Altered Inflammatory Response Is Associated With an Impaired Autonomic Input to the Bone Marrow in the Spontaneously Hypertensive Rat

Jasenka Zubcetic, Joo Yun Jun, Seungbum Kim, Pablo D. Perez, Aqeela Afzal, Zhiying Shan, Wencheng Li, Monica M. Santisteban, Wei Yuan, Marcelo Febo, Jay Mocco, Yumei Feng, Edward Scott, David M. Baekey, Mohan K. Raizada

Abstract—Autonomic nervous system dysfunction, exaggerated inflammation, and impaired vascular repair are all hallmarks of hypertension. Considering that bone marrow (BM) is a major source of the inflammatory cells (ICs) and endothelial progenitor cells (EPCs), we hypothesized that impaired BM–autonomic nervous system interaction contributes to dysfunctional BM activity in hypertension. In the spontaneously hypertensive rat (SHR), we observed a >50% increase in BM and blood ICs (CD4.8+) and a >50% decrease in EPCs (CD90+.CD4.5.8-) when compared with the normotensive Wistar–Kyoto rat. Increased tyrosine hydroxylase (70%) and norepinephrine (160%) and decreased choline acetyltransferase (30%) and acetylcholine esterase (55%) indicated imbalanced autonomic nervous system in SHR BM. In Wistar–Kyoto rat, night time–associated elevation in sympathetic nerve activity (50%) and BM norepinephrine (41%) was associated with increased ICs (50%) and decreased EPCs (350%) although BM sympathetic denervation decreased ICs (25%) and increased EPCs (40%). In contrast, these effects were blunted in SHR, possibly because of chronic downregulation of BM adrenergic receptor α2a (by 50%–80%) and β2 (30%–45%). Application of norepinephrine resulted in increased BM IC activation/release, which was prevented by predentinibution of acetylcholine. Electrophysiological recordings of femoral sympathetic nerve activity showed a more robust femoral sympathetic nerve activity in SHR when compared with Wistar–Kyoto rat, peaking earlier in the respiratory cycle, indicative of increased sympathetic tone. Finally, manganese-enhanced MRI demonstrated that presynaptic neuronal activation in SHR was associated with an accelerated retrograde transport of the green fluorescent protein–labeled pseudorabies virus from the BM. These observations demonstrate that a dysfunctional BM autonomic nervous system is associated with imbalanced EPCs and ICs in hypertension. (Hypertension. 2014;63:542-550.) • Online Data Supplement

Key Word: bone marrow • EPCs • hypertension • inflammation • rats, inbred SHR • sympathetic drive

A utonomic dysfunction, characterized by increased sympathetic and decreased parasympathetic activity, is a hallmark of neurogenic hypertension.1,2 Recent evidence has indicated a direct interaction of the autonomic nervous system with the immune system to regulate normal cardiovascular homeostasis. Thus, a dysfunctional neural-immune communication has been implicated in the pathogenesis of cardiovascular diseases and hypertension.3,4 Evidence of increased sympathetic nervous system activity to immune organs in hypertension supports this contention.5 Moreover, atherosclerotic vasculature is further compromised by mobilization of the bone marrow (BM)–derived inflammatory cells (ICs) after myocardial infarction, characterized by increased sympathetic nerve activity (SNA).6 However, the anti-inflammatory effects of the vagus nerve (i.e., parasympathetic) stimulation are demonstrated by lowered levels of the inflammatory cytokines and suppressed activation of ICs.7,8 Therefore, the imbalance of the parasympathetic/sympathetic influence in hypertension contributes to the hypertensive phenotype by perpetuating the inflammatory response.9 In contrast to the ICs, decreased circulating levels of endothelial progenitor cells (EPCs) and their dysfunction are demonstrated in hypertension and cardiovascular diseases,10-11 suggesting EPCs’ impaired abilities in repairing vascular damage. The EPCs, like ICs, seem to be neuroregulated, as suggested by the diurnal pattern of their release into the circulation.12 As the EPC numbers and function are inversely correlated in patients and rat models of hypertension,13,14 the overactive sympathetic drive to the BM and the imbalance in the overall parasympathetic/
sympathetic tone in hypertension may also directly contribute to the dampened EPC numbers and function, leading to the impairment of the endothelial reparative processes and accelerating the vascular dysfunction and hypertension-associated pathophysiology. Taken together, these observations led us to hypothesize that the autonomic imbalance influences the release of the progenitor cells from the BM, thereby affecting the circulating IC and EPC levels. Spontaneously hypertensive rat (SHR), a model of neurogenic hypertension that exhibits an early onset of autonomic and endothelial dysfunctions and increased inflammatory response, has been used in this study to evaluate this hypothesis. We present direct evidence that an altered autonomic regulation of BM is associated with an imbalance in the EPC and IC levels in hypertension.

**Methods**

All experimental protocols are presented in the Methods section and are available in the online-only Data Supplement. All animal procedures were approved by the University of Florida Institute Animal Care and Use Committee.

**Results**

**Circadian Regulation of BM ICs and EPCs**

First, we investigated whether increased sympathetic drive affected the BM activity in the SHR by comparing the levels of EPCs and ICs in the BM at the times of lowest (ie, 11 am, day) and highest (ie, 8 pm, night) sympathetic drive. We observed a 50% increase in the low frequency of systolic blood pressure (SBP), LF:SBP, in the Wistar–Kyoto (WKY) rat and a 130% increase in the LF:SBP in the SHR at night versus day (Figure 1A). This was associated with increased BM norepinephrine (WKY, 41%; SHR, 38%) at night versus day (Figure 1B). Furthermore, the overall sympathetic drive as measured by LF:SBP was higher in the SHR versus the WKY rats at both day and night (Figure 1A and 1B). Furthermore, a 158% increase in the density of tyrosine hydroxylase (TH) immunoreactivity (Figure 4A), a 30% decrease in the density of choline acetyl transferase (Figure 4B), and a 55% decrease in the density of acetylcholine esterase (Figure 4C) immunoreactivity around the femoral BM blood vessels were observed in the SHR when compared with the WKY, suggesting impaired BM autonomic nervous system input. Increased sympathetic drive at night in the WKY rats was associated with a 50% and 33% increase in the IC levels in the BM and blood, respectively (Figure 1C and 1D, left), and a 350% decrease in the blood EPCs (Figure 1F, left). In comparison, the ICs were higher and the EPCs were lower in the BM of the SHR both at day and night (Figure 1C and 1D) and in the blood of the SHR at day (Figure 1E and 1F, left) when compared with the WKY rat. Furthermore, there was a lack of the night time–associated increase in the ICs and decrease in the EPCs in the SHR (Figure 1C and 1F, right), suggesting a dysfunctional response of the SHR BM to the circadian-related sympathetic drive changes. To investigate this further, we performed BM sympathetic denervation by dissection of the superior cervical sympathetic nerve in the WKY and SHR rats.
ganglion in the WKY and SHR as previously described.\textsuperscript{15} Forty-eight hours after the superior cervical ganglion, we observed a 50% and 30% decrease in the BM norepinephrine in the WKY and SHR, respectively (Figure 2A) when compared with the naïve controls. This was associated with a 25% decrease in the BM IC levels and a 40% increase in the BM EPC levels in the WKY (Figure 2B, left), similar to the decrease in ICs and increase in EPCs observed from night to day in the WKY (Figure 1C and 1D). However, BM ICs showed a trend toward an increase but produced no significant change after superior cervical ganglion, whereas we observed ≈18% increase in the BM EPC levels in the SHR (Figure 2B, right), suggesting attenuated responsiveness of the BM HSPCs to the BM sympathetic changes in the SHR when compared with the WKY. Quantitative polymerase chain reaction showed a significant decrease in both the $\alpha_2a$ (by ≈50 at day and ≈80% at night) and $\beta_2$-adrenergic receptors (by ≈45% at day and ≈30% at night) in BM mononuclear cells of SHR when compared with the WKY. This suggested a possible mechanism for loss of circadian regulation of BM cells in the SHR (Figure 3).

To investigate the mechanism of increased mobilization of the BM ICs at high sympathetic drive, we used in vivo real-time imaging of the tibial BM (Figure 5) to determine whether increase in the local norepinephrine in the BM would influence mobilization of inflammatory BM cells by studying the mobilization of green fluorescent protein (GFP)–labeled ICs in response to administration of 6 µg/kg norepinephrine, in the absence and in the presence of 80 mg/kg acetylcholine. A significant increase in the movement of ICs in response to norepinephrine was observed (Figure 5; Movies in the online-only Data Supplement). This movement was significantly attenuated by preadministration of acetylcholine (Figure 5).

**Loss of EPC Function in the SHR**

The overall decrease in the EPC numbers in the SHR when compared with the WKY was accompanied with the loss

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**Figure 2.** Effects of superior cervical ganglion denervation (SCGx) on the bone marrow (BM) endothelial progenitor cell (EPC) and inflammatory cell (IC) levels in spontaneously hypertensive rat (SHR) and Wistar-Kyoto (WKY) rats. A, A significant reduction in norepinephrine (NE) protein levels was observed in the BM cell supernatant in both the SHR and the WKY after SCGx ($P<0.05$; $n=3$). B and C, Examples of raw FACS (fluorescence activated cell sorting-area) data for WKY (B) and SHR (C), showing the changes in CD4.8\textsuperscript{+} (top, highlighted in red box) and CD90\textsuperscript{-}.CD4.5.8\textsuperscript{-} BM cells (bottom, highlighted in red box) in control and SCGx rats. D, A significant decrease in the ICs in the WKY and an increase in EPCs in both WKY and SHR were observed in the BM after SCGx ($P<0.05$ vs naïve control; $n=3$). FSC-A indicates forward scattered light; and SSC-Area, side scattered light.
of cell function: the angiogenic ability of the BM-derived cells was also reduced in the SHR when compared with the age-matched WKY, as evidenced by a 65% decrease in the formed tube length, a 50% reduction in tube width formation ex vivo (Figure S1A–S1C in the online-only Data Supplement), and a 35% decrease in the SHR BM EPC’s ability to proliferate in response to SDF (Figure S1D).

SNA to the BM Is Altered in the SHR

Next, we characterized the activity of the sympathetic nerve innervating the femoral bone. Respiratory cycle triggered averages of simultaneously recorded phrenic and femoral sympathetic nerve activities (fSNA) were made in the decerebrate artificially perfused rat (Figure 6A) as established previously. This in situ decerebrate artificially perfused rat revealed a classic phrenic nerve activity pattern (Figure 6B, second and third panels) and a robust fSNA (Figure 6B, fourth and fifth panels). The influence of respiration on fSNA was enhanced by 9% CO₂ (Figure 6D, third panel, red arrows) and was completely blocked by hexamethonium (Figure 6D, fourth panel). Next, we compared the respiratory modulation of fSNA between the hypertensive and normotensive rat. The peak firing of fSNA in the SHR occurred earlier in the phrenic nerve activity cycle (ie, at the end of the inspiration [I] phase; Figure 6E, third panel, red arrow) and was 25% more robust when compared with the normotensive control, the peak of which occurred in the postinspiration (P-I) phase (Figure 6E, second panel, red arrow), typical of respiratory-sympathetic patterning. These responses were consistent and repeatable over several preparations (n=4 per strain).

Retrograde Viral Tracing Reveals Dysfunctional Autonomic-BM Communication in the SHR

Finally, MRI and GFP-pseudorabies virus (PRV) retrograde labeling experiments were performed to investigate increased autonomic-BM communication in hypertension further. Mn²⁺-enhanced MRI, a technique commonly used to visualize elevated cellular activity in vivo, showed a 20% to 25% higher neuronal activity in the hypothalamic paraventricular nucleus (PVN) of the SHR when compared with the WKY rats (Figure 7). In addition,
the rate of the PVN retrograde labeling by the GFP-PRV injected in the BM was significantly faster in the SHR when compared with the WKY rats (Figure 7B). For example, 7 days after GFP-PRV administration, the PVN neurons from the SHR showed robust GFP fluorescence, whereas little fluorescence was seen in the PVN of the WKY rats (Figure 7B). A similar increase in the labeling of neurons in other SHR autonomic brain regions (such as the NTS [nucleus of the solitary tract], RVLM [rostral ventrolateral medulla], SFO [subfornical organ]) was observed (Figures S2 and S3). These responses were consistent and repeatable over several preparations (n=3 per strain for MRI; n=3 per strain per time point for GFP-PRV retrograde labeling).
Discussion

Our study is novel in a number of ways: (1) we are the first to establish the electrophysiological recordings of the sympathetic nerve innervating the femoral BM (ie, the fSNA; Figure 6). The electric properties of fSNA in the SHR are similar to those of the thoracic SNA in the SHR, in that its peak activity occurs earlier in the phrenic cycle and it is more robust when compared with the normotensive control (Figure 6); (2) norepinephrine and TH levels in the BM of the SHR are increased. In addition, night time norepinephrine levels were significantly higher than daytime in the BM of both the WKY and SHR, when the overall sympathetic drive was higher in these animals; (3) IC levels increased and EPC levels decreased in the SHR when compared with the WKY rats and correlated with the increased sympathetic drive; (4) direct application of norepinephrine into the BM activated the BM ICs; (5) regular circadian regulation of the BM ICs and EPCs, which is present in the WKY, is significantly compromised in the SHR; (6) α2a- and β2-adrenergic receptor levels are significantly decreased in the SHR BM, suggesting a possible mechanism behind the loss of circadian regulation of ICs/EPCs in the SHR BM; (7) a decreased BM parasympathetic tone, as demonstrated by decreased BM choline acetyl transferase and acetylcholine esterase in the SHR, may contribute to the inflammatory activation of the BM in the SHR. Taken together, we suggest that hypertension in the SHR is associated with a persistent increase in the sympathetic drive to the periphery, including the BM. This is associated with an increase in the BM norepinephrine and TH levels, resulting in impaired BM cell activity, reflected in the increased ICs and decreased EPCs in the SHR. Chronically high fSNA eventually leads to downregulation of adrenergic receptors in the BM, leading to the loss of circadian regulation of the BM cells in the SHR. This loss of circadian regulation, coupled with persistent increase in ICs and decrease in EPCs, compromises the ability of vasculature to repair the damage induced by hypertension, which is accentuated by the residual EPCs that become dysfunctional in the SHR.

We show here that the typical diurnal increases in the sympathetic vasomotor drive at night are accompanied by similar
changes in the BM norepinephrine; however, both the overall sympathetic vasomotor drive, as indicated by LF:SBP, and the BM norepinephrine are significantly higher in the SHR, indicating an increased sympathetic drive to the BM of the SHR. This is corroborated by elevated BM TH protein levels in the SHR (Figure 4), as well as our electrophysiological recordings showing activity changes in the BM fSNA in the SHR, which are similar to the changes observed in the thoracic SNA of the SHR and are indicative of elevated SNA. Other evidence supports this contention. For example, GFP-PRV retrograde labeling of the neurons in the PVN and other cardioregulatory brain regions from the BM is accelerated and more robust in the SHR when compared with the WKY. This is not because of the genetic diversity between the 2 rat strains but is rather associated with hypertension because accelerated labeling of the PVN neurons by BM administration of GFP-PRV is also observed in chronic angiotensin II–infused rat model of hypertension (not shown) and is in agreement with the heightened neuronal activity in the PVN of the SHR, as demonstrated by elevated manganese-enhanced MRI signals in the SHR (Figure 7).

In the WKY, the night time–associated increase in the BM sympathetic drive was accompanied by elevated BM and blood CD4.8 T cells and decreased EPC levels, suggesting circadian regulation of the BM cells (Figure 1). This concept is not new because both animal and human studies have shown that the release of BM cells follows a regular circadian pattern, and that in rodents; the immune cells (ie, the surveillance cells) are released at night, whereas the repair cells (including the EPCs) are released during the day. This circadian regulation of BM activity seems to be dependent on the sympathetic innervation, interruption of which results in pathological situations, as evidenced in diabetes mellitus. Our observations in the SHR are consistent with this because the circadian regulation of the BM cells seems to be impaired in the SHR (Figure 1). Thus, in the SHR, the IC levels remain chronically high, whereas the EPC levels remain low. Therefore, one can postulate that the presence of the functioning circadian rhythmic regulation of the highs and lows in ICs and EPCs at night and day, associated with diurnal sympathetic changes, is the reason that the WKY rat does not develop hypertension. In contrast, loss of circadian regulation in the SHR results in persistently higher ICs and lower EPCs throughout, which, combined with the reduction in the EPC function in the SHR (Figure S1) may contribute to increased inflammation and compromised repair of the vascular damage, thereby perpetuating the hypertension-related cardiovascular pathophysiology in the SHR. The loss of circadian regulation of the BM cell activity in the SHR is also reflected in the lack of BM cell response after the BM denervation. As the BM norepinephrine levels are reduced after the BM sympathetic denervation in both rat strains, this results in decreased ICs and increased EPCs but only in the WKY and not in the SHR (Figure 2D). This may be because of the remaining high norepinephrine levels in the BM of SHR when compared with
the WKY rats (Figure 2A). However, the lack of the BM cell response after BM denervation in the SHR, as well as the loss of circadian regulation of the BM cell activity discussed above, is more likely because of chronic downregulation of specific adrenergic receptors in the BM of the SHR, perhaps occurring in compensation for the chronically high sympathetic drive. Alternatively, it is pertinent to point out that the BM denervation was a relatively short-term experiment (<48 hours), which may not be sufficient time to correct the chronic adrenergic receptor dysfunction in the BM of SHR.

BM adrenergic receptors are crucial in the BM cell responses. In line with this, we observed that norepinephrine applied locally to the BM activated the BM ICs, confirming a direct effect of norepinephrine on the BM cells. Interestingly, this effect of norepinephrine was blocked by preapplication of acetylcholine in the BM, suggesting that the parasympathetic influence in the BM may counteract/dampen the effects of the SNA. As phrenic nerve activity is generally reduced in the SHR, and, as it appears, in the BM too, as demonstrated by reduced acetylcholine esterase and choline acetyltransferase protein levels in the BM of SHR when compared with the WKY rats, it may be that this also contributes to dysfunctional BM cell activity in the SHR. This is consistent with previous data showing that stimulation of the vagus nerve ameliorates experimental inflammatory diseases. However, further experiments, perhaps with the use of genetically modified animals where chimeric mice are generated by adoptive BM transfer from the nicotinic AchR knockout mice, are needed to elaborate the involvement of parasympathetic influence in the BM in hypertension. We also recognize a limitation of this study in that it does not distinguish the effects of the BM norepinephrine, delivered directly via the increased BM fSNA, from the peripheral norepinephrine delivered via the BM blood vessels. Availability of adrenergic receptor knockout mice may be useful in chimeric experiments to address this issue in the near future.

In summary, we propose that there is a loss of circadian regulation of the BM cells because of dysfunctional sympathetic/adrenergic mechanisms in the BM, resulting in chronically high ICs and low EPCs in the SHR. This study raises key questions: do changes in the BM sympathetic drive precede the development of high BP or are they involved in the establishment of the hypertensive pathophysiology? Although both animal and human studies implicate prehypertensive elevation in the sympathetic drive, further experiments to measure changes in fSNA before development of high BP are warranted. Nonetheless, the present study demonstrates a dysfunctional BM activity in hypertension, which is associated with changes in fSNA.

**Perspectives**

In this study, we present the first direct evidence for an impaired circadian regulation of the BM cell activity in the SHR. We present the hypothesis that an impaired sympathetic input to the BM promotes imbalance in the BM ICs and EPCs, which may result in an increased inflammatory-dependent vascular injury, and compromises the vascular repair in hypertension. This hypothesis is supported by the following: (1) enhanced functional neural connections between the presynaptic brain regions and the BM in hypertension; (2) norepinephrine directly activates the BM ICs, which is attenuated by preapplication of acetylcholine; (3) elevated TH and norepinephrine in the BM of SHR; (4) circadian control of the BM activity was impaired in the SHR, which exhibited increased ICs and decreased EPCs when compared with the WKY because of chronic downregulation of adrenergic receptor levels in the SHR BM. Thus, targeting the sympathoadrenergic mechanisms, the BM presents a novel strategy for consideration in neurogenic hypertension.

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**Disclosures**

None.

**References**


### Novelty and Significance

**What Is New?**
- This article presents direct evidence of altered sympathetic drive to the BM, which is associated with the dysfunctional BM-derived endothelial progenitor cells and inflammatory cells in the rat model of neurogenic hypertension.
- Retrograde labeling using green fluorescent protein–labeled pseudorabies virus shows increased neuronal communication between the brain presympathetic nuclei and the BM in the spontaneously hypertensive rat.
- Increased sympathetic drive to the BM is associated with increased BM tyrosine hydroxylase and norepinephrine. Decreased choline acetyltransferase and acetylcholine esterase suggests impaired parasympathetic influence to the BM. Furthermore, local delivery of norepinephrine to the BM increases the mobilization of the inflammatory cells in vivo which can be antagonized by similar localized delivery of acetylcholine.

**What Is Relevant?**
- Increased inflammation and reduced vascular repair are hallmarks of hypertension and cardiovascular diseases.

**Summary**

The study is the first direct evidence of elevated sympathetic drive to the BM in hypertension, which is associated with decreased BM-derived endothelial progenitor cell counts and function and increased BM-derived inflammatory cell counts and mobilization. We suggest that repairing the balance in the EPCs and inflammatory cells presents a novel antihypertensive target in drug-resistant hypertension.
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Altered inflammatory response is associated with an impaired autonomic input to the bone marrow in the SHR

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Short title: Brain-bone marrow communication in hypertension

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Methods

Animals

Adult male Wistar-Kyoto (WKY) and spontaneously hypertensive rats (SHR) aged 12-21 weeks (Charles River Laboratories) were housed in a temperature-controlled room (22°C to 23°C) with a 12:12-hour light-dark cycle. Additional male Sprague-Dawley (S-D) rats and SHRs aged 4-5 weeks were used for the in situ decerebrated artificially perfused rat (DAPR) preparation. All experimental procedures were approved by the University of Florida Institute Animal Care and Use Committee.

Decerebrate Artificially Perfused Rat (DAPR) Preparation and Sympathetic Nerve Recording from the Femoral Bone Marrow

Preparation Set Up

The experimental set up was performed as previously described. SHR and Sprague-Dawley rats (male, 4-6 weeks old, 80-160g) were used for this preparation. The rats were anesthetized, exsanguinated, decerebrated pre-collicularly, and immediately submerged into ice-chilled artificial cerebrospinal fluid (aCSF) (composition in mM: 125 NaCl, 24 NaHCO₃, 3KCl, 2.5 CaCl₂, 1.25 MgSO₄ and 1.25 KH₂PO₄, 10 dextrose, pH 7.3) (Sigma-Aldrich and Fisher, USA). The skin, viscera, and lungs were removed and the left phrenic nerve was isolated. The preparation was then transferred to an acrylic perfusion chamber, cannulated through the left ventricle with a double lumen catheter (Braintree Scientific). The preparation was immediately perfused with warmed Ringer’s solutions (32-33°C) containing Ficoll PM70 (1.25%, Sigma-Aldrich, USA) bubbled with 95% O₂/5% CO₂ and pumped through two in-line bubble traps and a filter (polypropylene mesh; pore size: 40 µm, Millipore) using a peristaltic pump. Neuromuscular paralysis was produced by addition of vecuronium bromide (2-4 µg/ml, Bedford Laboratories, USA) directly to the perfusate. Perfusion pressure
was measured via one lumen of the double-lumen catheter using a pressure transducer connected to an amplifier. Simultaneous recordings of the phrenic nerve activity (PNA) and the femoral nutrient foramen sympathetic nerve activity (fSNA) were obtained using glass suction electrodes (tip diameter, 0.2–0.3 mm), amplified (20K–50K) and filtered (3-30K), sampled at 5 kHz (CED, Cambridge) and monitored using Spike2 (CED). The perfusate flow (19-24 ml/min) was adjusted until an augmenting (i.e. eupneic) pattern of phrenic nerve activity was achieved. Vasopressin (1.25-4 nM final concentration, Sigma-Aldrich, USA) was added to the perfusate to increase vascular resistance and maintain perfusion pressure.

**fSNA Recording and Comparison in the Normotensive vs. Hypertensive Rats**

In order to characterize the fSNA, we monitored the effects of high CO₂ on the PNA and fSNA in the normotensive and hypertensive rats. To confirm activity on the fSNA, we blocked sympathetic transmission at the ganglia by addition of hexamethonium into the perfusate (100-300 µM final concentration). Phrenic-triggered averaging of integrated fSNA was performed as previously described for thoracic SNA characterization²,³ across 100 phrenic cycles (time constant=100 ms). This allowed for the classification of averaged fSNA into the phases related to the respiratory cycle: inspiration (I), post-inspiration (P-I), mid-expiration (M-E) and late expiration (L-E)²,³. To account for variations in cycle length between preparations, I phase was determined as time between the beginning of the PNA burst to end inspiration, and individual P-I and M-E were of a fixed duration (0.4 ms), whilst the L-E phase was estimated as the remainder of the phrenic cycle. Thus, the peak levels of fSNA during each respiratory phase could be compared across preparations.

**MNC isolation from BM and blood**

Male WKY and SHR (21 weeks old, n=12 per group) were euthanized at two different time points: at 10 am (corresponding to the lowest overall sympathetic
activity (SNA) point, labeled as ‘Day’), and at 8 pm (one hour after ‘lights off’, corresponding to the highest overall SNA point, labeled as ‘Night’). For isolation of MNCs from the bone marrow, intact femur and tibia were collected into PBS+2% FBS+1 mM EDTA buffer, followed by cleaning and removal of muscle and fat. The tips of the bones were cut to flush bone marrow cells with 20 ml of the PBS+2% FBS+1mM EDTA buffer, using a 10 ml syringe into a 50ml conical tube. Cells were spun down at 1200 rpm for 15mins at room temperature. To remove the residual red blood cells (RBCs), ammonium chloride (STEM CELL technology, Cat # 07850) was added and cells were incubated for 10 min on ice followed by 2 times washing with PBS+2% FBS+1mM EDTA, to remove the residual RBCs. The resulting MNCs were re-suspended in 1 ml of PBS+2% FBS+1mM EDTA and kept on ice until use. For isolation of MNCs from blood, blood was collected in 10 ml syringe lined with PBS+2% FBS+1 mM EDTA 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 on top of the layer of Ficoll-Paque (2:1 ratio), and spun at 1200 rpm for 25 minutes at RT to obtain the buffy coat. The buffy coat was then transferred to a fresh tube and pelleted down by spinning (1200 rpm, 15 minutes at RT). The supernatant was discarded and the cells were resuspended and washed in 15 ml of PBS (Ca^{2+} - and Mg^{2+} - free), spun down again at the same speed and resuspended in 400 µl of PBS. To remove the RBCs, ammonium chloride (STEM CELL technology, Cat # 07850) was added and cells were incubated for 10 min on ice followed by 2 times washing with PBS+2% FBS+1mM EDTA. The cell pellet was resuspended in 500 µl of PBS+2% FBS+1mM EDTA and kept on ice until use.

**Tube formation assay**

Isolated MNCs were plated in fibronectin pre-coated 6-well plates, and maintained with endothelial basal medium for up to 3 weeks, or until they differentiated into endothelial cells. Cells were then transferred to 96-well Matrigel matrix plate (BD BioCoat™ Angiogenesis System Endothelial Cell Tube
Formation, Cat #: 354149) at 2.5~3x10^4 cells/ml and incubated for 12 hours at 37°C, 5% CO₂. Cells were monitored under microscope (bright field) every 2-3 hours to identify the ability of tube formation. The length of tubes and the number of branches from the images were measured using image J software.

**Pseudo Rabies Virus Tracing**

Pseudo Rabies Virus (PRV-152; Virus Center grant no. P40RR018604) tagged with GFP was a gift from Dr. J Patrick Card, University of Pittsburgh, and was used as a retrograde tracer. The replication-competent virus was injected into the femur bone marrow (3 µl), and green fluorescence was examined in the autonomic brain regions (SFO, PVN, RVLM, NTS, IO) and the spinal cord (IML) 1-7 days later. The surgical site was shaved and prepared with sterile scrub and the left femur was exposed using a scalpel blade. The bone surface was cleaned using 3% H₂O₂ and a hole was burred into the distal epiphysis. To avoid any viral contamination during injections, the femoral bone was isolated from the surrounding tissue using sterile gauze soaked with 3% H₂O₂. PRV was injected into the bone marrow using a 5 µl Hamilton syringe {PRV-152: (3 µl of 4.86 x 10^8 PFU/ml viral recombinants)}. The needle was kept in place for 2 min to avoid the reflux of the inoculums along the needle track. The place of injections was stamped with Ethicon bone wax and the surface of the bone was wiped with 70% ethanol. The muscle surrounding the femur was sutured, and the skin was closed with surgical nylon. Analgesics were administered prior to surgery and for 48 hours after as needed. PRV-injected WKY and SHR were anaesthetized with isoflurane, and perfused with 200 ml heparinized saline followed by 100 ml of 10% formaldehyde solution, on days 1, 3, 5 and 7 following the PRV injections. Two additional experiments were performed in the WKY: (i) BM PRV injection in a separate group of WKY rats (n=4) which were euthanized 14 days after the injection; and (ii) BM PRV injection in a separate group of WKY rats (n=4), following a 4-week long s.c. infusion with 200 ng/kg/min of angiotensin II using s.c. infusion pump (Alzet); these rats were euthanized 7 days following the PRV
injection. Two separate control experiments were performed in which the virus was delivered either to the adjacent muscle or in the BM after the ablation of sciatic and femoral nerves. No positive GFP cells were observed in any of the CV relevant regions in the brain following the control experiments.

**Manganese-Enhanced Magnetic Resonance Imaging (MEMRI) in the Hypothalamus of WKY and SHR**

*Magnetic Resonance Imaging*

Brain images were collected in isoflurane-anesthetized rats on an actively shielded 4.7-Tesla Magnex Scientific MR scanner controlled by Agilent Technologies VnmrJ 3.1 console software. A 38-mm quadrature transmit/receive radiofrequency (RF) coil tuned to 200MHz was used (Insight NeuroImaging Systems, LLC, Leominster, MA). Anesthesia was initially induced under 2.0-2.5% isoflurane (0.1 mL/min) delivered in 100% oxygen for 30-60 seconds, and the levels were maintained with 1.0-1.25% throughout the experiment. Rats were placed in a plastic cylindrical frame with a respiratory monitor pad secured at the level of the diaphragm. The head was fixed in place with ear bars and a bite bar secured to the coil frame to minimize the motion\(^4\). The head was then placed inside a cylindrical holder with a built-in quadrature transmit/receive volume coil (*Insight NeuroImaging Systems, Leominster, MA, USA*). Body temperature was maintained using a warm air recirculating system that received feedback from a fiber optic thermocouple microprobe (SA Instruments, Inc., New York). Respiratory rates were monitored continuously and maintained between 50-60 beats per minute by adjusting isoflurane levels. Images were acquired using a \(T_1\)-weighted spin-echo multi-slice sequence with the following parameters: repetition time (TR) = 275.57 ms, echo time (TE) = 16.38 ms, data matrix 256 x 256 (along read x phase directions), size 30 x 30 mm (resolution 117 x 117 µm along the read and phase directions), 12 consecutive slices with 1.2 mm thickness and no gap. The images were averaged 30 times, and the total scan time per rat was 35 minutes.
Data Processing and Statistical Analysis

Accumulation of Mn\(^{2+}\) within brain tissue shortens its \(T_1\) recovery time, leading to enhanced signal intensity\(^5,6\). To normalize for scan-to-scan variability, we used a voxel-wise Z-score normalization procedure similar to Cross et al.\(^7\), which facilitates within and between groups comparisons at a set threshold level. Image processing was carried out using itk SNAP (http://www.itksnap.org) and Matlab custom code. A whole brain mask was generated per each scan and used to first crop out the non-brain portions of 3D MR scans (non-brain voxels were set to 0). A Gaussian filter was applied to the cropped images to reduce the high frequency noise. Each scan was converted to a Z score map through a voxel-wise normalization procedure. The median signal intensity across the entire extracted brain volume (\(\bar{x}\)) was subtracted from each voxel (\(x_i\)) and then divided by the variance (\(\sigma\)):

\[
Z\text{Score} = \frac{x_i - \bar{x}}{\sigma}
\]

A pre-set threshold of \(Z \geq 1\) was selected based on \textit{a priori} observation of individual datasets and a close inspection of their intensity distribution histograms. Normalized voxel intensity values (in Z scores) and the number of voxels equal to or above the threshold value were extracted for the regions of interest (ROIs).

Radiotelemetry Blood Pressure Measurements

Radiotelemetry implantation (DSI), and blood pressure and spectral analysis were performed as previously described\(^8\).

Tyrosine Hydroxylase (TH), Choline Acetyltransferase (ChAT) and Acetylcholinesterase (AchE) Immunohistochemistry in the Bone Marrow of SHR and WKY
The femur bones were taken from 21 week old WKY and SHR (n=6 per strain) and placed in 4% paraformaldehyde for 24 hours. The bones were then rinsed in ddH₂O, and placed in EDTA (12-15%) solution and shaken at RT for 5 days – 2 weeks, or until the decalcification was completed. Decalcified bones were then rinsed in 70% EtOH, and cryoprotected in 30% sucrose, before cutting. The bone slides were deparaffinized, peroxidase-blocked and rehydrated by submerging the slides in the following sequence of reagents: 2x 5 min in xylene; 2x3 min in 100%ETOH; 1X10 min in freshly prepared 30%hydrogen peroxide diluted to 3% in 100% Methanol; 1x 3min in 95%ETOH; 1x1min in 70%ETOH; 2x1min in dd ddH₂O. Immunohistochemistry was performed with rabbit anti-TH primary antibody (1:150 dilution; Millipore, Cat# AB152), rabbit anti-AchE (1:100 dilution; Novus Biologicals, Cat# NBP1-59170), and anti-ChAT (1:100 dilution; Millipore, Cat# AB143), incubated overnight at 4°C in a humidity chamber, followed by a secondary antibody incubation for 30 minutes at RT. Immunolabeling was detected by the Vector Labs Elite Rabbit (Cat# PK-6101) detection kit, according to the manufacturer’s instructions. The slides were mounted using CytoSeal XYL (Thermo Fisher). The photographs are taken using bright field microscopy under similar conditions for all slides. The images are processed and quantified for immune stain ‘density’ around the blood vessels in the bone marrow using Image J.

Superior Cervical Ganglion Denervation (SCGx)

Superior cervical ganglion denervation (SCGx) was performed in the male 21 weeks old WKY and SHR as described previously⁹. Briefly, under 2-4% isoflurane anaesthesia, a 2 cm vertical incision was made in the ventral neck region skin, and the salivary glands were retracted to expose the underlying muscle. The omohyoid muscle was dissected to expose the common carotid artery, and the SCG was identified behind the carotid bifurcations. To perform the SCGx, the SCG was gently pulled and dissected. After SCGx, the skin was closed with suture clips. The rats were allowed to recover for 36-48 hrs, to allow
for maximal reduction of NE in the BM⁹. The BM samples were collected at 8 pm (one hour after ‘lights off’, corresponding to the highest overall SNA point, labeled as ‘Night’), and NE ELISA and FACS analysis was performed as described elsewhere in the manuscript. The samples were compared to the samples from non-SCGx (naïve) rats, and between the WKY and SHR.

Direct flow cytometry (FACS) analysis

To profile the levels of BM and blood ICs and EPCs, the MNCs from BM and blood were prepared in a concentration of 0.5-1x10⁶ cells/100ul in PBS+2% FBS+1mM EDTA mixture media. CD90⁺/CD4.5.8⁻ cells were used as representative for EPCs, as these have previously shown angiogenic and endothelial reparative properties in the rat¹⁰. CD3⁺/45⁺, CD4.8⁺, CD4.8⁺/CD25⁺ were used as representative of T cells prominent in Ang II-induced hypertension¹¹, and CD68⁺ cells were used as representative of macrophages. Antibodies were purchased from AbD Serotec (Alex647 conjugated CD4/5/8/3/68, RPE conjugated CD25, FITC conjugated CD45, Percp-cy5.5 conjugated CD90), and used as recommended by the company. Cells were incubated with antibodies for 45 minutes at 4°C. Individual antibodies were prepared in each cell suspension and used as control. After spinning down and washing twice, cells were fixed with 2% paraformaldehyde for later analysis. All samples were read on an LSR-II (BD Biosystems) in University of Florida Interdisciplinary Center for Biotechnology Research (ICBR) and the data were analyzed with FACS Diva software, version 6.1.2.

CD90⁺/CD4.5.8⁻ Endothelial Progenitor Cell Selection

Selection (enrichment) of CD90⁺/CD4.5.8⁻ endothelial progenitor cells (EPCs)¹⁰ from BM and blood was performed using the Stem Cell Technologies Immunomagnetic positive and negative selection kit (Cat#18098), according to the manufacturer’s protocol. Negative selection was performed first, to exclude
all CD4.5.8+ cells from the total BM and blood MNCs. 50 µl of Easy Sep negative selection cocktail was added per 1 ml of 5x10^7 cells/ml mixture in PBS+2%FBS+1mM EDTA buffer, and incubated for 10 minutes at RT. Following this, 50 µl of Easy Sep Magnetic Nanoparticles were added to the cell mixture, and incubated for 10 minutes at RT. Additional PBS+2%FBS+1mM EDTA buffer was added to the tube to make up to 2.5 or 10 ml, depending on the size of the magnet, and the tube was placed inside the Easy Sep Magnet for 10 minutes at RT, followed by pouring off the desired fraction into a new 12x75 mm tube. The magnetically-labeled unwanted cells (CD4.5.8+) remain bound on the walls of the tube, held by the magnetic field. This step is repeated one more time, and the remaining wanted cells are resuspended in 1 ml of the PBS+2%FBS+1mM EDTA buffer. Next, positive selection was performed, to select for the CD90+ cells from the remaining BM and blood MNCs. The positive selection cocktail, including the mouse anti-CD90+ antibody, was added to the cell suspension (1:10), and incubated for 15 minutes at RT, followed by the Easy Sep Immunomagnetic selection as described above, three times in total. The resultant wanted magnetically-labeled cells (CD90+) remain inside the tube held by the magnetic field. These cells are then resuspended in 2.5 ml of the PBS+2%FBS+1mM EDTA buffer, and kept on ice until use.

**CD90+.CD4.5.8- Endothelial Progenitor Cell Migration and Proliferation Functional Assays**

Functional assays were performed to establish the ability of EPCs to migrate and proliferate upon stimulation. For the migration assay, enriched EPCs were subjected to an in vitro migration assay per manufacturer’s protocol (EMD Millipore Corporation, Billerica, MA). Briefly, 20,000 cells were placed in the top of a Boyden chamber and migrated towards 100nm SDF1-A (R&D Systems; Minneapolis, MN). Migrated cells were stained with CyQuant GR dye (Life Technologies, Grand Island, NY), which binds to cellular nucleic acids. The resulting Relative Fluorescence Units (RFU’s) were quantified using a 480/520
nm filter set on a Synergy micro plate reader (BioTek, Winooski, VT). For the proliferation assay, enriched EPCs were cultured in either STEM SPAN (Stem Cell Technologies, Vancouver, CA) media alone, or in the presence of 100nM SDF1-A. Proliferation of the cells was assessed using Vialight (Lonza, Walkersville, MD) per manufacturer's protocol. End point luminescence was measured in Relative Luminescence Units (RLU’s) using a one-second integrated time on a Synergy micro plate reader (BioTek, Winooski, VT).

**In vivo imaging of T cells in the bone marrow**

For the *in vivo* imaging of the bone marrow, a tibia window was installed as described previously\(^\text{12}\). Briefly, C57BL6 mice were anesthetized by intraperitoneal injections of Avertin (600 mg/kg; Aldrich, St Louis, MO, USA) and the tibia bone was carefully exposed. A sterilized drill attached to the Dremel tool was used to gently grind the bone surface to expose a ~4 mm of the bone marrow area. CD4\(^+\) and CD8\(^+\) T cells were sorted from UBC-GFP donor mice by FACS, and 2X10\(^7\) cells were injected into the each recipient C57BL6 mouse through the retro-orbital sinus. Six-eight hours following the cell injections, the animals were anesthetized with isofluorane and placed on a custom designed microscope for the cell imaging. Time-lapse images were acquired at 10X magnification using LEICA DM5500B microscope, a Hamamatsu 3CCD camera and Volocity 5.3 software (Perkin Elmer). After initial observation of the control values, a single norepinephrine (NE; 6\(\mu\)g/kg) injection was administered intra-arterially in the caudal femoral artery, for direct delivery into the imaging tibia window. The same area of the tibial window was imaged for one minute to monitor the effect of NE injection on the movement of the GFP-labeled CD4\(^+\) and CD8\(^+\) T cells. In a separate experiment, a single intra-arterial injection of acetylcholine (Ach; 80 mg/kg) was administered immediately prior to NE, in order to determine the effect of Ach on NE-induced movement of CD4\(^+\) and CD8\(^+\) T cells in the tibia.
Norepinephrine (NE) ELISA in the Bone Marrow Supernatant of SHR and WKY

Male WKY and SHR (21 weeks old, n=10 per group) were euthanized at two different time points: at 10 am (i.e. ‘Day’, corresponding to 5 hours following ‘lights on’ and the lowest overall sympathetic drive point), and at 8 pm (i.e. ‘Night’, corresponding to 15 hours following ‘lights on’, and the highest overall sympathetic drive point). 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 100 µl of NE ELISA buffer. The bones were then spun at the highest spin level for 30 minutes at 4°C, in order to extract the bone marrow. The bones were then removed, and the spun-down bone marrow was briefly vortexed and incubated for 2 hours on ice. Following this, the bone marrow was spun at 1200 rpm for 10 minutes at 4°C, and the bone marrow supernatant was collected and stored at -80°C for future use. The NE levels from the BM supernatant were measured using a commercially available kit (Labor Diagnostika Nord GmbH & Co.KG, Germany) following the manufacture’s instruction. The ELISA plate was evaluated spectrophotometrically at 405 nm (SynergyMx multi-mode microplate reader, Biotek). The quantification of NE level is achieved by comparing their absorbance with a reference curve prepared with a known standard concentration. All experiments were run in duplicates. The NE level was normalized for total protein level in the BM suprenatant. The protein concentration of bone marrow supernatant was determined by Bio-Rad protein assay method following the manufacturer’s instruction.

RNA isolation and Real-Time PCR

To analyze the mRNA levels, whole BM MNC total RNA was prepared using RNeasy kit (Qiagen) according to the manufacturer’s instruction. About 200 to 300 ng of purified RNA were reverse transcribed using high-capacity cDNA reverse transcription kit (Bio-Rad Laboratories). Quantitative Real-Time PCR was
performed with commercially-available specific primers and probes for adrenergic receptors alpha2a, alpha2b, alpha2c, alpha1d, beta2 and beta3, and an acetylcholine nicotinic receptor alpha7, by using PRISM 7000 sequence detection system (Applied Biosystem). Data were normalized to 18s ribosomal RNA or GAPDH. Undetectable levels of alpha2b, alpha2c, alpha1d, beta3, and nicotinic alpha7 receptors were found.

**Data and Statistical Analysis**

Data were expressed as mean±SEM 2-way ANOVAs or 1-way ANOVAs, and the Bonferroni post-test was used to allow multiple comparisons of cardiovascular variables across time and between different groups. Paired/unpaired Student t tests were used for further comparisons between 2 groups where applicable, with $P<0.05$ considered significant.
References


Figure S1: Angiogenic and proliferative abilities of the BM EPCs are reduced in the SHR. A-C: Significantly decreased angiogenic ability of the SHR BM MNCs is exhibited by the ~50% reduction in both the length and width of the tubes formed ex vivo (P<0.05 vs. WKY; n=6 per strain). D: Significantly lowered ex vivo proliferative ability of the BM EPCs in the SHR compared to the WKY (P<0.05 vs. WKY, clear bars; n=6 per strain).
Figure S2. Retrograde labeling of autonomic brain regions and spinal cord with PRV-GFP from the femoral bone marrow in SHR and WKY. A: GFP fluorescence reveals robust retrograde labeling in the inferior olive (IO) and the spinal cord (SC) in the SHR (left panel) with no GFP stain present in the WKY (right panel) at day 5 following the BM PRV injection. B: GFP staining reveals retrograde labeling in the SC in the SHR (left panel) with very little GFP stain present in the SC of WKY (right panel) at day 7 following the BM PRV injection. C: Neuronal cell death and some GFP staining exhibited in the PVN of the WKY at day 14 following the BM PRV injection.
Figure S3. Retrograde labeling of autonomic brain regions and spinal cord with PRV-GFP from the femoral bone marrow in SHR and WKY. GFP fluorescence reveals robust retrograde labeling in the NTS, RVLM, SFO and IO (bottom panel), with no GFP fluorescence present in the WKY (top panel) at day 7 following the BM PRV injection.