tDCS on motor learning may be attributed to specific enhancement of rotarod running skill or improvement of general

motor coordination. To address this issue, we introduced beam-walking learning task, in which the mouse was given a short familiarization period for walking along a wide beam (25 mm), followed by 3 trials of walking on a narrow beam (7 mm, day 1 & 2; 3 mm, day 3 & 4) (Fig. 2A). The learning was shown by the gradual reduction of the mean transverse time and the mean number of hindlimb slips during the beam walking, and the learning rate was quantified by the normalized difference of the mean number of slips between the last and the first beam-walking trial on the 3-mm beam. In the first set of experiments, we compared beam walking learning before and after 4 days of rotarod learning, and found no effect of rotarod training on learning the beam walking, as shown the reduction of the average total number of hindlimb slips (fig. S7, A to C), implying that the learning was specific to rotarod running. In the second set of experiments, we trained the mice to perform both rotarod running and beam walking ("dual tasks") on each day for 4 days, and found that the extent of learning for the beam walking was not affected by the rotarod learning, and reached a level comparable to that of beam-walking training alone (fig. S8, A to C). Thus, there was no transfer of learning from rotarod running to beam walking. Importantly, when we enhanced the rotarod learning by online anodal tDCS, no elevated learning of beam walking was observed during the dual-task training (Fig. 2, B to E). Conversely, when the learning of beam walking was enhanced by online anodal tDCS (fig.

S9, A and B, and movie S3 to S6), no enhanced learning of rotarod running was observed (fig. S10, A to D). Thus, the learning enhancement of online anodal tDCS during rotarod learning was specific to the rotarod task, rather than a general improvement of motor skill. In contrast to this specific anodal tDCS effect, we found that both online and offline *cathodal* tDCS during rotarod training had suppressive effects on learning both rotarod running (Fig. 2, F and H) and beam walking (Fig. 2, G and I).

We next examined the action of tDCS on the activity of M1 neurons using transcranial *in vivo* two-photon calcium imaging. We used *thy-1* transgenic mice expressing the calcium-sensitive fluorescent protein GCaMP6s in cortical neurons, and monitored spiking activity of individual neurons by measuring transient elevation of GCaMP6s fluorescence (*11*). After the skull-thinning procedure, we monitored the activity of a population of cortical neurons in the superficial layers of M1 in head-fixed mice on a rotating treadmill (at a constant rotation speed of 15 rpm), with tDCS applied through the thinned skull covered by a saline pool (Fig. 3A). We observed substantial spontaneous activity of M1 neurons (Fig. 3B), as reflected by pulsatile changes of fluorescence signals (ΔF / F_0). Furthermore, anodal tDCS resulted in a gradual increase of firing activity in many neurons during two consecutive 5-min application of anodal tDCS (Fig. 3, C and D, and movie S7). The tDCS-induced increase in spiking

activity was significantly higher for mice running on the rotating treadmill ("task") than those in a quiet resting state ("rest", Fig. 3, D and E). The lower current magnitude (25 μA) was used, based on current density estimates at the cortical surface under the thinned-skull condition. In contrast, cathodal tDCS (50 μA) applied to the same mice suppressed the firing of the same group of neurons during both "task" and "rest" periods (Fig. 3, D and F, and movie S8).

The results from all transgenic GCaMP6s mice (n=6) were summarized by plotting the average fluorescence change ($\Delta F / F_0$) during the 2-min baseline period prior to tDCS and the last 2-min of tDCS, for two consecutive trials under the task and rest conditions. Significant elevation and suppression of fluorescence signals were found for anodal and cathodal tDCS during the task period, respectively (Fig. 3, E and F). We noted that changes in the average fluorescence signal subsided gradually after each tDCS offset, and that the suppressive effect of cathodal tDCS persisted for a longer duration than the enhancement effect of anodal tDCS (Fig. 3G). This may account for the offline suppressive effect on the rotarod learning described above using only 5-min ITI in the present paradigm. Taken together, these results support the notion that anodal and cathodal tDCS modulate neuronal firing by inducing depolarization and hyperpolarization of cortical neurons, respectively, consistent with previous findings on

isolated brain slices (*12-14*). When applied at the time of specific motor circuit activation, as that occurred during rotarod running, anodal and cathodal tDCS could facilitate and reduce learning-associated modification of specific motor circuits in M1, via enhancing and reducing correlated firing that induces Hebbian long-term potentiation (LTP) of synapses within these circuits, respectively.

There are previous reports on the use of tDCS in alleviating neurologic disorders, including stroke (*4, 15*). Meta-analyses of published reports (*16, 17*) have shown a large variability in the clinical efficacy of tDCS that may be attributed to differences in the tDCS protocol and stroke condition. We thus examined the effect of tDCS on motor learning in a relatively defined mouse model of stroke. A standard middle cerebral artery occlusion (MCAO) in the mouse' left hemisphere was used to induce a large lesion within the left somatosensory cortex and part of the motor cortex at one day after 60 or 90 min of MCAO (Fig. 4A). When these mice were subjected to rotarod learning 14 days after MCAO (Fig. 4A), we found their motor coordination was significantly impaired, as shown by overall reduction in the time on the rotarod and the rate of rotarod learning, as compared to control mice that underwent MCAO surgery without sustained artery occlusion (Fig. 4, B and C). Furthermore, online anodal tDCS at the left perilesional M1 region (Fig. 4A) largely restored the learning of motor

coordination and running on the rotarod (Fig. 4, B, C and E, movie S9 and S10). In contrast, offline *anodal* tDCS and online or offline *cathodal* tDCS at the same site had no effect on learning motor coordination and rotarod running in this stroke model (fig. S11, A and B, and S12, A and B). Furthermore, online cathodal tDCS at the opposite site (contralateral to the infarct area) also had no effect on rotarod learning in stroke model mice (fig. S12, C and D).

In the absence of tDCS, 90-min MCAO impaired motor learning of both rotarod running and beam walking, as compared to control mice (Fig. 4, B to F). However, the mice that showed rotarod learning restoration by online anodal tDCS did not exhibit any improvement in learning beam walking, as compared to those subjected to sham tDCS treatment during rotarod running (Fig. 4, B to F). In contrast, offline anodal tDCS during rotarod training had no effect on learning both rotarod running and beam walking (Fig. 4, G and H, and fig. S13, A to D). Therefore, the restoration of rotarod learning in MCAO mice by anodal tDCS was task-specific, rather than a general restoration of learning for motor skills. Based on the above finding of elevated neuronal firing induced by anodal tDCS, the restoration of rotarod learning may involve specific enhancement of residual neural circuits after MCAO that were activated during rotarod running, without affecting those underlying beam walking.

The timing of tDCS relative to the targeted task performance has been addressed in previous studies of healthy human subjects and stroke patients, but conflicting results have been reported, as summarized by various meta-analyses (*16, 17*). For examples, online but not offline anodal tDCS of M1 during motor sequence task was found to enhance motor learning, while online cathodal tDCS had no or opposite effects (*18, 19*). However, another study using offline anodal tDCS prior to the motor task showed enhancement effect on motor learning (*20*). In cases of prolonged tDCS, the effects on human motor cortex could last for hours (*21*) and even days (*22*), and the timing of tDCS becomes less irrelevant. Our present results underscore the importance of online application of anodal tDCS during task performance when brief episodes of tDCS was used.

Previous studies on healthy human subjects have shown that anodal tDCS enhanced cognition or motor learning (*23-26*) and these effects were specific to different level of task difficulty (*27, 28*) or the site (*26, 29*) of tDCS. We found that tDCS on M1 specifically enhanced learning of rotarod task, without affecting learning of beam walking. Thus, even within the motor domain, conjunctive tDCS could exert modulation of specific motor functions. Taken together, our results strongly support the notion that functional specificity of tDCS depends on not only the spatiotemporal

pattern of electric fields applied (*30*) but also on the temporal conjunction of tDCS with the activation of specific circuits underlying the targeted function.

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SUPPLEMENATARY MATERIALS

Materials and Methods

Figs. S1 to S13

Movies S1 to S10

References (*31-39*)

Figure Legends

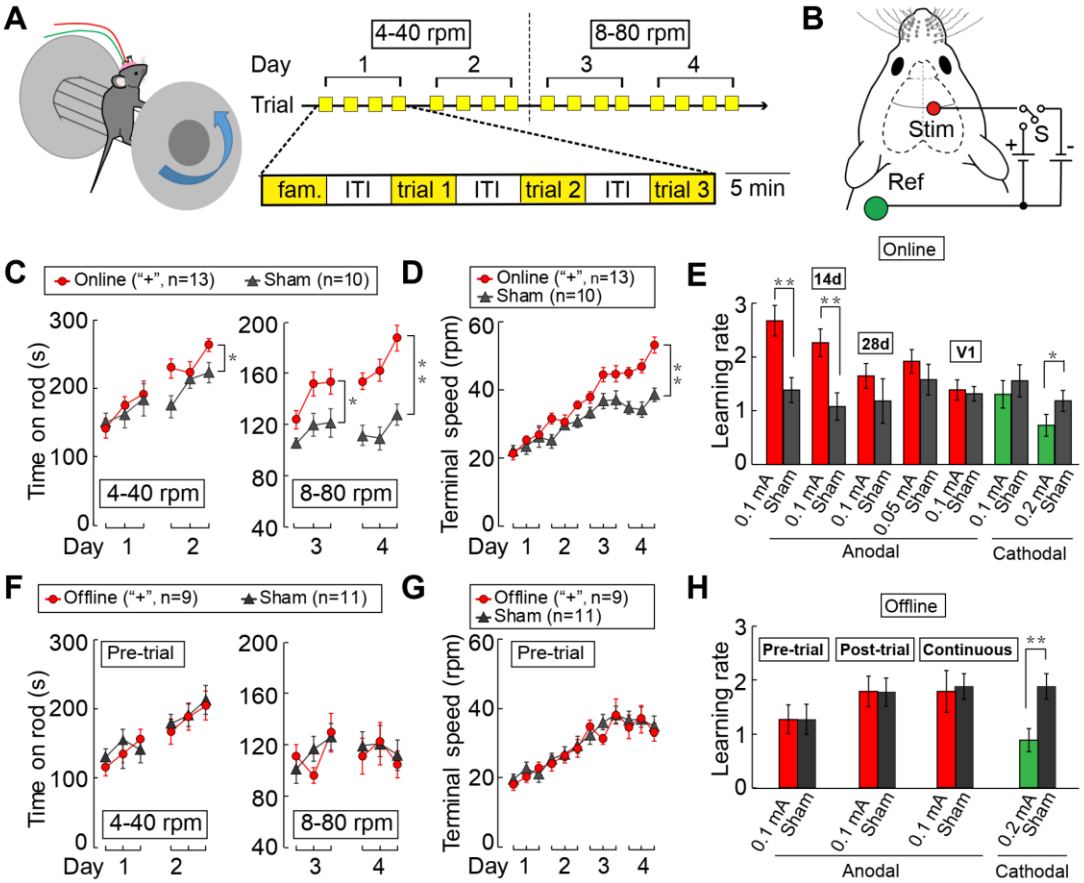

Fig. 1. Effects of tDCS on mouse learning of the rotarod running task. (**A**) Training Protocol: The mouse was subjected each day to a 5-min familiarization trial at a constant low speed, followed by three 5-min trials (separated by 5 min inter-trial interval) at linearly increasing rotation speed. (day 1 & 2: 4 - 40 rpm; day 3 & 4: 8-80 rpm). (**B**) Schematic diagram depicting the electrode configuration. "**Stim**": tDCS electrode. "**Ref**": reference electrode. "**S**": sham (no current). "+": anodal. "-": cathodal. (**C**) The average time of staying on the rotarod during each trial. (**D**) The

terminal rotation speed at which mice fell off the rotarod during each trial. "**Online**": anodal tDCS (0.1 mA) was applied during each trial. "**n**": total number of mice examined. (**E**) Summary of results showing the learning rate, as defined by the normalized difference of terminal speed between the last and the first trial of the entire training period. Data depict standard 4-day training with (color bars) and without (sham, black bars) online anodal (or cathodal) tDCS, applied to M1 at different current amplitudes. "**14d**" and "**28d**" refer to results obtained with 3 additional training trials at 14 and 28 days after training. "**V1**": the tDCS was applied to V1 instead of M1. (**F** to **H)** The results from experiments in which tDCS was applied during ITIs, presented in the same manner as those described above for C-E. "**Pre-trial**" and "**Post-trial**" refer to the average values obtained with tDCS applied during ITIs before and after each trial, respectively. "**Continuous**", 20-min continuous tDCS applied before the familiarization trial. Error bars, SEM. Significant difference was found between the data sets connected by lines ("*", $p < 0.05$; "**" , $p < 0.01$; C, D, F, G: two-way ANOVA; E, H: unpaired *t* test).

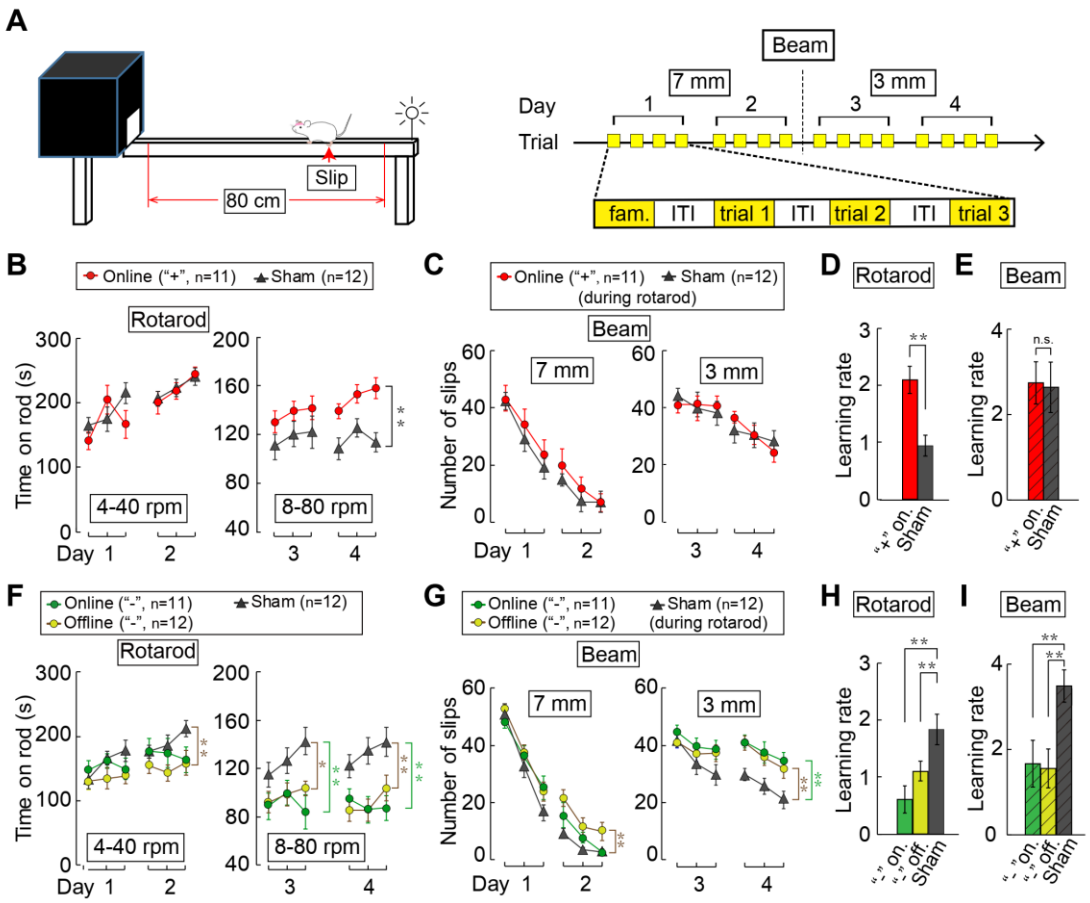

Fig. 2. Effects of tDCS-induced modulation of rotarod learning on the learning of beam walking. (**A**) Experimental protocol of beam walking. The mice were subjected to anodal online tDCS in the same manner as that described in Fig. 1C, except that rotarod task was followed by a beam walking learning task in the absence of tDCS. The mice were subjected to wide beam (25 mm) familiarization, followed by three trials on the thinner beam (day 1 & day 2, 7 mm; day 3 & day 4, 3 mm). (**B** and **C**) Data from dual-task experiments. Average time on the rotarod (in B) was presented as that in Fig.1C. The reduction in the average frequency of hindlimb slips (in C) during the 4-day training of beam walking. Note that online anodal tDCS during rotarod running improved rotarod learning (in B), but had no

effect on learning beam walking (in C). (**D** and **E**) Summary of results showing rotarod learning rate (see Fig.1E) and beam walking learning rate, as defined by normalized difference of the slip frequencies between the last and the first trial of the beam walking on the 3-mm beam. (**F** to **I**) Results of rotarod learning and beam walking learning by cathodal online (or offline) tDCS during rotarod learning. Error bars, SEM. Significant difference was found between the data sets connected by lines (“*”, $p < 0.05$; “**” , $p < 0.01$; B, C, E, F: two-way ANOVA; D, G: unpaired *t* test).

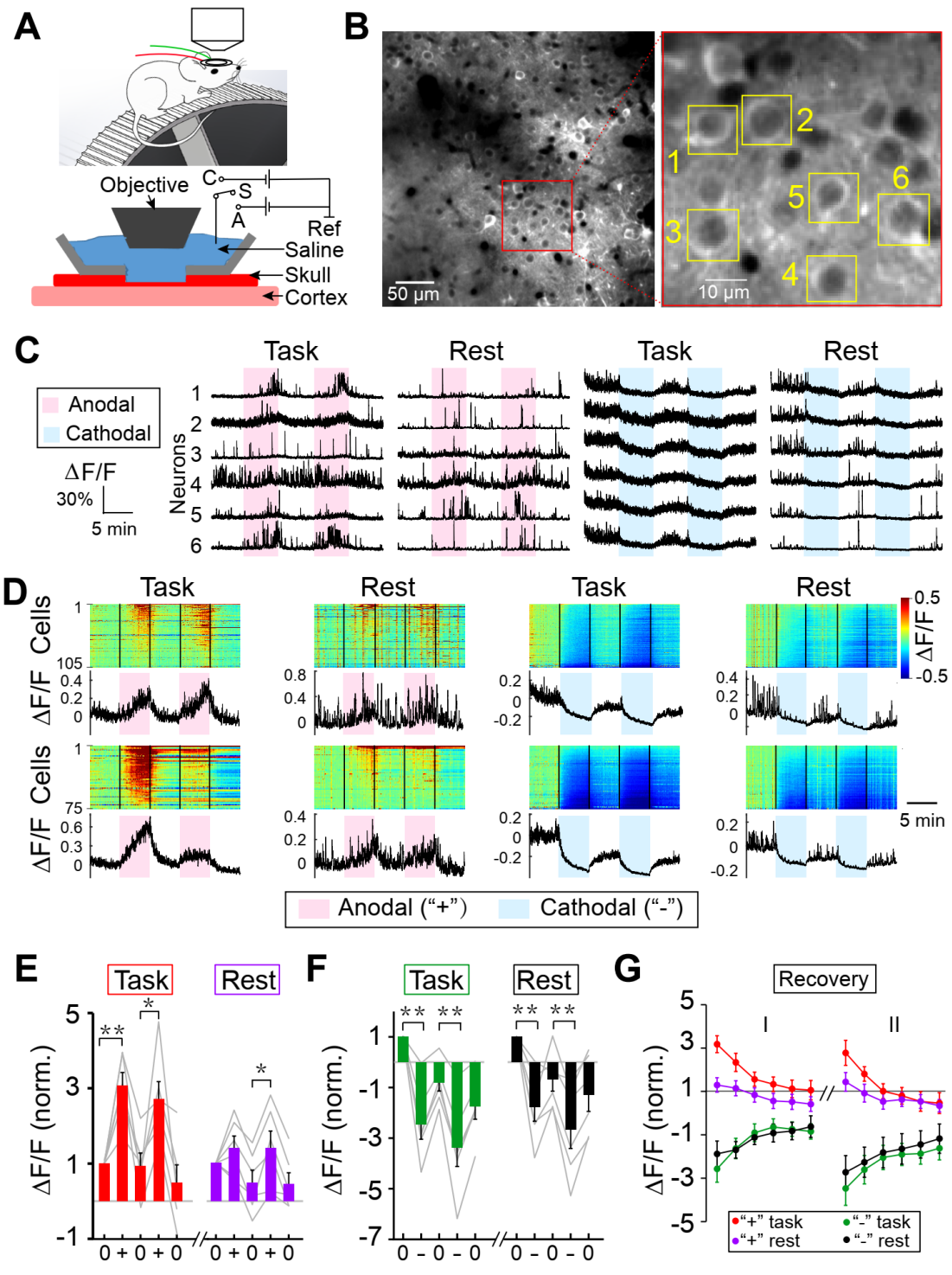

Fig. 3. Transcranial two-photon imaging of tDCS-induced modulation of cortical neuronal activity. (**A**) Schematic diagram depicting the optical window over the thinned skull, for two-photon imaging of M1 neurons in a head-fixed mouse on the rotating treadmill. (**B**) Example

images of Thy1-GCaMP6s-expressing neurons in M1, viewed through the optical window. Scale bar: 50 μm. Image on the right: higher resolution image of the red-boxed region on the left, showing fluorescence of individual layer 2/3 neurons. Scale bar: 10 μm. (**C**) Changes of GCaMP6s fluorescence ($\Delta F / F_0$) with time monitored at six M1 neurons (marked by yellow squares in B). Pink area: time of anodal tDCS (at 25 μA); blue area: time of cathodal tDCS (at 50 μA); "**Task**": walking on steady rotating treadmill; "**Rest**": mouse in a stereotaxic frame. (**D**) Fluorescence changes of all fluorescently labelled cells within the imaged field, recorded from two example mice. The amplitude of $\Delta F / F_0$ for each cell with time was color-coded with the scale shown on the right. The cells were ordered according to the peak values of $\Delta F / F_0$. Traces below represent average $\Delta F / F_0$ for all cells shown above. The same group of cells were monitored before and after two episodes of anodal and cathodal tDCS sequentially, under the task and rest conditions. (**E**) Summary on tDCS-induced GCaMP6s fluorescence changes for data from all mice (n=6). Average fluorescence changes ($\Delta F / F_0$) during the last 2-min of tDCS were normalized by the average values during the 2-min baseline period prior to tDCS, for two consecutive trials under the task and rest conditions. Data for the same set of neurons in each mouse were connected by lines. Significant difference was marked ("*", $p < 0.05$; "**", $p < 0.01$; paired *t* test). (**F**) Post-treatment persistence of tDCS effects was shown by the

average fluorescence changes with time, normalized by the values at the time of termination of anodal or cathodal tDCS, for task and rest conditions (n = 6 mice). Note the slower decay of the inhibitory effect after cathodal tDCS.

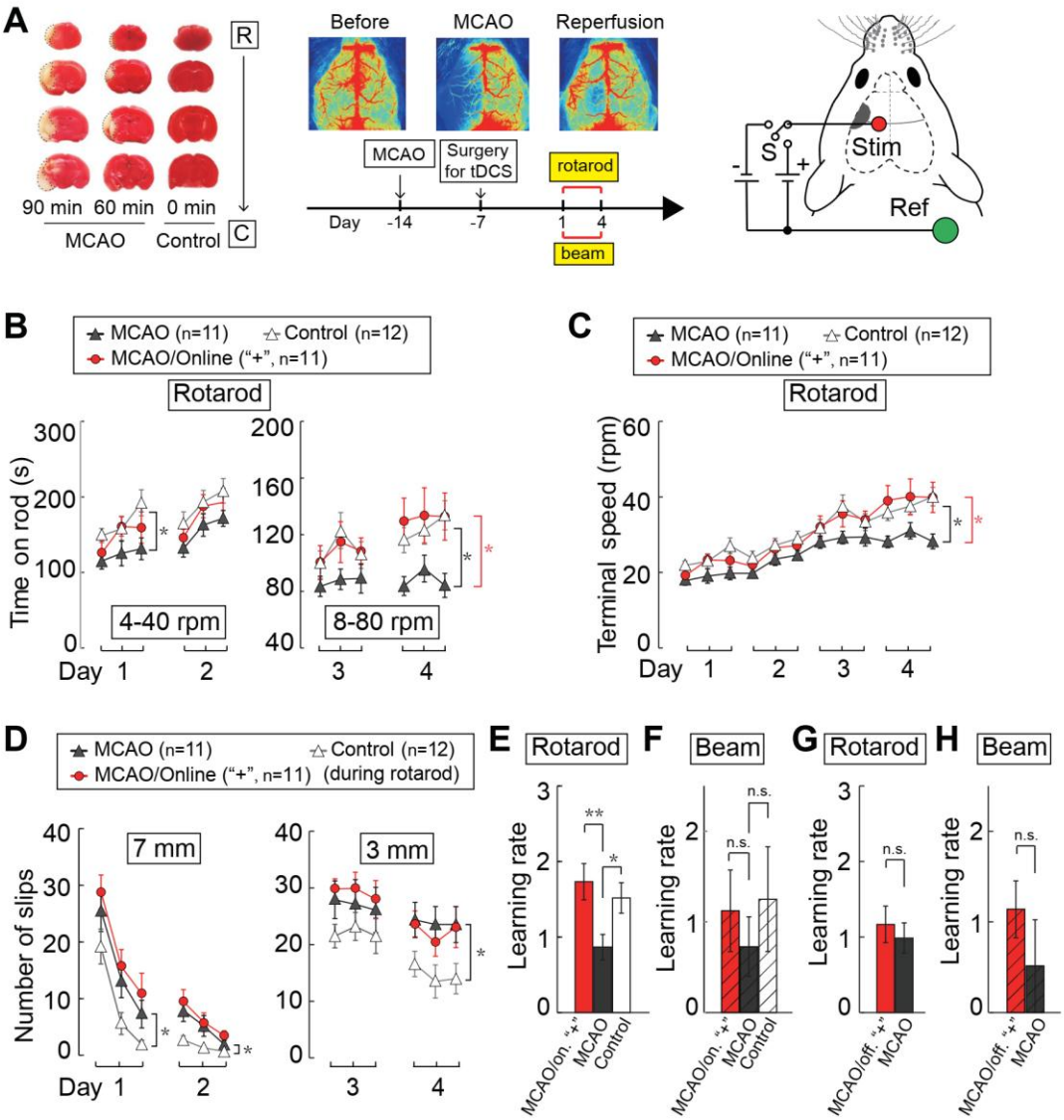

Fig. 4. Task-specific restoration of rotarod learning by online anodal

tDCS in MCAO mice. (**A**) TTC staining (left) and laser speckle contrast imaging (LSCI) (right) showing the lesion induced by MCAO, which was performed for 90, 60 or 0 min prior to reperfusion. The time schedule of MCAO, surgery for tDCS, and training for rotarod running and beam walking is shown. Schematic diagram on the right depicts the placement of tDCS electrodes in MCAO mice. Infarct area marked in gray, and the stimulation electrode ("**Stim**") covered parts of M1 and somatosensory cortex. (**B** and **C**) The average time on and terminal speed of the rotarod, for MCAO mice treated with online anodal tDCS, sham-stimulation, and Sham-MCAO surgery (control) in dual-task experiments, in which tDCS was applied only during rotarod running. Data presented in the same manner as in Fig.1, C and D. "**MCAO**": mice subjected to 90-min occlusion in MCA; "**Control**" (Sham-MCAO): mice subjected to the same surgery with no occlusion in MCA; "**MCAO/online**": MCAO mice subjected online tDCS during rotarod running. (**D**) The average frequency of hindlimb slips (contralateral to the lesion) during beam walking. (**E** and **F**) Learning rate for rotarod running and beam walking in MCAO mice subjected to online anodal tDCS. (**G** and **H**) Learning rate of rotarod and beam walking in MCAO mice subjected to offline anodal tDCS. Error bars, SEM. Significant difference was found between the data sets connected by lines ("*", $p < 0.05$; "**", $p < 0.01$; C, D, E: two-way ANOVA; F, G, H, I: unpaired *t* test).

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