Extremely low frequency electromagnetic stimulation reduces ischemic stroke volume by improving cerebral collateral blood flow.

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Running title: ELF-EMS as ischemic stroke therapy

Supplemental Methods

Permanent Distal Middle Cerebral Artery Occlusion (dMCAO)

Ten-week-old male C57Bl/6J and BALB/c mice were placed in a lateral position under 2% isoflurane anesthesia (IsoFlo, Abbot Animal Health, Belgium). Body temperature of the animals during surgery was maintained using a heating pad. Eye ointment (Duratears®, Alcon, Geneva, Switzerland) was applied to protect the eyes from dehydration. After shaving and disinfecting the surgical area, a vertical skin incision of ± 1cm was made between the ear and the eye of the mouse. Next, the temporal muscle was carefully dissected without removal of the muscle to expose the skull. The temporal bone was thinned at the site of the bifurcation of the middle cerebral artery (MCA) using a microdrill (Stoelting, Dublin, Ireland). The thinned skull and meninges overlying the MCA were carefully removed with surgical forceps. The MCA was permanently occluded by electrocoagulation of the artery proximally to the bifurcation using bipolar coagulation forceps (0.4mm tip; ERBE, Tuebingen, Germany) with the electrosurgical unit (ERBE ICC 50) set at 8 W. The wound was regularly rinsed with 0.9% NaCl solution. Occlusion of the MCA was visually confirmed by checking for discontinuation of the blood flow. The wound was then sutured and disinfected with isobetadine. Mice received 0.05 mg/kg buprenorphine (Temgesic, Val d'Hony Verdifarm, Belgium) subcutaneously as analgesic and were placed in a heating cage at 32°C to recover after surgery. Mice that developed intracranial bleeding during surgery were excluded before the start of the treatment.

Exactly 1 h after dMCAO, mice were anesthetized using ketamine/xylazine (ketamine: 120 mg/kg and xylazine: 10 mg/kg; IP) and placed in a restrainer to receive treatment. Mice were exposed to either sham or ELF-EMS treatment (60 Hz, 13.5 mT) for 20 min over 4 consecutive days. Sham-treated mice were anesthetized and placed in the restrainer under the ELF-EMS device without exposure to the electromagnetic field. After 7 days, mice were sacrificed to analyze infarct size. Sample size was calculated using GPower software (estimated effect size:

0.70, power: 80%). The mortality rate for the assessment of lesion size was 9% in C57Bl/6J mice and 16% in BALB/c mice.

Staining of brain sections with 2,3,5-Triphenyltetrazolium chloride (TTC)

Seven days after dMCAO, C57Bl/6J and BALB/c mice were sacrificed using cervical dislocation. Brains were carefully isolated using scissors and forceps, and 1 mm coronal brain slices were prepared using Double Edge, Stainless Steel blades (Ted Pella, California, USA) and Alto Steel brain matrix (Stoelting, Dublin, Ireland). The slices were stained for 30 min at room temperature using a 2% TTC solution (Sigma-Aldrich, St-Louis, USA). Stained brain slices were scanned and the infarct volume was quantified blinded using Image J (The National Institute of Health, MD, USA).

Laser Speckle Contrast Imaging

Ten-week-old male C57Bl/6J and BALB/c mice were anesthetized using ketamine/xylazine (ketamine: 120 mg/kg and xylazine: 10 mg/kg, IP) and placed in a restrainer. Prior to imaging, a midline incision was made on the scalp to expose the skull. After carefully removing the skin and cleaning the skull using cotton swabs, the cranium was moisturized using light and heavy mineral oils (Sigma-Aldrich, St-Louis, USA) to prevent the skull from drying. The lens was focused on the blood vessels of the entire cerebral cortex. Mice received either ELF-EMS (60 Hz, 13.5 mT) or sham treatment for 20 min. Speckle images were collected at a rate of 50 frames/sec and were acquired before (as baseline) and immediately after each treatment. For the assessment of cerebral blood flow following ischemic stroke, male C57Bl/6J mice were subjected to dMCAO as described above. dMCAO mice were similarly prepared to acquire perfusion maps and were imaged 1 h and 24 h after stroke induction. At both time points, the animals received either sham or ELF-EMS treatment and flux images were acquired pre- and post-treatment. Sample size was calculated using GPower software (estimated effect size: 0.97,

power: 80%). The mortality rate for the experiment including Laser Speckle Contrast imaging of dMCAO C57Bl/6J mice was 19%.

According to the manufacturer's manual (Moor Instruments), LSCI assessment of tissue blood flow (Flux) occurs as follows:

The speckles are mapped over a small grid of detector pixels (typically 5x5) and the contrast is assessed as the standard deviation (SD) of pixel intensities (average pixel intensity = I); SD is low for fast moving speckles (high blood flow) where the image is blurred, and SD is high for slow moving speckles (low blood flow) where the image is not so blurred.

The basic formula for LSCI assessment of tissue blood flow (Flux) is:

$$\text{Flux} \propto \left(\frac{\langle I \rangle}{SD}\right)^2$$

For all experiments, blood fluxes were recorded during 3 min before treatment (as baseline) and 5 min after sham or ELF-EMS application. Speckle images were quantified blinded using moorFLPI Full-Field Laser Perfusion Image Review V6.0 software (Moor Instruments, Axminster, UK). Regions of interest (ROI) were drawn in the left and right hemispheres in baseline and post-treatment images at exactly the same location. Mean flux values were calculated in both hemispheres individually over a time period of 2 min for baseline images and 3 min for post-treatment images. All obtained flux values were normalized to the area analyzed. Flux values acquired from the post-treatment images were normalized to baseline perfusion values. After correcting for baseline perfusion, cerebral blood flow values measured in the ELF-EMS-treated group were normalized to the mean flux value of the sham-treated group.

Laser Doppler Imaging

Ten-week-old male NMRI RjHan mice were anesthetized using an intraperitoneal injection (IP) of ketamine/xylazine (ketamine: 84 mg/kg and xylazine: 10 mg/kg) after which both hind limbs

were either exposed to ELF-EMS (60 Hz, 13.5 mT) or sham treatment for 20 min. Sham or extremely low frequency electromagnetic stimulation (ELF-EMS) application on the left or right hind limb was randomized. Perfusion maps were acquired before (as baseline) and immediately after each treatment. Blood flow was quantified on the basis of colored histogram pixels normalized for the surface analyzed using image analysis software (Laser Doppler Perfusion Measure, version 3.0.8, Moor Instruments). After correcting for baseline perfusion, the mean perfusion measured in the hind limb that received ELF-EMS treatment was normalized to the mean flux value of the hind limb that received sham treatment in each mouse.

Nitrosyl-hemoglobin (Hb-NO) quantification by Electron Paramagnetic Resonance spectroscopy (EPR)

Following Laser Doppler Imaging, venous blood was obtained from vehicle-, L-NAME- and 1400W-treated NMRI RjHan mice by puncture of the right heart ventricle under ketamine/xylazine anesthesia. Samples were collected immediately after the last perfusion measurement and were frozen for EPR measurements of nitrosylated (at heme-FeII) hemoglobin. Samples were stored at -80°C.

Circulating Hb-NO was measured by EPR signal of 5-coordinate-α-Hb-NO in blood of mice with a Magnettech Miniscope (MS400) as previously described[1]. In brief, samples were put in a Quartz Deware filled with liquid nitrogen and placed into the EPR spectrometer. The EPR signal was recorded with the following instrument settings: modulation amplitude= 0,7 mT; BO-Field=329 mT; Mw-attenuation=7 dB; Gain=500. The EPR spectrum for Hb-NO exhibits the well-resolved triplet hyperfine structure (hfs) at g_z-value ~ 2.02. For Hb-NO quantification, the peak-to-peak amplitude of the hfs component was used. EPR spectra were analyzed using ESR MPlot software (Magnettech) and results were normalized to the vehicle-treated group.

Transmission Electron Microscopy (TEM)

HMEC-1 cultured on plastic Thermanox coverslips were prepared for ultrastructural analysis. Immediately or 24 h after application of ELF-EMS for 20 min, cells were fixed with 2% glutaraldehyde and subsequently postfixated in 2% osmium tetroxide for 1 h at 4°C. Samples were dehydrated by consecutive exposure to increasing concentrations of acetone. Impregnation of the samples was performed overnight at room temperature in a 1:1 mixture of acetone with araldite epoxy resin. Thereafter, samples were embedded in araldite at 60°C using the pop-off method[2]. Embedded samples were transferred to 0.7% formvar-coated copper grids (Aurion, Wageningen, The Netherlands) and contrasted using 2% uranyl acetate (Sigma-Aldrich, St-Louis, USA) and lead citrate. TEM images were acquired with a Philips EM208 S electron microscope (Philips, Eindhoven, The Netherlands) equipped with the Morada Soft Imaging System camera and the corresponding iTEM-FEI software (Olympus SIS, Münster, Germany).

Protein Sample Preparation

In all cell experiments, protein samples were prepared by incubating HMEC-1 for 4 h in serum- and phenol red-free Dulbecco's Modified Eagle Medium (DMEM, ThermoFisher Scientific). In case of pathway analyses, LY294002 (20 µM, Sigma-Aldrich) was incubated 30 min prior to ELF-EMS treatment. HMEC-1 were either left unstimulated or stimulated by ELF-EMS for 20 min (60 Hz, 13.5 mT), after which they were immediately lysed on ice with RIPA lysis buffer supplemented with protease (cOmplete ULTRA Tablets, Roche, Basel, Switzerland) and phosphatase inhibitors (Sigma-Aldrich).

Femoral arteries were isolated from 10-week-old NMRI RjHan mice immediately after Laser Doppler imaging. Mice were euthanized by an overdose of ketamine/xylazine after which the femoral arteries were carefully dissected using forceps and surgical scissors. Isolated arteries

were then snap frozen in liquid nitrogen and stored at -80°C. To extract an adequate amount of proteins, 4 femoral arteries were pooled and lysed on ice with RIPA lysis buffer supplemented with protease (cOmplete ULTRA Tablets, Roche) and phosphatase inhibitors (Sigma-Aldrich).

All lysates were centrifuged and stored at -80°C. Protein concentration was determined with the Pierce BCA assay kit according to manufacturer's guidelines.

Cell Culture of HMEC-1

The human microvascular endothelial cell-1 (HMEC-1) line was kindly provided by the Centers for Disease Control and Prevention (Atlanta, GA). HMEC-1 were cultured in MCDB 131 medium (Gibco, Paisley, UK) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Biowest, Riverside, USA), 100 U/ml Penicillin and 100 µg/ml Streptomycin (P/S, Sigma-Aldrich), 10 mM L-glutamine (Sigma-Aldrich), 10 ng/ml human epidermal growth factor (EGF, Gibco) and 1 µg/ml hydrocortisone (Sigma-Aldrich). This medium is further referred to as standard HMEC-1 culture medium. The cells were maintained at 37°C in a humidified atmosphere with 5% CO_2 and used until passage 9. Every 2-3 days, culture medium was changed and HMEC-1 cultures were monitored daily with a Nikon Eclipse TS100 inverted phase-contrast microscope (Nikon Co., Tokyo, Japan). When the cultures reached 80-90% confluence, cells were harvested using 0.05% trypsin/EDTA (Sigma-Aldrich) and sub-cultured for further experiments.

Cell Culture and Nitrite Measurements of hCMEC/D3 cells

Human brain endothelial capillary cell line hCMEC/d3 were kindly provided by Prof. B. Broux (Hasselt University). Cells were cultured at 37°C and 5% CO, on collagen-I-coated flasks in EGM-2 medium (Lonza, Walkersville, MD, USA, category number CC-3156) supplemented with singleQuots Supplement Pack (Lonza, category number CC-4147) containing hydrocortisone, hFGFB, VEGF, R3-IGF-1, ascorbic acid, hEGF, GA, and 2.5% FBS. To assess

nitrite production, cells were seeded at a density of 78.95 x 10^3 cells/cm^2 on collagen-I-coated wells of a 24-well plate. After adherence of the cells, 300 µl fresh standard HMEC-1 culture MCDB medium was added and then subjected for 20 min with ELF-EMS (60 Hz, 13.5 mT) (or left untreated for the same amount of time). 24 h later, medium was collected, centrifuged and stored at -80°C. Nitrite levels were measured using the Griess Reagent System (Promega Benelux B.V.) according to manufacturer's guidelines.

Supplemental Tables

Supplementary Table 1. siRNA sequences used for siRNA transfection experiments.

ON-TARGETplus siRNA	Target Sequence
ON-TARGETplus SMARTpool siRNA J-006490-05, NOS3	CAGCACAAGAGUUAUAAGA
ON-TARGETplus SMARTpool siRNA J-006490-06, NOS3	CACAGGAAAUGUUCACCUA
ON-TARGETplus SMARTpool siRNA J-006490-07, NOS3	GCUCGGCCAUCACAGUGUU
ON-TARGETplus SMARTpool siRNA J-006490-08, NOS3	GCUCCCAACUUGACCAUCU
ON-TARGETplus Non-targeting siRNA #1 D-001810-01-05	UGGUUUACAUGUCGACUAA

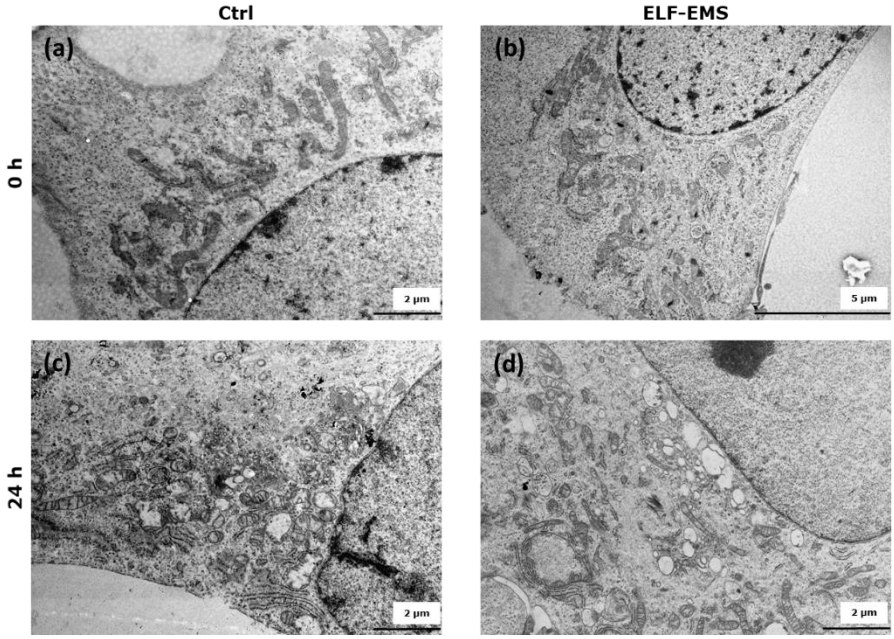
Supplementary Table 2. List of primary antibodies for western blotting.

Antibody	Reference number	Species	Company
Anti-(human) phospho-eNOS Ser1177	ab184154	Rabbit	Abcam, Cambridge, UK
Anti-(human) phospho-eNOS Thr495	9574S	Rabbit	Cell Signaling Technology, Leiden, The Netherlands
Anti-(human) eNOS	5880S	Mouse	Cell Signaling Technology, Leiden, The Netherlands
Anti-(mouse) eNOS	ab76198	Mouse	Abcam, Cambridge, UK
Anti-nNOS	AF2416	Goat	R&D Systems, Oxfordshire, UK
Anti-iNOS	ab202417	Rabbit	Abcam, Cambridge, UK
Anti-phospho-Akt Ser473	AF887	Rabbit	R&D Systems, Oxfordshire, UK
Anti-Akt	9272S	Rabbit	Cell Signaling Technology, Leiden, The Netherlands
Anti-β-actin	sc-47778	Mouse	Santa Cruz, Heidelberg, Germany

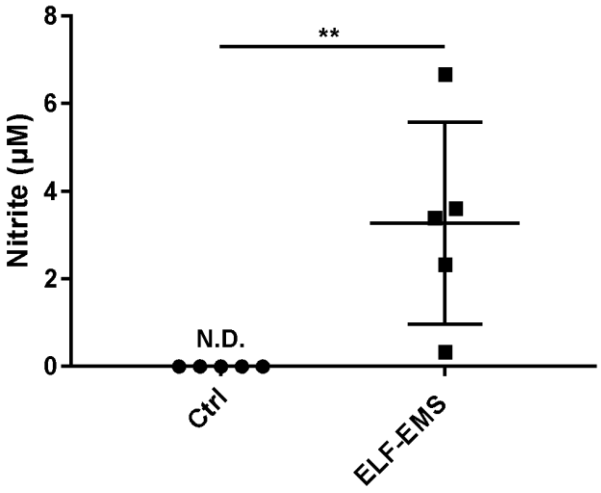
Supplementary Table 3. L-NAME, 1400W, ARL 17477 and LY294002 treatment does not affect baseline perfusion of the hind limb compared to vehicle-treated animals. Mean flux values were determined from the relative flux units normalized to the surface area. Data are represented as mean ± SD.

Inhibitors	Baseline perfusion vehicle group (a.u.)	Baseline perfusion inhibitor group (a.u.)	p-value
L-NAME	125.9 ± 40.5	121.8 ± 32.3	0.73
1400W	134.2 ± 35.7	124.9 ± 37.1	0.49
ARL 17477	107.7 ± 22.9	120.3 ± 23.3	0.10
LY294002	128.3 ± 36.6	128.3 ± 32.4	0.93

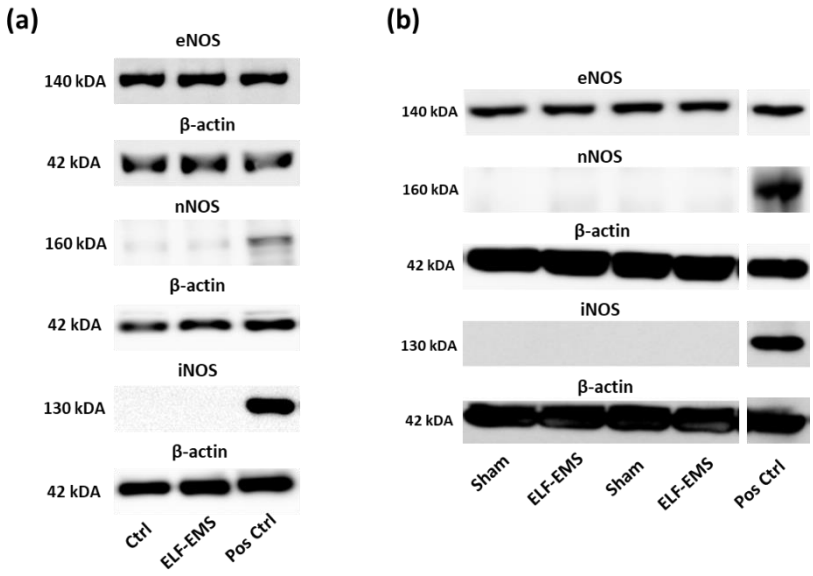
Supplemental Figures



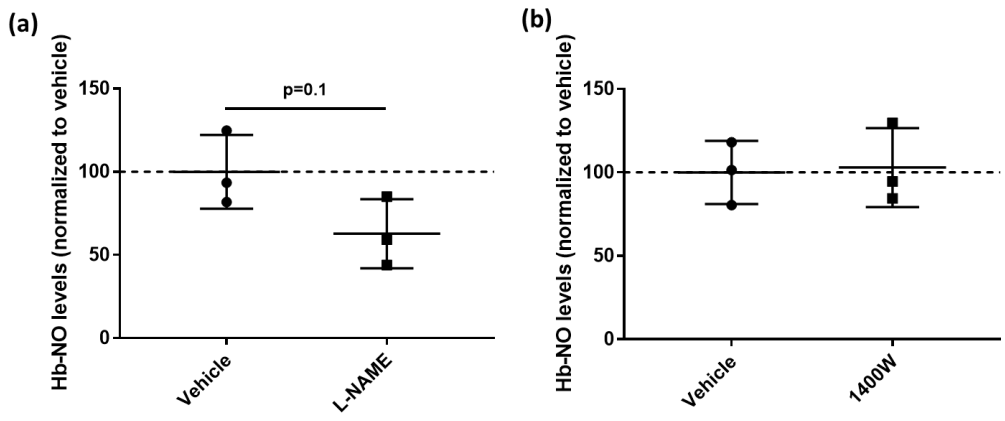
Supplementary Figure 1. ELF-EMS does not induce ultrastructural changes in HMEC-1. HMEC-1 cells were either left unstimulated or stimulated with ELF-EMS for 20 min. Cells were either immediately (0 h) or 24 h after ELF-EMS application fixated for ultrastructural analysis. (a) Ultrastructural analysis of untreated and (b) ELF-EMS-treated HMEC-1 immediately after ELF-EMS stimulation (0 h). (c) Ultrastructural analysis of unstimulated and (d) ELF-EMS-stimulated HMEC-1 24 h after ELF-EMS stimulation. No gross ultrastructural changes were observed in ELF-EMS-treated HMEC-1 after 0 h and 24 h. Scale bars: ((a), (c), (d))= 2 µm; (b)= 5 µm.



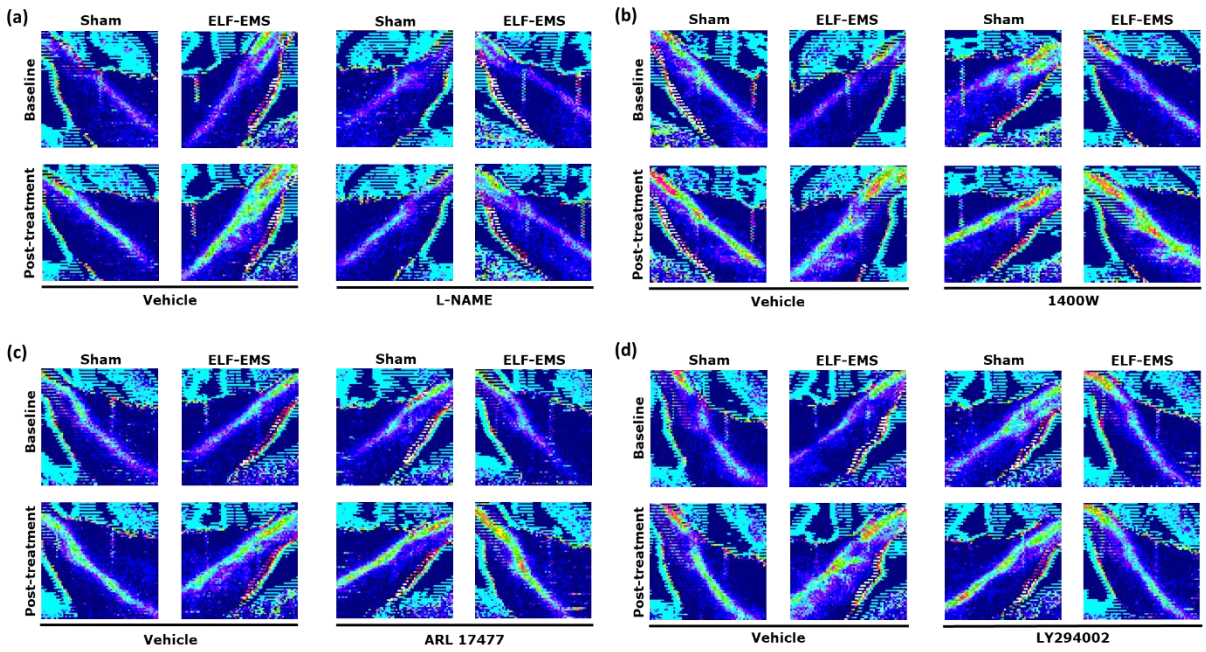
Supplementary Figure 2. ELF-EMS enhances NO synthesis in brain-derived hCMEC/D3 cells. hCMEC/D3 cells were either left unstimulated or stimulated by ELF-EMS (13.5 mT, 60 Hz) for 20 min. Cells were incubated for 24 h after ELF-EMS application after which medium was collected to analyze nitrite levels via Griess assay. ELF-EMS enhanced nitrite production (n=5; p=0.004) in hCMEC/D3 cells. Data are represented as mean ± SD. **P<0.01. N.D.: Not detectable.



Supplementary Figure 3. Expression of eNOS, nNOS, and iNOS in sham- and ELF-EMS-treated HMEC-1 and femoral arteries. (a) Representative blots corresponding to eNOS, nNOS, iNOS, and β-actin expression in unstimulated and ELF-EMS-stimulated HMEC-1. (b) Representative blots corresponding to eNOS, nNOS, iNOS, and β-actin of femoral arteries isolated from sham- or ELF-EMS-stimulated hind limbs of NMRI RjHan mice. Positive controls (Pos Ctrl) for eNOS, nNOS, and iNOS blots were EGF-stimulated HMEC-1 (500 ng/ml), SH-SY5Y neuroblastoma cells and bone marrow-derived macrophages (BMDM) stimulated with LPS (200 ng/ml), respectively. Sham- and ELF-EMS-treated HMEC-1 and femoral arteries expressed eNOS, whereas nNOS and iNOS expression was negligible or not detectable.



Supplementary Figure 4. Effect of L-NAME and 1400W treatment on Hb-NO production *in vivo*. Following Laser Doppler imaging, venous blood was collected from either vehicle-, L-NAME- or 1400W-treated NMRI RjHan mice and analyzed for Hb-NO content by EPR spectroscopy. (a) L-NAME-treated mice showed a trend towards a reduction in Hb-NO levels indicating successful inhibition of NO production by L-NAME (n=3; p=0.1). (b) No difference in Hb-NO formation was observed between vehicle and 1400W-treated mice (n=3). Since iNOS expression has to be induced in order to being activated[3], it is suggested that NO production by iNOS is negligible in healthy mice. Consequently, 1400W treatment would not affect Hb-NO levels if iNOS expression is not induced. Data are represented as mean ± SD and are normalized to the vehicle group.



Supplementary Figure 5. Representative perfusion maps acquired from sham- or ELF-EMS-stimulated hind limbs of L-NAME-, 1400W-, ARL 17477-, and LY294002-treated mice. Baseline images of blood flow were acquired before the start of either sham or ELF-EMS treatment. Post-treatment images were obtained immediately after 20 min of sham or ELF-EMS application. Colored pixels illustrate blood flow variations from minimal (dark blue) to maximal (red) values. (a) Mice received either vehicle or L-NAME (1 g/500 ml) in the drinking water for 5 consecutive days. Shown are representative images of blood perfusion maps obtained by Laser Doppler imaging of either sham or ELF-EMS-stimulated hind limbs of both experimental groups. (b) Mice were injected with either vehicle or 1400W (10 mg/kg) IP for 6 consecutive days. Representative images of blood perfusion maps obtained by Laser Doppler imaging of either sham or ELF-EMS-stimulated hind limbs of both experimental groups. (c) Mice received either vehicle or ARL 17477 IP injections (10 mg/kg) for 6 consecutive days. Representative images of blood perfusion maps obtained by Laser Doppler imaging of either sham or ELF-EMS-stimulated hind limbs of both experimental groups. (d) Mice were treated (IP) with either vehicle or LY294002 (10 mg/kg) for 3 consecutive days. Representative images of blood perfusion maps obtained by Laser Doppler imaging of either sham or ELF-EMS-stimulated hind limbs of both experimental groups.

References

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