SUPPLEMENTAL MATERIAL

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Supplemental Methods

Chronic Stress Procedure
The chronic stress procedure was carried out as described by Strekalova et al. with minor modifications. The procedure consists of exposure to rat, restraint stress, and tail suspension which were applied in the following order: days 1-7: exposure to a rat; days 8-10: restraint stress; days 11-14: tail suspension; days 15-21: exposure to rat; days 22-25: restraint stress; and days 26-28: tail suspension

Exposure to rat: At the beginning of the dark phase of the light cycle two mice were placed inside a cage with diameters of 16x14x22cm which was then placed inside a rat cage with diameters of 33x19x55cm. Subsequently a male wistar rat (Charles River) was introduced into the rat cage and remained there for 15 hours (19:30 – 10:30). To improve olfactory contact between the animals, mice cages contained holes (diameter 0.7 cm) in the side wall. After the termination of the procedure animals were housed in their home cages for the rest of the day.

Restraint stress: Plastic restrainers were constructed by making air holes on 50 ml syringes. Animals were placed inside the restraining syringe (internal diameter 30 mm) for 2.5 h during the dark phase of the light cycle.

Tail suspension stress: Approximately 1 cm from the end, each mouse's tail was taped (3M Durapore tape) to a piece of metal tubing fixed to a wall. Mice were suspended by the tail approximately 80 cm above the floor for 6 min/day during the dark phase of the light cycle.

Heart rate and blood pressure were measured by a computerized tail-cuff system after termination of the stress protocol as described previously (BP-2000, visitech Systems, Apex, NC) in conscious animals.

Aortic Ring Preparations and Tension Recording
After excision of the descending aorta, the vessel was immersed in Tyrode solution containing, in mmol/L, NaCl 118.0, CaCl₂ 2.5, KCl 4.73, MgCl₂ 1.2, KH₂PO₄ 1.2, NaHCO₃ 25.0, Na EDTA 0.026, D(+)-glucose 5.5, pH 7.4. Adventitial tissue was carefully removed. Three-millimeter rings were mounted in organ baths filled with the above-described buffer (37°C; continuously aerated with 95% O₂ and 5% CO₂) and were attached to a force transducer, and isometric tension was recorded. The vessel segments were gradually stretched over 60 minutes to a resting tension of 10 mN, which was maintained throughout the experiment, and were allowed to equilibrate for another 30 minutes. Drugs were added in increasing concentrations to obtain cumulative concentration-response curves: KCl (20 and 40 mmol/L), phenylephrine (1 nmol/L to 10 µmol/L), carbachol (1 nmol/L to 100 µmol/L), and glyceroltrinitrate (1 nmol/L to 10 µmol/L). The drug concentration was increased when vasoconstriction or vasorelaxation was completed. Drugs were washed out before the next substance was added. The relaxing effect of carbachol was abolished by adding L-NAME (1 µmol/L).

Vascular and brain gene expression
RNA from aortic and brain (block of brain from bregma 0 to bregma 3.14) homogenates were isolated with RNA-clean. 1 µg of the isolated total RNA was reverse transcribed using random primers and MMLV reverse transcriptase for 60 min at 42°C and 10 min at 75°C. Aortic and brain mRNA expressions of endothelial NO synthase (eNOS) and angiotensin II
type 1 (AT1) receptor were quantified with the TaqMan PCR system (Abi Prism 7700 Sequence Detection System, PE Biosystems) using TaqMan probes Mn00435204_m1Nos3 and Mm00616371_m1 provided by Applied Biosystems. Real-time RT-PCR using SYBR Green (Applied Biosystems, Darmstadt, Germany) was performed for mmMCP-1 (forward 5'-TGGCCCTAAGGTCTTCAACAC-3', reverse 5'-CCGAGTCCACACCTTG-3'), mmICAM-1 (forward 5'-ttcagctgtaggtgta-3', reverse 5'-ccttcgtagtgtagtata-3') and mmVCAM-1 (forward 5'-tctcttctgttcctgtg-3', reverse 5'-acctccacctgtggtg-3'). Expression was normalized to 18S ribosomal RNA.

Immunofluorescence analysis
Immunofluorescence studies were performed on paraffin-embedded 5 µm brain sections (block of brain from bregma 0 to -2.12) applying a monoclonal antibody against CD31 (PECAM-1, CD31, Santa Cruz). An anti-goat antibody coupled peroxidase (Sigma, Germany) was used as a secondary antibody. The signal was enhanced by thymidine-streptavidin amplification method (Perkin Elmer, USA and Dianova, Germany). Nuclei were counterstained with 4′,6 diamidino-2-phenylindole (DAPI Calbiochem, Germany) as described previously.

Cultured brain endothelial cells
Brain endothelial cells (bEnd.3, ATCC, CRL-2299) were cultured and grown to confluence using DMEM (ATCC-30-2002). Cellular viability was determined by cell count, morphology, and trypan blue exclusion. Cellular mRNA expression of endothelial NO synthase (eNOS) was quantified with the TaqMan PCR system (Abi Prism 7700 Sequence Detection System, PE Biosystems) using TaqMan probe Mn00435204_m1Nos3. Expression was standardized to 18S ribosomal RNA. For 18S the primers were forward 5'-TCAACACGGAACCTTCAC-3', reverse 5'-ACCAGACAAATCGCTCCAC-3'.

Cerebral Ischemia and Measurement of Physiological Parameters
After 6 weeks of treatment including 4 weeks of stress procedures mice were anesthetized with 1.0 volume percent isoflurane in 69% nitrous oxide (N₂O) and 30% oxygen (O₂) and subjected to left middle cerebral artery occlusion (MCAo) for 30 minutes followed by reperfusion (72h) as described previously. Regional cerebral blood flow (CBF) measured using laser Doppler-flowmetry (Perimed, Järfälla, Sweden) fell to less than 20% during ischemia and returned to approximately 80% to 100% within 5 minutes after reperfusion in all groups (p>0.05). Core temperature was maintained at 36.5±0.5°C. In randomly assigned animals, the left femoral artery was cannulated for arterial blood pressure monitoring and blood withdrawal. Arterial blood samples were analyzed for pH, arterial oxygen pressures, and partial pressure of carbon dioxide as described. After 72 hours, animals were euthanized by a pentobarbital overdose, and brains were quickly removed from the skull and snap-frozen in isopentane on dry ice for cryostat sectioning. Direct and indirect lesion volumes were quantified by computer assisted volumetry on 20 µm hematoxylin-stained cryostat sections as described previously.
Supplemental References


