Molecular Neurobiology (2019) 56:157–173
https://doi.org/10.1007/s12035-018-1067-z

Therapeutic Potential of a Combination of Electroacupuncture and TrkB-Expressing Mesenchymal Stem Cells for Ischemic Stroke

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Received: 4 December 2017 / Accepted: 8 April 2018 / Published online: 22 April 2018

Abstract

We prepared and grafted tropomyosin receptor kinase B (*TrkB*) gene-transfected mesenchymal stem cells (TrkB-MSCs) into the ischemic penumbra and investigated whether electroacupuncture (EA) treatment could promote functional recovery from ischemic stroke. For the behavioral test, TrkB-MSCs+EA resulted in significantly improved motor function compared to that obtained with MSCs+EA or TrkB-MSCs alone. At 30 days after middle cerebral artery occlusion (MCAO), the largest number of grafted MSCs was detected in the TrkB-MSC+EA group. Some differentiation into immature neuroblasts and astrocytes was detected; however, only a few mature neuron-like cells were found. Compared to other treatments, TrkB-MSCs+EA upregulated the expression of mature brain-derived neurotrophic factor (BDNF) and neurotrophin-4/5 (NT4) and induced the activation of TrkB receptor and its transcription factor cAMP response element-binding protein (CREB). At 60 days after MCAO, EA highly promoted the differentiation of TrkB-MSCs into mature neuron-like cells compared to the effect in MSCs. A selective TrkB antagonist, ANA-12, reverted the effect of TrkB-MSCs+EA in motor function recovery and survival of grafted MSCs. Our results suggest that EA combined with grafted TrkB-MSCs promotes the expression of BDNF and NT4, induces the differentiation of TrkB-MSCs, and improves motor function. TrkB-MSCs could serve as effective therapeutic agents for ischemic stroke if used in combination with BDNF/NT4-inducing therapeutic approaches.

Keywords Brain-derived neurotrophic factor · Electroacupuncture · Ischemic stroke · Mesenchymal stem cells · Neurotrophin-4 · Tropomyosin receptor kinase B

Abbreviations

BDNF	Brain-derived neurotrophic factor
BMP	Bone morphogenetic protein
CREB	cAMP response element-binding protein
DAPI	4′,6-Diamidino-2-phenylindole
Dcx	Doublecortin
EA	Electroacupuncture
FGF	Fibroblast growth factor
GFAP	Glial fibrillary acidic protein
MSCs	Mesenchymal stem cells
NeuN	Neuronal nuclei
NTFs	Neurotrophic factors
NT4	Neurotrophin-4/5
TrkB	Tropomyosin receptor kinase B
VEGF	Vascular endothelial growth factor

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s12035-018-1067-z) contains supplementary material, which is available to authorized users.

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Introduction

Stroke is one of the leading causes of death and serious, long-term disability worldwide and has a profound socio-economic impact. Despite extensive research efforts, brain injury due to cerebral ischemia remains a vexing public health problem because of the resultant severe motor and cognitive function deficits. Current therapeutic possibilities for post-stroke

rehabilitation are limited [1, 2]. An attractive therapeutic approach to restore neurological function after stroke is a stem cell-based strategy involving stimulation of endogenous stem cells and exogenous administration [3]. In particular, exogenous administration of mesenchymal stem cells (MSCs) or neural stem cells, which have self-renewal and multipotent-differentiation capabilities, offers great promise in improving functional recovery from stroke [4–7].

MSCs have emerged as promising vehicles to deliver or produce beneficial proteins for therapeutic purposes, because these cells are relatively easy to collect, expand, and maintain for a relatively long time after being transplanted [8, 9]. Transplantation of MSCs has been shown to improve functional recovery after stroke by enhancing endogenous repair via the secretion of trophic molecules such as neurotrophic factors (NTFs) and cytokines [10, 11]. However, transplanted MSCs show poor ability to improve functional recovery in the ischemic brain in terms of long-term survival, migration, and differentiation [5]. To improve the potential of MSCs, it has been suggested that MSCs could be used to treat specific preconditions such as hypoxia/reoxygenation or as genetic carriers of target molecules such as NTFs [8, 12, 13].

Electroacupuncture (EA) is a technique that combines traditional acupuncture with low-level electricity. It has been used for stroke and post-stroke rehabilitation because it is relatively straightforward, cheap, and safe to administer in comparison with other conventional therapies, and has no obvious serious adverse effects [14]. Therefore, both EA treatment and MSC grafting are promising clinical therapies for stroke rehabilitation. Interestingly, MSCs produce a variety of trophic factors (e.g., NTFs) that support the survival of damaged brain tissue, thus playing a key role in the efficacy of transplantation [15–17]. The effects of EA also mediate NTFs in the central nervous system by modulating sensory stimulation [18, 19].

EA may offer an alternative treatment that promotes the expression of NTFs in the brain to enhance functional recovery after stroke [20, 21]. Our previous results have shown that EA enhances the neurological repair mechanisms via brain-derived neurotrophic factor (BDNF), vascular endothelial growth factor (VEGF), and stromal cell-derived factor-1 signaling pathways in ischemic stroke models [22, 23]. Further, EA promotes the recovery of memory function via a mechanism associated with neurotrophin-4/5 (NT4)/tropomyosin receptor kinase B (TrkB) signaling in a vascular dementia model [24].

MSCs can differentiate into mature neural cells in the host brain via alteration of their gene expression profiles in response to the surrounding environment [25]. Here, we show that EA stimulation can increase the levels of BDNF and NT4 in the ischemic brain. Therefore, EA acts as an extrinsic signal to enhance the activity of NTFs in the brain by stimulating specific points on the body surface, subsequently promoting the differentiation of transplanted MSCs. However, dividing, undifferentiated MSCs do not express the BDNF/NT4 receptor TrkB [26]. We hypothesized that EA specifically stimulates the expression of BDNF and NT4 in the brain after ischemic stroke, and that more MSCs could differentiate into mature functional neurons if MSCs would express TrkB, ultimately contributing to restoration of neurological function. Therefore, we attempted to graft MSCs transfected with the BDNF/NT4 receptor TrkB into the ischemic penumbra and to investigate whether EA could induce the grafted MSCs to differentiate into mature neuron-like cells, thus improving functional recovery from ischemic stroke.

Materials and Methods

Experimental Animals

Male C57BL/6 mice (5 and 8 weeks of age) were obtained from DooYeol Biotech (Seoul, Korea). The mice were housed at 22 °C under alternating 12-h light:dark cycles and were fed a commercial diet and allowed tap water ad libitum throughout the study. All experiments were approved by the Pusan National University Animal Care and Use Committee in accordance with the National Institutes of Health Guidelines (Approval No. PNU-2017-1448). Each group consisted of eight mice and all treatments were administered under isoflurane (Choongwae, Seoul, Korea) anesthesia, which was provided using a calibrated vaporizer (Midmark VIP3000; Orchard Park, OH, USA). Mice were randomly divided into six groups: (1) the sham group, (2) the MCAO group, (3) the MCAO+MSC group, (4) the MCAO+MSC+EA group, (5) the MCAO+TrkB (T)-MSC group, and (6) the MCAO+TrkB (T)-MSC+EA group.

Mouse Model of Transient Focal Cerebral Ischemia

Focal cerebral ischemia was induced by occluding the middle cerebral artery (MCA) using the intraluminal filament technique. A fiber-optic probe was affixed to the skull over the MCA for measurement of regional cerebral blood flow using a PeriFlux Laser Doppler System 5000 (Perimed, Stockholm, Sweden). Baseline values were measured prior to internal carotid artery ligation (considered to be 100% flow). Middle cerebral artery occlusion (MCAO) was induced by a silicon rubber-coated 7-0 monofilament (Doccol Corporation, Sharon, MA, USA) in the internal carotid artery and the monofilament was advanced to occlude the MCA. The filament was withdrawn 40 min after occlusion, and reperfusion was confirmed using laser Doppler.

EA Stimulation

Under light isoflurane anesthesia, two stainless-steel needles (0.18 mm in diameter and 30 mm in length) were inserted to a depth of approximately 2 mm at the acupoints corresponding to Baihui (GV20, the midpoint of the line connecting the apexes of both ears on the parietal bone) and Dazhui (GV14, the posterior midline and in the depression below the spinous process of the seventh cervical vertebra) in men and were connected to a Grass S88 electrostimulator (Grass Instrument Co., West Warwick, RI, USA). EA stimulation was performed for 20 min at a frequency of 2 Hz, and output voltage was set at 2 V in accordance with our previous studies [22, 24]. For gene expression profiling, EA treatment was administered once daily for 10 days from day 5 after MCAO. After MSC transplantation, EA treatment was administered thrice a week, and the duration of EA treatment was limited to 10 or 22 days after MCAO. Subjects in the non-EA groups received only light isoflurane anesthesia for 20 min.

Gene Expression Profiling and Real-time Polymerase Chain Reaction

We profiled gene expression at day 15 after the final EA stimulation session according to the experimental methods of our previous study [24]. Eighty-four target genes were analyzed using the Mouse Growth Factors RT^2 Profiler PCR Array in Rotor-Disc 100 format (PAMM-041Z, Hilden, Germany). Differences in gene expression were analyzed using Qiagen's web-based RT^2 Profiler PCR Array Data Analysis software, version 3.5 (http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php). Data were normalized using multiple housekeeping genes and analyzed by comparing the $2^{-\Delta CT}$ values of normalized data. These results were confirmed using real-time polymerase chain reaction (PCR). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a housekeeping control gene and all experiments were performed in triplicate for each sample. Gene expression was quantified by comparing the $2^{-\Delta CT}$ values of normalized data. All primers for SYBR green reactions are listed below:

- *BDNF* forward, 5′- AGGTGAGAAGAGTGATGACC ATCC -3′
- *BDNF* reverse, 5′- CAACATAAATCCACTATCTT CCCC -3′
- *NT4* forward, 5′- CAAGGCTAAGCAGTCCTATGT -3′
- *NT4* reverse, 5′- CAGTCATAAGGCACGGTAGAG -3′
- *GAPDH* forward, 5′- CACCATCTTCCAGGAGCGAG -3′
- *GAPDH* reverse, 5′- CCTTCTCCATGGTGGTGAAG AC -3′

Construction of the pEGFP-C1-mTrkB Plasmid

Total RNA from mouse brain was reverse-transcribed to cDNA for reverse-transcription PCR. Briefly, DNA fragments encoding TrkB were amplified from brain cDNA using the following primer sets: TrkB-EcoRI forward, 5′-CCGGAATTCAATGTCGCCCTGGCTGAAGTG -3′; and TrkB-KpnI reverse, 5′- CGGGGTACCCTAGCCTAGGA TATCCAGG -3′. cDNA was then digested with *EcoR* I and *Kpn* I restriction endonucleases (New England BioLabs, Ipswich, MA, USA) and ligated into the enhanced green fluorescent protein vector (pEGFP-C1; BD Biosciences Clontech, Palo Alto, CA, USA). After transformation, positive clones were picked for mini plasmid preparation, and identified by double restriction enzyme digestion and sequencing. Plasmid purity was evaluated for high transfection efficiency.

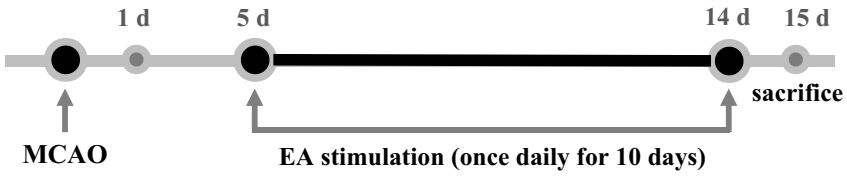
Transfection of MSCs with the *TrkB* Gene

We isolated bone marrow MSCs using a modified version of a previously described method [27]. Passage 2 MSCs were seeded on 6-well plates in a complete culture medium and grown to 70–90% confluence prior to transfection. A transfection mixture containing 7.5 μL Lipofectamine® 3000 (Life Technologies Corporation, Carlsbad, CA, USA) and 2.5 μg pEGFP-C1-mTrkB or empty pEGFP-C1 (mock without DNA) was prepared in 250 μL Opti-MEM (Gibco-Invitrogen, Paisley, UK) and incubated for 15 min at room temperature according to the manufacturer's instructions. The cells were incubated with the transfection mixture for 24 h at 37 °C. The effects of gene overexpression were determined by fluorescence confocal microscopy and western blot experiments at 24 h following transfection. The specific bands were detected using a primary antiserum against TrkB (Abcam, Cambridge, UK), and the signal intensity of each band was normalized to values for β-actin (Sigma-Aldrich, St. Louis, MO, USA).

MSC Transplantation

All MSCs used for transplantation were at passage 3. Five days after MCAO, mice were fixed on a stereotaxic apparatus under general isoflurane anesthesia. Before transplantation, 5 μL/mL Vybrant™ CM-DiI cell-labeling solution (Molecular Probes, Eugene, OR, USA) was used to label MSCs for 20 min. The total volume of MSC suspension (1×10^6/2 μL phosphate-buffered saline [PBS]) was injected at an infusion rate of 0.5 μL/min into the peri-infarct site: anteroposterior, 0.0 mm from the bregma, mediolateral, − 2.5 mm from the midline, and dorsoventral, − 4.0 mm below the dura. Stereotaxic coordinates were chosen for a target area based on *The mouse brain in stereotaxic coordinates* [28]. In

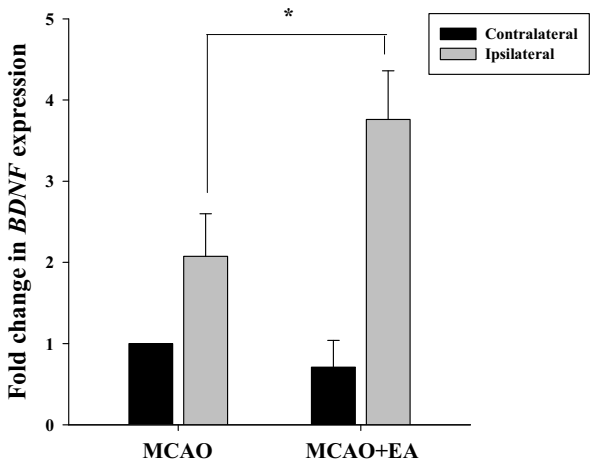
a



b

Gene	Description	Fold upregulation (MCAO+EA vs. MCAO)
BDNF	*Brain derived neurotrophic factor*	**2.62**
NT4	*Neurotrophin 4/5*	**2.53**

c



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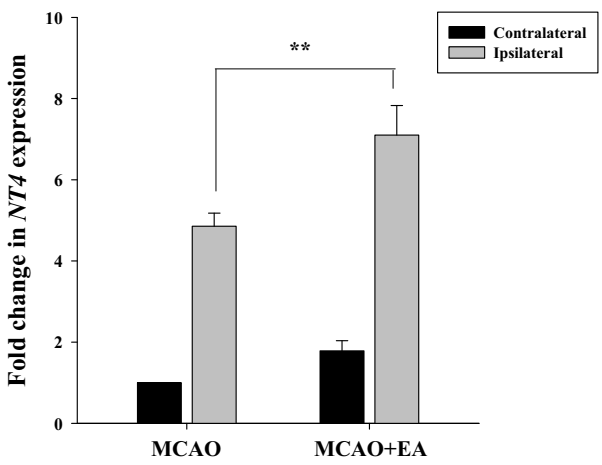

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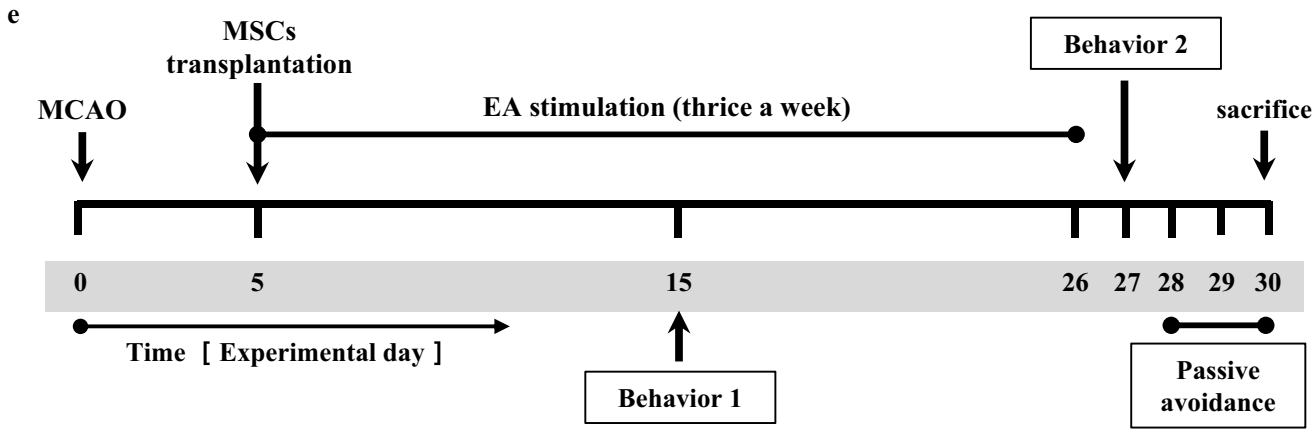

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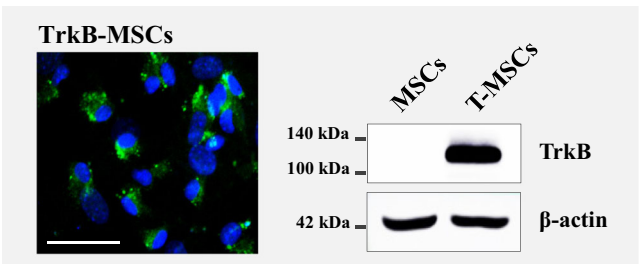


◀ **Fig. 1** **a** Experimental procedures for the RT^2 Profiler PCR Array. All animal experiments were performed on the indicated day. **b** Gene expression profiles in the extracts of the ipsilateral core and penumbra from the MCAO and MCAO+EA groups. Gene expression profiles were evaluated using the Mouse Growth Factors RT^2 Profiler PCR Array. The table shows the selected genes, including *BDNF* and *NT4*, that demonstrated differential expression between MCAO and MCAO+EA groups. Numbers in bold indicate upregulated genes. **c**, **d** Fold change in the expression of the two selected genes as detected by quantitative real-time PCR analysis. Data are expressed as the mean (±SEM). $*P < 0.05$ and $**P < 0.01$ vs. the MCAO group. **e** Schematic diagram for experimental procedures. All animal experiments were performed on the indicated day. **f** At 24 h after transfection, the expression of TrkB in MSCs was evaluated by confocal microscopy (left; green) and western blot analysis (right). Immunoblots were run under the same experimental conditions and β-actin was used as a control. Scale bar = 50 μm

the sham and MCAO groups, 2 μL of PBS was injected instead of cell suspension.

Behavioral Assessment

The corner, cylinder, rotarod, and wire grip tests were performed at days 15 and 27 after MCAO, and a retention trial of the passive avoidance test was performed at day 30 after MCAO. The corner test was used to evaluate neurological function in terms of sensorimotor and postural asymmetries. The mouse was placed between two angled boards facing the corner. When the mouse reached the wedge of the corner, it would then rear and turn either to the right or the left. Ten trials were performed for each mouse, and only turns involving full rearing along either board were recorded. The percentage of ipsilateral turns was calculated. The cylinder test was used to investigate spontaneous forelimb use and locomotor asymmetry. Each mouse was placed inside a transparent cylinder (15 cm in height and 9 cm in diameter). While in the cylinder, the mice were observed rearing to a standing position and exploring the surface wall with one or both forelimbs. We recorded the movement of mice with forelimb use upon first contact against the wall following rearing and during the vertical exploration. A total of 20 movements were recorded during the test. The percentage of non-impaired (ipsilateral) forelimb use was calculated.

Motor coordination and equilibrium were measured using a rotarod apparatus (Panlab S.L.U., Barcelona, Spain). After adaptation trials, each mouse was placed on the rotating rod for three trials per day at a speed of 16 rpm for 3 min and the time that the animal was able to hold itself on the rod was recorded. Vestibulo-motor function was assessed using a modified method of wire grip test. Mice were placed on a thin, horizontal, metal wire (45 cm in length) that was placed between two vertical poles 45 cm above a protective padding. Then, the mice were allowed to traverse the wire for 60 s. The wire grip score was quantified using the following 4-point scale: grade 0, failure to remain on the wire for less than 30 s; grade 1, failure to hold on to the wire with the fore and hind paws together; grade 2, holding on to the wire with the fore and hind paws but not the tail; grade 3, moving along the wire on fore paws plus tail as well as holding on to the wire using the tail along with the fore and hind paws. An average of three trials was calculated for each mouse on a test day.

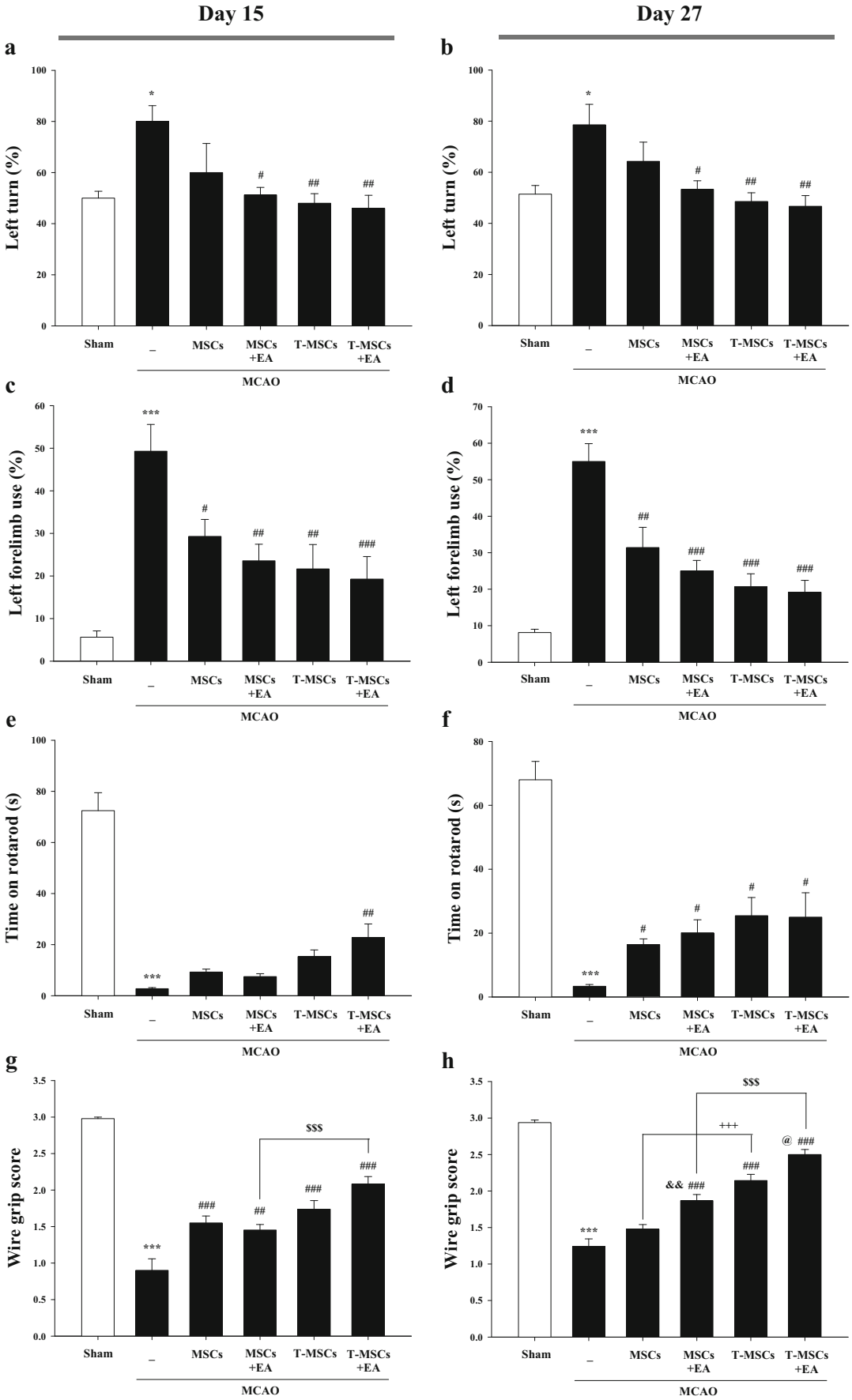
A passive avoidance test was used to assess short-term memory function. The chamber consisted of one illuminated compartment and one dark compartment separated by an automatic guillotine door (Med-Associates, Inc., St. Albans, VT, USA). All animals received daily training in passive avoidance chambers for 3 consecutive days prior to acquisition and retention trials. During acquisition trials, mice that crossed into the dark compartment received a 0.5-mA electric foot shock that lasted 3 s. Twenty-four hours later, a retention trial was administered by placing the mice in the light compartment and recording the step-through latency. Animals that failed to enter the dark compartment within 600 s were assigned a maximum test latency score of 600 s. Results of the experiment were recorded using MED-PC software interfaced with the test apparatus.

Immunohistochemistry

All brain samples were subjected to serial sagittal cryosectioning (section thickness, 25 μm) for each ipsilateral hemisphere (2.0–3.0 mm lateral from the midbrain). Frozen sections were incubated with primary antibodies against sex-determining region Y-box 2 (Sox2; Abcam), doublecortin (Dcx; Abcam), neuronal nuclei (NeuN; Millipore Corporation), glial fibrillary acidic protein (GFAP; DAKO, Glostrup, Denmark), S100A6 (Abcam), S100β (Abcam), mature BDNF (mBDNF; Novus Biologicals, Littleton, CO, USA), NT4 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), phospho (p)-TrkB (Tyr505) (Abcam), and p-cAMP response element-binding protein (CREB, Ser133; Santa Cruz Biotechnology). After incubation with an appropriate fluorescent secondary antibody (Molecular Probes), slides were mounted in the mounting medium with 4′,6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Inc., Burlingame, CA, USA). They were captured using a fluorescence microscope (Carl Zeiss Imager M1; Carl Zeiss, Inc., Gottingen, Germany) and a laser scanning confocal microscope (LSM 510; Carl Zeiss, Inc.).

TrkB Antagonist Study

Mice were randomly distributed into TrkB-MSC+EA and TrkB-MSC+ANA-12+EA treatment groups. For blockade of overexpressed TrkB signaling in MSCs, the specific TrkB


Day 15
Day 27
a
b
c
d
e
f
g
h
Left turn (%)
Left forelimb use (%)
Time on rotarod (s)
Wire grip score
Sham
–
MSCs
MSCs +EA
T-MSCs
T-MSCs +EA
MCAO
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◀ **Fig. 2** TrkB-MSC grafting combined with EA stimulation improves motor function at days 15 and 27 after ischemic stroke ($n = 6–8$). Quantification of the results of the corner test (**a**, **b**), cylinder test (**c**, **d**), rotarod test (**e**, **f**), and wire grip test (**g**, **h**). Data are expressed as the mean (±SEM). $^{*}P < 0.05$ and $^{***}P < 0.001$ vs. the sham group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ vs. the MCAO group; $^{\&\&}P < 0.01$ and $^{+++}P < 0.001$ vs. the MCAO+MSC group; $^{@}P < 0.05$ vs. the MCAO+T-MSC group; and $^{\$\$\$}P < 0.001$ vs. the MCAO+MSC+EA group

antagonist ANA-12 (0.5 mg/kg, i.p.; Tocris Bioscience, Bristol, UK) was administered 1 h prior to each EA session.

Statistical Analyses

All data were expressed as the mean ± standard error of the mean (SEM) and analyzed using the SigmaStat statistical program version 11.2 (Systat Software, San Jose, CA, USA). Statistical comparisons were performed using a one-way analysis of variance (ANOVA) with repeated measures and Tukey's post hoc test of least significant difference. A p value < 0.05 was interpreted as statistically significant.

Results

EA Treatment Upregulates *BDNF* and *NT4* Gene Expression After Ischemic Stroke

We evaluated the expression of growth factor genes in the ipsilateral core and penumbra of the ischemic brain following the final EA stimulation. Using the arbitrary cutoff of greater than twofold change, we selected two genes, namely *BDNF* and *NT4*, among the 84 genes that were significantly changed by EA treatment. The results were confirmed using real-time PCR. We observed a change greater than twofold in the expression of *BDNF* and *NT4* genes in the MCAO+EA vs. the MCAO group (Fig. 1b). Real-time PCR analysis confirmed that EA treatment increased the expression of *BDNF* and *NT4*

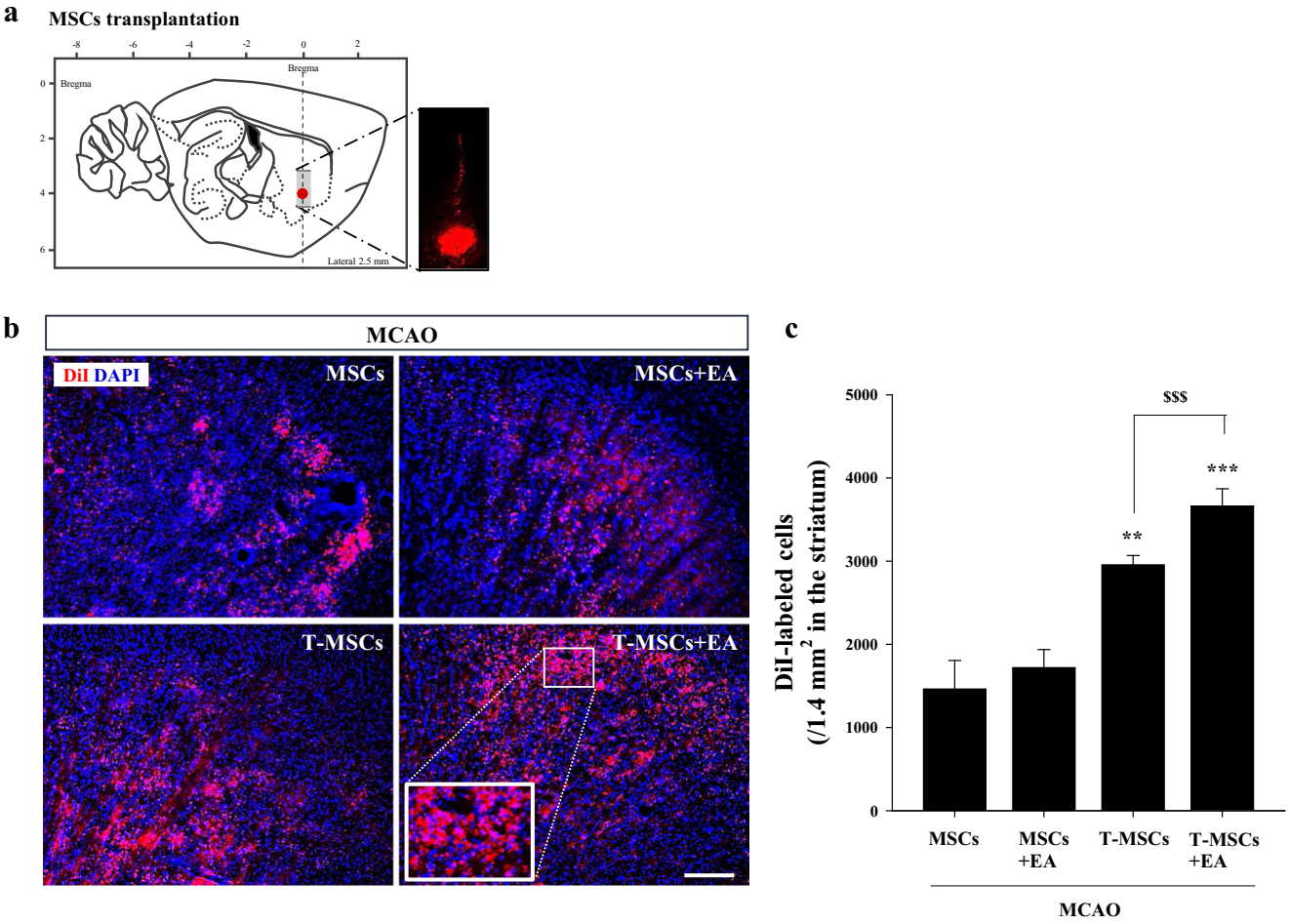
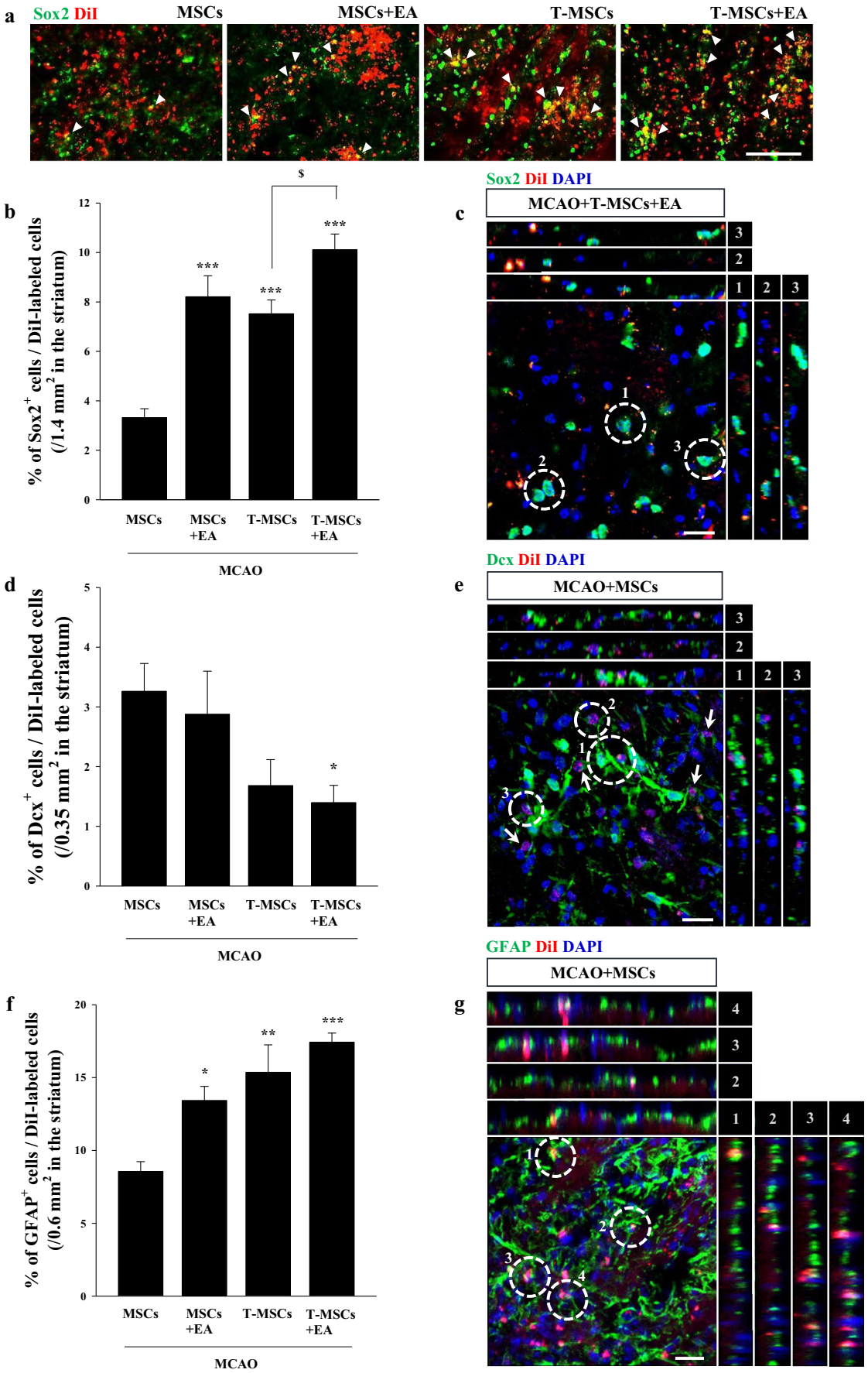


Fig. 3 TrkB-MSC grafting combined with EA stimulation increases the migration of MSCs toward the injured sites at day 30 after ischemic stroke. **a** Transplantation of MSCs into the ischemic penumbra. **b** Detection of MSCs labeled with CM-DiI fluorescent dye in the ipsilateral striatum. Scale bar = 200 μm. **c** Histogram analysis of DiI-labeled MSCs ($n = 5–6$). Data are expressed as the mean (±SEM). $^{**}P < 0.01$ and $^{***}P < 0.001$ vs. the MCAO+MSC group; $^{\$\$\$}P < 0.001$ vs. the MCAO+T-MSC group


a
Sox2 DiI
MSCs
MSCs+EA
T-MSCs
T-MSCs+EA
b
% of Sox2+ cells / DiI-labeled cells (/1.4 mm2 in the striatum)
$
***
***
***
MSCs
MSCs +EA
T-MSCs
T-MSCs +EA
MCAO
c
Sox2 DiI DAPI
MCAO+T-MSCs+EA
d
% of Dcx+ cells / DiI-labeled cells (/0.35 mm2 in the striatum)
*
MCAO
e
Dcx DiI DAPI
MCAO+MSCs
f
% of GFAP+ cells / DiI-labeled cells (/0.6 mm2 in the striatum)
*
**
***
MCAO
g
GFAP DiI DAPI
MCAO+MSCs

◀ **Fig. 4** Transplanted MSCs differentiate into Dcx-positive neuroblasts or GFAP-positive astrocytes in the ischemic penumbra. **a** TrkB-MSC grafting combined with EA stimulation increases the expression of Sox2 in DiI-labeled MSCs at day 30 after ischemic stroke. Scale bar = 100 μm. **b**, **d**, **f** Representative histograms of Sox2-positive ($Sox2^+$), Dcx-positive (Dcx^+), or GFAP-positive ($GFAP^+$) MSCs ($n = 5–6$). Data are expressed as the mean (±SEM). $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ vs. the MCAO+MSC group; $^{\$}P < 0.05$ vs. the MCAO+T-MSC group. **c**, **e**, **g** Confocal fluorescence microscopic image of $Sox2^+$, Dcx^+, or $GFAP^+$ MSCs in the striatum. Scale bar = 20 μm

genes in the ipsilateral site of the ischemic brain in comparison with the expression in the MCAO group (Fig. 1c, d). These data suggest that EA treatment enhances the expression of specific growth factors after cerebral stroke.

TrkB Gene-Transfected MSCs Express TrkB Protein In Vitro

Based on the results of gene expression profiling, we transfected the MSCs with the BDNF/NT4 receptor TrkB. High levels of TrkB were expressed in vitro at 24 h after transfection. TrkB protein levels were detected using western blotting in *TrkB* gene-transfected MSCs,

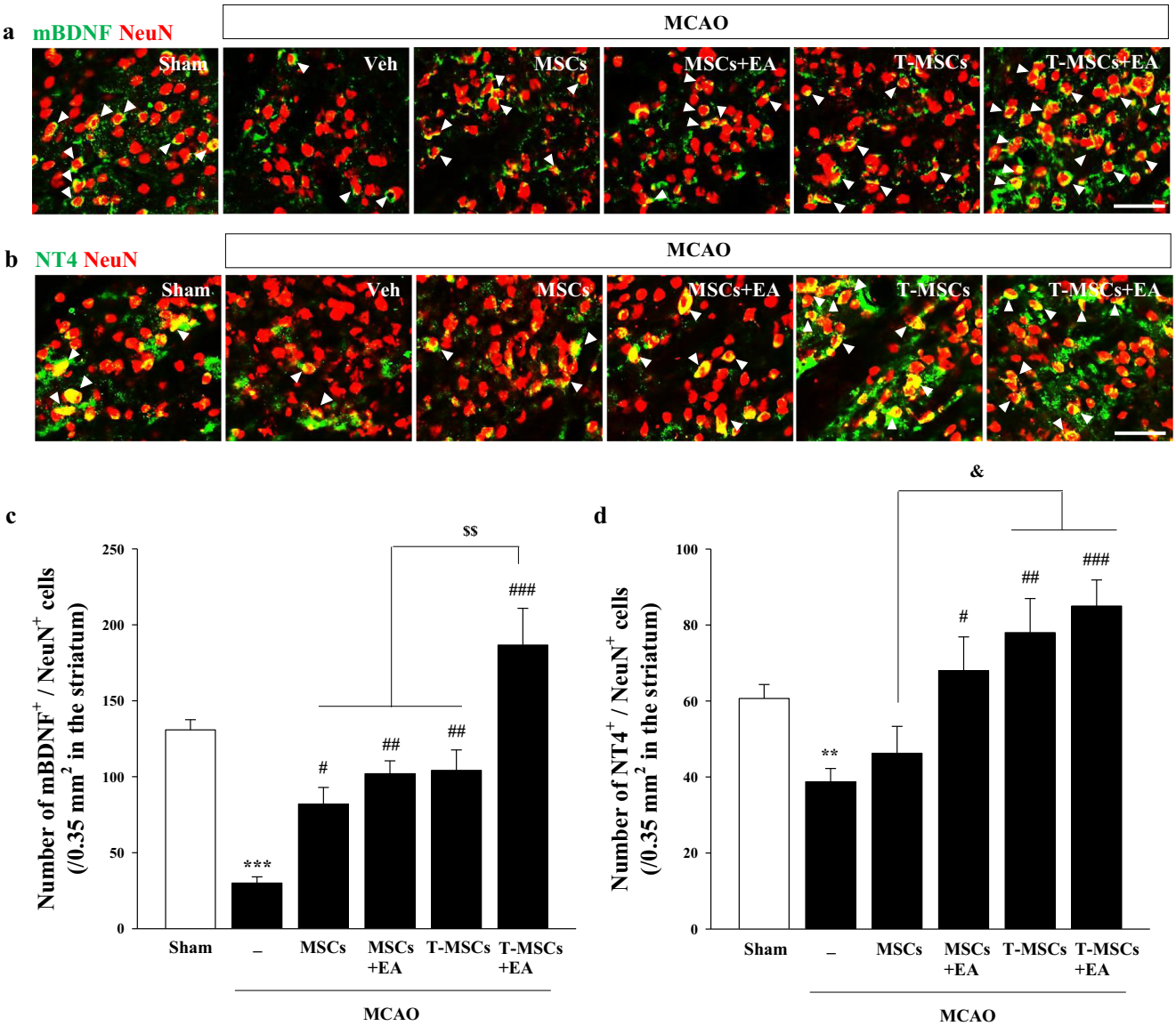


Fig. 5 TrkB overexpression and EA stimulation promote the expression of mBDNF and NT4 in NeuN-positive striatal neurons after cerebral ischemia. **a**, **b** Representative fluorescence microscopic images of mBDNF/NeuN or NT4/NeuN double-labeled cells (arrowheads) in the ipsilateral striatum at day 30 after ischemic stroke. Scale bar = 50 μm. **c**, **d** Numbers of mBDNF/NeuN-positive ($mBDNF^+/NeuN^+$) or NT4/NeuN-positive ($NT4^+/NeuN^+$) cells at day 30 after ischemic stroke ($n = 6–8$). Data are expressed as the mean (±SEM). $^{**}P < 0.01$ and $^{***}P < 0.001$ vs. the sham group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ vs. the MCAO group; $^{\$\$}P < 0.01$ vs. MCAO+MSC, MCAO+MSC+EA, and MCAO+T-MSC groups; and $^{\&}P < 0.05$ vs. the MCAO+MSC group

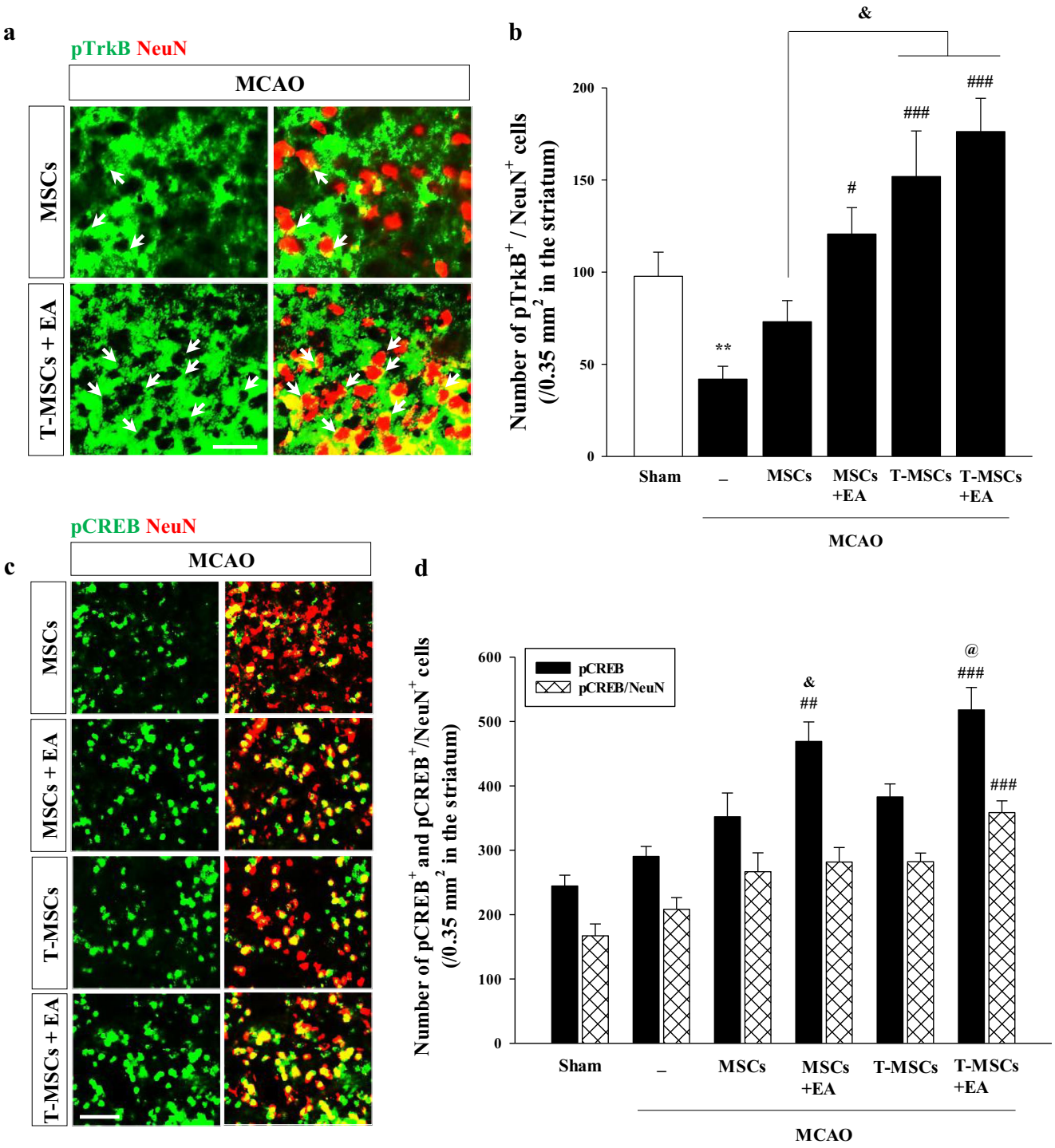


Fig. 6 TrkB overexpression and EA stimulation promote the activation of neurotrophin signaling after cerebral ischemia. **a** Representative fluorescence microscopic images of pTrkB/NeuN double-labeled cells (arrow) in the ipsilateral striatum at day 30 after ischemic stroke. Scale bar = 25 μm. **b** Histogram analysis showing the number of pTrkB/NeuN-positive ($pTrkB^+/NeuN^+$) cells ($n = 6$). Data are expressed as the mean (±SEM). $^{**}P < 0.01$ vs. the sham group; $^{\#}P < 0.05$ and $^{\#\#\#}P < 0.001$ vs. the MCAO group; $^{\&}P < 0.05$ vs. the MCAO+MSC group. **c** Representative fluorescence microscopic images of pCREB/NeuN double-labeled cells in the ipsilateral striatum at day 30 after ischemic stroke. Scale bar = 50 μm. **d** Histogram analysis showing number of pCREB-positive ($pCREB^+$) cells or pCREB/NeuN-positive ($pCREB^+/NeuN^+$) cells ($n = 6$). Data are expressed as the mean (±SEM). $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ vs. the MCAO group; $^{\&}P < 0.05$ vs. the MCAO+MSC group; $^{@}P < 0.05$ vs. the MCAO+T-MSC group

but not in mock MSCs (i.e., without the *TrkB* gene) (Fig. 1f). These data suggest that *TrkB* gene-transfected MSCs (hereinafter TrkB-MSCs) stably expressed TrkB protein in vitro. Therefore, MSCs and TrkB-MSCs were

used for transplantation into the ischemic penumbra of mice in the following experiments.

Combined EA and Grafted MSCs Improve Motor Function After Ischemic Stroke

Both corner (Fig. 2a, b) and cylinder (Fig. 2c, d) tests showed that MCAO mice developed movement disorders in comparison with sham mice. Mice in the MSC+EA and TrkB-MSC+EA groups showed an improvement in movement at days 15 and 27 after MCAO, but there was no significant difference between the two groups. In the rotarod test, only the TrkB-MSC+EA group showed a significant improvement in motor coordination compared to the MCAO group at day 15 after MCAO (Fig. 2e), whereas all groups with grafted MSCs—alone or combined with EA treatment—showed improved motor function compared to that in the MCAO group at day 27 after MCAO (Fig. 2f). In the wire grip test, MSC+EA and TrkB-MSC+EA groups significantly showed improved motor function at days 15 and 27 after MCAO (Fig. 2g, h). In particular, the TrkB-MSC+EA group showed a significant change in functional recovery as compared with the MSC+EA group at days 15 and 27 after MCAO. In the passive avoidance test, no significant inter-group differences in the step-through latency were observed during the acquisition trials, but a significant effect was observed in the retention trials. MCAO mice exhibited a significantly shorter step-through latency than sham mice in the retention trials on day 30 after ischemic stroke. MSC+EA and TrkB-MSC+EA groups showed significantly longer latencies than MCAO mice in these same trials. However, there was no significant difference between the two groups (Fig. S1). Taken together, these behavioral studies indicate that EA treatment may improve motor and cognitive functions by supporting grafted MSCs after ischemic brain injury.

EA Treatment Increases the Survival and Migration of Grafted TrkB-MSCs to Injured Sites After Ischemic Stroke

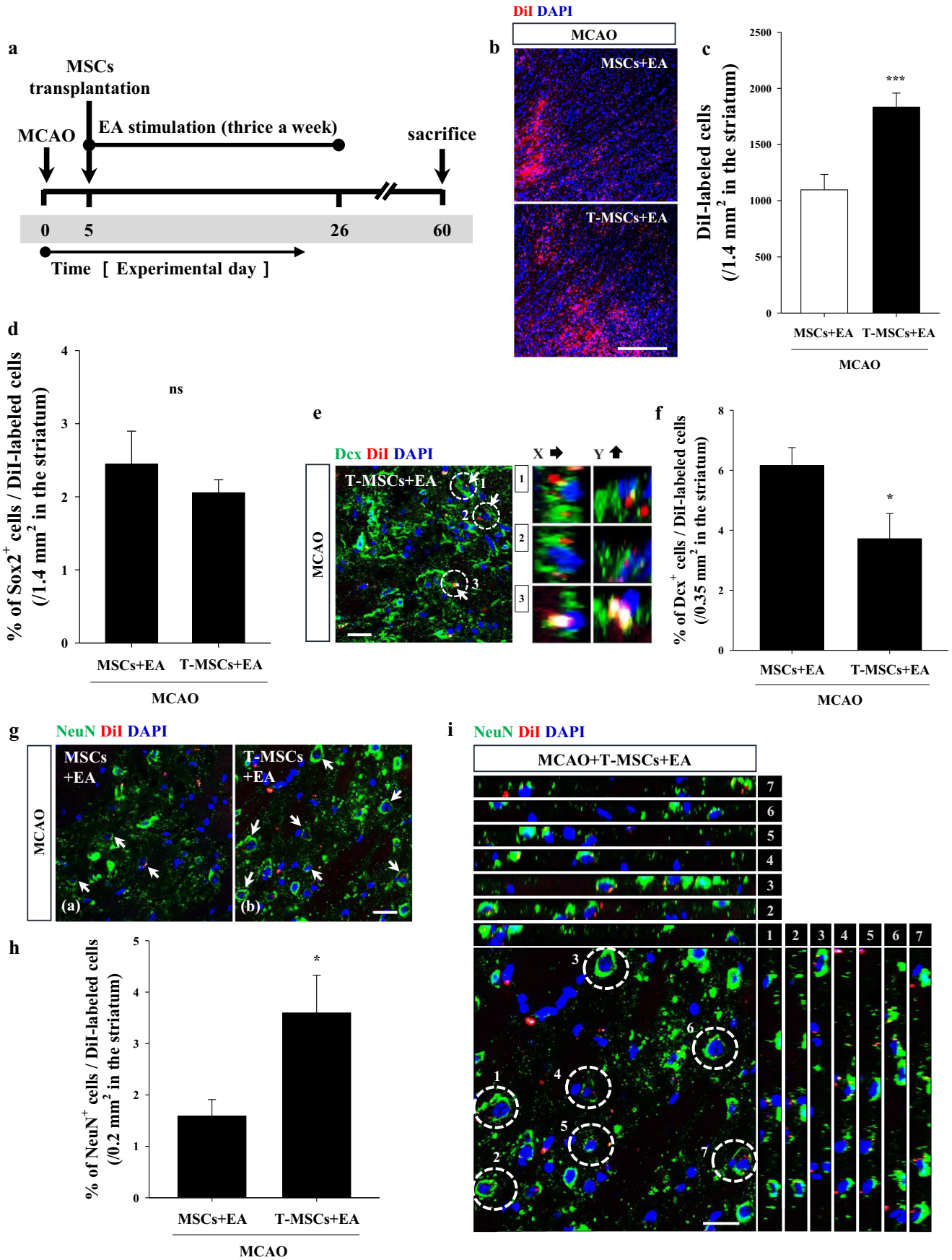
MSCs or TrkB-MSCs were transplanted into the ischemic penumbra (lateral 2.5 mm from bregma) after MCAO as shown in Fig. 3a. At day 30 after MCAO, we detected DiI-labeled MSCs in the ipsilateral striatum using fluorescence microscopy (Fig. 3b). TrkB-MSCs showed significantly higher survival and migration to injured sites than MSCs. The survival and migration of TrkB-MSCs was even higher in the TrkB-MSC+EA group (Fig. 3c). These results indicate that TrkB-MSC survival and migration to injured sites are further promoted by EA treatment.

EA Treatment Promotes Differentiation of Grafted MSCs into Neuron- or Astrocyte-Like Cells

In order to evaluate the effect of EA treatment on MSC differentiation, we stained brain tissues with markers for neural stem cells (Sox2), immature neuroblasts (Dcx), mature neurons (NeuN), and astrocytes (GFAP). MSC differentiation was observed under a confocal microscope (Fig. 4). Immunopositivity was expressed as the percentage of marker-positive cells to DiI-positive cells. First, Sox2/DiI-positive MSCs were found in the ipsilateral striatum of all the MSC groups at day 30 after MCAO. MSC grafting combined with EA treatment increased the percentage of Sox2/DiI-positive cells in comparison with that in the MSC group. For example, the TrkB-MSC+EA group showed significantly more Sox2/DiI-positive cells than the TrkB-MSC group (Fig. 4a, b), which was confirmed using confocal microscopy (Fig. 4c). Dcx/DiI-positive cells were observed in all the MSC groups (Fig. 4d, e), and the percentage of neuroblast-like cells was lower in the TrkB-MSC+EA group than in the MSC group. However, MSCs differentiated into neuron-like (NeuN/DiI-positive) cells were rarely found at day 30 after MCAO in the TrkB-MSC+EA group (Fig. S2a). Finally, GFAP/DiI-positive cells were observed in all the MSC groups, and their number tended to show a gradual increase in other groups (MSC+EA < TrkB-MSC < TrkB-MSC+EA group) in comparison with that in the MSC group (Fig. 4f, g). To confirm the mature development of GFAP-expressing MSCs, we used a specific astrocyte precursor marker (S100A6) and a mature astrocyte marker (S100β). The percentage of cells expressing these markers tended to be higher in the TrkB-MSC+EA group compared to the MSC group, but the differences were not statistically significant (Fig. S2b–e). Results showed that grafted MSCs were mainly neural stem- or astrocyte-like cells; MSCs differentiated into neuroblast-like cells at day 30 after MCAO, with only a few mature neuron-like cells being observed in the TrkB-MSC+EA group. These data suggest that EA treatment promotes differentiation of grafted MSCs into neuron- or astrocyte-like cells, while it is not easy to support the differentiation into mature types at day 30 after ischemic stroke.

TrkB Overexpression Combined with EA Treatment Promotes the Activation of BDNF/NT4/TrkB Signaling After Ischemic Stroke

The expressions of BDNF and NT4 were evaluated in the ipsilateral striatum. At day 30 after MCAO, the number of mBDNF-positive cells in neurons was increased in all the MSC groups compared to that in the MCAO group; in particular, the expression was the highest in the TrkB-MSC graft combined with EA treatment (Fig. 5a, c). The number of NT4-positive cells in neurons showed a significant increase in the TrkB-MSC and TrkB-MSC+EA groups in comparison with that in the MSC group (Fig. 5b, d). Moreover, the number of


a
MSCs transplantation
MCAO
EA stimulation (thrice a week)
sacrifice
0
5
26
60
Time [Experimental day]
b
DiI DAPI
MCAO
MSCs+EA
T-MSCs+EA
c
DiI-labeled cells (/1.4 mm2 in the striatum)
***
MSCs+EA
T-MSCs+EA
MCAO
d
% of Sox2+ cells / DiI-labeled cells (/1.4 mm2 in the striatum)
ns
MSCs+EA
T-MSCs+EA
MCAO
e
Dcx DiI DAPI
X
Y
MCAO
T-MSCs+EA
f
% of Dcx+ cells / DiI-labeled cells (/0.35 mm2 in the striatum)
*
MSCs+EA
T-MSCs+EA
MCAO
g
NeuN DiI DAPI
MCAO
MSCs +EA
T-MSCs +EA
(a)
(b)
h
% of NeuN+ cells / DiI-labeled cells (/0.2 mm2 in the striatum)
*
MSCs+EA
T-MSCs+EA
MCAO
i
NeuN DiI DAPI
MCAO+T-MSCs+EA

◀ **Fig. 7** TrkB-MSC grafting combined with EA stimulation promotes the differentiation of MSCs into Dcx- or NeuN-positive cells in the ischemic penumbra as well as motor function improvement at day 60 after cerebral ischemia. **a** Schematic diagram for experimental procedures. All animal experiments were performed on the indicated day. **b** Detection of MSCs labeled with CM-DiI fluorescent dye in the ipsilateral striatum at day 60 after ischemic stroke. Scale bar = 200 μm. **c** Histogram analysis of DiI-labeled MSCs (n = 5–7). Data are expressed as the mean (±SEM). ***$P < 0.001$ vs. the MCAO+MSC+EA group. **d** Representative histogram of Sox2-positive ($Sox2^+$) MSCs (n = 6–7). **e** Representative confocal fluorescence microscopic images of Dcx-positive (Dcx^+) MSCs (arrow) in the MCAO+T-MSC+EA group and **f** histogram of Dcx^+ MSCs (n = 6). Scale bar = 20 μm. Data are expressed as the mean (±SEM). *$P < 0.05$ vs. the MCAO+MSC+EA group. **g** Representative confocal fluorescence microscopic images and **h** histogram of NeuN-positive ($NeuN^+$) MSCs (arrow) (n = 6). Scale bar = 20 μm. Data are expressed as the mean (±SEM). *$P < 0.05$ vs. the MCAO+MSC+EA group. **i** Magnified image of **g**(b)

mBDNF-positive cells in the subventricular zone (SVZ) was higher in the TrkB-MSC group vs. the MSC group, and in the TrkB-MSC+EA group vs. the MSC+EA group. Notably, the number of mBDNF/NeuN-positive cells was the highest in the TrkB-MSC+EA group (Fig. S3a, b). On the other hand, the number of NT4-positive cells in the SVZ was increased only in the TrkB-MSC+EA group in comparison with that in the MCAO group (Fig. S3c, d). The activation of BDNF/NT4 receptor (TrkB) was confirmed using a specific antibody against pTrkB. The number of pTrkB-positive cells in neurons significantly increased in the TrkB-MSC groups—with or without EA treatment—in comparison with that in the MSC group (Fig. 6a, b). In the SVZ, the number of pTrkB/NeuN-positive cells only increased in the TrkB-MSC+EA group in comparison with that in the MCAO group (Fig. S4a, b).

Neurotrophin signaling (BDNF/NT4/TrkB) leads to phosphorylation of the transcription factor CREB. At day 30 after MCAO, the total number of pCREB-positive cells was significantly higher in the two EA treatment groups (MSC+EA and TrkB-MSC+EA) than in the MCAO group, but there was no significant difference between the two former groups (Fig. 6c, d). Importantly, the number of pCREB-positive cells in neurons only increased in the TrkB-MSC+EA group. In the SVZ, the number of pCREB/NeuN-positive cells similarly significantly increased in the TrkB-MSC+EA group (Fig. S4c, d). pTrkB and pCREB were also expressed in grafted MSCs (Fig. S4e). These data indicate that EA treatment in the TrkB-MSC group may promote grafted MSC survival and differentiation after ischemic stroke via a BDNF/NT4-TrkB-CREB mechanism.

TrkB Overexpression Combined with EA Treatment Promotes the Differentiation of Grafted MSCs into Mature Neuron-Like Cells

At day 30 after MCAO, transplanted MSCs did not fully differentiate into mature neuron-like cells. To confirm the differentiation status of MSCs in vivo, we compared the MSC+EA and TrkB-MSC+EA groups at day 60 after MCAO (Fig. 7a). The number of DiI-labeled MSCs was significantly higher in the TrkB-MSC+EA group than in the MSC+EA group (Fig. 7b, c). However, the percentage of Sox2/DiI-positive cells was not significantly different between the two groups (Fig. 7d). The percentage of Dcx/DiI-positive neuroblast-like cells was reduced in the TrkB-MSC+EA group compared to that in the MSC+EA group (Fig. 7e, f). Importantly, the percentage of NeuN/DiI-positive mature neuron-like cells was significantly higher in the TrkB-MSC+EA group than in the MSC+EA group at day 60 after MCAO (Fig. 7g–i). These data suggest that TrkB overexpression combined with EA treatment may promote the differentiation of grafted MSCs into mature neuron-like cells.

Blocking TrkB Inhibits the Beneficial Effects of TrkB Overexpression Combined with EA Treatment

To evaluate the specific effect of TrkB overexpression and EA treatment on behavioral functions as well as on MSC survival and migration, we employed a potent TrkB antagonist, ANA-12, and analyzed the results at day 15 after MCAO (Fig. 8a). While ANA-12 administration produced no significant behavioral changes in the corner and cylinder tests (Fig. 8b, c), this antagonist attenuated the motor function recovery induced by TrkB overexpression and EA stimulation in the rotarod and wire grip tests (Fig. 8d, e). Consistent with the impairment of motor function, ANA-12 administration reduced the survival and migration of DiI-labeled MSCs in the ipsilateral striatum (Fig. 8f, g). Besides, the percentage of Sox2/DiI-positive cells was significantly reduced in the TrkB-MSC+ANA+EA group (Fig. 8h, i). These data suggest that TrkB signaling may be involved in the mechanisms of the EA-mediated motor function improvement via the migration of surviving MSCs to injured tissues following ischemic stroke.

Discussion

Stem cell-based therapies for ischemic stroke are considered to be promising because of the diverse roles of MSCs in brain disorders [29]. However, their therapeutic efficacy and underlying mechanisms remain to be elucidated. Moreover, strategies to improve the poor survival and differentiation of MSCs after transplantation are still under investigation. Here, we present evidence that EA treatment promotes the secretion of BDNF and NT4 in the brain, working through their common receptor, TrkB. We used genetically modified *TrkB* gene-transfected MSCs (TrkB-MSCs) in a mouse model of ischemic stroke. EA treatment promoted the survival, migration, and neural differentiation of TrkB-MSCs by modulating the expression of both BDNF and NT4, ultimately enhancing functional improvement after stroke.

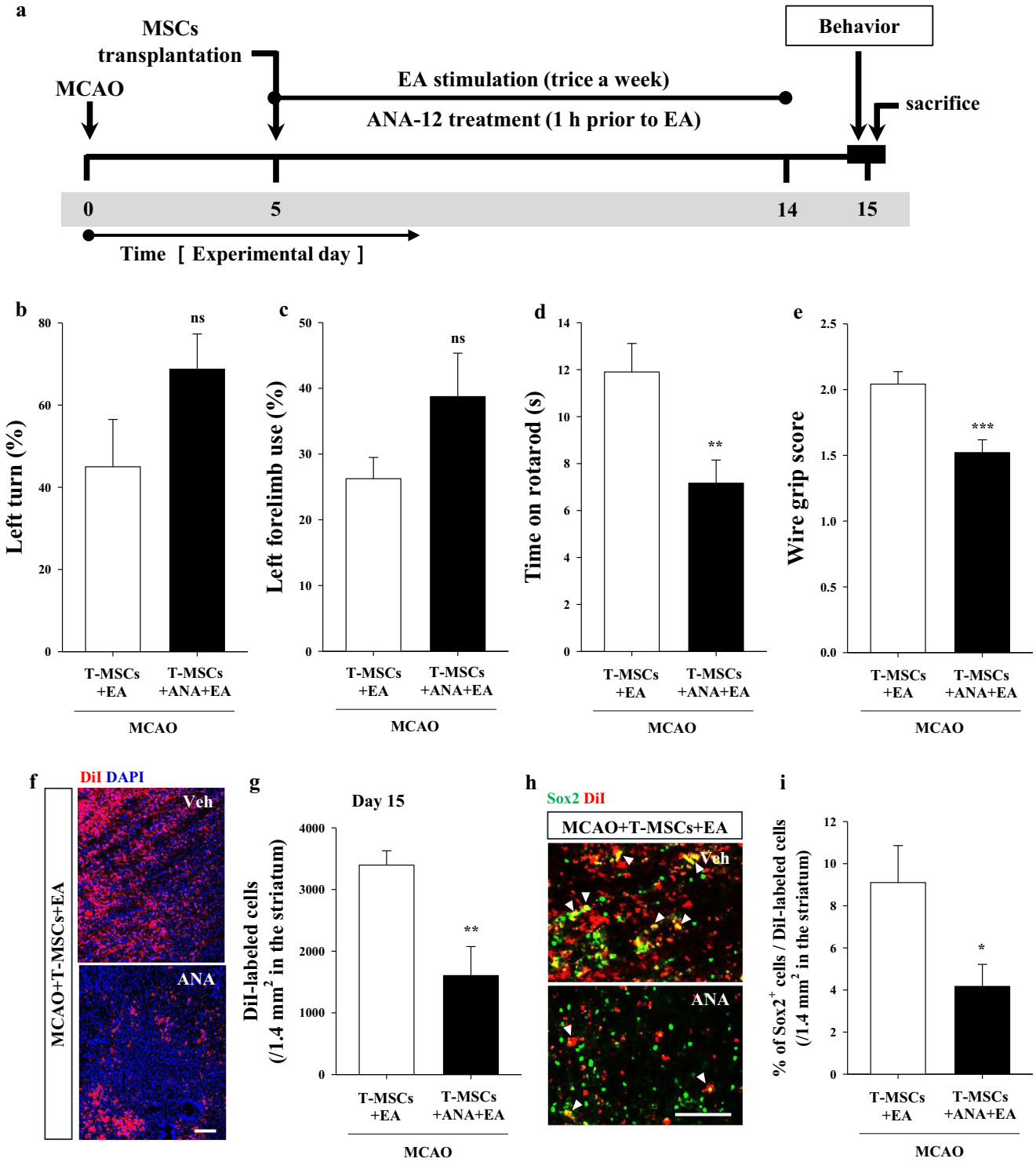


Fig. 8 TrkB antagonist ANA-12 inhibits the beneficial effects of EA at day 15 after ischemic stroke ($n = 8$). **a** Schematic diagram for experimental procedures. All animal experiments were performed on the indicated day. Quantification of the results of the corner test (**b**), cylinder test (**c**), rotarod test (**d**), and wire grip test (**e**). $^{**}P < 0.01$ and $^{***}P < 0.001$ vs. the MCAO+T-MSC+EA group. **f** Detection of MSCs labeled with CM-DiI fluorescent dye in the ipsilateral striatum and **g** histogram analysis of DiI-labeled MSCs ($n = 6$–7). Scale bar = 200 μm. Data are expressed as the mean (±SEM). Scale bar = 100 μm. $^{**}P < 0.01$ vs. the MCAO+T-MSC+EA group. **h** Representative fluorescence microscopic images of Sox2-positive ($Sox2^+$) MSCs in the MCAO+T-MSC+EA group and **i** histogram analysis of $Sox2^+$ MSCs (arrowheads) ($n = 6$–7). Scale bar = 100 μm. Data are expressed as the mean (±SEM). $^{*}P < 0.05$ vs. the MCAO+T-MSC+EA group

EA treatment-induced brain NTFs mediate therapeutic effects in ischemic stroke models [18, 19]. The beneficial effect of EA treatment for functional recovery is associated with the signaling pathways of specific NTFs, including BDNF, VEGF, and NT4 [22, 24]. In this study, we used the optimal duration of EA stimulation at a low frequency of 2 Hz (ten consecutive stimulations from day 5 after MCAO) and confirmed significant expression of *BDNF* and *NT4* genes in the brain among 84 genes. Although the expression levels of some bone morphogenetic protein (BMP) and fibroblast growth factor (FGF) family members showed about twofold upregulation, the results were not consistent between the data sets of the MCAO+EA and MCAO groups (data not shown). BDNF/NT4-mediated TrkB signaling is one of the key regulators of proliferation and neurogenesis in the central nervous system [30–33]. Therefore, we prepared *TrkB* gene-transfected MSCs because undifferentiated MSCs do not express the TrkB receptor [26, 34].

Hence, we grafted MSCs overexpressing TrkB into the ischemic penumbra of the brain and investigated whether EA treatment upregulated BDNF and NT4. A combination of grafted MSCs and EA treatment significantly improved the functional recovery of motor and cognitive deficits compared to the findings for the MCAO group. Transplantation of TrkB-expressing MSCs along with EA treatment particularly enhanced the recovery of motor function in rotarod and wire grip tests compared to the recovery in the other groups. These results show that grafted TrkB-MSCs may promote functional recovery from motor impairments after ischemic stroke through EA-induced upregulation of BDNF and NT4.

Transplantation of MSCs into the brain has been discussed with regard to two aspects: their ability to transdifferentiate into functional neural cells and to produce NTFs [13, 35]. Although transplantation of MSCs improves functional recovery in neurological disorders, the survival rate and cellular replacement of grafted MSCs in the central nervous system are fairly poor [36–38]. Overexpression of the TrkB receptor enhances neuronal survival, axonal growth, and neuronal plasticity [39, 40]. Therefore, we first evaluated the survival, migration, and differentiation of grafted MSCs. Grafted TrkB-MSCs showed higher survival and migration in the ischemic brain than MSCs. This effect was further increased by EA treatment. Our results were confirmed by the percentage of DiI/Sox2 double-labeled MSCs, suggesting that TrkB-MSC survival may be enhanced via EA treatment-induced BDNF/NT4 upregulation.

BDNF induces the neural differentiation of MSCs via TrkB-mediated signaling, and TrkB expression of MSCs elevates the neuronal regeneration [41]. At day 30 after stroke, grafted MSCs had differentiated into lineages of neuron-like (Dcx- or NeuN-positive cells), astrocyte-like (S100A6-, S100β-, or GFAP-positive cells), and oligodendrocyte (platelet-derived growth factor receptor A [PDGFRα]- or 2′,3′-cyclic-nucleotide 3′-phosphodiesterase [CNPase]-positive cells; data not shown) cells in the ischemic striatum. Treatment with either EA or TrkB-MSCs significantly induced the differentiation of MSCs into astrocyte-like cells, but not into oligodendrocyte-like cells (data not shown). Unlike astrocyte-like cells, the number of Dcx-positive neuroblast cells in the TrkB-MSC+EA group was lower than that in the MSC group, but a few mature neuron-like cells (NeuN-positive) were observed in the latter. The differentiation into mature neuron-like cells could be detected in the TrkB-MSC+EA group. Therefore, it is possible that the Dcx-positive neuroblast cells in the TrkB-MSC+EA group may have differentiated into mature neuron-like cells.

Transplantation of MSCs promotes cell proliferation and survival via the secretion of a variety of growth factors and cytokines [13, 42]. Genetically modified grafted MSCs also secrete many specific growth factors that are neurotrophic, and aid in repair of the ischemic brain [43, 44]. The use of grafted TrkC-expressing MSCs with EA treatment activates the expression of NT3 in a spinal cord injury model. In the present study, transplantation of MSCs increased the expression of both mBDNF and NT4 in the ischemic striatum and SVZ. Notably, grafted TrkB-MSCs combined with EA treatment promoted the expression of mBDNF, which was consistent with the phosphorylation levels of the TrkB receptor, while NT4 levels remained unchanged. Supporting these results, previous studies have shown that BDNF can be more effective than NT4 for stroke recovery in vivo because of the different biologic activities of the neurotrophins BDNF and NT4 induced by TrkB receptors and their downstream signals [45, 46].

BDNF/NT4 and their receptor TrkB activate the transcription factor CREB in pathological conditions involving genetic regulation of neuron survival and differentiation [47–49]. Similar to previous data, CREB phosphorylation was increased in the ipsilateral striatal neurons of the TrkB-MSC+EA group. These expression patterns were also similarly observed in the SVZ, which is a known site of neurogenesis and self-renewing neurons in the adult brain. Moreover, expressions of pTrkB and pCREB were also observed in grafted MSCs, suggesting that grafted MSCs might be activated by BDNF/NT4/TrkB signaling.

The differentiation of MSCs into mature neuron-like cells was low at 30 days after MCAO. Therefore, we investigated MSC differentiation after a longer time period (60 days) to confirm whether EA treatment affects the differentiation of TrkB-MSCs into mature neuron-like cells. We clearly found that EA treatment promoted the differentiation of TrkB-expressing MSCs into neuron-like cells as well as their survival. Moreover, the number of Dcx-positive neuroblast cells decreased, similar to the findings noted 30 days after MCAO, indicating differentiation of TrkB-MSCs into mature neurons. Furthermore, to confirm whether TrkB signaling was

associated with the beneficial effects of EA treatment in ischemic stroke, we administered a potent TrkB antagonist, ANA-12 (ANA). EA treatment following TrkB blockade suppressed the EA-induced motor function recovery and survival and migration of grafted TrkB-MSCs.

To summarize, our findings indicated that TrkB-expressing MSCs combined with EA treatment promoted neural survival and differentiation via the stimulation of the BDNF/NT4-TrkB signaling pathway. As a result, stroke-associated motor and cognitive dysfunction was improved. Collectively, EA treatment activates the secretion of specific NTFs such as BDNF and NT4 and promotes TrkB-expressing MSC survival, migration, and differentiation into mature neuronal cells. Our results suggest that EA treatment combined with TrkB-MSCs provides a good treatment option for functional recovery from injuries caused by cerebral ischemia. Other therapeutic techniques that induce the secretion of NTFs are also expected to serve as valid clinical approaches when used in combination with cells genetically modified to express the receptors for those NTFs.

Author Contributions All authors contributed extensively to this work. S.M.A. and B.T.C. conceived and designed the experiments; S.M.A., Y.R.K., and S.Y.L. performed the experiments; S.M.A., Y.I.S., K.T.H., H.K.S., and B.T.C. analyzed the data; and S.M.A. and B.T.C. wrote the manuscript.

Funding Information This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korean government (MSIP) (2014R1A5A2009936). This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Science, ICT and future Planning (2015R1A2A2A03006712).

Compliance with Ethical Standards

The study was approved by the Pusan National University Animal Care and Use Committee in accordance with the National Institutes of Health Guidelines (Approval No. PNU-2017-1448).

Conflict of Interest The authors declare that there are no competing interests.

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