SUPPLEMENTAL MATERIAL

Involvement of Bone Marrow Cells and Neuroinflammation in Hypertension

Monica M. Santisteban[1], Niousha Ahmari[2], Jessica Marulanda Carvajal[1], Michael B. Zingler[1], Yanfei Qi[3], Seungbum Kim[1], Jessica Joseph[1], Fernando Garcia-Pereira[2], Richard D. Johnson[2], Vinayak Shenoy[4], Mohan K. Raizada[1*], and Jasenka Zubcevic[2*]

Department of Physiology and Functional Genomics, College of Medicine University of Florida, Gainesville, FL 32610[1]; Department of Physiological Sciences, College of Veterinary Medicine University of Florida, Gainesville, FL 32610[2]; Department of Cardiology, College of Medicine University of Florida, Gainesville, FL 32610[3]; Department of Pharmacodynamics, College of Pharmacy University of Florida, Gainesville, FL, 32610[4]

Methods

Animals

All experimental procedures were approved by the University of Florida Institute Animal Care and Use Committee and complied with the standards stated in the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Rats were housed in a temperature-controlled room (22°C to 23°C) with a 12:12-hour light-dark cycle, in specific-pathogen free cages, and had access to standard rat chow and water *ad libidum*.

Adult Wistar-Kyoto (WKY) rats and spontaneously hypertensive rats (SHR) aged 12-14 weeks (Charles River Laboratories) were used for the initial BM qPCR and CCL2 ELISA.

Six-week-old WKY and SHRs were used for BM reconstitution experiments described below.

Six-week-old Sprague Dawley (SD; Charles River Laboratories) and SD-Tg(UBC-EGFP) (Rat Resource & Research Center strain 65; bred in house) were used for BM reconstitution experiments described below. This hemizygous transgenic strain contains a single enhanced green fluorescent protein gene under the control of human ubiquitin-C promoter located at Chromosome 14.

Eight-week-old SD rats were used for all other experiments.

Bone Marrow (BM) Ablation and Reconstitution

We generated WKY and SHR BM chimeras by lethal irradiation of six-week-old WKY and SHR male rats (950 cGy of X-rays; service provided by University of Florida Animal Care Services) followed by BM reconstitution with $1x10^7$ whole BM cells from young age-matched male WKY and SHR. The four groups generated are as follows: normotensive WKY rats reconstituted with the SHR BM (WKY-SHR), and hypertensive SHR reconstituted with the WKY BM (SHR-WKY), as well as their appropriate control groups (WKY-WKY and SHR-SHR). Design scheme is presented in Online Figure 1 (n=12-14 per group). Initial BM irradiation and reconstitution experiments were performed in the adult female SHR, and the success of reconstitution with

male WKY and SHR whole BM cells was confirmed by Y-FISH in BM MNCs (Online Fig. 2). Successful reconstitution was defined as >90% Y chromosome-stained MNCs isolated from the blood. In all subsequent experiments, adult age-matched male chimeric WKY and SHR were used to investigate the role of BM in hypertension. Only male-to-male transplant data is shown in the main manuscript.

Following irradiation, animals were allowed to recover for 2.5 months before the initiation of experiments. During this time, they received 0.4% Baytril antibiotic (enrofloxacin; Bayer) in the drinking water, moist chow, and Nutrical during the first 3 weeks. Subcutaneous injections of sterile 0.9% saline were performed as necessary to mildly dehydrated animals.

In a second experiment, six-week-old SD rats were lethally irradiated (950 Gy of X-Rays) and reconstituted with age matched $1x10^7$ eGFP-SD whole bone marrow cells. The same recovery procedures were implemented in this group of animals. BM reconstitution was confirmed by GFP+ FACS in blood mononuclear cells (MNCs).

MNC isolation from BM and blood

For isolation of MNCs from the bone marrow, intact and thoroughly cleaned of adjacent tissue femur bones were collected into MNC isolation buffer (PBS+2% FBS+1mM EDTA buffer). Both tips of the epiphyses were cut to flush bone marrow cells with 20 ml of the MNC isolation buffer, using a 10 ml syringe and 20 gauge needle into a 50ml conical tube. Cells were centrifuged at 1200 rpm for 15 minutes at room temperature (RT). The supernatant was discarded and the cells were washed in 30 ml of sterile PBS, centrifuged (1200rpm x 15min) and resuspended in 400 µl of PBS. 4-5 ml of ammonium chloride (STEM CELL technology, Cat # 07850) was added for red blood cell (RBC) lysis and incubated for 15 min at RT, followed by washing with MNC isolation buffer twice. The resulting MNCs were re-suspended in 1 ml of MNC isolation buffer and kept on ice until use or 4°C if overnight.

For isolation of MNCs from blood, blood was collected in 10 ml syringe washed with MNC isolation buffer, and diluted in the same buffer at 1:1 ratio, at RT. The diluted blood was then slowly added to a 50 ml conical tube over a layer of Ficoll-Paque PLUS (2:1 blood to Ficoll ratio; GE Healthcare), and centrifuged at 1200 rpm for 25 minutes at RT to obtain the buffy coat. The buffy coat was then transferred to a fresh 15 ml conical tube and pelleted down by centrifuging (1200rpm x 15 minutes). The supernatant was discarded and the cells were washed in 15 ml of sterile PBS, centrifuged again (1200rpm x 15min) and resuspended in 400 µl of PBS. 4-5 ml of ammonium chloride (STEM CELL technology, Cat # 07850) was added for red blood cell (RBC) lysis and incubated for 15 min at RT, followed by washing with MNC isolation buffer twice. The resulting MNCs were re-suspended in 1 ml of MNC isolation buffer and kept on ice until use or 4°C if overnight.

Y-Fluorescence *in situ* Hybridization

Following full recovery, 100 µl of blood was collected from each rat under isoflurane anesthesia. MNCs were isolated and resuspended in sterile PBS. 5 µl of MNC saline solution was fixed onto a glass slide and immunostained for Y chromosome (Rat Idetect Chr Y Paint Probe RED; ID Labs, Ontario, Canada) using the manufacturer's protocol. Successful reconstitution was adjudged by >90% Y chromosome-stained MNCs isolated from the blood.

Radiotelemetry, Blood Pressure Measurements and Spectral Analysis

Radio-transmitter implantation (DSI) was performed as recommended by the manufacturer. Briefly, animals were anesthetized with 2% isoflurane. Rat telemeters (Data Sciences International; PA-C40) were implanted into the abdominal aorta. Animals received buprenorphine (0.1mg/kg) for pain management and allowed to recover for one week. Baseline mean arterial pressure (MAP), systolic blood pressure (SBP), diastolic blood pressure (DBP) and heart rate were recorded over 48 hours. Following the experimental protocol, blood

pressure and heart rate were recorded once a week for 48 hours. Spectral analysis of the SBP and pulse interval waveforms was performed at Zeitgeber (ZT) 12-13 at baseline and seven weeks of Ang II infusion in SD rats using the *Hey Presto* software[1]. Time point was chosen to represent highest sympathetic activation. Data is presented as a change from baseline. The same protocol for spectral analysis was used at end point in the waveforms of WKY and SHR irradiated animals, and data is presented and mean ± SEM.

Noninvasive Blood Pressure Measurements

Noninvasive blood pressure measurements were performed using the CODA System for Mice and Rats, following the manufacturer's protocol (Kent Scientific Corp).

Blood Flow Measurements

Animals were anesthetized with isoflurane and placed in a prone position with the feet positioned (plantar side up) on a platform warmed by circulating water. Body temperature was maintained at 37^{o} C using a rectal thermistor and a circulating water heating pad under the animal. Measurements of blood flow in the plantar region of the left and right hind-limb were taken over a five minute period using a laser speckle contrast imager (LSCI; PeriCamPSI, PeriMed, Inc.) interfaced with a dedicated computer. The laser generator probe was positioned 15.0 cm above the plantar surface. The LSCI sample rate was 53 samples/sec and the digitized blood flow values were stored in a data file. The analysis area on the foot (region of interest, ROI, see Figure 3) was set by the programmable software based on spatial landmarks in the foot, and the same ROI parameters (sample area and position) were used for all animals to validate inter-animal comparisons. Blood flow (perfusion) values were expressed in arbitrary units of intensity. After the 5 min measurement period was completed, the anesthesia was discontinued and the animal returned to the cage and vivarium. The blood flow data was analyzed in two different phases of the 5 min record. The first 1.5 minutes were analyzed for

the rate of blood flow change (slope) and the last 1.5 minutes, during which the perfusion had stabilized in all groups, was analyzed for mean perfusion. For each animal, data from both feet were combined after determining that there were no significant differences in blood flow between the left and right foot.

Chronic Angiotensin II infusion and Oral Minocycline treatment

HTN was established by chronic infusion of Ang II (200ng/kg/min) using mini osmotic pumps (ALZET 2004) implanted subcutaneously. Control animals received 0.9% saline in osmotic pumps. Oral minocycline (Sigma M9511) regimen was initiated 2 days prior to implantation of the Ang II pumps. Minocycline (50mg/kg) was dissolved in sterile distilled water to a final volume of 1ml per animal, which was delivered by oral gavage at the same time every day. Control animals received 1ml of vehicle (sterile distilled water) daily by oral gavage.

Flow Cytometry

MNCs from blood and BM were prepared in a concentration of 0.5-1x10^6 cells/100ul in PBS+2% FBS+1mM EDTA mixture media. $CD4^+/CD8^+$, $CD4^+/CD8^+/CD25^+$, and $CD3^+/CD45^+$ were used as representative of T cells prominent in Ang II-induced hypertension, $CD68^+$ was used as representative of monocytes/macrophages, and $CD4^-/CD5^-/CD8^-/CD90^+$ were used as representative of angiogenic progenitor cells[2-5]. Antibodies were purchased from AbD Serotec (Alex647 conjugated CD3, CD4, CD5, CD8; RPE conjugated CD25, CD68; FITC conjugated CD45; PerCP-cy5.5 conjugated CD90), and used as recommended by the manufacturer. Cells were incubated with antibodies for 45 minutes at 4^{o}C. Individual antibodies for each filter were prepared in each cell suspension and used as control. After centrifuging (1200rpm x 15min) and washing twice, cells were fixed with 2% paraformaldehyde for analysis. All samples were read using an LSR-II (BD Biosystems) at the University of Florida ICBR and the data were analyzed with FACS Diva software, version 6.1.2.

Immunohistochemistry

Rats were euthanized, under deep anesthesia, by perfusion with 300ml of PBS followed by 300ml of Formalin. Brain tissue was collected in formalin overnight, and then transferred to 30% sucrose for 3-4 days (or until drop to bottom of 50ml conical tube). Then, whole brain was cryoprotected with OCT Compound (Tissue-Tek), frozen and stored at -80°C until sectioning. Brain sections were cut at 30um and stained following a free-floating protocol. First, sections were blocked for 30 minutes in 10% goat serum in 0.3% Triton X-100. Immunohistochemistry was performed with rabbit anti-Iba1 primary antibody (1:600 dilution; Wako 079-19741) and/or chicken anti-GFP (1:1000 dilution; Abcam ab13970) in 0.3% Triton X-100 incubated overnight at 4°C, followed by a secondary antibody in 0.3% Triton X-100 incubation (1:1000 dilution; Invitrogen; A-11008 or A-11012) for 60 minutes at RT. Slides were mounted with VECTASHIELD mounting medium with DAPI (Vectorlabs). The micrographs were taken using spinning disk fluorescent confocal microscopy under equal conditions for all slides. The images are processed and quantified for cell body area, number of microglia cells, number of double positive Iba1+/GFP+ cells, using Image J by 2-3 blinded researchers.

RNA isolation and RT-PCR

RNA was isolated from BM MNCs using RNeasy Plus Mini Kit (Qiagen) and from brain PVN tissue using TRIzol Reagent (Ambion), both as per manufacturer's protocols. Purity of RNA was evaluated spectrophotometrically by 260/280 ratio. Reverse transcription was accomplished using High Capacity Reverse Transcription kit (Applied Biosystems) and 500ng RNA from previous step. RT-PCR was performed using Taqman universal PCR master mix and Taqman gene expression assay primers (Applied Biosystems): Gapdh (Rn01775763_g1), Ccl2 (Rn00580555_m1), Ccr2 (Rn01637698_s1), Csf2 (Rn01456850_m1), Hif1a (Rn00577560_m1), Ifng (Rn00594078_m1), Il1b (Rn00580432_m1), Il6 (Rn01410330_m1), Il12b

(Rn00575112_m1), Tlr4 (Rn00569848_m1), Tnf (Rn01525859_g1), Itgam (Rn00709342_m1), Nos2 (Rn00561646_m1), Nos3 (Rn02132634_s1). Real-time PCR was run using ABI Prism 7600 sequence detection system. All cDNA samples were assayed in duplicated. Data were normalized to GAPDH.

Chemokine (C-C motif) ligand 2 (CCL2) and Norepinephrine (NE) ELISA

Femur bones were collected, trimmed at the distal epiphysis end, and placed in a 15 ml conical tube, with the trimmed epiphysis facing the bottom and immersed in 200 µl of sterile PBS (CCL2 ELISA) or NE ELISA buffer (NE ELISA). The bones were then centrifuged at 4000rpm for 30 minutes at 4°C to extract the bone marrow. Bones were removed, and the pelleted bone marrow was briefly vortexed, and incubated for 30 minutes on ice. Bone marrow was then centrifuged at 1200 rpm for 10 minutes at 4°C, and the bone marrow supernatant was collected and used immediately (CCL2 ELISA) or stored at -80°C for later use (NE ELISA).

The CCL2 levels from the BM supernatant, serum, and cerebrospinal fluid (CSF) were measured using a commercially available kit (Life Technologies; KRC1012) following the manufacturer's instruction. The NE levels from the BM supernatant and plasma were measured using a commercially available kit (Labor Diagnostika Nord GmbH & Co.KG, Germany; Rocky Mountain Diagnostics; BA E-5200) following the manufacturer's protocol.

The ELISA plates were evaluated spectrophotometrically at 450 nm (SynergyMx multi-mode microplate reader, Biotek). The quantification of CCL2 and NE content was achieved by comparing their absorbance with a reference curve prepared with a known standard concentration (provided in kits). All experiments were run in duplicates. The CCL2 and NE content were normalized for total protein level in the BM supernatant and CSF, or serum volume. The protein concentration of BM supernatant and CSF was determined by Bio-Rad protein assay method following the manufacturer's instruction.

Cardiac Hypertrophy

Cardiac remodeling was determined by two measurements: (1) quantification of cardiomyocyte diameter in the left ventricular free wall of hematoxylin and eosin (H&E) stained cardiac tissue and (2) by normalizing whole cardiac weight to tibia length. Cardiac tissue was collected whole, rinsed with sterile PBS, dried of excess liquid, and weighed. It was fixed in formalin for 2 days, and given to the Molecular Pathology core to embed in paraffin and stain with H&E. Quantification of cardiomyocyte diameter was performed by two-three blinded researchers using ImageJ.

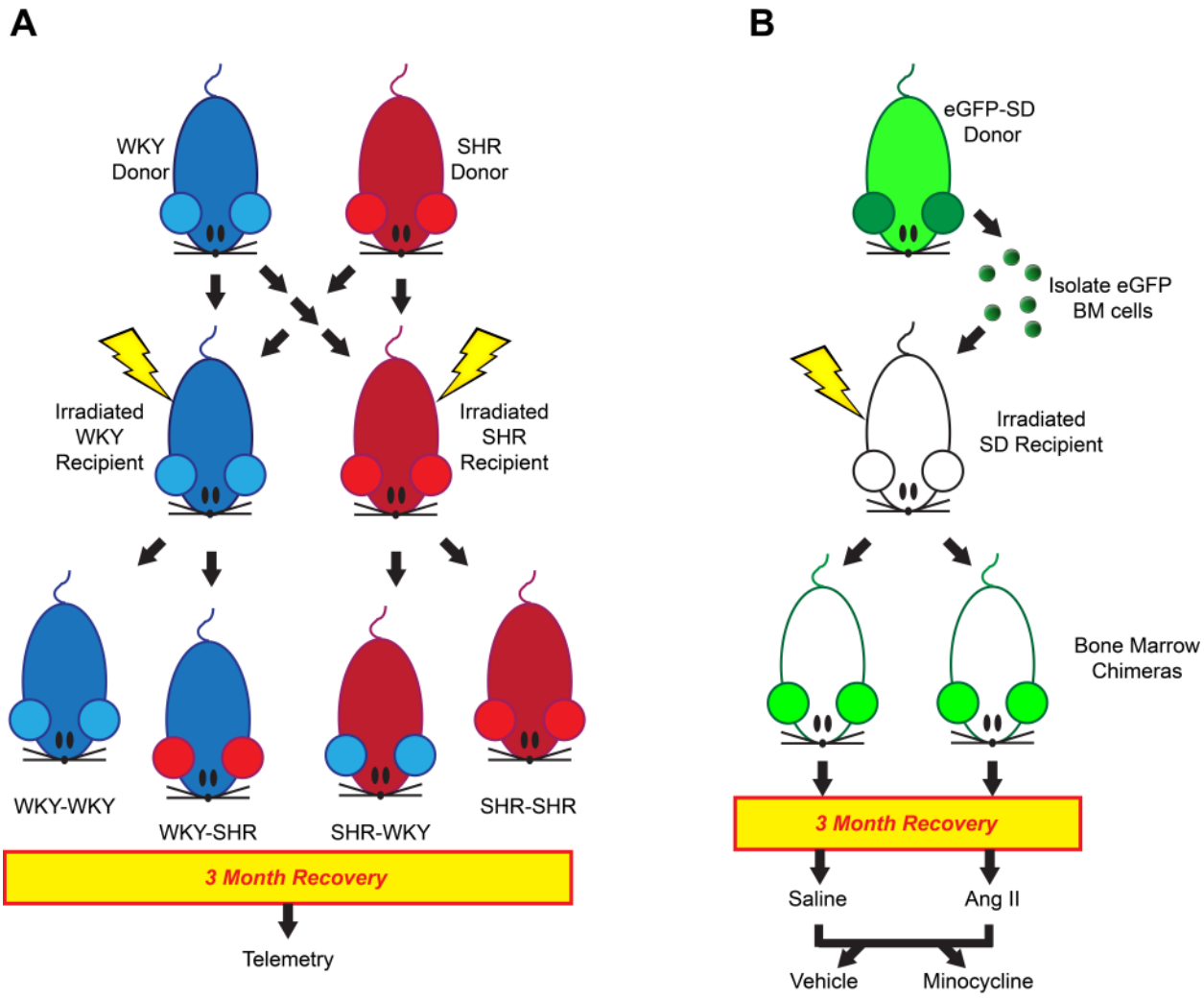
Primary Hypothalamic Neuronal Culture

Hypothalamic neurons in primary culture were established essentially as described previously[7]. These cultures contain more than 90% neurons with some astroglia. These cultures have extensively utilized in our previous studies to investigate Ang II signal transduction pathways[8, 9]. Neuronal cultures were established in 35mm 6 well plates for 10 days before use. Cultures were treated with 1uM Ang II in a serum-free media, and cell lysate and culture media were collected at 3, 6, 9, and 12 hours for analysis.

Data and Statistical Analysis

Data were expressed as mean±SEM. 2-way ANOVAs or 1-way ANOVAs, and Bonferroni post-tests were used to allow multiple comparisons of cardiovascular variables across time and between different groups. Paired/unpaired Student *t* tests were used for comparisons between 2 groups where applicable, with $p<0.05$ considered significant. GraphPad Prism 6 was used as the statistical software and for graph generation.

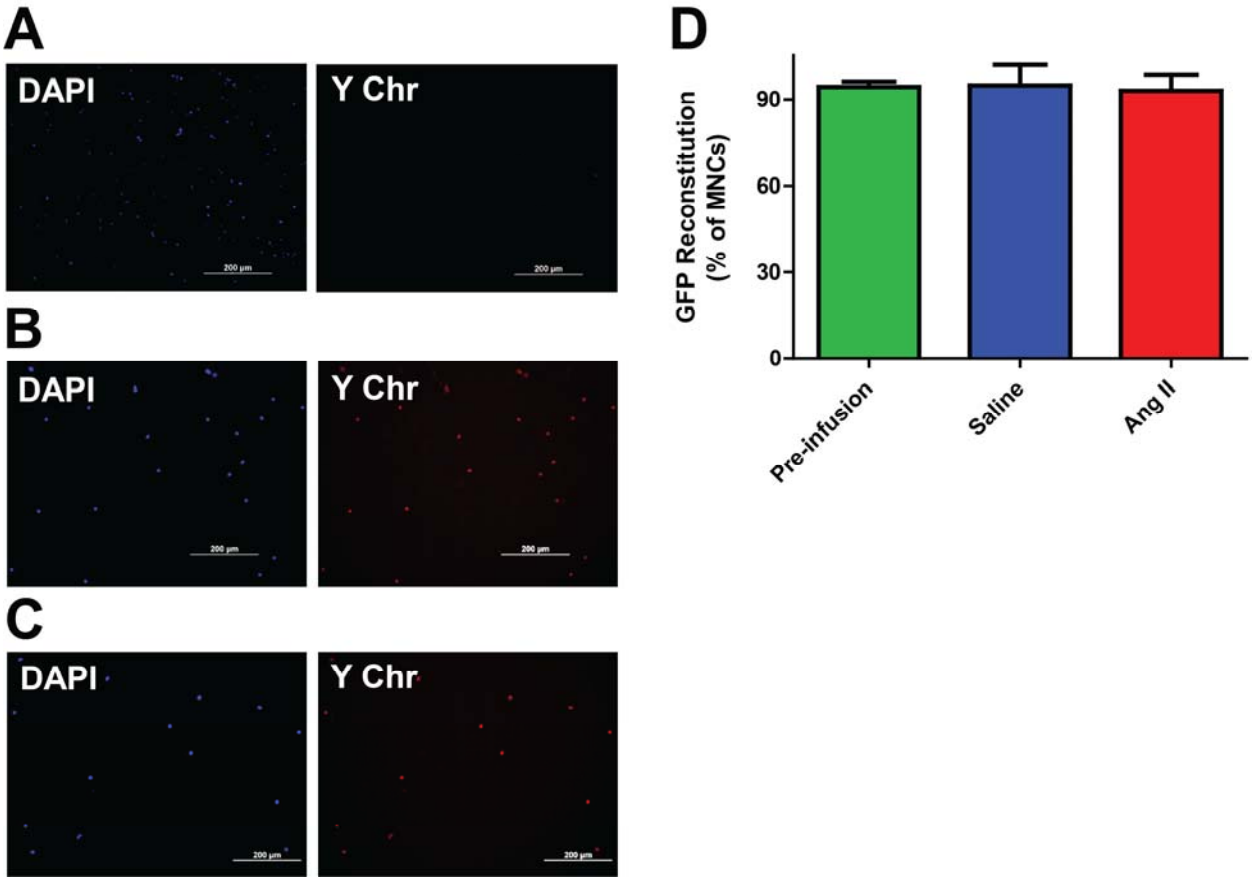
Online Figures



Online Figure I. Schematic design of bone marrow reconstitution experiments

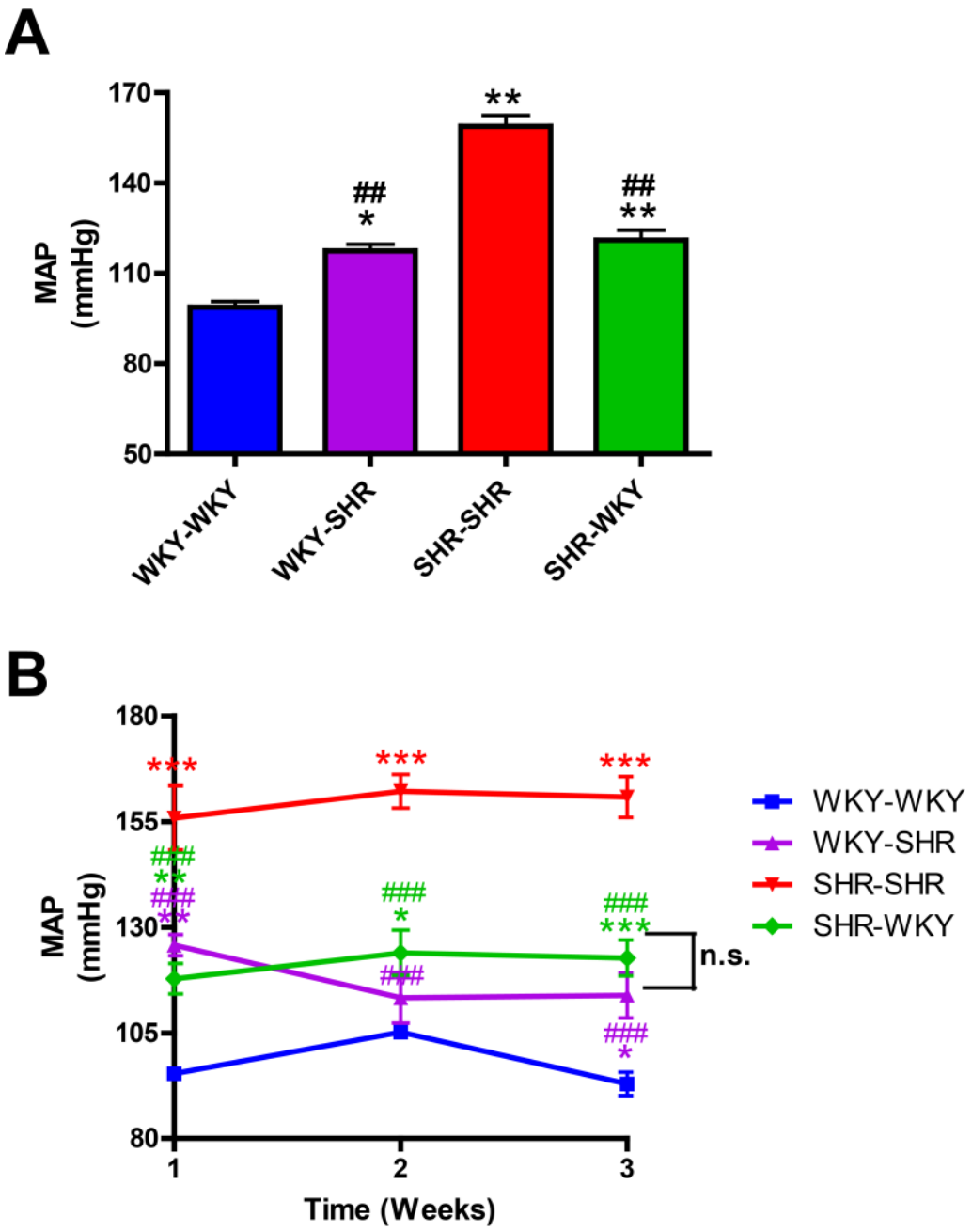
A. Design of WKY and SHR bone marrow (BM) chimeras. BM ablation was achieved by lethal irradiation, followed by reconstitution with whole BM MNCs from donor SHR and WKY rats.

B. Design of eGFP-BM chimeras and minocycline experiment. Donor eGFP BM MNCs were isolated from SD-Tg(UBC-EGFP) and injected via tail vein into irradiated SD recipients. All animals were allowed to recover for 3 months prior to initiation of experiments. For more details, see methods section.



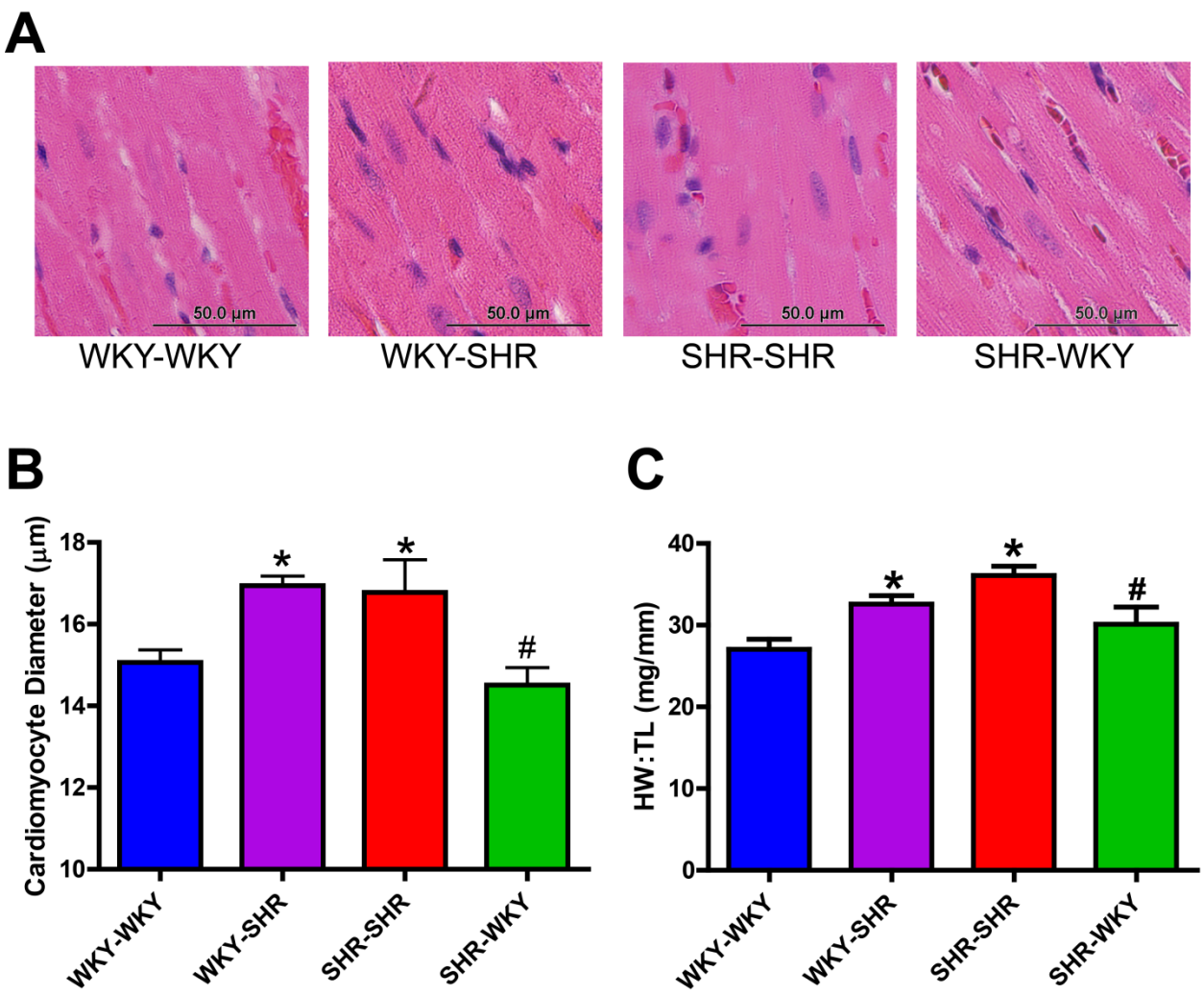
Online Figure II. Reconstitution of SHR and WKY and eGFP-SD bone marrow (BM)

A. Negative control Y chromosome staining of mononuclear cells (MNCs) from the blood of naïve female SHR, with DAPI-stained cell nuclei on the left and no presence of Y chromosome-stained cells on the right. **B.** Representative Y chromosome staining of MNCs from the blood of the female SHR reconstituted with the male WKY BM. **C.** Representative Y chromosome staining of MNCs from the blood of the female SHR reconstituted with the male SHR BM. Reconstitution was considered effective when >90% of blood MNCs stained positive for Y-FISH. **D.** Reconstitution of SD rats with eGFP BM was confirmed before beginning experiments, and again at the end of both saline and Ang II infusion.



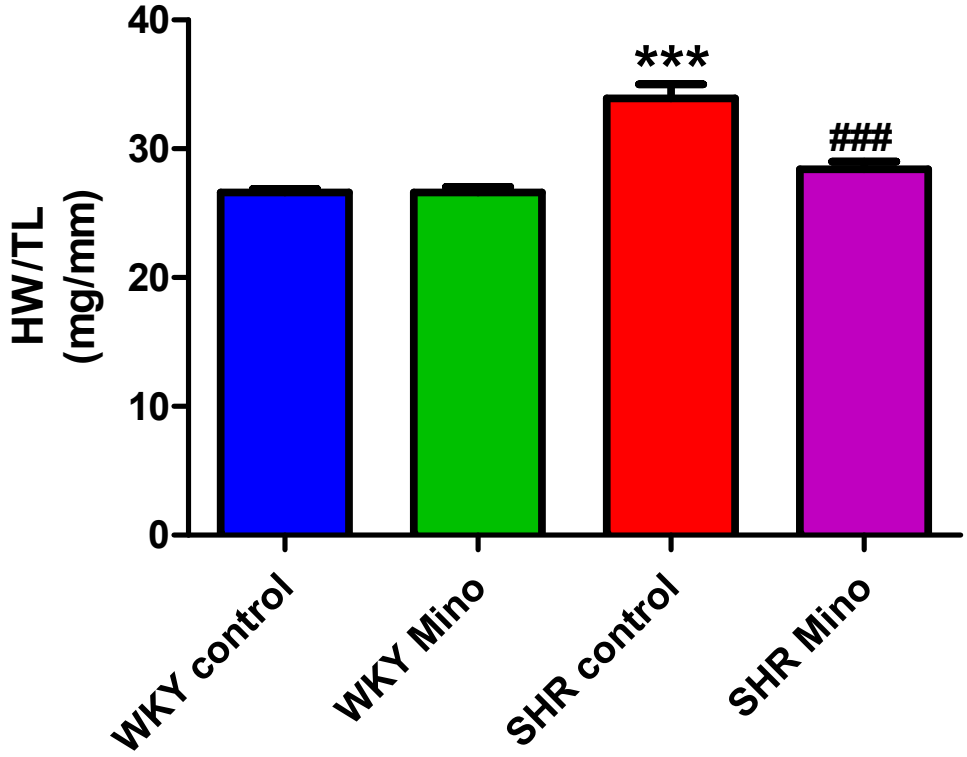
Online Figure III. Parallel experiment of WKY and SHR chimeric rats was carried out where mean arterial pressure (MAP) was measured by tail-cuff

A. MAP obtained by tail-cuff was comparable to that obtained by radiotelemetry and presented in the main manuscript. **B.** This effect was consistent and reproducible among animals and over time (n=7-9). *p<0.05, **p<0.01, ***p<0.001 vs WKY-WKY; ###p<0.001 vs SHR-SHR



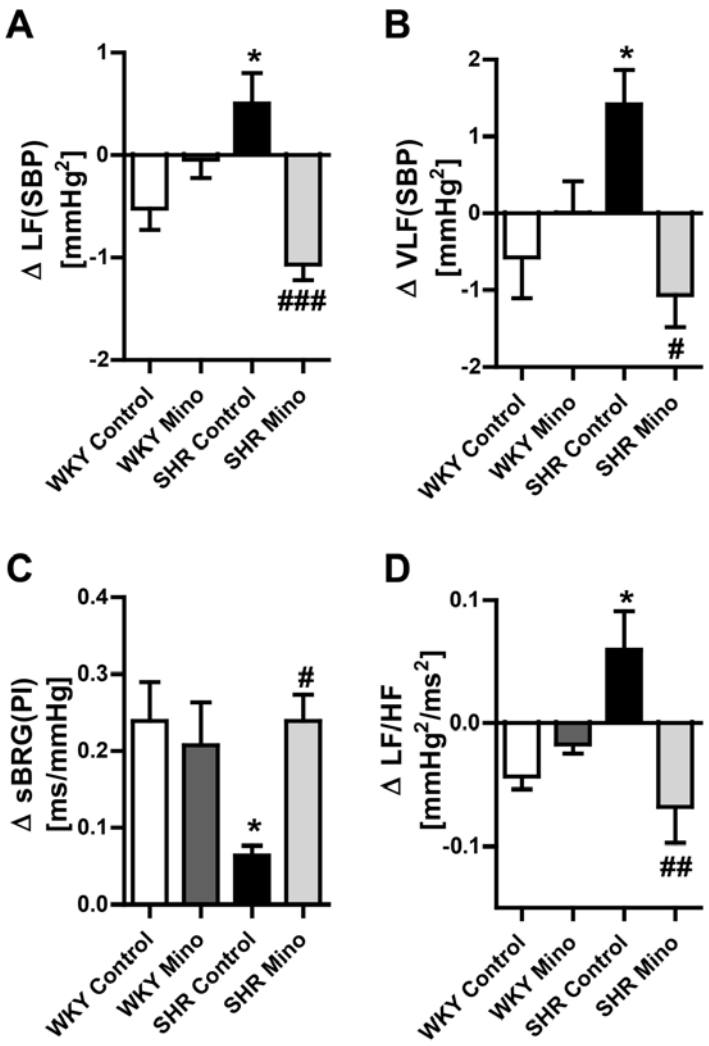
Online Figure IV. Cardiac remodeling was confirmed by cardiomyocyte diameter measured from the left ventricular free wall

A. H&E stained representative images of left ventricular free wall. **B**. Quantification of cardiomyocyte diameter confirmed findings of heart weight to tibia length ratio in main manuscript (n=3 per group). **C.** Cardiac hypertrophy was determined by heart weight to tibia length ratio (HW:TL; n=5 per group). WKY-SHR have higher HW:TL than WKY-WKY control; and SHR-WKY have lower HW:TL than SHR-SHR counterparts. *p<0.05 vs WKY-WKY; #p<0.05 vs SHR-SHR



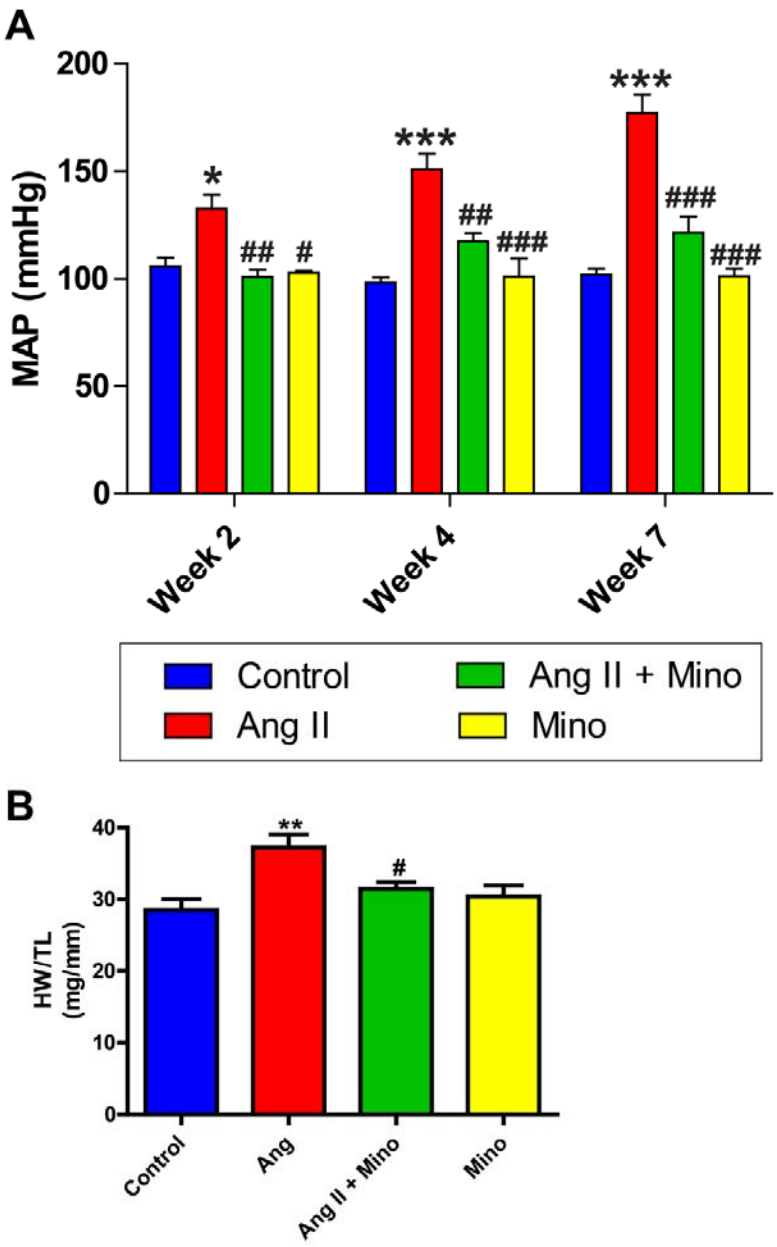
Online Figure V. Minocycline reduces cardiac hypertrophy in the SHR

A. Oral Mino for 6 weeks given to10 week old SHRs lowers HW:TL as an indicator of cardiac hypertrophy (n=5-6 per group). ***p<0.001 vs WKY control, ###p<0.001 vs SHR control.



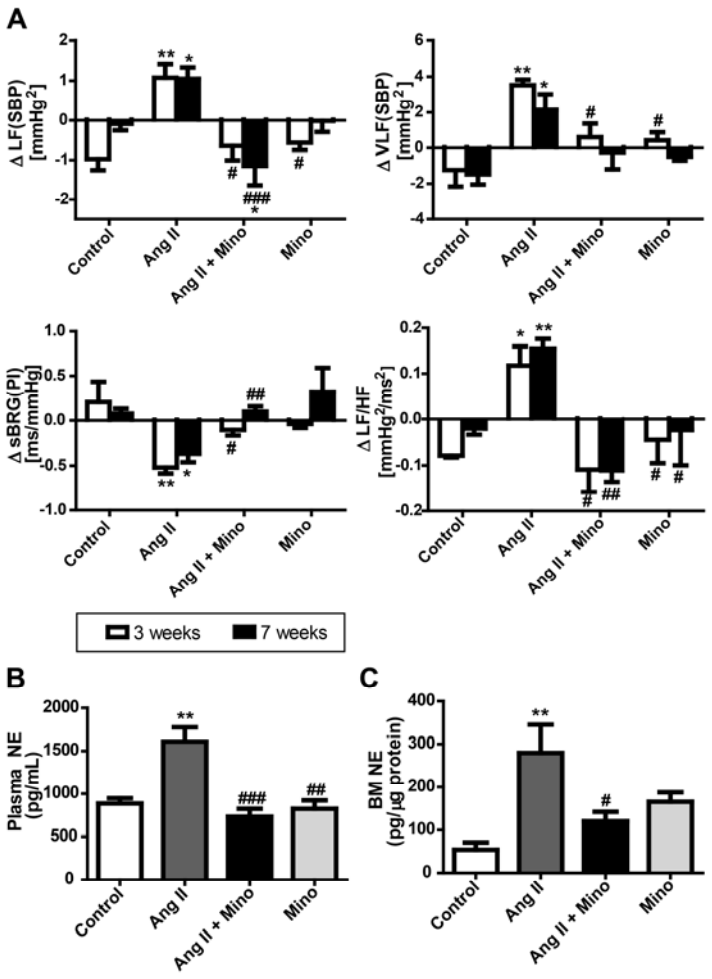
Online Figure VI: Oral minocycline (mino) restores autonomic balance in SHRs.

A. Spectral analysis of the systolic blood pressure (SBP) and pulse interval (PI) waveforms of telemetry revealed that cardiac spontaneous baroreflex gain [ΔsBRG(PI)] was dampened in SHR versus WKY rats. This effect was attenuated by mino treatment. **B.** Vasomotor sympathetic tone (ΔLF[SBP]) and **C.** the humoral modification of sympathetic tone (ΔVLF[SBP]) were both increased in SHRs, and were reversed by mino. **D.** Additionally, vasovagal balance (ΔLF[SBP]:HF[PI]) was restored following mino treatment (n=3-5 per group). LF: low frequency, VLF: very low frequency, sBRG: spontaneous baroreflex gain, HF: high frequency. *p<0.05, **p<0.01 vs WKY control; #p<0.05, ##p<0.01 vs Ang II, ###p<0.001 vs SHR control.



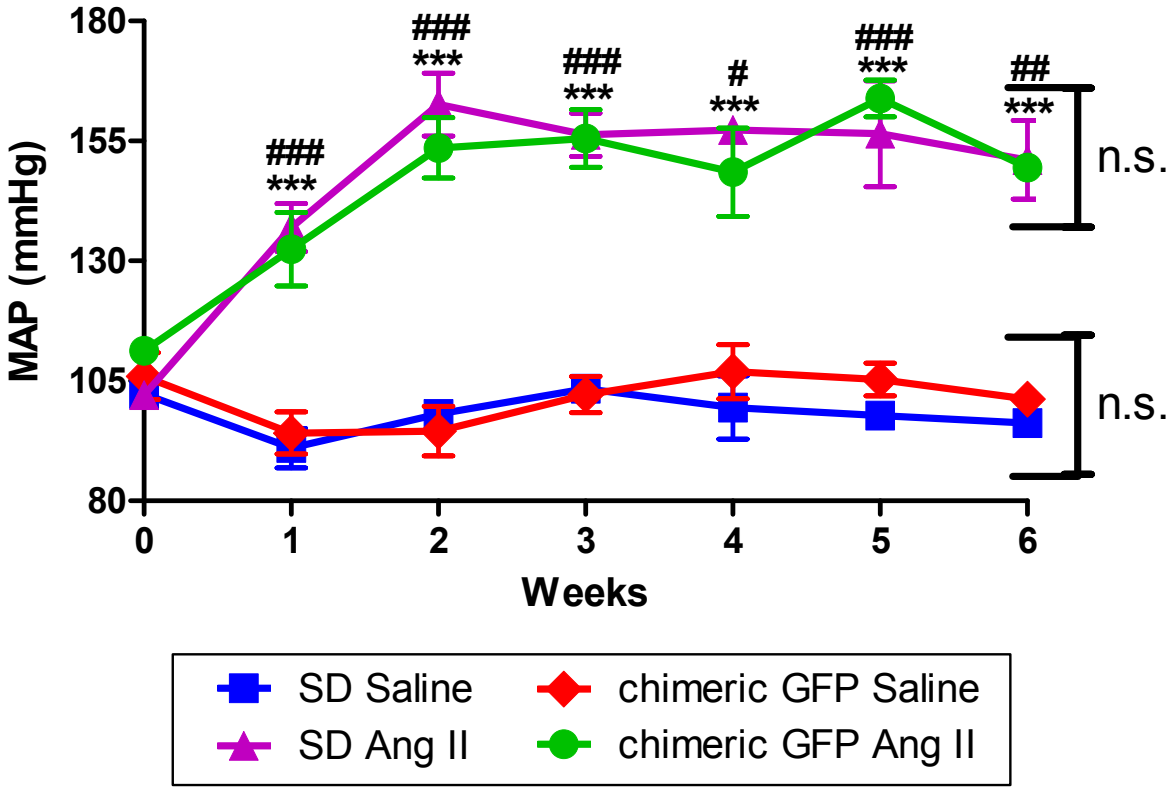
Online Figure VII. Minocycline lowers mean arterial pressure (MAP) and indirect measurement is comparable with the telemetry data presented in the main manuscript

A. A parallel experiment was performed with additional animals in which MAP was measured by tail-cuff (n=4-6 per group). The effects of minocycline are reproducible between animals and constant over time. **B.** Oral Mino lowers HW:TL in Ang II-dependent HTN as an indicator of cardiac hypertrophy (n=8 per group). *p<0.05, **p<0.01, ***p<0.001 vs Control; #p<0.05, ##p<0.01, ###p<0.001 vs Ang II



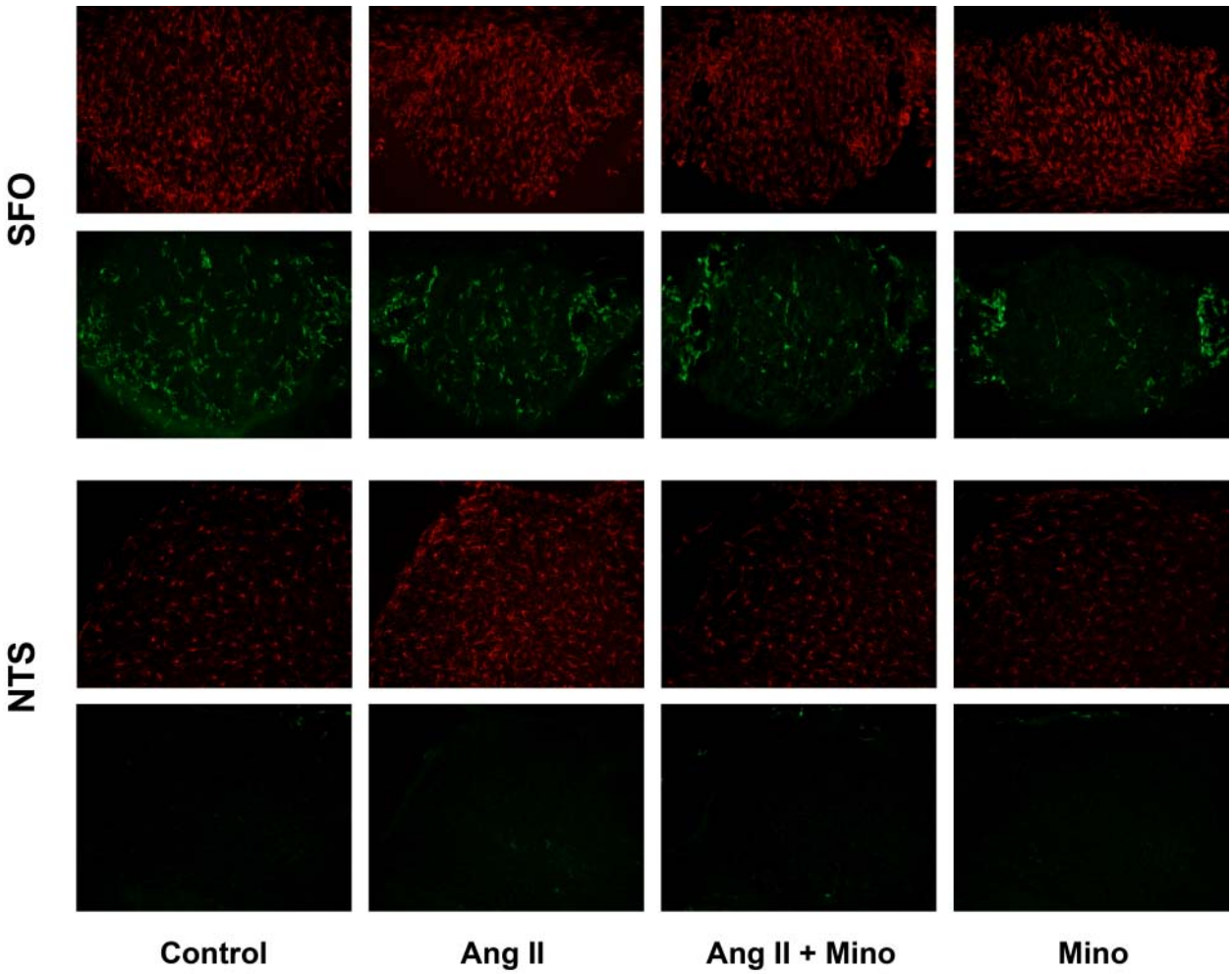
Online Figure VIII: Minocycline restores autonomic balance in chronic Ang II infusion.

A. Spectral analysis of the systolic blood pressure (SBP) and pulse interval (PI) waveforms of telemetry revealed that cardiac spontaneous baroreflex gain [ΔsBRG(PI)] was dampened in Ang II rats versus control. This effect was attenuated by mino treatment. Vasomotor sympathetic tone (ΔLF[SBP]) and the humoral modification of sympathetic tone (ΔVLF[SBP]) were both increased in Ang II infused rats, and were reversed by mino. Additionally, vasovagal balance (ΔLF[SBP]:HF[PI]) was restored following mino treatment (n=3-5 per group). **B.** Oral mino attenuates the elevation in plasma norepinephrine (NE) content (n=4-5 per group). **C.** Similarly, NE in the bone marrow (BM) supernatant was also decreased by mino (n=4-5 per group). LF: low frequency, VLF: very low frequency, sBRG: spontaneous baroreflex gain, HF: high frequency. *p<0.05, **p<0.01 vs control; #p<0.05, ##p<0.01 vs Ang II, ###p<0.001 vs Ang II.



Online Figure IX. Validation of the eGFP-SD chimeric rat model

These chimeric rats have similar response to Chronic Ang II infusion as naïve SD rats (n=8 per group). ***p<0.001 vs SD Saline; #p<0.05, ##p<0.01, ###p<0.001 vs chimeric GFP Saline.



Online Figure X. GFP+/Iba1+ microglia/macrophages in other autonomic brain regions

No distinct changes in GFP+/Iba+ microglia/macrophages were observed in either the subfornical organ (SFO) or the solitary nucleus (NTS). Red is Iba1 stain for microglia, green is GFP+ bone marrow derived cells.

References

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