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Jacqueline K. Limberg
University of Missouri

Elizabeth P. Ott
University of Missouri

Walter W. Holbein
Mayo Clinic

Sarah E. Baker
Mayo Clinic

Timothy B. Curry
Mayo Clinic

See next page for additional authors

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Authors

Jacqueline K. Limberg, Elizabeth P. Ott, Walter W. Holbein, Sarah E. Baker, Timothy B. Curry, Wayne T. Nicholson, Michael J. Joyner, and J. Kevin Shoemaker

RESEARCH ARTICLE | 50 Years of Microneurography: Insights into Neural Mechanisms in Humans

Pharmacological assessment of the contribution of the arterial baroreflex to sympathetic discharge patterns in healthy humans

Jacqueline K. Limberg,^{1,2} Elizabeth P. Ott,¹ Walter W. Holbein,² Sarah E. Baker,² Timothy B. Curry,² Wayne T. Nicholson,² Michael J. Joyner,² and J. Kevin Shoemaker³

¹Department of Nutrition and Exercise Physiology, University of Missouri, Columbia, Missouri; ²Department of Anesthesiology and Perioperative Medicine, Mayo Clinic, Rochester, Minnesota; and ³School of Kinesiology, University of Western Ontario, London, Ontario, Canada

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Limberg JK, Ott EP, Holbein WW, Baker SE, Curry TB, Nicholson WT, Joyner MJ, Shoemaker JK. Pharmacological assessment of the contribution of the arterial baroreflex to sympathetic discharge patterns in healthy humans. *J Neurophysiol* 119: 2166–2175, 2018. First published February 28, 2018; doi:10.1152/jn.00935.2017.—To study how changes in baroreceptor afferent activity affect patterns of sympathetic neural activation, we manipulated arterial blood pressure with intravenous nitroprusside (NTP) and phenylephrine (PE) and measured action potential (AP) patterns with wavelet-based methodology. We hypothesized that 1) baroreflex unloading (NTP) would increase firing of low-threshold axons and recruitment of latent axons and 2) baroreflex loading (PE) would decrease firing of low-threshold axons. Heart rate (HR, ECG), arterial blood pressure (BP, brachial catheter), and muscle sympathetic nerve activity (MSNA, microneurography of peroneal nerve) were measured at baseline and during steady-state systemic, intravenous NTP ($0.5\text{--}1.2\ \mu\text{g}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$, $n = 13$) or PE ($0.2\text{--}1.0\ \mu\text{g}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$, $n = 9$) infusion. BP decreased and HR and integrated MSNA increased with NTP ($P < 0.01$). AP incidence (326 ± 66 to 579 ± 129 APs/100 heartbeats) and AP content per integrated burst (8 ± 1 to 11 ± 2 APs/burst) increased with NTP ($P < 0.05$). The firing probability of low-threshold axons increased with NTP, and recruitment of high-threshold axons was observed (22 ± 3 to 24 ± 3 max cluster number, 9 ± 1 to 11 ± 1 clusters/burst; $P < 0.05$). BP increased and HR and integrated MSNA decreased with PE ($P < 0.05$). PE decreased AP incidence (406 ± 128 to 166 ± 42 APs/100 heartbeats) and resulted in fewer unique clusters (15 ± 2 to 9 ± 1 max cluster number, $P < 0.05$); components of an integrated burst (APs or clusters per burst) were not altered ($P > 0.05$). These data support a hierarchical pattern of sympathetic neural activation during manipulation of baroreceptor afferent activity, with rate coding of active neurons playing the predominant role and recruitment/derecruitment of higher-threshold units occurring with steady-state hypotensive stress.

NEW & NOTEWORTHY To study how changes in baroreceptor afferent activity affect patterns of sympathetic neural activation, we manipulated arterial blood pressure with intravenous nitroprusside and phenylephrine and measured sympathetic outflow with wavelet-based methodology. Baroreflex unloading increased sympathetic activity by increasing firing probability of low-threshold axons (rate coding) and recruiting new populations of high-threshold axons.

Baroreflex loading decreased sympathetic activity by decreasing the firing probability of larger axons (derecruitment); however, the components of an integrated burst were unaffected.

baroreflex; microneurography; modified Oxford technique; sympathetic outflow

INTRODUCTION

Sympathetic neural recordings obtained from skeletal muscle in humans [via the technique of microneurography (Vallbo et al. 1979; Salmanpour et al. 2010)] are often rectified and integrated to give multiunit muscle sympathetic nerve activity (MSNA) “bursts” that represent activity of several axons recorded simultaneously. With this, a fundamental question in motor unit physiology (Adrian and Bronk 1929; Henneman 1957) has also emerged in sympathetic nervous system activity: Are increases in the incidence and/or size of multiunit integrated MSNA bursts the result of an increased firing rate of previously active neurons [rate coding (Macefield and Wallin 1999; Salmanpour et al. 2011a)] and/or recruitment of additional neurons (Badrov et al. 2015; Steinback et al. 2010b; Wallin et al. 1994)? The frequency of neural discharge determines neurotransmitter release patterns (Lambert et al. 2011), and the number of active neurons can vary with each integrated, multiunit MSNA burst (Macefield and Elam 2003). In this way, altered sympathetic neuronal firing patterns may have consequences in the cardiovascular system that are not detected with integrated MSNA recordings.

The activity of the sympathetic nervous system can vary significantly when blood pressure increases and decreases. Despite robust alterations in sympathetic activity with manipulation of baroreceptor afferent activity, changes in blood pressure are more closely related to integrated, multiunit MSNA burst occurrence than burst size (Kienbaum et al. 2001). These data led researchers to hypothesize that the baroreflex is more important in determining whether a sympathetic burst occurs, while another unknown factor determines the number of axons recruited (Keller et al. 2006; Kienbaum et al. 2001; Malpas 2010). Consistent with this concept, Salmanpour and

Address for reprint requests and other correspondence: J. K. Limberg, Dept. of Nutrition and Exercise Physiology, Univ. of Missouri, 520 Hitt St., Columbia, MO 65211 (e-mail: limbergj@missouri.edu).

colleagues used wavelet-based methodology to show that small to medium-sized, low-threshold axons were present in most integrated, multiunit MSNA bursts at baseline and increased their firing rates during orthostatic stress (Salmanpour et al. 2011a, 2011b; Salmanpour and Shoemaker 2012). In contrast, the authors found that larger, faster-conducting axons had a low probability of firing at baseline and were minimally affected by baroreceptor pathways engaged during moderate orthostatic stress (Salmanpour et al. 2011a; Salmanpour and Shoemaker 2012).

These recent results support a relatively small impact of moderate lower body suction on sympathetic action potential (AP) recruitment, compared with other reflexes (Steinback et al. 2010a, 2010b). In contrast, during more severe orthostasis (-80 -mmHg lower body negative pressure), the majority of individuals studied recruited a new population of larger-amplitude, faster-conducting postganglionic axons (Badrov et al. 2015; Salmanpour et al. 2011a). However, until now, the study of baroreflex control of MSNA AP recruitment has occurred under stable baseline conditions and/or during nonhypotensive lower body negative pressure, where the baroreflex stimulus for elevations in MSNA involves changes in pulse pressure but little change in diastolic or mean pressures. To advance our understanding of the contribution of the baroreflex to sympathetic discharge patterns in humans, we systematically manipulated arterial blood pressure by using intravenous nitroprusside and phenylephrine to elicit a large range of primarily arterial baroreflex stress and measured sympathetic outflow with wavelet-based methodology (Salmanpour et al. 2010). We hypothesized that baroreflex unloading with nitroprusside would increase firing of low-threshold axons (rate coding) and would recruit latent axons. We further hypothesized that baroreflex loading with phenylephrine would decrease firing of low-threshold axons.

METHODS

Research subjects. All subjects were 18- to 45-yr-old, healthy (no acute or chronic conditions) nonsmokers with a body mass index <30 kg/m² and not taking any medications. All experiments were performed in the Clinical Research and Trials Unit (CRTU) at Mayo Clinic, were approved by the Institutional Review Board at the Mayo Clinic, and conformed to the Declaration of Helsinki. On a screening visit, each subject gave written informed consent, followed by a review of medical history and a brief physical exam performed by a laboratory physician that included baseline measurements of height, weight, blood pressure, and heart rate.

Instrumentation. On the study day, subjects arrived at the laboratory after a 4-h fast and after abstaining from exercise, caffeine, and alcohol for at least 24 h. Subjects were semirecumbent and were instrumented with a three-lead electrocardiogram to measure heart rate (Cardiacap/5; Datex-Ohmeda) and a nose clip/mouthpiece connected to a turbine for measurements of respiratory rate and tidal volume (Universal Ventilation Meter; Vacumetrics). A 20-gauge, 5-cm catheter was placed in the brachial artery under aseptic conditions after local anesthesia (2% lidocaine) to measure arterial blood pressure and for periodic blood sampling (epinephrine, norepinephrine). Standard blood assays were performed by the Immunochemistry Core Laboratory of the CRTU of the Mayo Clinic Center for Clinical and Translational Science.

Resting MSNA was recorded with the technique of microneurography (Salmanpour et al. 2010; Vallbo et al. 1979). Multiunit MSNA was recorded with a tungsten microelectrode (200- μ m shaft diameter tapering to 1–5 μ m at the uninsulated tip, 2.0 ± 0.4 M impedance; FHC, Bowdoin, ME) placed percutaneously into the peroneal nerve, posterior to the fibular head under direct two-dimensional live ultra-

sound guidance with a 12–15 MHz linear probe (Curry and Charkoudian 2011). A reference electrode was positioned subcutaneously ~ 4 cm from the recording electrode. The recorded signal was amplified 80,000-fold, band-pass filtered (700–2,000 Hz), rectified, and integrated (resistance-capacitance integrator circuit, time constant 0.1 s) by a nerve traffic analyzer (662C-3; Dept. of Bioengineering, University of Iowa). Sympathetic bursts in the integrated neurogram were identified with a custom-manufactured automated analysis program (Salmanpour et al. 2010). Multiunit MSNA is reported as bursts per minute (burst frequency), bursts per 100 heartbeats (burst incidence), and burst area (arbitrary units per min). Only sections of data without ectopic beats were included in the analysis.

Action potential detection and analysis. AP patterns were detected and extracted with wavelet-based methodology on the raw, band-pass-filtered neurogram (Salmanpour et al. 2010). Briefly, a continuous wavelet transform using a “mother wavelet” with the same morphology as a physiological postganglionic sympathetic AP was used for AP detection (Salmanpour et al. 2010). The continuous wavelet transform with the matched mother wavelet was applied to the filtered MSNA signal to provide a wavelet coefficient between the AP of interest and the mother wavelet such that the wavelet coefficient was largest in the presence of the APs and negligible when applied to noise. Wavelet coefficients related to APs and noise were separated on the basis of thresholding analysis (Salmanpour et al. 2010). Extracted APs were grouped as overlapped (summed signal of 2 co-occurring APs) or single APs, based on their morphology. Nonoverlapped APs were ordered on the basis of peak-to-peak amplitude (32-point *k*-means), and histogram analysis was performed to separate APs into amplitude-based clusters based on Scott’s rule (Scott 1979; proposes optimal histogram bin width based on the sample size and an estimate of the standard deviation of the data). The number of AP clusters in the neurogram varied between maneuvers. Thus bin characteristics (minimum histogram bin width, maximum bin center, and total number of bins) were normalized within each individual to ensure that corresponding clusters at baseline and pharmacological intervention contained APs with similar peak-to-peak amplitudes (Badrov et al. 2015). With this, an increase in the number of clusters is considered equal to increased recruitment of additional populations of neurons.

Outcome variables include APs per burst (reflecting firing rate), clusters per burst (reflecting recruitment), and conduction latency of each cluster (reflecting synaptic delays and verifying large amplitude clusters as new populations). Conduction latency was calculated as the time delay between the R wave of the electrocardiogram of the preceding cardiac cycle and the negative peak of the AP waveform. Because the number of total clusters varies by individual, each participants’ number of total clusters was normalized to 10 total clusters with methods published previously (Badrov et al. 2015). In this way, each cluster group contained a 10% range of the largest detected cluster (which was given a value of 100%). For example, cluster 0–10% contained an average of all cluster numbers that were 0–10% of the largest cluster (Badrov et al. 2015).

Intravenous infusions. An intravenous catheter was placed under local anesthesia for systemic intravenous infusion of nitroprusside and phenylephrine, administered separately. Nitroprusside (nitric oxide donor) was infused intravenously after 3 min of quiet rest at an individualized dose (0.5 – 1.2 μ g·kg⁻¹·min⁻¹) intended to achieve a blood pressure fall of 10 mmHg (Grassi et al. 1995, 2004, 2005; Grossman et al. 1991; Heindl et al. 2006). Phenylephrine (α_1 -adrenergic agonist) was infused intravenously after 3 min of quiet rest at a range of doses (0.2 – 1.0 μ g·kg⁻¹·min⁻¹) selected to achieve varied levels of baroreceptor activity (Cui et al. 2002; Heindl et al. 2006; Wilkins et al. 2008). For example, 1.5 μ g·kg⁻¹·min⁻¹ has been shown to increase mean blood pressure ~ 15 mmHg and silence MSNA via baroreceptor loading (Cui et al. 2002; Wehrwein et al. 2010). In contrast, low doses of phenylephrine (0.1 μ g·kg⁻¹·min⁻¹) may increase blood pressure only slightly (~ 1 mmHg) (Wilkins et al. 2008). With such small changes in baroreceptor stretch changes in

Table 1. *Cardiorespiratory and sympathetic responses to systemic nitroprusside*

	Baseline	Nitroprusside
Heart rate, beats/min	61 ± 2	80 ± 3*
Systolic blood pressure, mmHg	146 ± 3	136 ± 4*
Diastolic blood pressure, mmHg	77 ± 2	73 ± 2*
Mean blood pressure, mmHg	99 ± 2	91 ± 2*
Respiratory rate, breaths/min	11 ± 1	11 ± 1
Tidal volume, l/breath	0.70 ± 0.14	0.81 ± 0.16
Minute ventilation, ml/min	6.7 ± 0.8	7.9 ± 1.0
Epinephrine, pg/ml	67 ± 11	82 ± 16
Norepinephrine, pg/ml	174 ± 14	215 ± 26
Burst frequency, bursts/min	25 ± 3	41 ± 3*
Burst incidence, bursts/100 heartbeats	41 ± 5	53 ± 4*
Mean burst area, AU/min	1.4 ± 0.2	2.8 ± 0.4*
Action potential frequency, APs/min	191 ± 35	439 ± 90*
Action potential incidence, APs/burst	8 ± 1	11 ± 2*
Action potential incidence, APs/100 heartbeats	326 ± 66	579 ± 129*
Mean cluster incidence, clusters/burst	4.5 ± 0.5	5.5 ± 0.7*
Max cluster incidence, clusters/burst	9.5 ± 1.0	10.9 ± 1.0*
Max cluster number	21.6 ± 2.7	24.4 ± 3.0*

Data are means ± SE from $n = 13$. Average final dose was $0.90 \pm 0.05 \mu\text{g}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$. Data were analyzed with a 1-way repeated-measures ANOVA. Normality was assessed with the Shapiro-Wilk test. If not normally distributed, a Friedman repeated-measures ANOVA on ranks was conducted [respiratory rate, minute ventilation, action potential (AP) incidence (APs/burst), max cluster number]. AU, arbitrary units. Pairwise comparisons were done with the Holm-Sidak method. * $P < 0.05$ vs. baseline.

multiunit MSNA may be undetectable, but changes in the firing of individual axons may be observed with the AP analysis approach.

Study visit. Subjects completed two protocols separated by a minimum of 30 min. Protocol order was randomized and counterbalanced. For each protocol, respiratory rate was held at baseline levels with a metronome. *Protocol 1* consisted of intravenous infusion of sodium nitroprusside. All individuals started at a dose of 0.8

$\mu\text{g}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$, which was increased $0.1 \mu\text{g}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ every 2–3 min until a 10-mmHg fall in blood pressure and an increase in MSNA were observed. If blood pressure fell >10 mmHg, the nitroprusside dose was reduced in $0.1 \mu\text{g}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ increments until a stable 10-mmHg fall in blood pressure was reached (time to achieve desired effect = 6.2 ± 1.1 min; average final dose $0.90 \pm 0.05 \mu\text{g}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$). Data are reported as a 5-min average during the final dose (total infusion time = 11.3 ± 1.1 min).

Protocol 2 consisted of intravenous infusion of phenylephrine at $0.2 \mu\text{g}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ for 3 min. The dose of phenylephrine was increased by $0.2 \mu\text{g}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ every 3 min until arterial blood pressure was increased 5–10 mmHg and multiunit MSNA signal was silenced. After the final 3-min infusion, phenylephrine infusion was stopped and the MSNA signal was allowed to return, ensuring that the fall in MSNA was not the result of signal decay. Data are reported as a 3-min average during the final dose (phenylephrine *dose 2*, range 0.4–1.0, average 0.62 ± 0.08) and during approximately half of the maximum dose (phenylephrine *dose 1*, range 0.2–0.6, average 0.29 ± 0.05).

Three individuals from *protocol 1* and six naive individuals were recruited to complete a third protocol. On a separate study day, subjects arrived to the laboratory after a 12-h fast and after abstaining from exercise, caffeine, and alcohol for at least 24 h. In the three returning individuals, visits were separated by 18 ± 2 mo (range 14–20 mo). Instrumentation for *protocol 3* was identical to *protocols 1* and *2*, although subjects wore a mask rather than a nose clip/mouthpiece and respiratory rate was not controlled. After instrumentation, individuals completed a 5-min quiet resting period. An intravenous bolus of sodium nitroprusside ($100 \mu\text{g}$) was then administered, followed by an intravenous bolus of phenylephrine ($150 \mu\text{g}$) (modified Oxford technique) (Ebert 1990; Rudas et al. 1999; Smyth et al. 1969). This approach is currently considered the “gold standard” to examine sensitivity of the arterial baroreflex (Rudas et al. 1999). By incorporating the modified Oxford technique, we are able to examine the robustness of observations made under more transient decreases/increases in blood pressure. Data are reported as an average during the last 1 min of rest; after nitroprusside infusion, when blood pressure

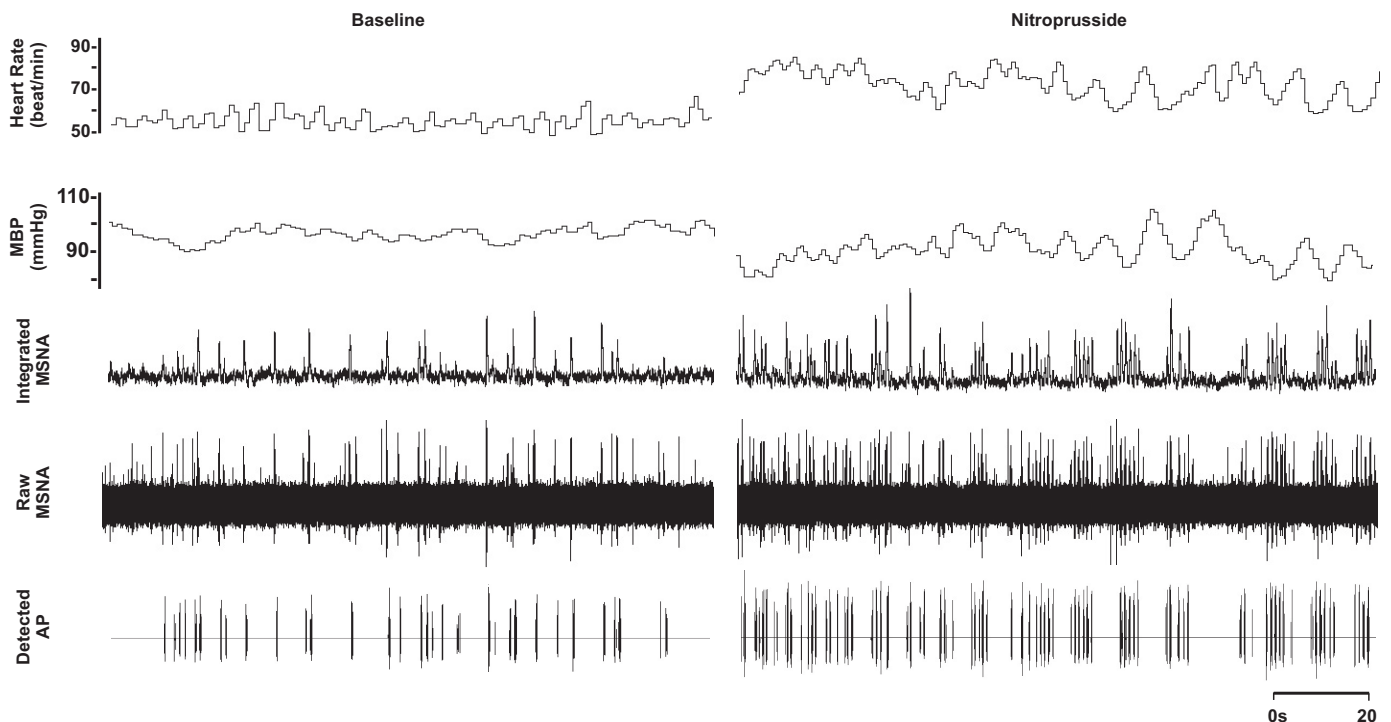


Fig. 1. Representative sample of data from 1 subject (26 yr, $21 \text{ kg}/\text{m}^2$) collected at baseline and during steady-state nitroprusside infusion ($1.1 \mu\text{g}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$). MBP, mean blood pressure.

was falling; and during the subsequent phenylephrine infusion, when blood pressure was increasing but MSNA integrated bursts could still be detected.

Data analysis. Data were recorded at 10,000 Hz with a computer data acquisition system (PowerLab; ADInstruments) and stored for off-line analysis (Salmanpour et al. 2010). Statistical analyses were conducted with SigmaPlot version 14.0 (Systat Software). Data presented in Tables 1–3 were analyzed by using a one-way repeated-measures analysis of variance (ANOVA) to determine the main effect of condition (baseline, drug) on main outcome variables. Normality was assessed with the Shapiro-Wilk test. If not normally distributed, a Friedman repeated-measures ANOVA on ranks was conducted. Pairwise comparisons were done by the Holm-Sidak method. Data presented in Figs. 2, 4, 6, and 7 were analyzed by using a two-way repeated-measures ANOVA to determine the main effect of relative cluster size (10–100% of total clusters) and condition (baseline, drug) and the interaction of cluster and condition. Pairwise comparisons were done with the Holm-Sidak method. All data are presented as means \pm SE. P values ≤ 0.05 were considered statistically significant.

RESULTS

Subject demographics. The initial protocol recruited 19 individuals, of which high-quality nerve recordings were made in 14 individuals. Thirteen male subjects (30 ± 2 yr, 181 ± 2 cm, 82 ± 3 kg, 25 ± 1 kg/m²) successfully completed *protocol 1* (1 subject was excluded because of failure to achieve desired fall in blood pressure). Of those 13 individuals, 9 individuals successfully completed *protocol 2* (30 ± 2 yr, 181 ± 2 cm, 82 ± 3 kg, 25 ± 1 kg/m²) (data from 4 individuals were excluded because of shifts in the MSNA baseline during the infusion protocol). Three individuals returned for a second visit to complete *protocol 3*. An additional six male subjects were recruited to complete *protocol 3* only ($n = 9$ total; 31 ± 2 yr, 183 ± 2 cm, 89 ± 3 kg, 26 ± 1 kg/m²).

Nitroprusside. Compared with baseline, blood pressure decreased and heart rate increased with nitroprusside ($P < 0.01$; Table 1 and Fig. 1). Nitroprusside elicited a progressive increase in integrated MSNA bursts ($P < 0.01$; Table 1 and Fig. 1). Underlying nitroprusside-mediated increases in integrated MSNA were increases in neuronal discharge rate (APs/min, APs/100 heartbeats) and AP content per integrated burst ($P < 0.05$; Table 1). The probability of APs from the same cluster firing once per integrated MSNA burst (single firing; Fig. 2A) and more than once per burst (multiple firing; Fig. 2B) increased with nitroprusside, and recruitment of latent, high-threshold axons was observed (max cluster number; max clusters/burst) ($P < 0.05$ for all; Table 1).

Phenylephrine. No changes in neuronal recruitment and/or firing patterns were observed at the lower dose (*dose 1*) of phenylephrine, despite an ~ 2 -mmHg increase in diastolic blood pressure (Table 2). Compared with baseline, blood pressure increased and heart rate decreased with the highest dose (*dose 2*) of phenylephrine ($P < 0.01$; Table 2 and Fig. 3). The highest dose of phenylephrine elicited a reduction in integrated MSNA ($P < 0.05$; Table 2 and Fig. 3). Underlying the phenylephrine-mediated decreases in integrated MSNA at the highest dose were decreases in neuronal discharge rate (APs/min, APs/100 heartbeats) and fewer unique clusters (max cluster number, max clusters/burst), suggesting derecruitment of axons ($P < 0.05$ for all; Table 2). The firing probability (single and multiple) of low-threshold axons was not altered with phenyl-

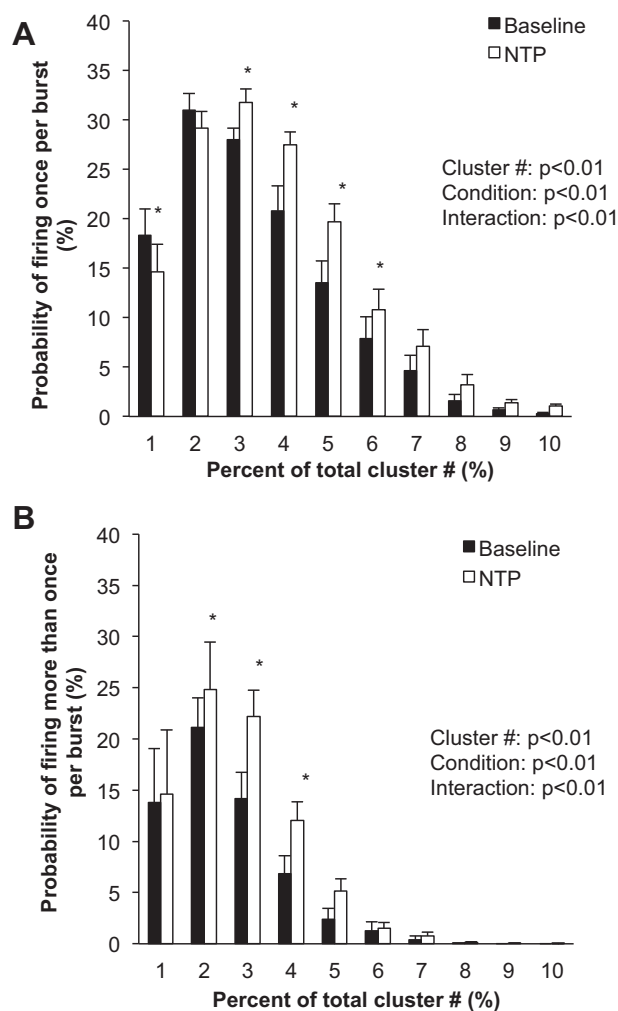


Fig. 2. Sympathetic responses to steady-state systemic nitroprusside (NTP). Data are means \pm SE from $n = 13$. Data were analyzed with a 2-way repeated-measures ANOVA to determine the main effect of relative cluster size (10–100% of total clusters) and condition (baseline, drug) and the interaction of cluster and condition. *A*: probability of a relative cluster (10–100% of total clusters) firing once per integrated MSNA burst. *B*: probability of a relative cluster (10–100% of total clusters) firing more than once per integrated MSNA burst. * $P < 0.05$ vs. baseline.

ephine (Fig. 4), and the components of an integrated burst were not altered (APs/burst, clusters/burst, $P > 0.05$; Table 2).

Modified Oxford procedure. Blood pressure decreased and heart rate increased with nitroprusside, and values returned to baseline levels with subsequent phenylephrine (main effect, $P < 0.01$; Table 3 and Fig. 5). Nitroprusside elicited a robust increase in integrated MSNA bursts that returned to baseline levels with phenylephrine (main effect, $P < 0.01$; Table 3 and Fig. 5). Underlying changes in integrated MSNA were increases in neuronal discharge rate with nitroprusside (APs/min, APs/100 heartbeats) that returned to baseline with subsequent phenylephrine (main effect, $P < 0.05$; Table 3). No changes were observed in AP recruitment (max cluster number $P = 0.69$, max clusters/burst $P = 0.25$; Table 3), and components of a burst were not altered (APs/burst $P = 0.22$; mean clusters/burst $P = 0.36$) during the modified Oxford procedure. Furthermore, the firing probabilities (single and multiple) were not altered (P value range 0.14–0.56; Fig. 6).

Table 2. *Cardiorespiratory and sympathetic responses to systemic phenylephrine*

	Baseline	Phenylephrine Dose 1	Phenylephrine Dose 2
Heart rate, beats/min	61 ± 2	60 ± 2	56 ± 3*†
Systolic blood pressure, mmHg	145 ± 4	144 ± 4	150 ± 4*†
Diastolic blood pressure, mmHg	77 ± 2	79 ± 2*	83 ± 2*†
Mean blood pressure, mmHg	98 ± 2	100 ± 2	106 ± 3*†
Respiratory rate, breaths/min	12 ± 1	12 ± 1	12 ± 1
Tidal volume, l/breath	0.51 ± 0.08	0.51 ± 0.08	0.57 ± 0.07
Minute ventilation, ml/min	5.8 ± 0.8	6.0 ± 0.7	6.7 ± 0.8
Epinephrine, pg/ml	58 ± 10	60 ± 7	54 ± 9
Norepinephrine, pg/ml	160 ± 18	132 ± 19*	132 ± 20
Burst frequency, bursts/min	24 ± 2	23 ± 2	11 ± 2*†
Burst incidence, bursts/100 heartbeats	41 ± 3	39 ± 4	21 ± 3*†
Mean burst area, AU/min	1.3 ± 0.4	1.2 ± 0.4	0.5 ± 0.1
Action potential frequency, APs/min	238 ± 73	222 ± 63	91 ± 25*†
Action potential incidence, APs/burst	8.9 ± 2.3	8.9 ± 2.0	7.7 ± 1.8
Action potential incidence, APs/100 heartbeats	406 ± 128	377 ± 102	166 ± 42*†
Mean cluster incidence, clusters/burst	4.6 ± 0.7	4.6 ± 0.7	3.9 ± 0.5
Max cluster incidence, clusters/burst	8.6 ± 1.0	8.6 ± 1.0	6.9 ± 0.7*†
Max cluster number	14.6 ± 2.3	15.1 ± 2.2	8.9 ± 0.8*†

Data are means ± SE from $n = 9$ unless otherwise noted (*dose 2*: epinephrine and norepinephrine, $n = 6$). Data are reported as a 3-min average during the final dose (phenylephrine *dose 2*, range 0.4–1.0, average $0.62 \pm 0.08 \mu\text{g}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$) and during approximately half of the maximum dose (phenylephrine *dose 1*, range 0.2–0.6, average $0.29 \pm 0.05 \mu\text{g}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$). Data were analyzed with a 1-way repeated-measures ANOVA. Normality was assessed with the Shapiro-Wilk test. If not normally distributed, a Friedman repeated-measures ANOVA on ranks was conducted [minute ventilation, mean burst area, action potential incidence (AP/burst), max cluster incidence]. Pairwise comparisons were done with the Holm-Sidak method. * $P < 0.05$ vs. baseline. † $P < 0.05$ vs. phenylephrine *dose 1*.

Synaptic delays. AP cluster latency decreased with cluster size in all protocols ($P < 0.01$) but was not altered with nitroprusside ($P = 0.42$) or phenylephrine ($P = 0.39$) or during the modified Oxford procedure ($P = 0.40$) (see Fig. 7).

DISCUSSION

The main new finding in this study is that baroreflex unloading via a reduction in arterial pressure can elicit recruitment of

larger, faster-conducting sympathetic neurons. Conversely, baroreflex loading via increases in arterial pressure can derecruit neurons that are present at baseline. In both cases, we confirm prior observations demonstrating that ongoing rate coding of actively firing sympathetic neurons is under baroreflex control and is especially important during transient decreases/increases in blood pressure. We also show synaptic delays, which have been observed previously to be reduced

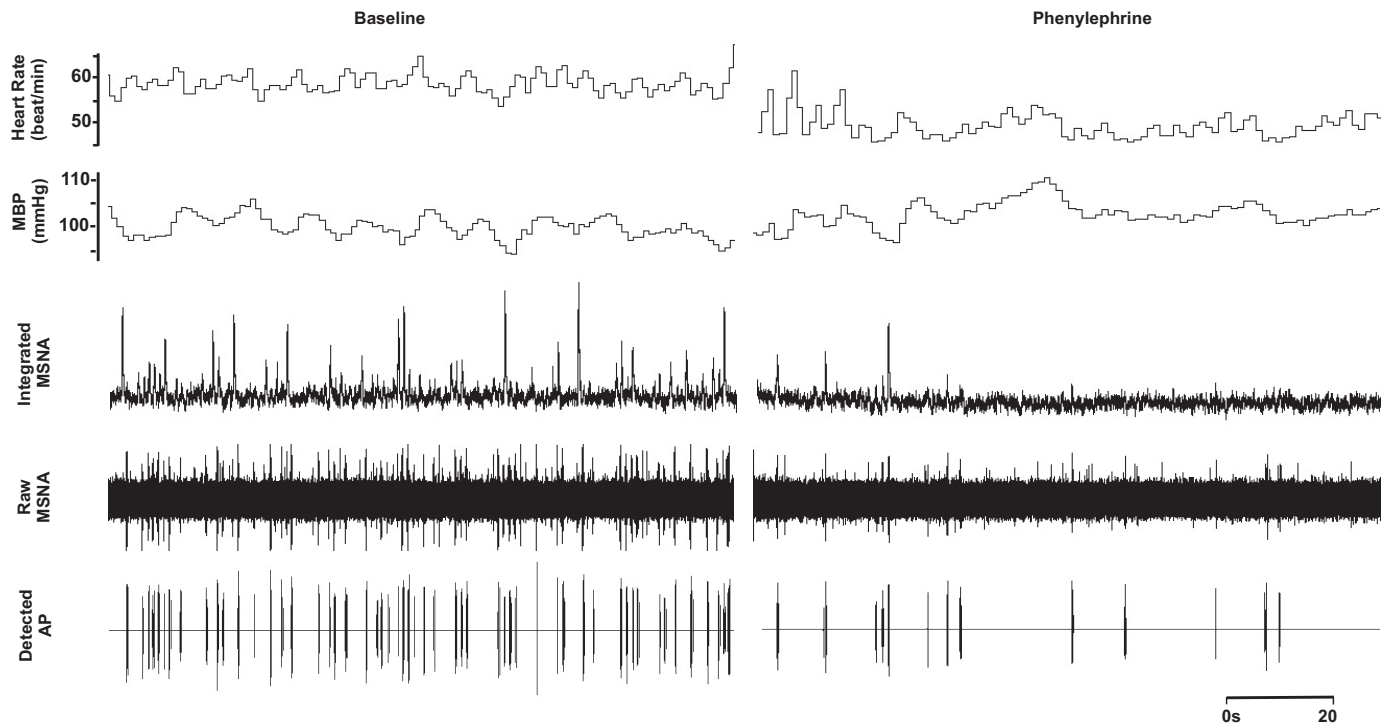


Fig. 3. Representative sample of data from 1 subject (26 yr, 21 kg/m²) collected at baseline and during phenylephrine infusion at the highest dose ($0.6 \mu\text{g}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$).

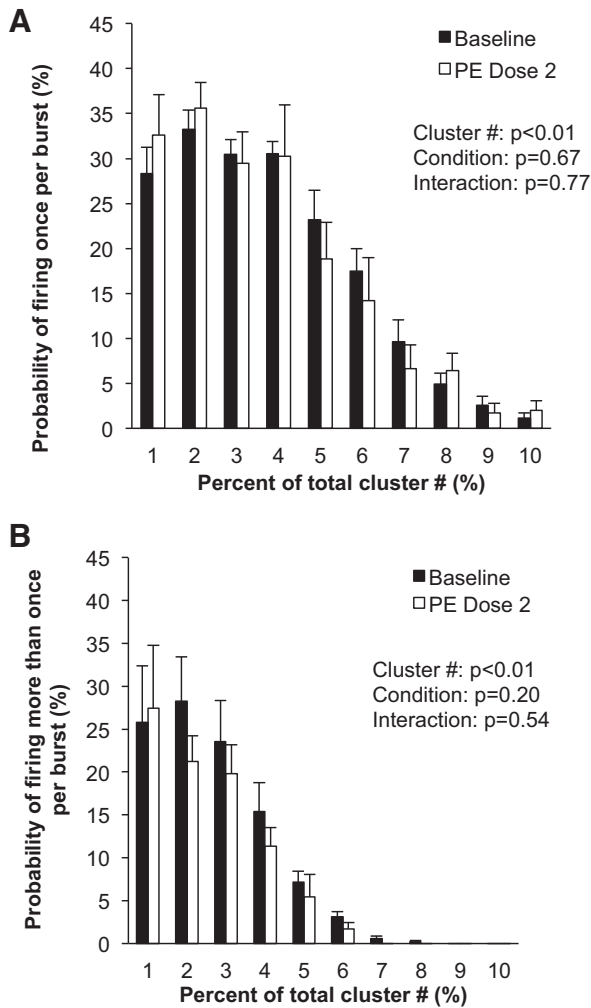


Fig. 4. Sympathetic responses to steady-state systemic phenylephrine (PE). Data are means \pm SE from $n = 9$. Data were analyzed with a 2-way repeated-measures ANOVA to determine the main effect of relative cluster size (10–100% of total clusters) and condition (baseline, drug) and the interaction of cluster and condition. *A*: probability of a relative cluster (10–100% of total clusters) firing once per integrated MSNA burst. *B*: probability of a relative cluster (10–100% of total clusters) firing more than once per integrated MSNA burst.

during physical stress [handgrip exercise (Badrov et al. 2016a), voluntary apnea (Steinback et al. 2010a)] and somewhat prolonged during -80 -mmHg lower body negative pressure (Badrov et al. 2015), are unaffected by baroreflex loading/unloading by pharmacological methods.

Nitroprusside. In the present investigation, we found that pharmacological baroreflex unloading with systemic nitroprusside increases sympathetic neuronal discharge rate (rate coding) by increasing the firing probability of low-threshold axons (Fig. 2), in addition to recruitment of latent, higher-threshold axons (Table 1). This is similar to what has been observed previously during severe chemoreflex stress (Steinback et al. 2010a, 2010b) but is in contrast to previous work from Salmanpour and colleagues showing that larger, faster-conducting axons are minimally affected by baroreceptor pathways (Salmanpour et al. 2011a, 2011b; Salmanpour and Shoemaker 2012). However, the majority of these data were obtained under baseline conditions, thus relying on spontaneous fluctuations and/or low-level lower body negative pressure. In con-

trast, during more severe orthostasis (-80 -mmHg lower body negative pressure), the majority of individuals studied recruited a new population of larger-amplitude, faster-conducting post-ganglionic axons (Badrov et al. 2015; Salmanpour et al. 2011a).

Comparisons of outcomes of the present pharmacological approach with observations using nonpharmacological models (i.e., orthostatic stress evoked by lower body negative pressure; Badrov et al. 2015; Salmanpour et al. 2011a) suggest that a consistent physiological response can be achieved under conditions of relatively stable baroreflex stress. With this, in the present study we show that an ordered, hierarchical pattern of neuronal recruitment is possible during intravenous infusion of nitroprusside in healthy male adults and further support the concept that subpopulations of larger-amplitude, faster-conducting sympathetic axons that are not present at baseline can be recruited during steady-state baroreflex stress. This pattern of recruitment is not unlike recruitment of motor units during muscular contraction (Adrian and Bronk 1929; Henneman 1957). It is important to acknowledge, however, that although recruitment occurs, it expresses interindividual variations. For example, previous work found that only 60–65% of individuals recruit latent subpopulations of axons during severe orthostasis (-80 mmHg lower body negative pressure; Badrov et al. 2015; Salmanpour et al. 2011a). Similarly, the present data show an increase in maximum clusters per burst (indicating recruitment) in 75% of individuals ($n = 10$ of 13) studied with pharmacological methods.

We also observed an increase in the probability of multiple firing (APs from the same cluster firing more than once per burst) during steady-state nitroprusside infusion (Fig. 2B). Macefield and colleagues have shown, using single-unit MSNA recordings, that neurons typically fire only once per integrated burst at rest (Macefield et al. 1994; Macefield and Wallin 1999) and the same neuron can fire multiple times within a single integrated burst during stress (Macefield and Wallin 1999). Although the present analysis approach cannot infer single-axon behavior, we show a relatively low probability of multiple firing at baseline, which is augmented during nitroprusside infusion.

Given that the frequency of neural discharge determines neurotransmitter release patterns (Lambert et al. 2011), these data may have important implications for neurovascular transduction. More specifically, one would expect an increase in release of norepinephrine and colocalized neurotransmitters (i.e., neuropeptide Y) with an increase in multiple firing—a concept that may be supported by the present data. With this, it would be especially interesting to examine similar responses in a group of individuals with known impairments in sympathetic baroreflex sensitivity and/or neurovascular transduction. For example, older individuals have a reduced capacity to recruit latent neural subpopulations compared with young adults (Badrov et al. 2016a). When combined with our new observations, it is reasonable to speculate that impairments in sympathetic baroreflex sensitivity observed with aging may be related to attenuated recruitment of latent, high-threshold axons in response to baroreflex unloading.

Phenylephrine. To our knowledge, this is the first study of its kind to examine derecruitment strategies of sympathetic axons in humans. As noted above, a key strategy of the sympathetic nervous system to alter neuronal activity is to change the

Table 3. *Cardiorespiratory and sympathetic responses during modified Oxford test*

	Baseline	Nitroprusside	Phenylephrine
Heart rate, beats/min	58 ± 2	73 ± 4*†	61 ± 3
Systolic blood pressure, mmHg	138 ± 4	130 ± 4*†	139 ± 5
Diastolic blood pressure, mmHg	75 ± 3	65 ± 3*†	75 ± 3
Mean blood pressure, mmHg	96 ± 3	86 ± 3*†	95 ± 3
Respiratory rate, breaths/min	13 ± 1	12 ± 1	12 ± 1
Tidal volume, l/breath	0.58 ± 0.10	0.77 ± 0.07	0.98 ± 0.17*
Minute ventilation, ml/min	7.1 ± 1.0	8.9 ± 1.1	11.5 ± 2.1*
Burst frequency, bursts/min	21 ± 1	36 ± 2*†	22 ± 4
Burst incidence, bursts/100 heartbeats	37 ± 4	53 ± 4*†	37 ± 7
Mean burst area, AU/min	1.1 ± 0.2	2.1 ± 0.2*†	1.2 ± 0.2
Action potential frequency, APs/min	222 ± 36	454 ± 71*†	254 ± 74
Action potential incidence, APs/burst	10.3 ± 1.2	12.2 ± 1.8	10.7 ± 1.6
Action potential incidence, APs/100 heartbeats	398 ± 75	678 ± 120*	431 ± 127
Mean cluster incidence, clusters/burst	5.3 ± 0.6	5.4 ± 0.6	5.0 ± 0.5
Max cluster incidence, clusters/burst	9.8 ± 0.8	10.0 ± 0.9	10.3 ± 1.0
Max cluster number	10.7 ± 1.2	11.3 ± 1.2	10.6 ± 1.2

Data are means ± SE from $n = 9$. Data were analyzed with a 1-way repeated-measures ANOVA. Normality was assessed with the Shapiro-Wilk test. If not normally distributed, a Friedman repeated-measures ANOVA on ranks was conducted (heart rate). Pairwise comparisons were done with the Holm-Sidak method. * $P < 0.05$ vs. baseline. † $P < 0.05$ vs. phenylephrine.

number of active neurons and/or the firing probability of neurons (Macefield and Wallin 1999). Previous work in motor units has shown that the firing rate of earlier-recruited motor units is greater than that of units recruited later (Henneman et al. 1965). In support of this idea, our data show that the firing

probability of larger-diameter, higher-threshold axons is attenuated during pharmacological loading of the baroreflex. Specifically, there were fewer unique clusters present during the highest dose of phenylephrine infused, compared with baseline, and AP incidence was reduced (Table 2). In light of this,

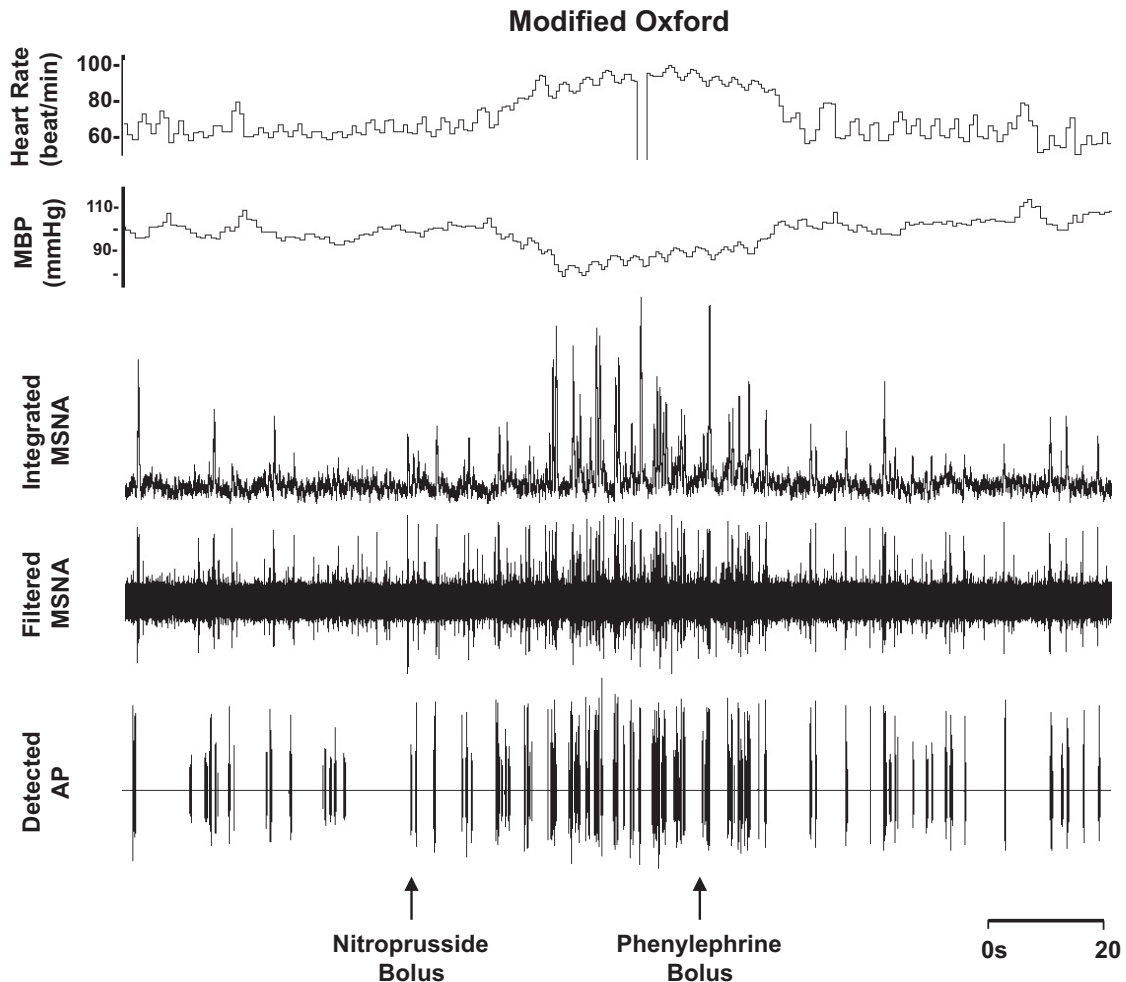


Fig. 5. Representative sample of data from 1 subject (24 yr, 24 kg/m²) collected at baseline and during the modified Oxford procedure.

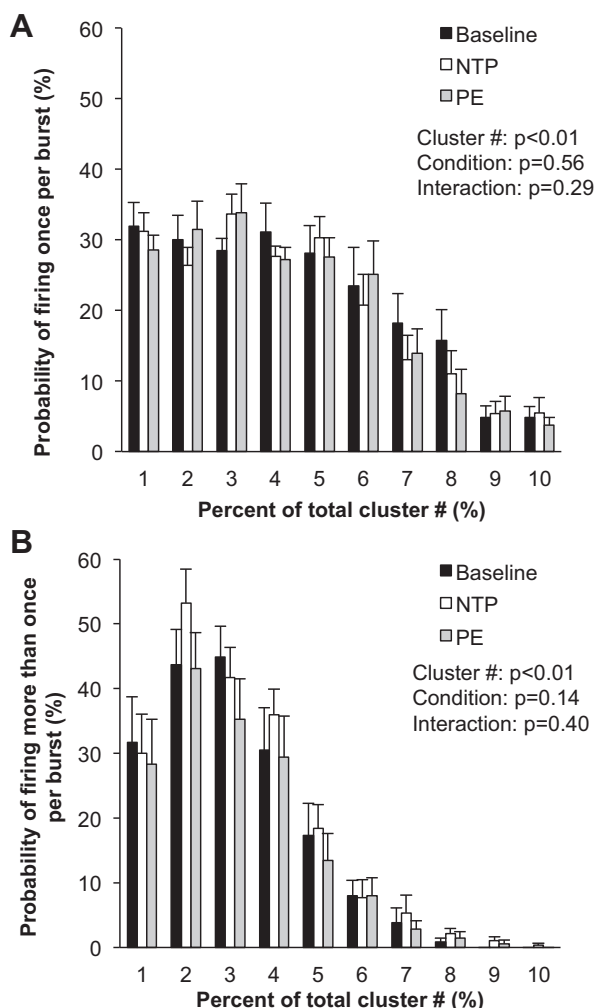


Fig. 6. Sympathetic responses to the modified Oxford procedure. Data are means \pm SE from $n = 9$. Data were analyzed with a 2-way repeated-measures ANOVA to determine the main effect of relative cluster size (10–100% of total clusters) and condition (baseline, drug) and the interaction of cluster and condition. *A*: probability of a relative cluster (10–100% of total clusters) firing once per integrated MSNA burst. *B*: probability of a relative cluster (10–100% of total clusters) firing more than once per integrated MSNA burst.

we can conclude that neurons present at baseline exhibiting the highest threshold activation (larger diameter, shorter latency) are the first lost under conditions of baroreflex loading. Based on the understanding that sympathetic neurons directed to

skeletal muscle vasculature are vasoconstrictor signals, we surmise that, although changes in cluster number are relatively subtle in the present model, they should have an impact on regulation of vascular tone. Such findings may have important implications for clinical conditions such as hypertension, where one could speculate that patients may be unable to derecruit high-threshold axons with increases in arterial blood pressure. However, it is important to acknowledge that, contrary to our hypothesis, any changes in neuronal firing and recruitment were only observed at the highest dose of phenylephrine and relatively small (~ 2 mmHg) changes in blood pressure had minimal effect on AP recruitment.

Modified Oxford procedure. Three returning subjects and six naive individuals completed a third protocol that consisted of a bolus infusion of nitroprusside followed immediately by a bolus infusion of phenylephrine (modified Oxford technique) (Ebert 1990; Rudas et al. 1999; Smyth et al. 1969). This approach is often used experimentally to examine sensitivity of the arterial baroreflex and is considered the “gold standard” (Rudas et al. 1999). With this, results from the modified Oxford approach vary from conclusions obtained during individual infusions in the following areas: 1) bolus nitroprusside did not increase recruitment of latent axons and 2) bolus phenylephrine did not decrease firing of larger, faster-conducting axons. The severity of the stimulus in terms of a change in blood pressure is relatively consistent between the protocols, and thus we speculate that differences in the response may be due to differing lengths of time over which the stress is occurring (seconds vs. minutes). From this, we can conclude that transient changes in blood pressure rely on rate coding rather than recruitment to achieve changes in sympathetic activation, which is in contrast to responses observed during steady-state infusions, where axonal recruitment/derecruitment was observed. It is also possible that recruitment/derecruitment occurs briefly in one or two bursts at the nadir/peak blood pressure during the modified Oxford procedure that are not detectable by current analysis methods. However, previous published work has shown that short (8–47 s) sections of data do not significantly impact detectable clusters (Badrov et al. 2016a), and thus this is a minor limitation.

Synaptic delays. Wallin and colleagues have shown an inverse relationship between burst latency (measured as the delay of an integrated MSNA burst from a representative ECG R wave) and burst size, with larger bursts exhibiting shorter reflex latencies (Wallin et al. 1994). Consistent with this, we

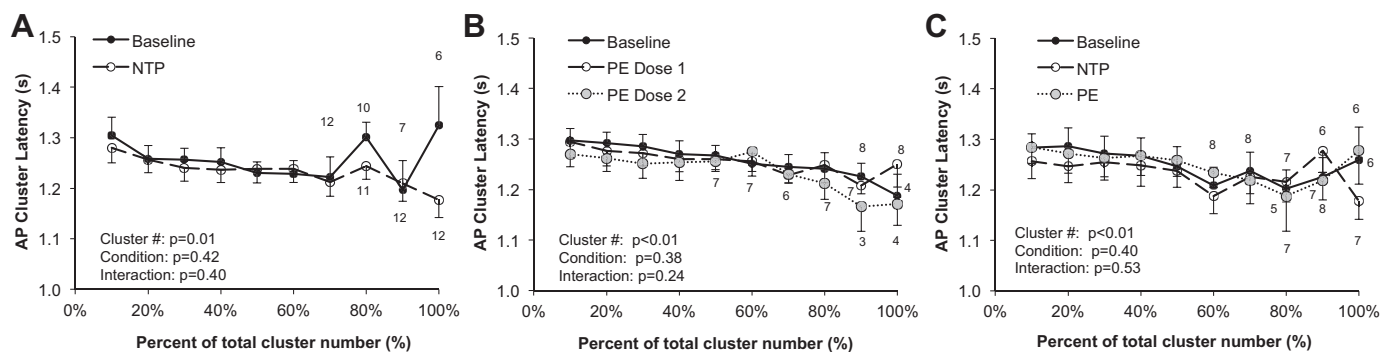


Fig. 7. Synaptic delays during systemic infusions. *A*: steady-state nitroprusside (NTE; $n = 13$). *B*: steady-state phenylephrine (PE; $n = 9$). *C*: modified Oxford ($n = 9$). Sample sizes for clusters in which not all subjects are included (clusters were not present) are indicated. Data are means \pm SE. Data were analyzed with a 2-way repeated-measures ANOVA to determine the main effect of relative cluster size (10–100% of total clusters) and condition (baseline, drug) and the interaction of cluster and condition.

observed an inverse relationship between AP cluster amplitude and conduction velocity, with larger clusters exhibiting a shorter latency (Fig. 7). Macefield and colleagues have also shown that the latency of a single axon is highly variable at rest (Macefield et al. 1994), and in situations of severe physiological stress altered synaptic delays and/or central processing times have been suggested (Badrov et al. 2015; Coote and Macleod 1974; Fagius et al. 1987; Salmanpour et al. 2011b; Wallin et al. 1994). Along these lines, Badrov and colleagues found that AP clusters were recruited more slowly (longer latency) during severe orthostasis (Badrov et al. 2015). In the present investigation, synaptic delays were unaffected by pharmacological baroreflex loading/unloading. We hypothesize that clear shifts in cluster latency profiles may be primarily the result of changes in central processing and thus likely occur only in situations where an individual level of perceived stress and/or tolerance is present (Badrov et al. 2015, 2016a, 2016b, 2017); however, future work in this area is needed.

Experimental considerations. Fagius and colleagues (Fagius et al. 1985) showed by blocking baroreceptor afferent activity that the normal cardiac rhythmicity of MSNA was disrupted and impulses instead occurred in a slow, irregular rhythm. Thus it may be expected that nitroprusside infusion, via arterial baroreflex unloading, would reduce the pulse-synchronous nature of MSNA and augment observed latencies. We did not observe augmented latencies in the present study; however, APs that are not pulse synchronous or not comprised within a burst are not included in the present analysis. Future work will be necessary to examine changes in activity of APs that are not pulse synchronous or incorporated into a multiunit MSNA burst.

The present approach classifies the contribution of multiple vasomotor neurons to multiunit integrated MSNA bursts morphologically based on peak-to-peak amplitude (Salmanpour et al. 2010). This approach compliments the single-unit approach (Macefield et al. 1994; Macefield and Wallin 1999), although it cannot determine whether APs with the same size/morphology are from the same or different neurons (Salmanpour et al. 2010). Furthermore, detection of APs by this method requires adequate signal-to-noise ratio. The average signal-to-noise ratio for the data was between 4.25 ± 0.21 (phenylephrine) and 4.51 ± 0.22 (nitroprusside). Based on previous work (Salmanpour et al. 2010), this level of signal-to-noise ratio would produce a correct detection rate of ~90% and a false positive detection rate of <3%. Finally, these studies were all conducted in young, healthy male participants with relatively normal baroreflex function. Whether these findings translate to women and/or individuals with impaired baroreflex function has yet to be assessed.

Conclusions. In the present investigation we used a pharmacological approach (the “gold standard”) to elicit targeted increases/decreases in arterial blood pressure. Our data point to an important role for baroreflex mechanisms in modifying AP rate coding patterns. In addition, we show that the sympathetic nervous system has the ability to recruit latent subpopulations of large APs during steady-state unloading of the baroreceptors in healthy male participants and derecruit during baroreflex loading, with some interindividual variation. Conversely, recruitment was not apparent during large, but transient, fluctuations in arterial pressure. Further work will be needed to see

whether these results translate to conditions with known baroreflex impairments.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

J.K.L., T.B.C., W.T.N., M.J.J., and J.K.S. conceived and designed research; J.K.L., W.W.H., S.E.B., T.B.C., W.T.N., M.J.J., and J.K.S. performed experiments; J.K.L. and E.P.O. analyzed data; J.K.L., E.P.O., W.W.H., S.E.B., M.J.J., and J.K.S. interpreted results of experiments; J.K.L. and E.P.O. prepared figures; J.K.L. drafted manuscript; J.K.L., W.W.H., S.E.B., M.J.J., and J.K.S. edited and revised manuscript; J.K.L., E.P.O., W.W.H., S.E.B., T.B.C., W.T.N., M.J.J., and J.K.S. approved final version of manuscript.

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