Supplement Material

Material and Methods

Generation of transgenic mice. A fusion transgene (syn-hACE2) consisting of 4.4 kb of the rat synapsin promoter and a cDNA encoding the full open-reading frame of the human ACE2 (NCBI accession number: AF291820) was constructed (Figure 1A). The transgene segment was obtained by digestion with *XhoI* and *SpeI*, separated by agarose gel electrophoresis, and recovered using the Qia-Quick gel purification kit (Qiagen Technologies, Hilden, Germany). Transgenic mice were generated by microinjection into fertilized C57BL/6JxSJL/J (B6SJLF2) mouse embryos at The University of Iowa Transgenic Facilities, positive founders were backcrossed to C57BL/6J beyond 7 generations and bred for study as described previously.[1] All mice were fed standard mouse chow and water *ad libitum*. All procedures were approved by the Institutional Animal Care and Use Committee at the University of Iowa and Louisiana State University Health Science Center.

RNA isolation and reverse transcription PCR. Total RNA was isolated from various tissues from both transgenic and NT mice, using a standard RNA extraction procedure (TRIZOL, Invitrogen, Carlsbad, CA) as described.[2] Isolated RNA was treated with RNase-free DNase I (Qiagen Technologies, Hilden, Germany) to remove any contaminating genomic DNA. Total RNA was quantified using a Biophotometer (Beckman counter DU 640). Complementary DNA synthesis was carried out using AMV Reverse Transcriptase and Random primers (Biorad laboratories, Hercules, CA). This cDNA was used as template for PCR analysis. RNA in the absence of reverse transcriptase was used as a negative control. Specific primers for human ACE2 (Fwd: 5'-TGA AAA ATG AGA TGG CAA GAG-3'; Rev: CAT TTC ATT GTC GTT CCA TTC ATA-3'; amplicon length: 1364 bp) and internal control mouse β-actin (Fwd: 5'-TGT GAT GGT GGG AAT GGG TCA GAA-3'; Rev: 5'-TGT GGT GCC AGA TCT TCT CCA TGT-3'; amplicon length: 140 bp) were designed using PrimerQuest Software (IDT, Coralville, IA). The PCR was performed with 1 µl samples in a total volume of 20 µl consisting of 5U/µl *Taq* DNA polymerase (Qiagen Technologies, Hilden, Germany), 10 m for each dNTP, 25 mmol/L $MgCl_2$, PCR buffer, 20 µmol/L each of forward and reverse primers. Cycling conditions were 95 ℃ for 5 min, and then 35 cycles consisting of 45 sec at 95 ℃, 45 sec at 56 ℃ and 1 min at 72 ℃, plus an additional extension at 72 ℃ for 5 min.

Western blotting. Tissue from brain cortex, hypothalamus and brainstem were collected separately and homogenized with a glass pestle in the cell lysis buffer (in mmol/L: HEPES: 10, NaCl: 150, $MgCl_2$: 5, EGTA: 1, 0.02% (w/v) NaN_3, pH 7.4) containing a protease inhibitors cocktail (Sigma, St Louis, MO). The lysate was centrifuged at 10,000 rpm, 4 ℃ for 15 min and the supernatant transferred to a clean tube. Proteins concentration was measured using a BCA assay kit (Pearce, Rockford, IL). Cell lysates (10 µg) were mixed with SDS-PAGE sample buffer (0.125 mol/L Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromophenol blue), heated at 100 ℃ for 5 min and loaded onto a 15% SDS–polyacrylamide gel for electrophoresis. Proteins were transferred to nitrocellulose membrane at 200 mA for 1 hr by semi-dry blot (Fisher Scientific, Houston, TX). Membranes were blocked with 5% non-fat milk in PBS-T (1.47 mmol/L NaH_2PO_4, 8.09 mmol/LNa_2HPO_4, 145 mmol/L NaCl, 0.05% (v/v) Tween-20®, 0.01% (w/v) thimerosal, pH 7.4) for 2 hr at room temperature and incubated with a goat-anti-human ACE2 antibody (AF933, R&D Systems, Minneapolis, MN) in 1:1000 dilution for 1 hr at room temperature. Membranes were washed with PBST 4 times for 5 min then incubated with donkey anti-goat IgG-HRP (ab6885, abCAM, Cambridge, MA, 1:5000) and goat anti-mouse beta-actin antibody (ab8229, abCAM, 1:5000) for 45 min at room temperature, as described.[3] Specific bands were detected by chemiluminescence according to the manufacturer's instructions (ECL®, Perkin Elmer, Boston, MA).

ACE2 activity. Tissue from brain cortex, hypothalamus and brainstem were collected separately and homogenized with a glass pestle in 1 mL ACE2 activity reaction buffer (in mmol/L: NaCl: 1000, Tris: 75, $ZnCl_2$: 0.5, pH 7.5) and centrifuged at 20,800 x g for 10 min and the supernatant transferred to a clean tube. Proteins concentration was measured using a BCA assay kit (Pearce, Rockford, IL). ACE2 activity measurement was carried out in the presence of captopril to eliminate any contribution by endogenous

ACE and based on the use of the Fluorogenic Peptide Substrate VI (FPSVI, 7Mca-Y-V-A-D-A-P-K(Dnp)-OH)(R&D systems, Minneapolis, MN) as described previously.[3] This substrate contains a C-terminal dinitrophenyl moiety that quenches the inherent fluorescence of the 7-methoxycoumarin group by resonance energy transfer. ACE2 removes the C-terminal dinitrophenyl moiety in the FPSVI thus increasing fluorescence emission at 405 nm under excitation at 320 nm. Tissue lysates containing 100 μg of protein, were incubated with FPSVI (100 μmol/L) and captopril (10 μmol/L) in reaction buffer (100 μL) at room temperature. Non specific enzyme activity was measured by including DX600 (1 μmol/L), a specific ACE2 inhibitor, (Phoenix Pharmaceutical, Belmont, CA). Fluorescence emission was monitored using a SpectraMax M2 Fluorescence Reader (Molecular Devices, Sunnyvale, CA). The noise to signal ratio for enzyme activity in the absence of substrate or cell extract was <5%. Specific ACE2 activity was calculated by subtracting the total activity in the presence of 10 μmol/L captopril from the activity in the presence of both 10 μmol/L captopril and 1 μmol/L DX600. Data (arbitrary fluorescence units, AFU) are presented as amounts of substrate FPSVI converted to product per minute and are normalized for total protein.

Radioimmunoassays (RIAs) of Angiotensin II and Ang 1-7 levels. Male syn-hACE2 and control littermates (n=8), 8-10 weeks old, were anesthetized and infused subcutaneously for 14 days using osmotic minipumps (Alzet) containing either 1) saline, 2) Ang-II (600 ng/kg.min) 3) the Ang-(1-7) receptor blocker D-Ala^7-Ang-(1-7) (600 ng/kg.min) or 4) Ang-II + D-Ala^7-Ang-(1-7). At the end of the 2-week infusion, blood was collected following decapitation, in the presence of 0.44 mmol/L 1,10-phenanthroline monohydrate (Catalog number P-1294, Sigma, St. Louis MO.), 0.12 mmol/L pepstatin (Peninsula Labs, Belmont CA), 1 mmol/L sodium p-hydroxymercuribenzoate (Catalog number H 0642, Sigma, St. Louis MO), 15% EDTA, and 0.01 mmol/L WFML-1, a rat renin inhibitor (AnaSpec Inc, San Jose, CA). Brain regions were quickly dissected over ice and frozen in liquid nitrogen. Blood and tissue samples were immediately shipped in dry ice to the Wake Forest University Hypertension Core Laboratory tissues for processing. Blood samples were centrifuged at 2000 rpm for 10 minutes at 4°C. Plasma was transfered into a pre-chilled centrifuge tube and spinned at 2000 rpm for 10 more minutes at 4 ℃. Brain regions were homogenized in acid/ethanol [80% vol/vol 0.1mol/L HCl] containing a cocktail of protease inhibitors including 0.44 mmol/L 1,10-phenanthroline monohydrate (Sigma, St. Louis MO.), 0.12 mmol/L pepstatin (Peninsula Labs, Belmont CA), 1 mmol/L sodium p-hydroxymercuribenzoate (Sigma, St. Louis MO), 15% EDTA, and 0.01 mmol/L WFML-1, a rat renin inhibitor (AnaSpec Inc, San Jose, CA), centrifuged at 12000 rpm for 20 minutes at 4 ℃ and stored overnight at 4 ℃. Samples were re-centrifuged at 12000 rpm for 20 min at 4 ℃, the supernatant was removed, added to 1% HFBA and refrigerated overnight at -20 ℃. The supernatant was extracted using Sep-Pak columns activated with 5 ml wash of a mixture of n-heptafluorobutyric acid (HFBA): methanol (0.1%:80%) and sequential washes of 0.1% HFBA. After the sample was applied to the column, it was washed with 0.1% HFBA and followed with a water wash. The samples were eluted with 3.3 ml washes of a mixture of acid methanol (0.1%:80%), reconstituted and splitted for the two RIAs. For Ang II, samples were reconstituted in assay buffer and for Ang-(1-7), a TRIS buffer with 0.1% BSA was used. Recoveries of radiolabeled angiotensin added to the sample and followed through the homogenization and extraction were 68% (n=23). Samples were corrected for recoveries. Ang II was measured using a radioimmunoassay kit (Alpco, Windham, NH) and Ang-(1-7) was measured using the antibody previously described.[4] The minimum detectable levels of the assays were 0.8 fmol/ml and 2.8 fmol/ml for Ang II, and Ang-(1-7), respectively. Values at or below the minimum detectable level (MDL) of the assay were arbitrarily assigned that value for statistical analysis. The intra-assay and inter-assay coefficient of variation was 12 and 22% for Ang II, and 8% and 20% for Ang-(1-7).

Immunohistochemistry. Detection of human ACE2 and NOS was performed as described previously.[3,5] Mice were deeply anesthetized with Nembutal and perfused transcardially with PBS (0.1 mmol/L, pH 7.4) for 2 min followed by 4% paraformaldehyde in PBS (0.1 mmol/L, pH 7.4) for 10 min. The brain was then removed, post fixed for 1 hr in 4% paraformaldehyde in PBS (0.1 mmol/L, pH 7.4) and then placed in 20%

sucrose solution overnight. Coronal sections (30 μm) were collected on slides and permeabilized with 0.2% Tween-20® (15 min), while non specific binding was blocked with 5% bovine serum albumin (BSA, 1 hr). Sequential sections were incubated with a rabbit anti-human ACE2 antibody (1:50, Catalog number sc-17719, Santa Cruz, CA) or a rabbit anti AT_1 receptor (1:100, Catalog number SC-1173, Santa Cruz Inc, CA), AT_2 receptor (1:100, Catalog number SC-9040, Santa Cruz Inc, CA), Mas (1:50, Catalog number ab13354, abCam), nNOS (1:100, Catalog number 4234, Cell signaling Inc, MA), eNOS (1:1, Catalog number ab15280, AbCam), and Ser^{1177}-Phos-eNOS (1:100, Catalog number 9571, Cell signaling Inc, MA), Thr^{495}-Phos-eNOS (1:100, Catalog number 9574, Cell signaling Inc, MA) antibody for 36 hr at 4 ℃. Sections were incubated at room temperature with the secondary antibody a donkey-anti-rabbit IgG-HRP (NEF812, Perkin Elmer) 1:100 dilution for 1 hr. At the end of the protocol, color was developed by incubation with DAB substrate, according to the manufacturer (Vector Laboratories, SK-4100). Images were captured using a brightfield microscope (Nikon Eclipse E600). Additionally, either or both primary and secondary antibodies were omitted in the incubation to check for unspecific staining.

Nitric oxide (NO) measurement. Male syn-hACE2 and NT mice (n=8/group), 8-10 weeks old, were anesthetized and infused subcutaneously for 14 days using osmotic minipumps (Alzet) containing either 1) saline, 2) Ang-II (600 ng/kg.min) 3) the Ang-(1-7) receptor blocker D-Ala^7-Ang-(1-7) (600 ng/kg.min) or 4) Ang-II + D-Ala^7-Ang-(1-7). At the end of the 2-week infusion, mice were anesthetized and NO levels were measured using a NO detection probe (NS-ISO-NOPF100-1MM WPI Inc, FL) as described by the manufacturer. The NO probe was polarized in 0.3 mmol/L CuCl2 at 37 ℃ for about 2 hours until stabilization of the current. A standard curve was generated by adding serial concentrations of S-nitroso-N-acetyl-l,l-penicillamine (SNAP). The NO probe was then lowered into the mouse lateral ventricle using previously reported coordinates[3] and the current was measured for at least 5 minutes post-stabilization. Conversion to nitric oxide concentrations (μ mmol/L) was performed according to the standard curve.

Physiological recordings. Male syn-hACE2 and control littermates (n=12), 8-10 weeks old, were anesthetized and instrumented with a radiotelemetry probe, as described.[3,6] After 14 days recovery, baseline BP was recorded for 4 days. Mice were then infused subcutaneously for 14 days using osmotic minipumps (Alzet) containing either 1) saline, 2) Ang-II (600 ng/kg.min) 3) the Ang-(1-7) receptor blocker D-Ala^7-Ang-(1-7) (600 ng/kg.min) or 4) Ang-II + D-Ala^7-Ang-(1-7). Water intake was recorded daily. Autonomic function was assessed in conscious freely moving mice, before and at the end of the infusion protocol, by using a standard pharmacological method involving random ip injection of a β-blocker (propranolol, 4 mg/kg), a muscarinic receptor blocker (atropine, 1 mg/kg) and a ganglionic blocker (hexamethonium, 10 mg/kg).[7] Each injection was separated by at least a 3-hour recovery period. Changes in HR were calculated following administration of the antagonists. Spontaneous baroreflex sensitivity was calculated using the sequence method as used previously.[3]

Statistical Analysis. Data are expressed as mean ±SEM. Data were analyzed by Student's *t* test or two-way ANOVA (Bonferroni post hoc tests to compare replicate means) when appropriate. Statistical comparisons were performed using Prism5 (GraphPad Software, San Diego, CA). Differences were considered statistically significant at $P<0.05$.

References

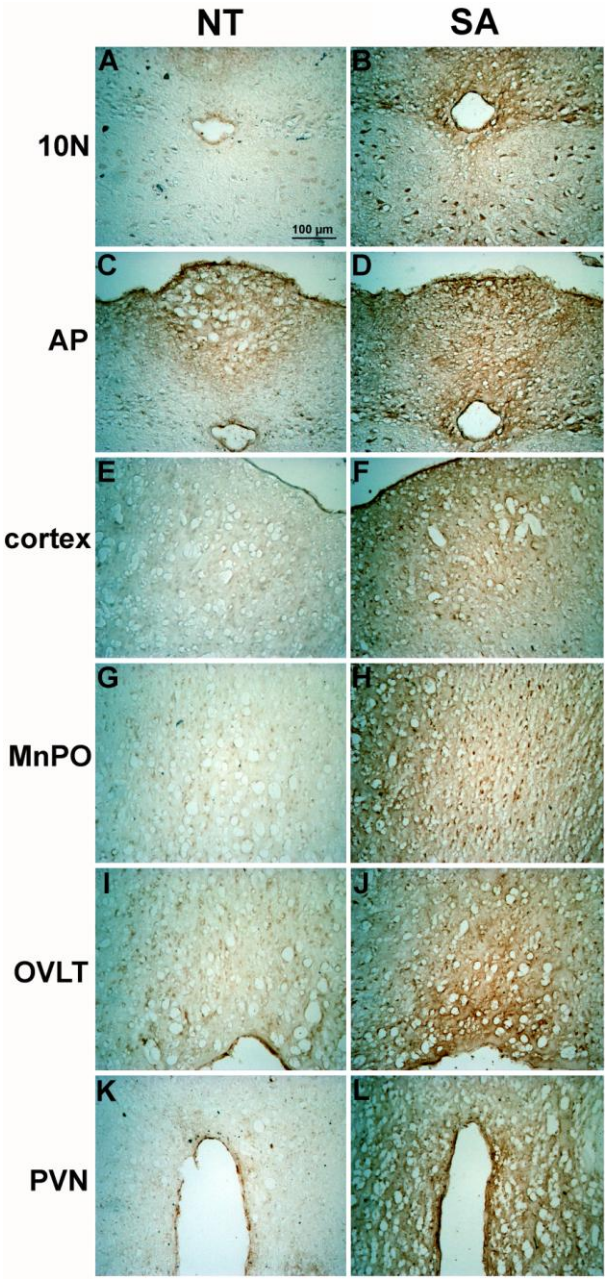
1. Sigmund CD. Major approaches for generating and analyzing transgenic mice. An overview. *Hypertension.* 1993;22:599-607.
2. Feng Y, Feng Y, Zhu X, Dang Y, Ma Q. Alkali burn causes aldehyde dehydrogenase 3A1 (ALDH3A1) decrease in mouse cornea. *Mol Vis.* 2004;10:845-850
3. Feng Y, Yue X, Xia H, Bindom SM, Hickman PJ, Filipeanu CM, Wu G, Lazartigues E. Angiotensin-Converting Enzyme 2 Overexpression in the Subfornical Organ Prevents the Angiotensin II-Mediated Pressor and Drinking Responses and Is Associated With Angiotensin II Type 1 Receptor Downregulation. *Circ Res.* 2008;102:729-736.

4. Senanayake PD, Moriguchi A, Kumagai H, Ganten D, Ferrario CM. Increased expression of angiotensin peptides in the brain of transgenic hypertensive rats. *Peptides.* 1994;15:919-926.
5. Doobay MF, Talman LS, Obr TD, Tian X, Davisson RL, Lazartigues E. Differential expression of neuronal ACE2 in transgenic mice with overexpression of the brain renin-angiotensin system. *Am J Physiol - Regul Integr Comp Physiol.* 2007;292:R373-R381.
6. Lazartigues E, Dunlay SM, Loihl AK, Sinnayah P, Lang JA, Espelund JJ, Sigmund CD, Davisson RL. Brain-selective overexpression of angiotensin (AT1) receptors causes enhanced cardiovascular sensitivity in transgenic mice. *Circ Res.* 2002;90:617-624.
7. Xia H, Feng Y, Obr TD, Hickman PJ, Lazartigues E. Angiotensin II Type 1 Receptor-Mediated Reduction of Angiotensin-Converting Enzyme 2 Activity in the Brain Impairs Baroreflex Function in Hypertensive Mice. *Hypertension.* 2009;53:210-216.

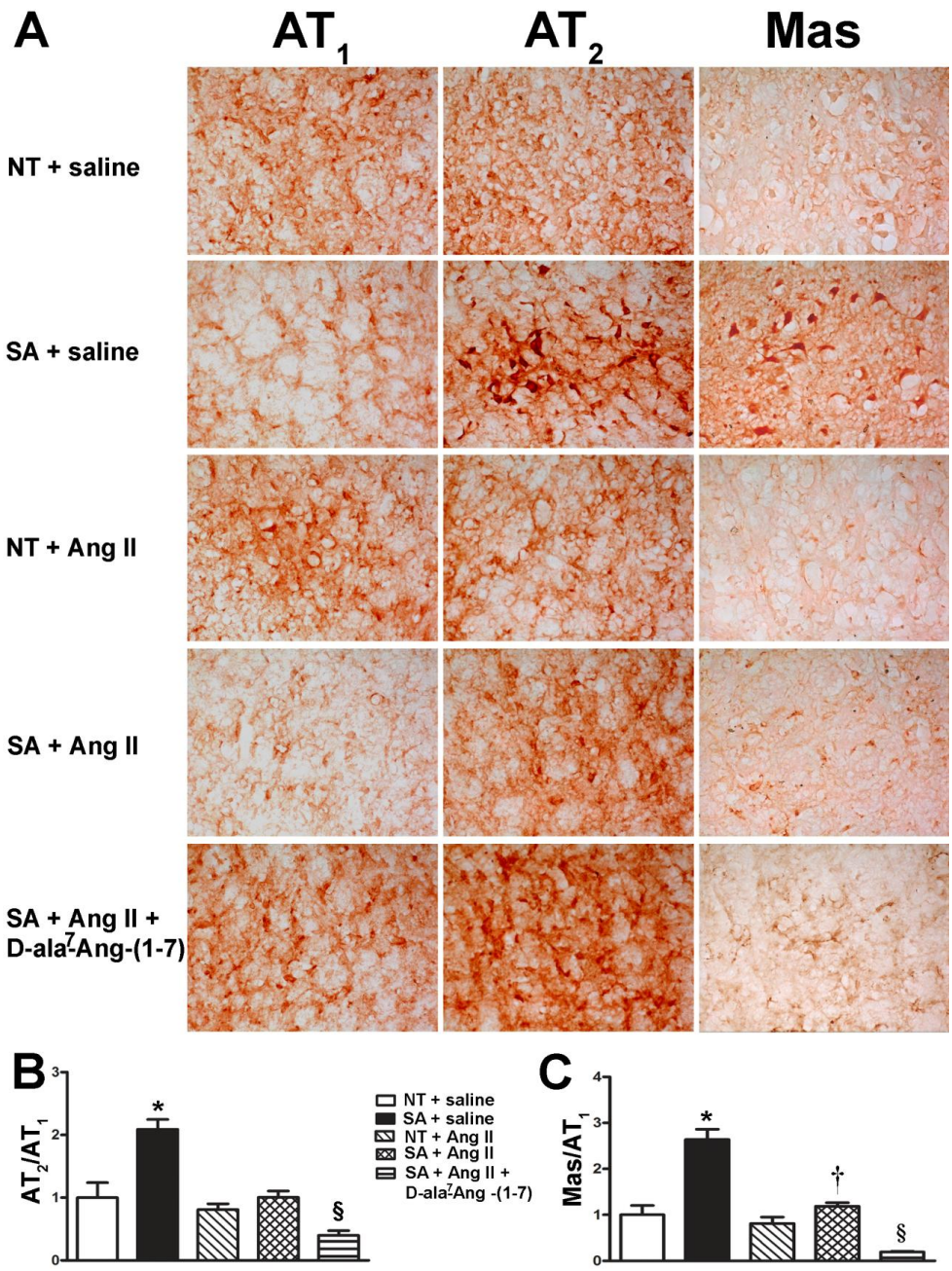
Online Table I. Baseline hemodynamic parameters and activity.

	MAP *(mmHg)*	SBP *(mmHg)*	DBP *(mmHg)*	PP *(mmHg)*	HR *(bpm)*	Activity *(AU)*
			24 hr (6 PM–6 PM)			
Control	97.2±2.2	106.9±3.1	83.2±2.0	23.6±3.5	527.7±8.4	6.3±0.8
Syn-hACE2	101.8±2.0	115.1±2.3	87.6±1.9	27.4±2.0	556.5±8.6	5.1±0.5
			Day (6 AM–6 PM)			
Control	95.2±4.7	104.8±3.5	81.3±2.3	23.4±3.6	515.5±7.8	5.2±0.8
Syn-hACE2	101.2±4.4	114.4±2.4	86.8±1.9	27.6±2.0	550.4±8.9	4.4±0.4
			Night (6 PM–6 AM)			
Control	99.1±7.2	109.4±2.7	85.4±1.8	23.9±3.4	535.4±9.6	7.6±0.8
Syn-hACE2	102.9±6.2	115.6±2.2	88.4±1.9	27.1±2.0	564.6±8.4	5.9±0.7

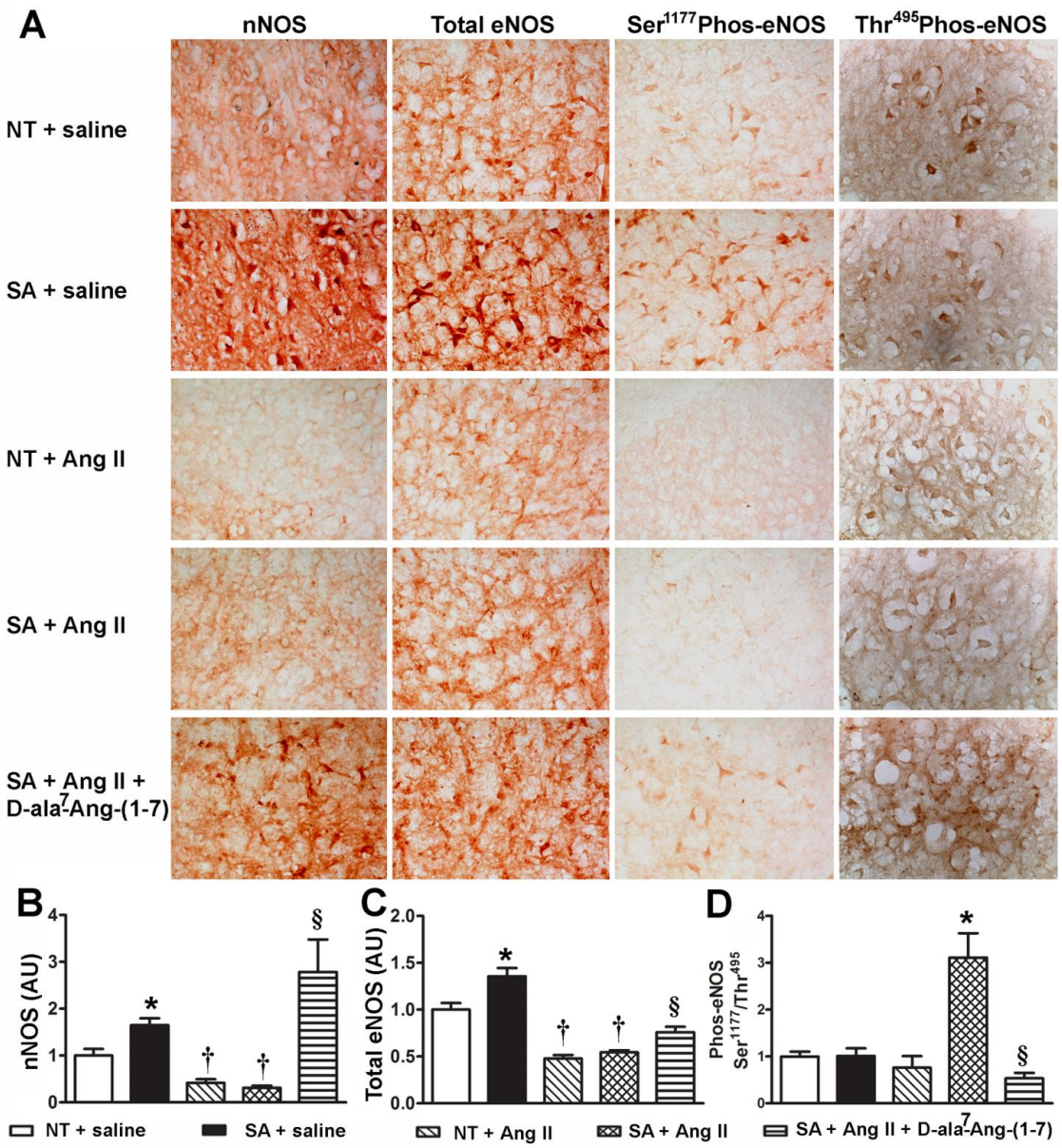
Data represent the average of the daily 24hr means in each animal. Values are mean ±SEM. Abbreviations: MAP, mean arterial pressure; SBP, systolic blood pressure; DBP, diastolic blood pressure; PP, pulse pressure; HR, heart rate; bpm, beats/min; AU, arbitrary units.



Online Figure I. Expression of hACE2 in transgenic mice. Representative immunohistochemistry pictures showing hACE2 expression in NT (left) and SA (right) mice. Low reactivity was observed in NT mice (A, C, E, G, I, K), while Widespread expression was detected throughout the brain of SA including in cardiovascular: 10N (B), AP (D) , MnPO (H) and PVN (L), and non-cardiovascular regions: cortex (F) and OVLT (J). Abbreviations: 10N, dorsal motor nucleus of the vagus nerve; AP, area postrema; MnPO, median preoptic area; OVLT, organum vasculosum of the lamina terminalis; PVN, paraventricular nucleus.



Online Figure II. Angiotensin AT_1, AT_2 and Mas receptors expression in the RVLM. (A) Representative immunohistochemistry pictures for AT_1, AT_2 and Mas receptors (n=3/group). In the RVLM, AT_2/AT_1 (B) and Mas/AT_1 (C) receptors ratios were significantly ($P<0.05$) increased in SA compared to NT mice. Ang II infusion reduced the ratios in SA mice to similar levels than in NT mice. They were further reduced ($P<0.05$) after Ang-II+D-ala^7-Ang-(1-7). *$P<0.05$ vs. NT; $^{\dagger}P<0.05$ vs. baseline and $^{\S}P<0.05$ vs. SA + Ang II.



Online Figure III. NOS expression in the RVLM. (A) Representative immunohistochemistry images for nNOS, total eNOS, phos-eNOS Ser^{1177} and phos-eNOS Thr^{495} (n=3/group). Baseline, nNOS (B) and total eNOS (C) were significantly (P<0.05) increased in SA compared to NT mice. Following Ang II infusion, nNOS and total eNOS expression were similarly decreased in SA mice and NT mice while phos-eNOS Ser^{1177}/Thr^{495} ratio (D), an index of phosphorylation vs. dephosphorylation, was significantly increased. Ang-II+D-ala^{7}-Ang-(1-7) significantly increased the nNOS (B) and eNOS (C) expression in SA mice (P<0.05) while the phos-eNOS Ser^{1177}/Thr^{495} ratio was dramatically (P<0.05) reduced after Ang-II+D-ala^{7}-Ang-(1-7). Statistical significance: *P<0.05 vs. NT; $^{\dagger}P$<0.05 vs. baseline and $^{\S}P$<0.05 vs. SA + Ang II.