Prenatal Hypoxia Leads to Increased Muscle Sympathetic Nerve Activity, Sympathetic Hyperinnervation, Premature Blunting of Neuropeptide Y Signaling, and Hypertension in Adult Life

William Rook, Christopher D. Johnson, Andrew M. Coney, Janice M. Marshall

Abstract—Adverse conditions prenatally increase the risk of cardiovascular disease, including hypertension. Chronic hypoxia in utero (CHU) causes endothelial dysfunction, but whether sympathetic vasoconstrictor nerve functioning is altered is unknown. We, therefore, compared in male CHU and control (N) rats muscle sympathetic nerve activity, vascular sympathetic innervation density, and mechanisms of sympathetic vasoconstriction. In young (Y)-CHU and Y-N rats (≈3 months), baseline arterial blood pressure was similar. However, tonic muscle sympathetic nerve activity recorded focally from arterial vessels of spinothoracic muscle had higher mean frequency in Y-CHU than in Y-N rats (0.56±0.075 versus 0.33±0.036 Hz), and the proportions of single units with high instantaneous frequencies (1–5 and 6–10 Hz) being greater in Y-CHU rats. Sympathetic innervation density of tibial arteries was ≈50% greater in Y-CHU than in Y-N rats. Increases in femoral vascular resistance evoked by sympathetic stimulation at low frequency (2 Hz for 2 minutes) and bursts at 20 Hz were substantially smaller in Y-CHU than in Y-N rats. In Y-N only, the neuropeptide Y Y1-receptor antagonist BIBP3226 attenuated these responses. By contrast, baseline arterial blood pressure was higher in middle-aged (M)-CHU than in M-N rats (≈9 months; 139±3 versus 126±3 mm Hg, respectively). BIBP3226 had no effect on femoral vascular resistance increases evoked by 2 Hz or 20 Hz bursts in M-N or M-CHU rats. These results indicate that fetal programming induced by prenatal hypoxia causes an increase in centrally generated muscle sympathetic nerve activity in youth and hypertension by middle age. This is associated with blunting of sympathetically evoked vasoconstriction and its neuropeptide Y component that may reflect premature vascular aging and contribute to increased risk of cardiovascular disease. (Hypertension. 2014;64:00-00.) • Online Data Supplement

Key Words: aging ■ fetal programming ■ hypertension ■ hypoxia ■ NPY

It is well established that adverse conditions in utero induce fetal programming and increase the risk of cardiovascular disease in the offspring.1 Notably, placental insufficiency, which leads to impaired delivery of oxygen and nutrients to the fetus, is a major cause of intrauterine growth restriction and has been associated with hypertension in adult life.2

Studies involving animal models of fetal programming have already shown that endothelial dysfunction occurs in the offspring when pregnant rats are exposed to systemic hypoxia during pregnancy to induce chronic hypoxia in utero (CHU) or nutrient restriction.3 However, the effects of CHU are more dramatic and occur earlier. Thus, male CHU offspring showed endothelial dysfunction at 3 to 4 months of age that was associated with reduced bioavailability of nitric oxide and oxidative stress and prevented by treating the pregnant dam with antioxidant.1,5 By contrast, endothelial dysfunction did not occur in nutrient restriction offspring until 7 months of age when nitric oxide still contributed to endothelium-dependent dilation.3

Endothelial dysfunction may contribute to development of hypertension4 as may enhanced myogenic tone, another characteristic of male CHU, but not nutrient restriction rats.7 However, elevated blood pressure in patients with essential hypertension is considered to be initiated by sympathetic hyperactivity.4 Accordingly, young men and women who were small for gestational age at birth had raised muscle sympathetic nerve activity (MSNA).9 Further, chick embryos made hypoxic during incubation showed augmented responses to tyramine, which releases norepinephrine (NE) and sympathetic hyperinnervation of femoral arteries.10 Whether similar changes occur in mammals is not known.

Sympathetic hyperactivity in essential hypertension in young humans and spontaneously hypertensive rats (SHRs)
is accompanied by exaggerated muscle vasoconstrictor responses to sympathetic activation and higher plasma levels of the cotransmitter neuropeptide Y (NPY). But chronically raised MSNA also occurs during aging and is accompanied by reduced reflex vasoconstriction and loss of NPY involvement in skin. Further, vasoconstrictor responses evoked by sympathetic activation were blunted in acute and chronic hypoxia in adult rats and humans; this was attributed to impaired vasoconstriction to NE and adenosine triphosphate, but preservation of the NPY component.

In view of these findings, we hypothesized that CHU rats have raised MSNA, develop hypertension early during aging, and show augmented muscle vasoconstrictor responses to sympathetic nerve activation, but preservation of the NPY component. By contrast, we hypothesized that control (N) rats show impaired sympathetic vasoconstrictor muscle with aging and loss of the NPY component. Thus, we performed experiments on male control rats and CHU rats at 10 to 12 and at 36 to 37 weeks of age, which may be compared with humans in young adulthood and early middle age (≈16–18 and 30–35 years, respectively).

Methods and Materials
For a complete description of the Materials and Methods, see the online-only Data Supplement.

Experiments were performed on male N and CHU Wistar rats; the latter were offspring of rat dams housed in a hypoxic chamber at 12% O2 from day 10 to 20 of pregnancy. All experiments were approved by the Biomedical Research Ethics Committee of the University of Birmingham and performed under the UK Animal (Scientific Procedures) Act.

Recordings of MSNA
Under anesthesia, MSNA was recorded from the surface of arterial vessels of spinotrapezius muscle, together with arterial blood pressure (ABP), heart rate (HR), and respiratory frequency (Rf) from 27 Y-N and 17 Y-CHU rats under baseline conditions (i) during progressive acute hypoxia and (ii) baroreceptor unloading induced by sodium nitroprusside.

Sympathetic Innervation Density
Tibial arteries isolated from 10 Y-N rats and 13 Y-CHU rats were stained with glyoxylic acid to reveal sympathetic noradrenergic fibers and visualized to quantify nerve density.

Responord Countses Evoked by Sympathetic Nerve Stimulation
In 12 Y-N rats and 11 Y-CHU rats, sympathetic nerve stimulation (SNS) was applied via the right sympathetic chain as a 60 sec train of 120 pulses at 2 Hz and as 6 bursts of 20 pulses at 20 Hz at 10 sec intervals (120 pulses in total). Changes in femoral vascular resistance (FVR) were recorded before and during infusion of the NPY Y1-receptor antagonist BIBP-3226 (10 mg/kg/min).

Statistical Analysis
All data are presented as mean±SEM. Single comparisons were made by Student’s paired or unpaired t test. Multiple within-group comparisons were made by repeated measures analysis of variance, with Dunnet’s post hoc. For responses evoked by SNS, the maximum change in FVR (ΔFVR Max) evoked by SNS was calculated. In addition, the integral of FVR during SNS, relative to a 60 sec period before stimulation (Δint FVR) was calculated for each pattern of stimulation. Comparisons were made before and after BIBP-3226 by using mixed model analysis of variance with Scheffés post hoc test. P<0.05 was considered statistically significant.

Results

MSNA Recordings
Cardiac and respiratory rhythmicity was present in all single units discriminated from MSNA in Y-N and Y-CHU rats. Mean ongoing firing rate recorded from single units was ≈70% higher in CHU than in N rats (0.56±0.075 versus 0.33±0.036 Hz). Impulses generally occurred singly in both Y-N and Y-CHU rats; couplets, with an interpulse interval of <0.1 sec, seldom occurred, that is, the instantaneous firing frequencies were low. However, there was a lower proportion of low instantaneous frequencies (<1 Hz) and greater proportion of higher instantaneous frequencies (1–10 Hz) in CHU than in N rats (Figure 1), reflecting more couplets of high instantaneous frequency in Y-CHU rats. The percent of spikes occurring within the phase-related peaks and nadirs of the cardiac and respiratory cycles were fully comparable in Y-N and Y-CHU rats. For further details of MSNA, see Figures S1–S4 and Table S1 in the online-only Data Supplement.

Progressive systemic hypoxia induced greater increases in R, and HR in Y-CHU than in Y-N rats; the fall in ABP was greater in Y-N rats (P<0.05; Figure 2A). Concomitantly, MSNA increased, the change in firing frequency evoked by 8% O2 being similar in Y-N and Y-CHU rats (see Figure 2A). When SNP was infused to induce a fall in ABP comparable to that of hypoxia; the reflex increase in MSNA firing frequency was similar in Y-N rats and Y-CHU rats, as was the increase in HR (Figure 2B).

Sympathetic Nerve Density
The density of sympathetic innervation on tibial arteries was ≈50% greater in Y-CHU than in Y-N rats (Figure 3).
Responses Evoked by Sympathetic Nerve Stimulation

Continuous SNS stimulation at 2 Hz and bursts of impulses at 20 Hz evoked smaller increases in FVR in Y-CHU than in Y-N rats; these differences reaching statistical significance for FVR Max (Figure 4, top right). The Y1-receptor antagonist attenuated the increases in IntFVR evoked by 2 Hz and by bursts at 20 Hz in Y-N, but not Y-CHU rats (Figure 4, top left). Baseline ABP was higher in M-CHU than in Y-CHU rats (139±3 versus 131±3 mmHg; \( P<0.05 \)), but not different between M-N and Y-N rats (126±3 versus 127±3 mmHg); it was also higher in M-CHU than in M-N rats (\( P<0.05 \)). IntFVR increases evoked in M-N rats by SNS at 2 Hz and bursts at 20 Hz were blunted relative to Y-N rats (\( P<0.05 \)), such that evoked increases in IntFVR were similar in M-N and M-CHU rats (Figure 4, bottom left). The Y1-receptor antagonist did not affect the sympathetically evoked responses in either group. For further details, see online-only Data Supplement.

Discussion

The present study demonstrates for the first time that prenatal hypoxia leads to a substantial increase in the frequency of single unit MSNA and in sympathetic nerve density of skeletal muscle arteries in young adult male rats (Y-CHU) relative to control (Y-N) rats. Muscle vasoconstrictor responses evoked by SNS at low and high frequencies were blunted in Y-CHU relative to Y-N rats, and the contribution of NPY was absent in Y-CHU rats. By early middle age, M-CHU rats had raised ABP relative to M-N rats, but muscle vasoconstrictor responses evoked by SNS were also blunted in M-N rats; NPY made no contribution in either M-N or M-CHU rats.

Prenatal Hypoxia and MSNA

We recorded MSNA from the surface of muscle arterial vessels; it displayed both cardiac and respiratory rhythmicities as expected of muscle vasoconstrictor nerves.\(^{22,25}\) We analyzed our data as single unit activity rather than as multiunit MSNA

![Image of graph showing ABP and MSNA responses](http://hyper.ahajournals.org/ by guest on August 15, 2017)
Hypertension December 2014

because direct comparisons between experimental groups are difficult when the number of fibers adjacent to the electrode or recruitment of silent fibers can affect gross activity.

In line with our hypotheses, mean frequency in single units was ≈70% greater in Y-CHU than in Y-N rats. Further, MSNA in Y-CHU rats contained a higher proportion of couplets at instantaneous frequencies of 1 to 5 Hz and 5 to 10 Hz and a lower proportion at <1 Hz. In view of these findings, it is tempting to suggest that raised MSNA recorded in young men and women who were small for gestational age at birth9 may arise partly from prenatal hypoxia.

Ours is the first direct evidence that basal sympathetic activity is raised in any experimental model of prenatal programming. In the only other study in which gross renal sympathetic nerve activity was recorded in male offspring of dams fed a low protein diet, baseline renal sympathetic nerve activity was comparable to that of controls, but showed larger increases during static muscle contraction.26

In the few reports of single unit MSNA in humans, mean frequency of single unit MSNA was low in normotensives (≈0.3 Hz), higher in established essential hypertensives (≈0.6 Hz), and higher still in borderline hypertensives (≈0.75 Hz), consistent with sympathetic hyperactivity preceding the full development of essential hypertension.27 Further, in normotensives, couplets or higher multiples of impulses were uncommon, instantaneous frequencies being <1 Hz in ≈50% of units,28 but far more common in essential hypertension.29 On the other hand, in obesity-related hypertension, there was raised multiunit MSNA but not single unit MSNA, indicating greater recruitment of individual sympathetic fibers. Patients with congestive heart failure, or obstructive sleep apnea (OSA) and hypertension, had raised single-unit MSNA (0.98, 0.96 Hz), but only those with OSA showed increased numbers
of multiple impulses. Thus, prenatal hypoxia shares some characteristics with essential hypertension and OSA: raised single-unit MSNA and impulses in couples or multiples.

Cross-correlograms of respiratory- and cardiac-related coupling of single-unit MSNA showed they were similar in Y-N and Y-CHU rats. Thus, there is no reason to suggest that the higher MSNA in Y-CHU rats reflected stronger respiratory modulation or weaker baroreceptor-reflex inhibition of MSNA. By contrast, in working heart–brain stem preparations, the augmented sympathetic activity in SHRs reflected amplified respiratory coupling of sympathetic nerve activity. Thus, our findings compare better with those of essential hypertension or chronic obstructive pulmonary disease, which did not show augmented respiratory coupling of MSNA even though they had raised MSNA.

Nevertheless, cardiac-related rhythmicity was lower in chronic obstructive pulmonary disease patients than hypertensives, suggesting the raised MSNA in chronic obstructive pulmonary disease reflects depressed baroreceptor inhibition and increased tonic drive from peripheral chemoreceptors. A similar suggestion was made for the raised MSNA and multiple firing in OSA. In our study, the increases in MSNA evoked by baroreceptor unloading and the hypoxia-induced fall in ABP were comparable in Y-N and Y-CHU rats, suggesting baro- and chemoreceptor reflex effects on MSNA were similar. Indeed, the simplest interpretation is that the augmented MSNA in Y-CHU rats is neurogenic, reflecting greater central neural output to individual muscle sympathetic neurons. The baroreceptor reflex effect on MSNA apparently operates around a raised set point, and the peripheral chemoreceptor input modulates, rather than contributes to, this raised discharge.

Long-term hypoxia in adult life, lasting from 20 minutes to several months, causes an increase in MSNA that outlasts return to normoxia. Further, chronic intermittent hypoxia induced experimentally and occurring in OSA causes a persistent increase in MSNA. It has been suggested that this arises because hypoxia induces a long-term increase in the activity of the premotor sympathetic neurons of rostroventrolateral medulla initiated by chemoaffective input and accompanied by resetting of the baroreceptor reflex effect on rostroventrolateral medulla to higher levels. The present findings raise the possibility that prenatal hypoxia evokes a similar long-term potentiation of rostroventrolateral medulla that raises MSNA in adult Y-CHU rats.

**Prenatal Hypoxia and Sympathetic Vasoconstriction**

Higher mean frequency of single unit MSNA with more couples at higher instantaneous frequencies would be expected to exert a greater tonic vasoconstrictor influence in Y-CHU rats. The greater sympathetic nerve density revealed in tibial arteries of Y-CHU might be expected to further augment the number of impulses reaching arterial smooth muscle. And yet, baseline FVR was not different between Y-N and Y-CHU rats.

As expected, SNS with bursts at 20 Hz evoked a greater increase in FVR than stimulation at 2 Hz in both Y-N and Y-CHU rats, but these vasoconstrictor responses were blunted, not exaggerated in Y-CHU rats (in contrast to our hypotheses). Moreover, blockade of NPY Y1-receptors attenuated responses evoked by both patterns of stimulation in Y-N rats, but not Y-CHU rats. Because the cotransmitters NPY, ATP, and NE facilitate one another’s actions, it is difficult to quantify contributions of individual transmitters with selective receptor antagonists. Nevertheless, as responses evoked by both patterns were similar in N and CHU rats after Y1-receptor antagonism, it is likely the blunted vasoconstrictor responses in Y-CHU rats reflected lack of an NPY component. Such a deficit could also explain why baseline FVRs were similar in Y-N and Y-CHU rats even though MSNA in Y-CHU rats contained more high instantaneous frequencies.

Clearly, these effects of prenatal hypoxia differ from acute hypoxia in adult life when the contribution of NPY to sympathetically evoked vasoconstriction is maintained while the contributions of ATP and NE are attenuated. At first sight, they also differ from essential hypertension in humans and SHRs. NPY has been implicated in the development of hypertension not only because plasma levels are raised in young SHRs, but because NPY is released by high sympathetic discharge, facilitates noradrenergic vasoconstrictor responses, and induces vascular hypertrophy. However, although at 4 weeks of age, NPY content was higher in mesenteric arteries of SHRs than Wistar Kyoto controls (WKY), by 4 months of age, it was lower in SHRs than Wistar Kyoto controls. Moreover, at ≈3 months, antioxidant treatment augmented NPY release from sympathetic nerve fibers in SHRs but not in Wistar Kyoto controls, such that NPY then contributed to sympathetic vasoconstriction in SHRs. Thus, it may be that oxidative stress in essential hypertension, SHRs, and prenatal hypoxia has the common effect of limiting NPY release such that, in Y-CHU rats, the NPY contribution to sympathetic muscle vasoconstriction is minimal. Oxidative stress may also blunt the NE-evoked component of sympathetic vasoconstriction in Y-CHU rats by limiting NE synthesis, as in aging skin.

**Effects of Aging**

M-N rats showed blunted vasoconstrictor responses to sympathetic stimulation relative to Y-N rats that were not attenuated by Y1-receptor blockade. This is consistent with the finding that, in humans, reflex muscle vasoconstriction was attenuated with aging, even though MSNA was increased and that cutaneous vasoconstriction was blunted by aging and no longer included an NPY component. To our knowledge, ours is the first evidence that even modest aging attenuates the NPY component of sympathetic muscle vasoconstriction. The mechanisms are not clear.

From the fragmentary evidence available, sympathetic innervation density on femoral arteries of rats increased from 10 to 12 weeks to 9 months, but the NPY content in arterial vessels was similar at 4 and 16 weeks. Moreover, NPY neuropeptide activity increased from 2 to 6 months of age in skeletal muscle arterioles of rats and greatly attenuated sympathetically evoked NPY release at 6 months, whereas constrictor responses evoked by NPY in rat mesenteric arteries in vitro were similar at 4 weeks and 6 months. However, vasoconstriction evoked in muscle circulation by α1-adrenoceptor agonists was smaller in old than young human, whereas in dogs, muscle vasoconstriction evoked by adrenoceptor, ATP,
or NPY Y1-receptor stimulation did not change with age, neither did receptor expressions.44 Given these findings, we simply propose that attenuation of sympathetically mediated vasoconstriction in M-N rats reflects loss of the NPY component and, possibly, reduced release or action of NE and ATP; the underlying mechanisms may include oxidative stress associated with aging.14,40

Aging in CHU Rats

The lack of NPY contribution to sympathetically evoked vasoconstriction persisted in M-CHU rats. Indeed, M-CHU rats showed no obvious age-related attenuation of sympathetically evoked vasoconstrictor responses. Rather, it seems Y-CHU rats (=3-months-old) showed premature aging, displaying sympathetically vasoconstriction comparable to M-N rats (=9-months-old). This is reminiscent of the finding that endothelium-dependent relaxation was blunted in CHU rats at 4 months and did not show the age-dependent attenuation seen in N rats between 4 and 7 months.3 Because the nitric oxide contribution to endothelium-dependent relaxation was prevented in CHU rats at 4 months by treating the pregnant dam with Vitamin C,5 although endothelium-dependent relaxation at 7 months was restored by acute antioxidant treatment,6 it may be that oxidative stress not only led to early endothelial dysfunction, but depressed sympathetically evoked vasoconstriction in Y-CHU and M-CHU rats by blunting release and action of NPY and NE.14,40 This should be tested in future studies.

Prenatal Hypoxia and Hypertension

As we hypothesized, the raised MSNA in Y-CHU rats was associated with a progressive increase in baseline ABP: ABP was higher in M-CHU than in Y-CHU rats with no age-related change in N rats. As far as we are aware, this is a novel finding. It will be important to establish whether MSNA is also raised in M-CHU rats and whether ABP is raised in nonasphyxiated Y-CHU and M-CHU rats. Whether raised MSNA in male Y-CHU rats and hypertension in M-CHU rats are causally linked remains speculative. Baseline FVR was not raised in M-CHU rats; therefore, increased vasoconstrictor tone in muscle did not directly contribute to the raised ABP. However, the relationships between MSNA, vascular resistance, and ABP in healthy men are not straightforward and do not change systematically with age or hypertension. Nevertheless, much evidence supports a sympathetic mechanism for the genesis of hypertension.8,21,27,45

Perspectives

We have provided novel evidence that fetal programming induced by prenatal hypoxia leads to increased MSNA in young adult males with no change in respiratory or cardiac-related rhythmicities, nor in the effects of baroreceptor unloading or peripheral chemoreceptor stimulation. We therefore suggest that prenatal hypoxia leads to an increase in centrally generated sympathetic outflow to skeletal muscle. Our findings also add to evidence1 that prenatal hypoxia induces premature vascular aging: vasoconstrictor responses evoked by sympathetic nerve activation were blunted in young CHU rats, apparently because the NPY Y1-receptor contribution was lacking, a change that occurred in control rats by middle-age. Because baseline hypertension was present in CHU rats by middle age, we propose that raised MSNA and altered NPY signaling, together with endothelial dysfunction,1–6,39,40 collude to increase the risk of cardiovascular disease in individuals who experience prenatal hypoxia.1

Sources of Funding

This study was supported by a British Heart Foundation PhD Studentship (FS/08/068/25798).

Disclosures

None.

References

16. Hansen J, Sander M. Sympathetic neural o


Novelty and Significance

What Is New?

- These are the first recordings of muscle sympathetic nerve activity and sympathetically evoked muscle vasoconstriction in young adult rats made hypoxic in utero rats.
- Resting muscle sympathetic nerve activity was raised while muscle vasoconstrictor responses to sympathetic activation were blunted in chronic hypoxia in utero rats and lacked a neuropeptide Y component.
- Similar changes in sympathetic vasoconstrictor responses did not occur in control rats until middle age, by which time chronic hypoxia in utero rats were hypertensive.

What Is Relevant?

- Adverse conditions during pregnancy increase risk of cardiovascular disease in adult life.
- Raised muscle sympathetic nerve activity in youth is causally implicated in the genesis of hypertension.
- Impaired NPY release and blunted sympathetic vasoconstriction have been associated with hypertension and aging.

Summary

Fetal programming induced by hypoxia in utero increases muscle sympathetic nerve activity, leads to hypertension by middle age, and modulates sympathetic vasoconstriction in ways that suggest premature aging.
Prenatal Hypoxia Leads to Increased Muscle Sympathetic Nerve Activity, Sympathetic Hyperinnervation, Premature Blunting of Neuropeptide Y Signaling, and Hypertension in Adult Life

William Rook, Christopher D. Johnson, Andrew M. Coney and Janice M. Marshall

Hypertension. published online September 29, 2014;

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2014 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/early/2014/09/29/HYPERTENSIONAHA.114.04374

Data Supplement (unedited) at:
http://hyper.ahajournals.org/content/suppl/2014/09/29/HYPERTENSIONAHA.114.04374.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Hypertension can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Hypertension is online at:
http://hyper.ahajournals.org//subscriptions/
Pre-natal hypoxia leads to increased muscle sympathetic nerve activity, sympathetic hyper-innervation, premature blunting of NPY signaling in sympathetic vasoconstriction and hypertension in adult life

William Rook, Christopher D Johnson¹, Andrew M Coney & Janice M Marshall

School of Clinical & Experimental Medicine, College of Medical & Dental Sciences, University of Birmingham, B15 2TT; ¹School of Medicine, Dentistry and Biomedical Sciences, Queens University, Belfast BT9 7AE UK.
Supplementary Materials and Methods

The CHU rats were offspring of pregnant rats that were mated in the University Biomedical Services Unit (BMSU) and then housed in a servo-controlled hypoxic chamber breathing 12% O₂ in N₂ from day 10-20 of pregnancy. On day 20, they were returned to room air and pups were born and raised in room air, under standard animal welfare conditions that were identical to those of N rats. All animals were provided with standard rat chow ad libitum throughout. All rats were purchased from the supplier Charles River Laboratories (Kent, UK). N Rats were housed in BMSU for at least 2 weeks prior to acute experimentation.

Recordings of MSNA

These experiments were performed on Y-N (n=27) and on Y-CHU rats (n=17) randomly selected from 10 different litters. They were anesthetized and prepared for MSNA recordings using procedures described recently. In brief, anesthesia was induced with 3.5% Isofluorane in 5L/min O₂ and maintained by continuous infusion of the steroid anesthetic Alfaxan (1.0-1.5ml.hr⁻¹ IV, Vetoquinol, Buckingham, UK). Arterial blood pressure (ABP) was recorded from the right femoral artery and heart rate was computed from the ABP signal. Tracheal pressure (TP) was recorded via the tracheal cannula, to give an index of respiration. At intervals, a sample was taken from cannula in the left femoral artery for assay of blood gasses (arterial partial pressure of O₂, CO₂ (PaO₂; PaCO₂ respectively) and pH.

The left spinotrapezius muscle was carefully exposed and blunt dissected from underlying muscle so that it could be reflected over a pedestal, ventral surface uppermost, with its neural and vascular supply intact as we described recently using the focal recording technique described by Johnson & Gilbey. Thus, water-tight organ bath was constructed around the muscle and it was superfused with normal saline (0.9% NaCl in dH₂O). MSNA was recorded with a focal recording electrode that was placed on the surface of a 2nd or 3rd order arterial vessel within the muscle, light suction being applied with a syringe. A silver reference electrode was placed in close proximity to the focal recording electrode.

On-going MSNA was recorded under stable baseline conditions and in some rats, MSNA recordings were maintained (i) during acute hypoxia when the inspirate was changed at 2 minute intervals from air to 12, 10, and 8%O₂ and then returned to air and (ii) when sodium nitroprusside (SNP, 60µg.kg⁻¹) was infused via the left femoral artery to reduce ABP and so cause baroreceptor unloading.

Physiological variables were recorded using a NeuroLog system (Digitimer Ltd, Hertfordshire, UK) connected to a computer running Windows XP (Apple Inc, California, USA). Nerve activity signals were amplified (10k gain), and filtered (150Hz-1kHz band pass). Raw data was digitized and sampled via at 20,000Hz, ABP and TP were sampled at 100Hz. Raw multiunit recordings of MSNA were analyzed using the wavemark facility with Spike2 software (Version 7, Cambridge Electronic Design, Cambridge, UK). Single unit MSNA was discriminated from raw data on the basis of amplitude, duration and shape of action potentials as described recently. In brief, templates were constructed from spikes that were triggered by raw data that passed below a set voltage. Spikes were considered to match a template when 70% of
points in a spike matched the template. New templates were created when a minimum of 8 spikes not already matched to a template were found to have 70% of points within similarity boundaries. During discrimination, the ‘auto-fix’ function was used, i.e. if the shape of a spike evolved with time due to slow movement of the blood vessel, or slow loss of suction, the shape of the template evolved to reflect this. Each ‘auto-fixed’ template was based on the previous 64 spikes matched to that template. Principal component analysis was used when spikes appeared to have different templates but similar shape, to test whether they represented two different units, or were the same unit. All units described here displayed cardiac and respiratory rhythmicity, as confirmed by stimulus triggered histogram analysis (see Figure S2).

**Sympathetic innervation density.**

Tibial arteries were isolated from 10 Y-N rats and 13 Y-CHU rats following cervical dislocation under isofluorane anesthesia (3.5% isofluorane in 5L/minO2). Sympathetic noradrenergic fibers were stained in stretch preparations of arterial vessels using established procedures. In brief, tibial arteries were isolated from 10 Y-N rats (416±9g) and 13 Y-CHU rats (5 litters, 359±13g). The arteries, were immediately placed in 2% (w/v) glyoxylic acid in 0.1M phosphate buffer, with pH adjusted to pH 7 with 10M NaOH and incubated for 45 min. Each artery was then placed onto a glass slide and air-dried as a whole vessel preparation with the aid of a cool fan before being placed in an oven for 4 minutes at 100°C. Each slide was then covered with mineral oil and a temporary coverslip was placed on the slide. Slides were kept in the dark at ≤4°C to maintain stability of catecholamine fluorescence.

Stained vessels were analyzed using an Olympus confocal microscope using a 40x oil immersion objective: excitation wavelength was 395-440nm, and emission bandwidth 450-550nm. The upper and lower boundaries of the perivascular nerve plexus were found by manually adjusting the focal plane. Images were acquired at 800x800 pixels at 1μm slice intervals, typically 80 slices being acquired from at least 3 different areas of each vessel. All slices of a single vessel were compiled into a z-stack using ImageX software and the resulting image saved as a TIFF file. Sympathetic nerve density was calculated as we described recently. A grid with horizontal and vertical lines spaced at 44 pixel intervals was overlaid on the image, such that each square represented 2000 pixels, the vertical lines running parallel to the border of the vessel. A count was made of the number of times sympathetic nerve fibers intersected the grid lines; nerve density was expressed as nerve intercepts per μm tissue.

**Responses evoked by sympathetic nerve stimulation**

These experiments were carried out on 12 Y-N rats (343±9g, 71±2 days old) and 11 Y-CHU rats (351±10g, 4 litters, 70±3 days) and on 13 M-N and M-CHU rats (252±2 days old; 36-37 weeks old, 666±24g and (264±7 days; 36-38 weeks old, 651±30g; 6 litters respectively). They were anesthetized and prepared for recording ABP as described above except that M-N and M-CHU rats were more sensitive to the anesthesia and required an infusion rate of Alfaxan at only 0.5-0.8ml.hr⁻¹ to achieve full anesthesia. The right sympathetic chain was exposed via a mid-line laparotomy, a bipolar silver wire electrode was looped under the chain at lumbar level L3-4 and fixed in place with silicon elastomer compound (Kwik-Sil Adhesive, WPI, UK). The laparotomy was then closed. A Transonic flow probe was placed on the right femoral artery to record femoral blood flow (FBF); femoral vascular resistance (FVR) was computed on line as ABP/FBF.
Protocol. Sympathetic nerve stimulation (SNS) was applied via the sympathetic chain as pulses of 1ms duration and 1mA, as a 60 sec train of 120 pulses at 2Hz, and as 6 bursts of 20 pulses at 20Hz at 10 sec intervals, (120 pulses in total). Each pattern was delivered twice in randomized order before and after infusion over 30 minutes of the NPY Y1 receptor antagonist BIBP-3226, at 10μg.kg⁻¹.min⁻¹ via a cannula in the caudal ventral artery; infusion of the Y1 antagonist was continued during SNS.

Results

Recordings were obtained in 27 N rats, 5 of which appeared to be single unit, and 39 multiunit, and in 17 CHU rats of which 5 were single unit and 30 multiunit; examples of original recordings of MSNA and discriminated single units are shown in Figure S1.

By stimulus triggered histogram analysis of single unit activity, each unit included in the analysis for N and CHU rats contained respiratory-related rhythmicity: activity that was augmented during the post-expiratory and pre-inspiratory phase, and suppressed during the inspiratory and post-inspiratory phases (Figure S2 left). Similar analysis using the cardiac cycle showed that each unit had cardiac rhythmicity comprising augmented activity during the diastolic phase, and lower activity during the systolic phase (Figure S2 right).

In total, 198 and 157 single units were discriminated from raw MSNA in N and CHU rats respectively. The mean ongoing firing frequencies were 0.33±0.03Hz (range 0.02-1.41Hz) and 0.56±0.07Hz (range 0.02-2.41) respectively, mean frequency being substantially greater in CHU than N rats (Table S1).

Further analyses were performed of cardiac and respiratory modulation to quantify the strength of these modulations: each unit was recorded for at least 5 minutes in order to be included in these analyses. To assess the cardiac modulation, mean phase histograms were obtained and the proportion of spikes falling within ±60° of the peak in activity and the proportion falling outside were computed (see Figure S3). Similarly, the proportion of the spikes falling within ±60° of the post-expiratory phase of the respiratory cycle and the proportion falling outside were computed (Figure S4). As can be seen from the histograms, the strength of respiratory and of the cardiac modulation was similar in single unit MSNA of CHU and N rats (Figures S3 and S4).

Progressive systemic hypoxia induced similar falls in PaO₂ to 28.6±4mmHg and 24.4±3mmHg during 8%O₂ in Y-N and Y-CHU rats (n=6 and 5 respectively); PaCO₂ fell to 29±2 and 23±1mmHg respectively as a consequence of hyperventilation. These changes were not significantly different between Y-N and Y-CHU rats. However, the increases in Rf and HR were greater in CHU than N rats (Figure 2A). Progressive hypoxia also induced a fall in ABP in Y-N and Y-CHU rats to 58±12 and 89±8mmHg during 8%O₂ in Y-N and Y-CHU rats, the fall being greater in Y-N rats (P<0.05, Figure 2A). Concomitantly, MSNA increased in both Y-N and Y-CHU rats; the change in firing frequency evoked in single units by the change from air breathing to 8%O₂ was similar in Y-N and Y-CHU rats (see Figure 2A).
Since the hypoxia-induced increase in MSNA must have included baroreceptor unloading as well as peripheral chemoreceptor stimulation, we infused SNP to induce a fall in ABP comparable to the hypoxia-induced fall in both Y-N and Y-CHU rats (see Figure 2B). Baroreceptor unloading evoked an increase in MSNA firing frequency in single units in both Y-N rats and Y-CHU rats (37, 28 single units respectively); the change in MSNA from their respective baselines was not different between N and CHU rats (P=0.23). Baroreceptor unloading with SNP also evoked a reflex increase in HR in both groups (Figure 2B) the change HR was not different between N and CHU rats (P=0.14).

Responses evoked by sympathetic nerve stimulation.

There were no significant differences between Y-N and Y-CHU rats in baseline levels of ABP, HR or FVR (Table S2). Continuous SNS stimulation at 2Hz evoked an increase in intFVR and in FVR Max indicating hind limb vasoconstriction that was smaller in Y-CHU than Y-N rats (Figure 4 top). Bursts of impulses at 20Hz evoked larger increases in IntFVR and Max FVR in both Y-N and Y-CHU rats (P<0.05), but again these responses were blunted in Y-CHU rats (Figure 4 top). BIBP-3226 had no effect on baseline ABP or FVR in Y-N or Y-CHU rats indicating no basal vasoconstrictor influence of NPY. However, in N rats, the Y1-receptor antagonist attenuated the increase in IntFVR evoked by SNS at 2Hz and bursts at 20Hz. By contrast, the Y1-receptor antagonist did not affect constrictor responses evoked in Y-CHU rats.

Baseline ABP was higher in M-CHU than Y-CHU rats (P<0.05), but not different between M-N and Y-N rats (see Table S2). Further, baseline ABP was higher in M-CHU than M-N rats (Table S2). Vasoconstrictor responses evoked in M-N rats by continuous SNS at 2Hz and by bursts at 20Hz were blunted relative to those evoked in Y-N rats such that the increases in IntFVR evoked in M-N and M-CHU rats were similar (c.f. Figure 4 top & bottom). The Y1-receptor antagonist did not affect baselines (Table S2) or SNS-evoked responses in either M-N or M-CHU rats (Figure 4 bottom).

References


<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>ABP (mmHg)</th>
<th>HR (beats/min)</th>
<th>Rf (breaths/min)</th>
<th>Single units</th>
<th>Mean firing rate (Hz)</th>
<th>Instantaneous frequency range (Hz)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y-N</td>
<td>Control</td>
<td>127±3</td>
<td>452±16</td>
<td>2.28±0.13</td>
<td>58±4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Y-N</td>
<td>+BIBP</td>
<td>122±3</td>
<td>441±11</td>
<td>2.57±0.13</td>
<td>51±4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Y-CHU</td>
<td>Control</td>
<td>131±3</td>
<td>461±15</td>
<td>2.28±0.13</td>
<td>58±3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Y-CHU</td>
<td>+BIBP</td>
<td>127±4</td>
<td>448±10</td>
<td>2.57±0.18</td>
<td>54±5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M-N</td>
<td>Control</td>
<td>126±3</td>
<td>430±5</td>
<td>2.30±0.20</td>
<td>65±7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M-N</td>
<td>+BIBP</td>
<td>123±3</td>
<td>397±10</td>
<td>1.90±0.10</td>
<td>61±4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M-CHU</td>
<td>Control</td>
<td>139±3††</td>
<td>432±12</td>
<td>2.50±0.20</td>
<td>59±4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M-CHU</td>
<td>+BIBP</td>
<td>126±3</td>
<td>396±13</td>
<td>2.00±0.20</td>
<td>60±5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table S1:** General characteristics of the N and CHU rats. †††: p<0.001 between Y and N groups.

**Table S2:** Baseline values of cardiovascular variables in Y-N and Y-CHU rats and M-N and M-CHU rats before control SNS and during BIBP 3226 infusion (+BIBP) and before the second set of SNS. ††: p<0.01 between Y and CHU groups at same age.
Figure S1. Recordings of ongoing multiunit MSNA and cardiorespiratory variables recorded from N rat under baseline conditions. A: From Bottom up: ABP, Trach pressure (Tracheal pressure), HR (beats/min), RF (breaths/min), “raw” MSNA signal, discriminated spikes (labeled “All units”) and rate histograms for 5 individual units. B: Spike templates and overdrawn spikes discriminated from the raw multiunit recording.
Figure S2. Cardiac and respiratory rhythms in MSNA recording from Y-N rat. A: Stimulus triggered histogram of baseline MSNA triggered from peaks in tracheal pressure (expiration), demonstrating respiratory rhythmicity in multiunit MSNA (bottom) and each discriminated unit (60 bins, 0.05s bin width). B: Waveform average of tracheal pressure triggered from peaks in tracheal pressure (1s width). C: Stimulus triggered histogram of baseline MSNA triggered from peak of arterial pressure wave, demonstrating cardiac rhythmicity in multiunit MSNA and each discriminated unit (100 bins, 0.01s bin width). D: Waveform average of ABP wave triggered from peak of arterial pressure (1s width).
Figure S3. Phase histogram analysis of cardiac rhythmicity in MSNA. A, B: multiunit MSNA triggered from peak of ABP wave (180 bins, 0.3 s max cycle). C, D: Waveform average of ABP pressure wave in same rats, triggered from peak in ABP (width 0.25 s). E, F: Mean phase histograms of all single units, realigned to peak at 180°, and displayed as percentage of total spikes analyzed in each bin (360 bins per cycle). Grey area represents ±60° of cycle peak. G: Proportion of MSNA within and outside ±60° of peak in histogram.
Figure S4. Phase histogram analysis of respiratory rhythmicity in MSNA. A, B: multunit MSNA triggered from peak of tracheal pressure (TP) wave (180 bins, 0.3s max cycle). C, D: Waveform average of TP wave in same rats, triggered from peak in TP (width 0.25s). E, F: Mean phase histograms of all single units realigned to peak at 180°, displayed as percentage of total spikes analysed in each bin (360 bins per cycle). Grey area represents ±60° of cycle peak. G: Proportion of MSNA falling within and outside ±60° of peak in respiratory-triggered phase histogram.