Stroke

BASIC AND TRANSLATIONAL SCIENCES

Transcranial Direct Current Stimulation Enhances Neuroplasticity and Accelerates Motor Recovery in a Stroke Mouse Model

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BACKGROUND: More effective strategies are needed to promote poststroke functional recovery. Here, we evaluated the impact of bihemispheric transcranial direct current stimulation (tDCS) on forelimb motor function recovery and the underlying mechanisms in mice subjected to focal ischemia of the motor cortex.

METHODS: Photothrombotic stroke was induced in the forelimb brain motor area, and tDCS was applied once per day for 3 consecutive days, starting 72 hours after stroke. Grid-walking, single pellet reaching, and grip strength tests were conducted to assess motor function. Local field potentials were recorded to evaluate brain connectivity. Western immunoblotting, ELISA, quantitative real-time polymerase chain reaction, and Golgi-Cox staining were used to uncover tDCS-mediated stroke recovery mechanisms.

RESULTS: Among our results, tDCS increased the rate of motor recovery, anticipating it at the early subacute stage. In this window, tDCS enhanced BDNF (brain-derived neurotrophic factor) expression and dendritic spine density in the peri-infarct motor cortex, along with increasing functional connectivity between motor and somatosensory cortices. Treatment with the BDNF TrkB (tropomyosin-related tyrosine kinase B) receptor inhibitor, ANA-12, prevented tDCS effects on motor recovery and connectivity as well as the increase of spine density, pERK (phosphorylated extracellular signal-regulated kinase), pCaMKII (phosphorylated calcium/calmodulin-dependent protein kinase II), pMEF (phosphorylated myocyte-enhancer factor), and PSD (postsynaptic density)-95. The tDCS-promoted rescue was paralleled by enhanced plasma BDNF level, suggesting its potential role as circulating prognostic biomarker.

CONCLUSIONS: The rate of motor recovery is accelerated by tDCS applied in the subacute phase of stroke. Anticipation of motor recovery via vicariate pathways or neural reserve recruitment would potentially enhance the efficacy of standard treatments, such as physical therapy, which is often delayed to a later stage when plastic responses are progressively lower.

GRAPHIC ABSTRACT: A graphic abstract is available for this article.

Key Words: biomarker ■ brain-derived neurotrophic factor ■ forelimb ■ ischemia ■ motor cortex ■ personalized medicine ■ transcranial direct current stimulation

Currently, stroke is the second most common cause of death and the third most common cause of chronic disability worldwide.[1]

Dramatic alterations induced by the ischemic insult on neural circuitry and vasculature trigger plastic phenomena that are believed to underlie spontaneous, but unfortunately often limited, restoration of function. These reparative changes include: (1) increased angiogenesis and neurovascular unit remodeling to restore blood flow[2]; (2) functional and structural changes at

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Nonstandard Abbreviations and Acronyms

Akt	serine/threonine kinase
BDNF	brain-derived neurotrophic factor
CaMKII	calcium/calmodulin-dependent protein kinase II
ERK	extracellular signal-regulated kinases
LFP	local field potentials
MEF	myocyte-enhancer factor
pAkt	phosphorylated serine/threonine kinase
pCaMKII	phosphorylated calcium/calmodulin-dependent protein kinase II
pERK	phosphorylated extracellular signal-regulated kinase
pMEF	phosphorylated myocyte-enhancer factor
PSD	postsynaptic density
tDCS	transcranial direct current stimulation
TotCoh	total magnitude squared coherence
TrkB	tropomyosin-related tyrosine kinase B
VEGF	vascular endothelial growth factor

neuronal networks in the injured hemisphere; and (3) changes in the interhemispheric modulation.[3–6] Indeed, brain-wide changes in functional connectivity in both hemispheres, well beyond the infarcted area, have been observed.[7,8] Efficacy of physical rehabilitative therapies in poststroke treatment has also been associated with enhanced brain and vascular plasticity.[5,9] However, the extent of motor function restoration is quite variable and scarcely predictable. One possible strategy to accelerate or improve recovery could be an earlier intervention after disease onset when plastic changes are more likely to occur. However, physical therapy in the acute phase—that is, within a stroke unit environment—is often not feasible, especially in the case of severe impairments. Therefore, novel approaches should be considered including the use of noninvasive brain stimulation techniques.

In the field of noninvasive brain stimulation, transcranial direct current stimulation (tDCS), namely low-intensity currents (typically ≈1–2 mA) applied to targeted brain areas through the scalp, has great therapeutic potential because of its ability to modulate cortical excitability and to promote plasticity mechanisms.[10–12] Additional advantages of tDCS are its low cost, easy use, and favorable tolerability profile.

Several clinical studies tested the therapeutic potential of tDCS in stroke recovery with encouraging results.[13] However, a deeper understanding of the mechanisms underlying tDCS effects is needed to provide more evidence on the efficacy and consistency of its use in stroke rehabilitation.

Therefore, we aimed at characterizing the effect of bihemispheric tDCS—the most common configuration in human stroke studies placing anode over the lesioned motor cortex and cathode over the contralateral side[14]—on forelimb motor recovery in a mouse model of ischemic stroke of the motor cortex induced by photothrombosis.[15] tDCS protocol started 72 hours after stroke to boost spontaneous plasticity mechanisms occurring in this early phase. The simultaneous stimulation with facilitating and inhibiting currents on affected and unaffected motor areas, respectively, is also providing a useful rebalance of interhemispheric mutual modulation via transcallosal connections which is one of the most important mechanisms for favoring recovery, as demonstrated by constraint therapy.[16]

METHODS

Data are available from the corresponding author on reasonable request. This article adheres to the American Heart Association Journals implementation of Transparency and Openness Promotion Guidelines. Detailed description of methodology is available in the Supplemental Material.

Animals

C57BL/6 male mice (5–6 weeks old) were used. Experiments and animal procedures were approved by the Catholic University Ethics Committee and were in line with Italian (Ministry of Health guidelines, Legislative Decree No. 116/1992) and European Union (Directive No. 86/609/EEC) legislations on animal procedures. Groups were balanced for age, weight, littermate conditions, and baseline performances in the motor tests. Randomization was performed assigning random numbers. Inclusion and Exclusion Criteria are detailed in the Supplemental Material.

Photothrombotic Stroke

Motor cortex infarct was induced by Rose-Bengal photothrombosis,[15] as detailed in the Supplemental Material. At the end of the stroke protocol, tDCS electrodes were implanted. Two epicranial plastic tubes (internal diameter 3 mm) were positioned over the motor cortex of both hemispheres.[12] To verify the consistency of the stroke procedure in targeting the motor cortex, some mice (n=4) were randomly included in the experimental batteries and used for immunohistochemical detection of the infarct area by 2,3,5-triphenyltetrazolium chloride staining.[17]

Transcranial Direct Current Stimulation

tDCS protocol consisted of 3 stimulation sessions at a current intensity of 250 μA for 20 min (current density: 35.4 A/m^2) delivered in 3 consecutive days, once per day, starting from day 3 after stroke.[10,12] Details are provided in the Supplemental Material. For sham stimulation, animals underwent the same manipulations (ie, surgery and electrode placement) as in the real stimulation condition, but the stimulator was turned off (ie, no current was delivered).

Two main experimental groups were used in the study: tDCS-stroke mice, subjected to real tDCS, and sham-stroke mice, subjected to sham stimulation. For local field potential (LFP) recordings, healthy mice were also used, which were subjected to real (tDCS-healthy) or sham stimulation (sham-healthy).

Behavioral Tests

Grid-walking test, grip strength test, and single pellet reaching task assessed motor performance according to previously published protocols.[12,17] Details are provided in the Supplemental Material.

LFP Recordings and Analysis

The day after last stimulation session, mice used for electrophysiological analyses underwent a second surgical procedure for chronic electrode implants.[18] Recordings electrodes were positioned over the primary motor and somatosensory cortices of both sides.[12] Data were acquired using Cereplex Direct system (Blackrock microsystem); LFP data were processed in Matlab for connectivity analysis using scripts based on EEGLAB toolbox.[19] In particular, the global functional coupling of the LFP rhythms was indexed by the mean of the Magnitude Squared Coherence for all combinations of electrode pairs, namely the total magnitude squared coherence (TotCoh), as detailed in the Supplemental Material.

Morphological, Immunofluorescence, and Molecular Analyses

Infarct size and cerebral edema measurements, immunofluorescence, Golgi-Cox staining, Western immunoblotting, quantitative real-time polymerase chain reaction, and ELISA were performed according to published protocols[2,10,12,17,20] and manufacturer's instructions (see details and Table S1).

Statistical Analysis

Sample sizes were chosen with adequate statistical power (0.8) according to results of prior pilot data sets or studies, including our own, using similar methods or paradigms. Analyses were performed using the SigmaPlot 14.0 software. The results are presented as mean±SEM. All experiments and analyses were performed in a blinded way. Data were first tested for equal variance and normality (Shapiro-Wilk test). The statistical test used is indicated in the main description for each experiment. All statistical tests were 2-tailed and the level of significance was set at 0.05.

RESULTS

tDCS Accelerates Motor Recovery After Stroke

Grid-walking, grip strength, and single pellet reaching tests were performed before and 3 days after unilateral photothrombotic stroke induction, to establish baseline motor performance and to evaluate motor deficits following stroke. Mice were then randomly assigned to the sham-stroke or tDCS-stroke group and subjected to sham stimulation or bihemispheric tDCS, respectively. The time-course of motor recovery was assessed reevaluating motor performance from 1 day to 4 weeks after the last stimulation session (Figure 1A). The specificity of unilateral photothrombotic ischemia was confirmed by triphenyltetrazolium chloride staining (Figure 1B). No differences between tDCS and sham groups were found 24 hours after the end of stimulation protocol with respect to infarct size (P=0.91; n=3 mice for each group; unpaired Student t test; Figure 1C) and brain water content (ie, edema; stimulation×hemisphere: $F_{1,4}$=0.77; P=0.42; 2-way repeated measures (RM) ANOVA, n=3 mice each group; Figure 1D). Based on literature reports,[21,22] we expected that negligible changes in intracranial pressure occurred in our stroke model. Nevertheless, any variations in this parameter would occur in both sham-stroke and tDCS-stroke mice equally, thereby not affecting our results on the effectiveness of tDCS treatment.

In the grid-walking test, all mice exhibited a significant increase in the percentage of foot faults of the forelimb contralateral to the damaged motor cortex compared to their own prestroke values (n=9 mice assigned to sham-stroke group: 9.36±0.65% versus 4.33±0.39%, P<0.001; n=9 mice assigned to tDCS-stroke group: 9.06±0.83% versus 4.21±0.68%, P<0.001; 2-way RM ANOVA, Bonferroni post hoc; Figure 2A). Conversely, no significant change was observed in foot fault percentage of the forelimb contralateral to the intact motor cortex (sham-stroke: 3.71±0.61% versus 4.16±0.52%, P=0.21; tDCS-stroke: 4.32±0.40% versus 4.05±0.48%; P=0.10; Table S2), indicating the effectiveness of unilateral photothrombotic ischemia.

As expected, partial spontaneous recovery was observed in sham-stroke mice, with percentage of foot faults decreasing progressively over time. Remarkably, mice subjected to tDCS achieved a faster recovery compared to sham-stroke mice (stimulation×time: $F_{5,80}$=4.03, P=0.003; 2-way RM ANOVA; Figure 2A). In particular, 24 hours after tDCS (ie, 6 days after stroke), the percentage of foot faults was significantly lower compared to that of sham-stroke mice (5.98±0.74% versus 8.51±0.58%, P=0.004; 2-way RM ANOVA, Bonferroni post hoc) and the difference between groups was statistically significant up to 2 weeks after stimulation (12 days after stroke: 5.42±0.74% tDCS-stroke versus 7.47±0.40% sham-stroke, P=0.018; 19 days after stroke: 5.16±0.56% tDCS-stroke versus 6.81±0.59% sham-stroke, P=0.050; 2-way RM ANOVA, Bonferroni post hoc; Figure 2A). Motor performance of tDCS-stroke mice at day 12 after stroke did not significantly differ from that recorded before stroke (P=0.220 versus baseline; 2-way RM ANOVA, Bonferroni post hoc), indicating that recovery was achieved at this early time point. No further improvements were observed at day 19 and day 33 of the follow-up. At day 33, sham-stroke mice show partial motor recovery (P=0.002 versus baseline; P=0.280 versus tDCS-stroke; 2-way RM ANOVA, Bonferroni post hoc).

Forelimb strength was also affected by stroke, with no significant recovery observed over the 4-week follow-up (P<0.01 at all time points in both groups versus baseline; 2-way RM ANOVA, Bonferroni post hoc; Figure 2B). However, mice subjected to tDCS showed significantly

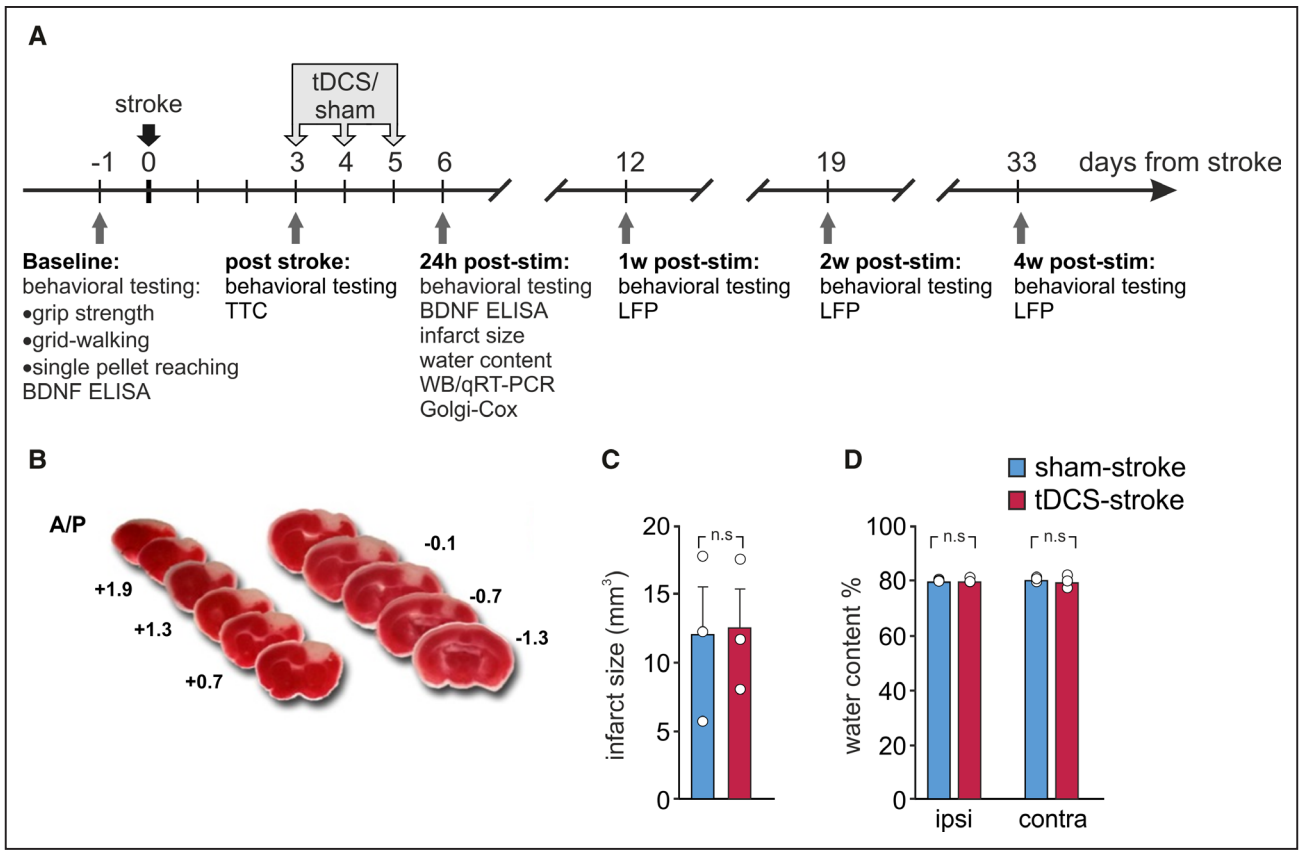

Figure 1. Study design and timeline of experiments.
A, Time schedule of behavioral testing, molecular, Golgi-Cox, infarct size, brain water content, and local field potential (LFP) analyses with respect to stroke induction (days from stroke) and to the end of transcranial direct current stimulation (tDCS) or sham treatment (hours or week post stimulation). All behavioral tests were performed up to 4 wk post stimulation, except single-pellet reaching test that was repeated up to 1 wk post stimulation. **B**, Representative triphenyltetrazolium chloride (TTC) stained coronal sections of mouse brain at day 3 after stroke (anterior-posterior bregma coordinates in mm [A/P]). **C** and **D**, Bar graphs showing (**C**) infarct size and (**D**) water content of ipsilesional (ipsi) and contralesional hemisphere (contra) at 6 d after stroke in tDCS-stroke and sham-stroke mice. Data are expressed as mean±SEM. BDNF indicates brain-derived neurotrophic factor; h, hours; n.s. not significant; post-stim, post stimulation; qRT-PCR, quantitative real-time polymerase chain reaction; w, weeks; and WB, Western immunoblotting.

higher grip strength values compared with sham-stroke mice starting from 24 hours after the completion of the tDCS protocol and lasting throughout the follow-up period (main factor stimulation: $F_{1,90}$=8.36; P=0.01; Figure 2B).

Regarding recovery in forelimb skilled reaching, our results showed an earlier motor improvement in tDCS mice compared with sham-stroke mice (n=12 tDCS-stroke mice; n=11 sham-stroke mice; main factor stimulation: $F_{1,19}$=4.652; P=0.04; 2-way RM ANOVA, Figure 2C). Particularly, 24 hours after stimulation (day 6 after stroke), success rate in the single pellet reaching task was 19.1±3.8% in tDCS-stroke mice versus 9.2±2.4% in sham-stroke mice (P=0.01; Bonferroni post hoc; Figure 2C). Functional improvement seen at 24 hours after tDCS was maintained, but not further improved, 1 week later (success rate at day 12: 20.3±3.3%; P>0.99 versus day 6 value; P=0.01 versus day 3 after stroke value). At day 12, spontaneous motor recovery brought success rates of sham-stroke mice to values slightly lower but not significantly different from those of tDCS-stroke mice (16.9±3.0%; P=0.75 versus tDCS-stroke; P=0.37 versus sham-stroke at day 3 after stroke, Figure 2C).

tDCS Increases Cortical Connectivity

Connectivity in the motor system network is initially reduced by stroke and undergoes progressive recovery both spontaneously and following therapy or training. Furthermore, increase in connectivity has been positively correlated with gain of motor function.[23] We, therefore, ascertained whether changes in brain connectivity occurred following tDCS in stroke mice by performing LFP recordings. Spontaneous cortical activity was recorded in awake, freely moving animals (n=10 sham-stroke, n=9 tDCS-stroke mice) using epidurally implanted electrodes placed over the motor and somatosensory cortices of both hemispheres (Figure 3A). LFPs were recorded in 30-minute sessions 1, 2, 3, and 4 weeks after the last tDCS or sham stimulation. For this set of experiments, healthy mice were also used to evaluate connectivity and tDCS effects on intact cortical networks (n=9 sham-healthy, n=11 tDCS-healthy

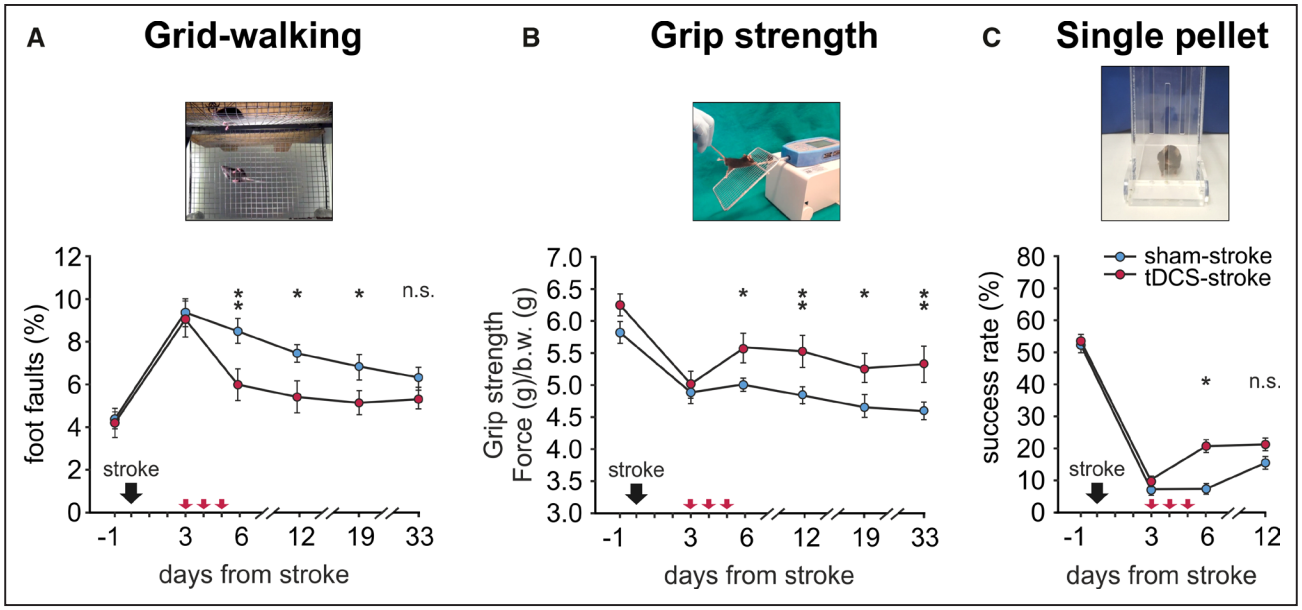

Figure 2. Transcranial direct current stimulation (tDCS) speeds up motor recovery after stroke.
A, Percentage of foot faults in the grid-walking test in mice subjected to tDCS or sham stimulation (red arrows) delivered from day 3 to day 5 after stroke. **B**, tDCS-stroke mice displayed higher strength values than sham-stroke mice starting from day 6 after stroke and throughout the entire follow-up period. **C**, In the single pellet reaching test, tDCS mice showed a faster improvement in success rate compared to sham-stroke mice. Photographs show mice performing the different tests. Data are expressed as mean±SEM. tDCS-stroke vs sham-stroke mice. b.w. indicates body weight; and n.s., not significant. *$P \leq 0.05$, **$P < 0.01$.

mice). A 3-way ANOVA design (condition×band×time) was performed to evaluate TotCoh—the index expressing global functional coupling of the LFP rhythms—followed by post hoc analysis with the Duncan's test. Our results showed that tDCS enhanced TotCoh in both healthy and stroke mice at all frequency bands independently of the time point of recording (condition×band: $F_{18,228}=3.00$; $P<0.001$; Figure 3A and Figure S1). As expected, sham-stroke mice showed significantly lower values of TotCoh than sham-healthy mice (theta, $P=0.02$; alpha1, $P=0.02$; alpha2, $P=0.04$). Moreover, a significant difference among the 4 conditions independently from time and band was observed (condition: $F_{3,38}=2.77$; $P=0.05$; Figure 3C). TotCoh was significantly higher in tDCS-stroke mice compared to sham-stroke mice ($P=0.05$) and no differences between tDCS-stroke and sham-healthy groups were found ($P=0.94$; Figure 3C). Collectively these results show that tDCS reduced stroke-induced impairment of sensory-motor networks, restoring the physiological level of overall connectivity among the motor and somatosensory cortices, as measured by TotCoh.

Molecular and Cellular Mechanisms of tDCS Involve Activation of BDNF/TrkB Signaling

Our next step was to investigate the molecular and cellular underpinnings of tDCS effects. Given the beneficial effects of tDCS on motor recovery in the subacute stroke phase and considering the potential relevance of motor improvement in this critical phase for rehabilitative purpose, we focused on this time window.

Among possible mediators of stroke recovery, we investigated the involvement of the neurotrophin BDNF (brain-derived neurotrophic factor) because of its strong association with tDCS effects in the motor cortex and other brain areas both in humans and rodents.[10–12,24] Our analyses revealed that 24 hours after tDCS, BDNF mRNA, and protein levels in the ipsilesional motor cortex were higher than those of sham-stimulated mice as revealed by quantitative real-time polymerase chain reaction and ELISA, respectively (BDNF mRNA: +355% versus sham-stroke; $P=0.004$; n=9 mice for each group; BDNF protein: +94% versus sham-stroke; $P<0.001$; n=9 sham-stroke, n=10 tDCS-stroke mice; unpaired Student *t* test; Figure 4A and 4B). BDNF plasma levels were also measured. Results showed that 72 hours after stroke, BDNF levels were not significantly different from prestroke baseline values in both groups (76.66±8.08 versus 107.65±8.82 pg/ml; $P=0.27$; n=3 mice assigned to sham-stroke group; 99.36±7.79 versus 109.10±8.83 pg/ml, $P=1.0$; n=4 mice assigned to tDCS-stroke group; 2-way RM ANOVA, Bonferroni post hoc). Remarkably, plasma BDNF levels were significantly increased 24 hours after tDCS (218.54±17.12 pg/ml; $P<0.001$ versus poststroke) but not after sham stimulation (75.80±13.09 pg/ml versus poststroke; $P=0.95$; 2-way RM ANOVA, Bonferroni post hoc; Figure 4C).

To shed light on the tDCS-induced BDNF-signaling cascade, we evaluated phosphorylation (ie, activation) of key signaling molecules downstream to BDNF/TrkB (tropomyosin-related tyrosine kinase B) activation, that is, pERK1/2 (phosphorylated extracellular

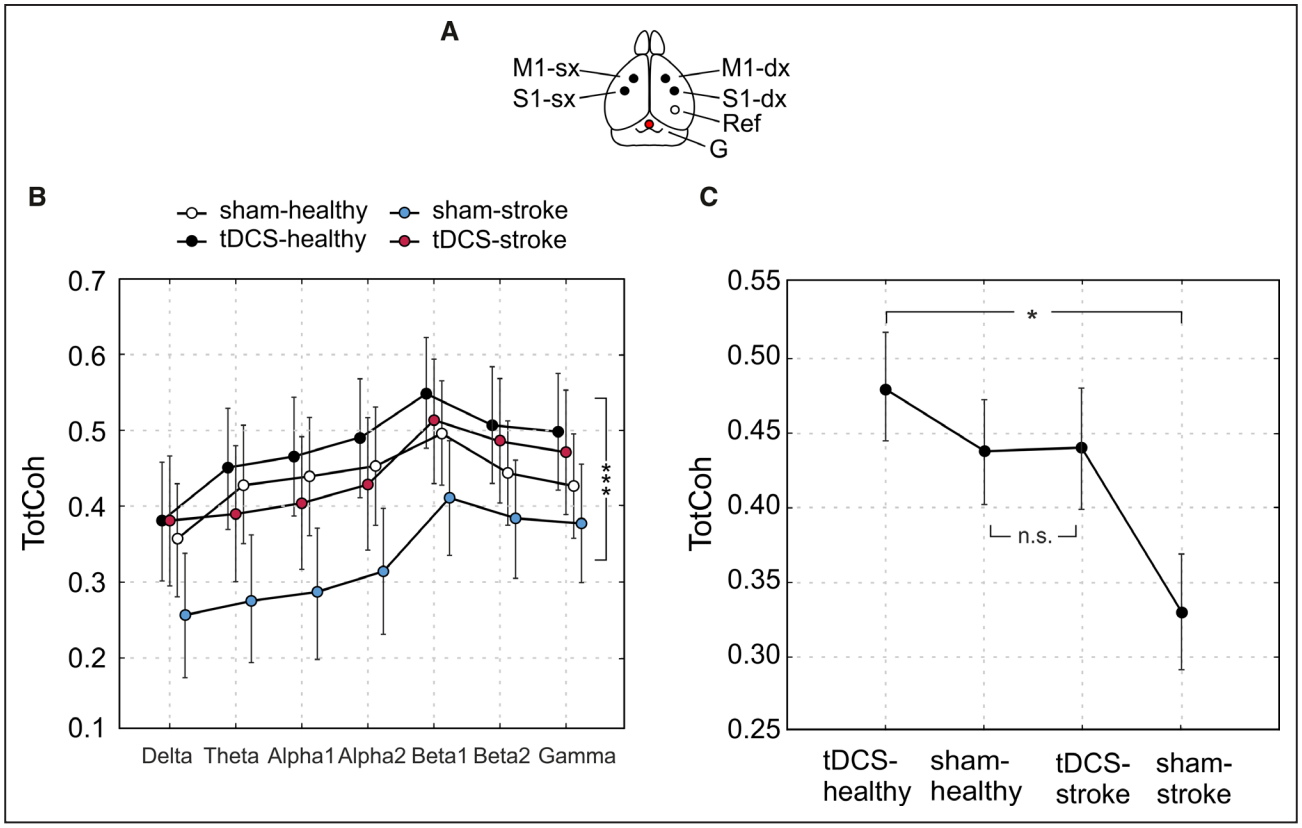

Figure 3. Transcranial direct current stimulation (tDCS) improves cortical connectivity.
A, Top view drawing of the mouse brain indicating the electrode positions for local field potential (LFP) recordings. **B**, Graph showing frequency band-specific coherence coefficients (total magnitude squared coherence [TotCoh]) in the different experimental groups (condition×band: $F_{18,228}$=3.00; ***P<0.001). **C**, Overall TotCoh graph highlighting differences among groups independently of time and band (condition: $F_{3,38}$=2.77; P=0.05). Data are expressed as mean±SEM. dx indicates right; G, ground; M1, primary motor cortex; Ref, reference electrode; S1, primary somatosensory cortex; and sx, left.

signal-regulated kinase 1/2)^{Tyr204}, pCaMKII (phosphorylated calcium/calmodulin-dependent protein kinase II)^{Thr286}, and pAkt (phosphorylated serine/threonine kinase)^{Ser473}. Western blot analysis, performed on peri-infarct tissue, revealed that, compared with the sham-stroke group, tDCS-stroke mice had a significantly higher

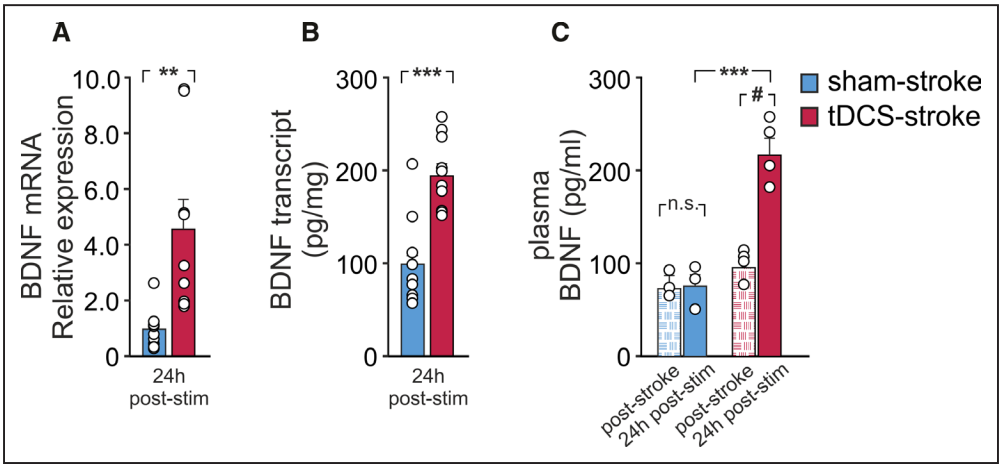

Figure 4. Transcranial direct current stimulation (tDCS) enhances BDNF (brain-derived neurotrophic factor) levels in brain and plasma of stroke mice 24 h after stimulation.
A, Changes in BDNF expression in perilesional brain tissue assessed by quantitative real-time polymerase chain reaction. Gene expression was normalized to TATA binding protein (TBP). **B** and **C**, Results from ELISA showing that tDCS-stroke mice had higher BDNF levels in both brain tissue (**B**) and plasma (**C**) compared to sham-stroke mice. Graph C shows BDNF levels measured from samples taken before (poststroke) and 24 h after tDCS or sham stimulation (poststim). Data are expressed as mean±SEM. n.s. indicates not significant; and post-stim, post stimulation. **P<0.01, ***P<0.001 between groups, #P<0.001 within group.

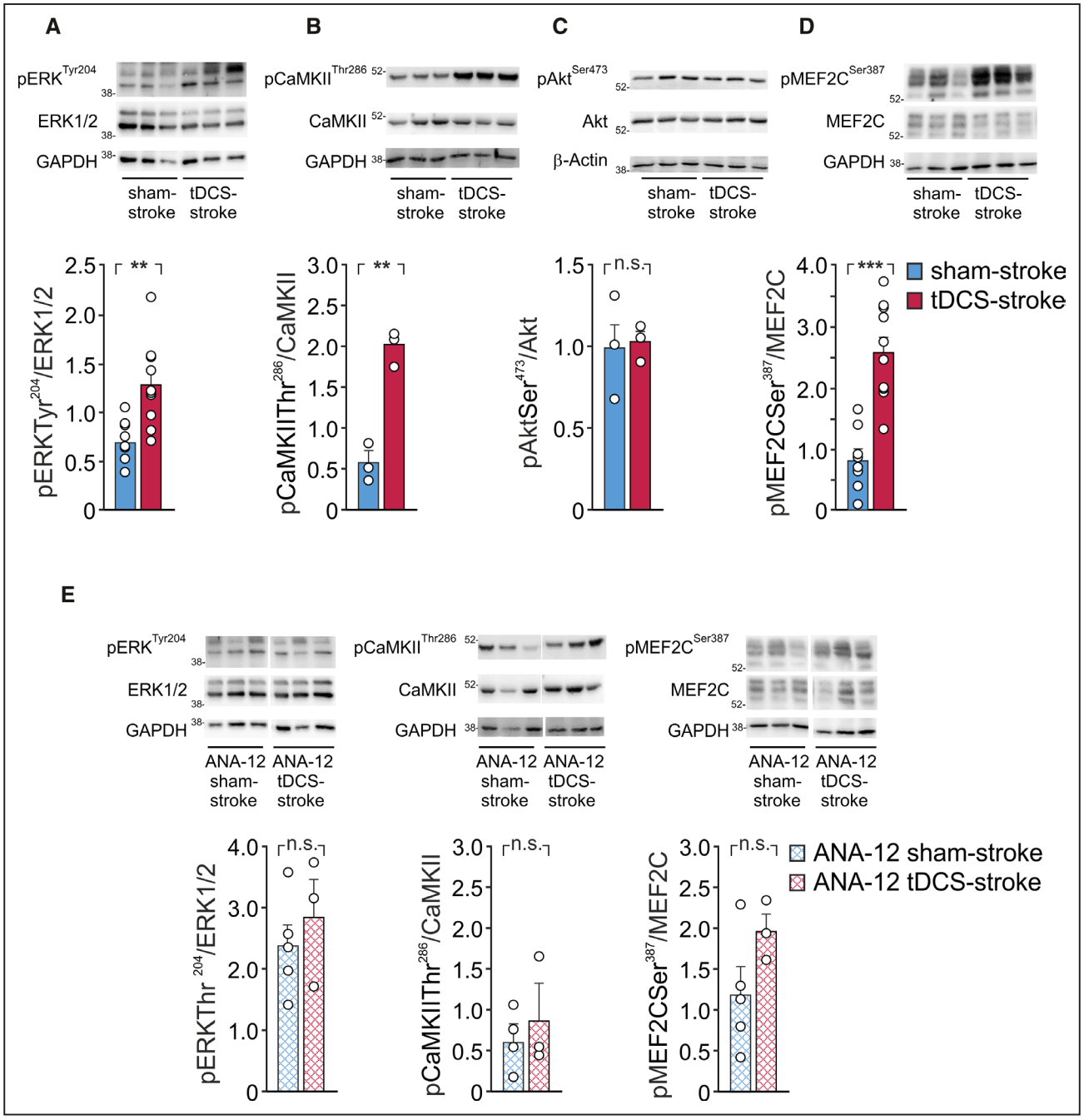

Figure 5. Molecular changes occur 24 h after transcranial direct current stimulation (tDCS) in the motor cortex of stroke mice. Representative Western immunoblots and band densitometry, normalized to both the corresponding total protein levels and GAPDH or β-actin as loading control, revealing tDCS-induced activation of (**A**) ERK1/2 and (**B**) CaMKII, but not (**C**) Akt pathways. **D**, Enhanced levels of pMEF2C^{Ser387} were also observed in tDCS-stroke mice compared to sham-stroke mice. **E**, tDCS-induced changes in pERK1/2^{Tyr204}, pCaMKII^{Thr286}, pMEF2C^{Ser387} were hindered by ANA-12. Data are expressed as mean±SEM. Akt indicates serine/threonine kinase; CaMKII, calcium/calmodulin-dependent protein kinase II; ERK, extracellular signal-regulated kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MEF, myocyte-enhancer factor; n.s, not significant; pAKT, phosphorylated serine/threonine kinase; pCAMKII, phosphorylated calcium/calmodulin-dependent protein kinase II; pERK, phosphorylated extracellular signal-regulated kinase; and pMEF, phosphorylated myocyte-enhancer factor. $^{**}P<0.01$, $^{***}P<0.001$.

level of pERK1/2^{Tyr204} (+84% versus sham-stroke; $P=0.001$; n=10 mice for each group; unpaired Student *t* test; Figure 5A) and pCaMKII^{Thr286} (+242% versus sham-stroke; $P=0.002$; n=3 mice for each group; unpaired Student *t* test; Figure 5B). Conversely, no changes between the 2 groups were observed in pAkt^{Ser473} levels ($P=0.86$; n=3 for each group; unpaired Student *t* test; Figure 5C). Of note, no changes in the level of BDNF-activated proteins were observed between sham-stroke and tDCS-stroke mice treated with the inhibitor of the BDNF TrkB receptor, ANA-12, (pERK1/2: $P=0.48$, n=3 tDCS-stroke and n=5 sham-stroke mice; pCaMKII:

P=0.46, n=3 tDCS-stroke and n=4 sham-stroke mice; 2-way ANOVA, Bonferroni post hoc; Figure 5E), providing a causal link among tDCS, BDNF, and downstream activation of ERK and CaMKII.

Phosphorylation of MEF (myocyte-enhancer factor)2C at Ser387 (pMEF2C^{Ser387}) was also investigated because it is activated by ERK1/2, and it is a crucial transcription factor of genes involved in neuroprotection and plasticity.[25,26] Results showed that pMEF2C^{Ser387} levels were higher in tDCS-stroke than sham-stroke mice (+209%; P<0.001; n=10 tDCS-stroke and n=8 sham-stroke mice; unpaired Student t test; Figure 5D), whereas they remained similar in both groups treated with ANA-12 (P=0.13; n=3 tDCS-stroke and n=5 sham-stroke mice; 2-way ANOVA, Bonferroni post hoc; Figure 5E) supporting the role of BDNF to activate MEF2C.

Finally, to ascertain how tDCS-induced BDNF stimulation contributed to poststroke network reorganization, Golgi-Cox staining was performed to assess spine density modifications in tDCS-stroke and sham-stroke mice treated or not with ANA-12. Morphological analysis on at least 55 pyramidal neurons per group (n=6 mice for each group) in layer II/III of the peri-infarct motor cortex was performed. Results showed that 24 hours after tDCS, spine density was increased in both apical and basal dendrites of tDCS-stroke compared to sham-stroke mice (apical dendrites: $F_{2,147}$=25.03; P<0.001; basal dendrites: $F_{2,154}$=18.26; P=0.012; 1-way ANOVA Bonferroni post hoc, Figure 6A). Comparison with healthy mice (n=3 mice, 30 pyramidal neurons) showed that stroke significantly reduced the number of spines at both apical and basal dendrites (P<0.001 sham-stroke versus sham-healthy) and that tDCS increased the number of spines in apical dendrites to value that were similar to those of healthy mice (tDCS-stroke versus sham-healthy mice; P=0.35; Figure 6A). The tDCS-rescue effect at basal dendrites was less pronounced (tDCS-stroke versus sham-healthy mice; P=0.002; Figure 6A). Of note, tDCS effect on spinogenesis was prevented by treatment with ANA-12 ($F_{1,58}$=0.33; P=0.57; ANA-12 tDCS-stroke versus ANA-12 sham-stroke; n=3 mice for each group, 30 pyramidal neurons per group; 1-way RM ANOVA; Figure 6B).

Consistent with BDNF-dependent spine density increase, we also found enhanced levels of the postsynaptic density protein marker, PSD (postsynaptic density)-95, in tDCS-stroke mice (n=3) compared with sham-stroke mice (+58% versus sham-stroke mice, n=3; P=0.03; 2-way ANOVA, Bonferroni post hoc), that was hindered by ANA-12 (P=0.71; Figure 6C).

To substantiate the link between tDCS effects on functional recovery and the activation of BDNF/TrkB pathway, we evaluated motor performance in the grid-walking test in stroke mice treated with ANA-12 (n=8 mice) or vehicle (1% dimethyl sulfoxide [DMSO], dissolved in 0.9% NaCl solution; n=5 mice). Results showed a significant difference between the 2 groups 24 hours after tDCS (ie, day 6 after stroke; ANA-12: 10.4±0.70% versus vehicle: 6.68±0.74%; P<0.001; 2-way RM ANOVA, Bonferroni post hoc; Figure 6D). Expectedly, amelioration of motor deficits was observed in vehicle-injected mice (P=0.017; day 6 versus 72 hours after stroke), whereas it was fully prevented in ANA-12 mice (P=0.74; day 6 versus 72 hours after stroke; Figure 6D).

Additionally, TotCoh was evaluated 1 week after tDCS in stroke mice treated with ANA-12 (n=6) and compared with results obtained at the same time point in the other experimental groups (same mice as in Figure 2 and Figure S1). Statistical comparison revealed significant difference between ANA-12–treated mice and tDCS-stroke mice (P=0.025; 2-way ANOVA, Duncan post hoc; Figure 6E and Figure S2). No significant differences were observed between ANA-12–treated mice and sham-stroke mice (P=0.40; Figure 6E and Figure S2), thus demonstrating that blockade of TrkB receptor hindered tDCS effect on connectivity.

Collectively, data obtained in ANA-12–treated mice highlight the crucial role of BDNF in mediating tDCS effects at functional, structural, and molecular levels.

We also investigated possible effects of tDCS in neovascularization, which plays an important role in stroke recovery.[2] Interestingly Western immunoblot analysis, showed significantly higher expression of VEGF (vascular endothelial growth factor)-A, in tDCS mice compared with sham-stroke mice, 24 hours after the end of the stimulation protocol (+29.0%; P=0.046; n=6 mice for each group; unpaired Student t test; Figure S3A). No changes in the expression of VEGF-C were, instead, found following tDCS (P=0.35; n=6 mice for each group; unpaired Student t test; Figure S3B). To evaluate neovascularization a cohort of sham-stroke and tDCS-stroke mice (n=3, for each group) received bromodeoxyuridine (100 mg/kg, single daily injections from day 3 to day 6 after stroke) to label recently proliferated cells; mice were killed 24 hours after tDCS or sham stimulation to evaluate immunoreactivity for bromodeoxyuridine and CD31, a marker of endothelial cells (details in Supplemental Methods). Results showed increased number bromodeoxyuridine$^+$/CD31$^+$ cells following tDCS treatment (43.6±6.0% over total bromodeoxyuridine$^+$ cells in tDCS-stroke mice versus 27.9±3.6% in sham-stroke mice; P=0.009; n=3 mice for each group; unpaired Student t test; Figure S3C). Collectively, these data support an effect of tDCS in neovascularization.

DISCUSSION

There is an urgent need to develop new therapeutic strategies to treat neurological sequelae in stroke survivors. We demonstrated that single daily sessions of bihemispheric tDCS, applied from day 3 to day 5 after stroke, induced a faster motor recovery of skilled and nonskilled performances, recruiting plasticity mechanisms that

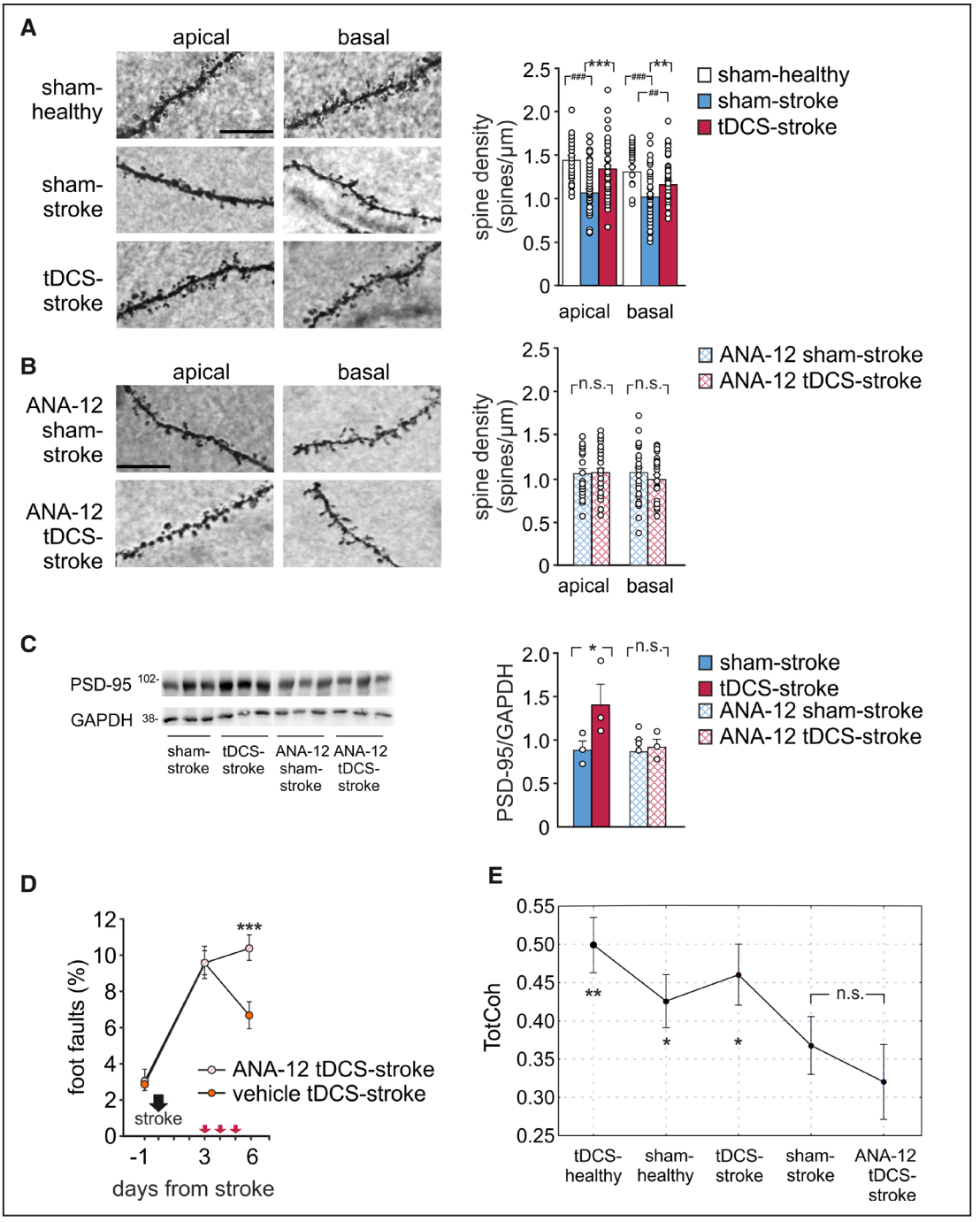

Figure 6. Transcranial direct current stimulation (tDCS) increases dendritic spine number and postsynaptic density in the motor cortex. **A**, Representative images of apical and basal dendrites analyzed in layer II/III pyramidal neurons of the peri-infarct area of sham-healthy, sham-stroke, and tDCS-stroke mice. Bar graph shows mean spine density values in the different experimental groups. **B**, Representative images of apical and basal dendrites in layer II/III pyramidal neurons of sham-stroke and tDCS-stroke mice treated with ANA-12. Bar graph shows mean values of spine density in all neurons examined. Scale bars: 10 µm. **C**, Western blots and bar graph of densitometric analysis revealing higher levels of PSD (postsynaptic density)-95 in tDCS-stroke mice that were not observed after ANA-12 treatment. **D**, Percentage of foot faults in the grid-walking test in mice subjected to tDCS and treated with ANA-12 or vehicle (red arrows) from day 3 to day 5 after stroke. **E**, Graph showing overall total magnitude squared coherence (TotCoh) in ANA-12 tDCS-stroke mice (n=6) compared to data obtained at the same time point in all the other experimental groups (same groups as in Figure 2; main effect of group, $F_{4,43}$=2.97, P=0.029; 2-way ANOVA, Duncan post hoc); asterisks indicate significant differences vs ANA-12–treated mice. Data are expressed as mean±SEM. n.s indicates not significant. *P<0.05, **P<0.01, ***P<0.001. In **A**: ##P<0.01, ###P<0.001 vs sham-healthy.

crucially involve the neurotrophin BDNF, which has been previously implicated in both protection and recovery following stroke in humans and animal models.[27–29]

The translational potential of our results is supported by a recent study on a small cohort of patients who showed increased rates of upper limb recovery following

bihemispheric tDCS over the motor cortex starting 48 to 72 hours after stroke onset.[30]

We recently showed that repeated anodal tDCS sessions over the motor cortex promoted motor cortex plasticity and enhanced motor performance in healthy mice.[12] Here we chose bihemispheric stimulation with the anode over the lesioned motor cortex and the cathode over the contralateral side, which should combine the effect of anodal stimulation, increasing excitability and favoring plasticity, and the effect of cathodal stimulation, decreasing the heightened contralesional inhibitory drive that is known to negatively impact motor recovery.[4,14,31]

In this scenario, to target specific functional networks rather than specific areas, it is necessary to understand how (or even whether) brain networks respond during or after application of tDCS. With regard to tDCS, the notion that stimulation effects are brought about by increases/decreases in activation of the stimulated area has been enriched by studies showing that tDCS effects are not restricted to the stimulated sites.[32] This is understandable given that brain areas which are remote anatomically, but functionally connected to the stimulated ones, can be activated by the same stimulus.

Our results showed that tDCS accelerates motor recovery. Indeed, performances of tDCS mice in both skilled and nonskilled motor tasks were significantly higher compared to those of sham-stimulated mice as early as 24 hours after stimulation (ie, 6 days after stroke). The considerable rate of motor recovery promoted by tDCS has potentially relevant clinical implications. Patients would benefit not only from the obvious early motor outcomes but also from the associated positive emotional impact that might prevent or ameliorate poststroke psychological side effects often responsible for delayed or partial recovery with chronic disability and increased mortality.[33] Additionally, early tDCS might represent a unique opportunity to avoid delaying rehabilitation to the chronic phase for those patients who cannot start physiotherapy in the subacute phase. Of equal importance, if motor recovery can be anticipated, tDCS would also reduce excessively long bed-ridden hospitalization periods with associated complication risks, including co-morbidity and increased mortality.

However, it is important to highlight some limitations of our experimental model when translating our findings into clinical application. Regarding tDCS, rodent epicranial stimulation uses higher currents and is regarded as a hypersensitive model, compared with human montages, being the current density across the mouse brain greater than human tDCS (ie, ≈7A/m^2 versus 0.1 A/m^2 in humans). Concerning the experimental stroke model, we used the photothrombotic ischemia, which has several advantages, including low invasiveness, high reproducibility of the lesion, and low mortality, but it does not fully recapitulate all features of human ischemic stroke.[34] Also further studies are needed to confirm the efficacy of tDCS treatment in promoting poststroke recovery in aged mice, which reportedly show differences in recovery mechanism and BDNF activity compared to younger animals.[35]

Of note, here, we provide greater and novel understanding of the cellular and molecular mechanisms behind tDCS effects that might be exploited to improve the efficacy of future therapies. Histopathologic analysis showed that 6 days after stroke, both tDCS and sham-stimulated mice had recovered from brain edema, in agreement with previous works,[17,20] and displayed similar infarct size. However, in line with large body of clinical and experimental evidence indicating that spontaneous and therapy-driven motor recovery after stroke depends on neuroplasticity and reorganization of motor cortical networks,[4,5,9] we found that tDCS increased cortical connectivity and structural plasticity, as revealed by changes in dendritic spine density. In particular, we performed LFP recordings to investigate relationships between functional improvement and electrophysiological changes in vivo focusing on TotCoh as a measure of connectivity, since its alterations or restoration have been previously linked to stroke behavioral deficits and recovery, respectively.[23] In keeping with this, we found that functional coupling between primary motor and somatosensory cortices of both hemispheres was decreased at all frequency bands and time points in stroke mice and, more importantly, tDCS significantly increased connectivity. Particularly, tDCS restored TotCoh to values similar to those observed in sham-healthy mice and further increased this parameter in healthy mice, suggesting that a structural network reorganization occurs following tDCS. Several works investigated tDCS and its capacity to induce changes in cortical electroencephalogram oscillations, suggesting that motor recovery might be enhanced by early stimulation that seeks to increase functional connectivity of motor relays and pathways.[36,37] Of note, changes in TotCoh occurred as early as 1 week after the end of stimulation protocol (Figure 6E and Figure S1), and no significant changes were observed within stroke groups over the 4-week follow-up time. On this basis, it can be argued that changes in TotCoh are early events in the recovery process triggering tDCS-dependent improvement at behavioral level. Increased TotCoh following tDCS might correlate with the timing of recovery; indeed, higher speed of motor improvement was consistently observed in all behavioral tests in tDCS-stimulated stroke mice, whereas sham-stimulated stroke mice showed slower spontaneous recovery accompanied by lower values of TotCoh. Our contention is in agreement with recent clinical studies,[19,38–41] demonstrating correlations between functional abnormalities of brain networks and poststroke clinical outcomes and suggesting that connectivity changes at baseline (ie, in days immediately following stroke) could be used as a predictive index of stroke recovery (measured by specific scales used clinically in stroke).

One possible substrate for rewiring in the peri-infarct area network could be the local increase of synaptic contacts. Indeed, in line with mounting evidence of tDCS-induced spinogenesis,[12,42] our Golgi-Cox staining of the peri-infarct cortex showed enhanced spine density in both apical and basal dendrites at layer II/III pyramidal neurons following tDCS. tDCS effect was more pronounced at apical dendrites, where it almost restored spine density at value similar to those of healthy mice. Increased expression of the synaptic protein PSD-95 supported the remarkable synaptogenic effect of tDCS that might account for its effect on functional recovery.[43]

Several lines of evidence suggest that neovascularization occurs around the infarct area and facilitates reestablishment of normal blood flow, promotes axonal sprouting, synaptogenesis, and dendritic/connectivity remodeling, enabling functional recovery.[2] Of note, we found that tDCS increased vascular proliferation in the peri-infarct cortex, and such effect was associated with enhanced expression of VEGF-A, a known mediator of angiogenesis,[44] in line with previous findings.[20] Although more detailed analysis by imaging techniques is needed to demonstrate a direct tDCS effect on regional cerebral blood flow and its contribution to the favorable functional outcomes in our model, increased angiogenesis following tDCS is likely to contribute to reestablishment of blood flow to peri-infarct regions, as demonstrated by other studies.[20,45] Of note, changes of cerebral perfusion after tDCS have been, previously reported in ischemic stroke models as well as in healthy adults as revealed by neuroimaging studies.[46,47]

Our study specifically addressed the possible involvement of the neurotrophin BDNF, which is known to play a key role in functional and structural plasticity.[48] Notably, in preclinical models of stroke intraventricular infusion of BDNF antisense oligonucleotides and local blockade of BDNF induction in peri-infarct cortex blocked behavioral recovery,[49,50] supporting a role of endogenous BDNF in promoting recovery. Additionally, systemic administration of BDNF promoted functional recovery and the underlying mechanisms, including axonal outgrowth, synaptogenesis, and neurogenesis.[29,51,52]

Unfortunately, immediate translation of such findings into clinical settings failed, due to the intrinsic problems of BDNF protein, such as its poor blood-brain-barrier permeability, off-target effects on the peripheral nervous system, and short half-life.[53] Systemic administration of BDNF-inducing ampakine, CX1837, was also successful in promoting behavioral recovery in animal models but, overall, therapeutic use of ampakines has also waned, due to inconsistent performance in clinical trials probably related to their low potency; side effects related to excessive and wide-spread activation of glutamate receptors are also concerns for their clinical use in stroke.[54] To overcome such limitations, given that previous studies, including our own, have shown that BDNF is involved in mediating tDCS effects in many brain areas, including the motor cortex[11,12,24]; here, we evaluated whether such noninvasive tool could promote induction of endogenous BDNF in the peri-infarct cortex, resulting in functional recovery.

We found that 24 hours after tDCS-stroke, mice showed higher BDNF levels in the peri-infarct tissue than sham-stroke mice. Such increase was accompanied by activation of TrkB receptor–dependent signaling pathways, namely, those involving pERK1/2^{Tyr204} and pCaMKII^{Thr286}, whose pivotal role in structural and functional plasticity is well known.[48] tDCS-promoted activation of CaMKII and CREB (cAMP response element–binding protein), a common downstream target of both the ERK and CaMKII pathways, was previously observed in the mouse motor cortex under physiological conditions.[12]

Interestingly, cAMP/CREB pathway has been involved in motor recovery[35,55] and it could be plausibly another possible mediator of tDCS effects in our experimental model. Here we identified a further effector of tDCS, MEF2C^{Ser387}, which has been previously linked to BDNF/ERK1/2 pathway and in addition to a well-known role in neuronal development and survival, also triggers activity-dependent plasticity mechanisms.[26] The third canonical pathway activated by BDNF, involving Akt activation, seems not to be recruited by tDCS in our experimental model. Whether this was related to experimental factors, such as the time window of our analyses, or to true specificity of treatment in activating different BDNF pathways, might warrant future investigation.

Although we cannot exclude the contribution of other pathways, our data clearly demonstrate a main role of BDNF/TrkB signaling in mediating tDCS effects. Indeed ANA-12, which selectively blocks TrkB receptor, prevented tDCS-mediated amelioration of motor deficits in the early subacute phase, as assessed by grid-walking test, providing a causal link between BDNF and tDCS-dependent improvements of functional outcomes. Furthermore, ANA-12 prevented tDCS-dependent increase of pERK1/2^{Tyr204}, pCaMKII^{Thr286}, and pMEF2C^{Ser387}, as well as the increase in spine density, PSD-95, and Tot-Coh, demonstrating their dependence on BDNF/TrkB activation and strongly suggesting the contribution of these pathways/mechanisms in stroke recovery. In addition, data from the literature support also possible link between BDNF/TrKB activation and increased VEGF expression and angiogenesis.[56,57]

Taken together, our results: (1) provide novel evidence that tDCS could be used as an effective and targeted therapeutic approach to boost BDNF-dependent endogenous facilitating mechanisms in stroke recovery, including signaling pathways involved in plasticity as well as changes at dendritic spines and vasculature; (2) further substantiate the role of this neurotrophin in stroke recovery; and (3) identify a favorable time

window for tDCS delivery, namely the early subacute phase after stroke. Such noninvasive approach would overcome limitations of exogenous delivery of BDNF or BDNF-inducing drugs, hopefully leading to an easier and more successful translation of results into clinical settings. Furthermore, we found that following tDCS, BDNF levels were also increased in blood samples, encouraging further studies to address whether such changes could be used as a novel biomarker to probe brain state or to predict the efficacy of treatment and overall poststroke functional recovery.

CONCLUSIONS

Bihemispheric tDCS applied during the subacute phase of stroke accelerates the rate of motor recovery. At cellular and molecular levels, tDCS enhanced BDNF expression and BDNF-dependent signaling pathways resulting in increased structural plasticity and connectivity. Our study opens perspectives for using tDCS as a tool to promote targeted BDNF-mediated neuroplasticity.

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Disclosures

None.

Supplemental Material

Supplemental Methods
Figures S1–S3
Tables S1–S2

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