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REVIEW | *50 Years of Microneurography: Insights into Neural Mechanisms in Humans*

Fifty years of microneurography: learning the language of the peripheral sympathetic nervous system in humans

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Shoemaker JK, Klassen SA, Badrov MB, Fadel PJ. Fifty years of microneurography: learning the language of the peripheral sympathetic nervous system in humans. *J Neurophysiol* 119: 1731–1744, 2018. First published February 7, 2018; doi:10.1152/jn.00841.2017.—As a primary component of homeostasis, the sympathetic nervous system enables rapid adjustments to stress through its ability to communicate messages among organs and cause targeted and graded end organ responses. Key in this communication model is the pattern of neural signals emanating from the central to peripheral components of the sympathetic nervous system. But what is the communication strategy employed in peripheral sympathetic nerve activity (SNA)? Can we develop and interpret the system of coding in SNA that improves our understanding of the neural control of the circulation? In 1968, Hagbarth and Vallbo (Hagbarth KE, Vallbo AB. *Acta Physiol Scand* 74: 96–108, 1968) reported the first use of microneurographic methods to record sympathetic discharges in peripheral nerves of conscious humans, allowing quantification of SNA at rest and sympathetic responsiveness to physiological stressors in health and disease. This technique also has enabled a growing investigation into the coding patterns within, and cardiovascular outcomes associated with, postganglionic SNA. This review outlines how results obtained by microneurographic means have improved our understanding of SNA outflow patterns at the action potential level, focusing on SNA directed toward skeletal muscle in conscious humans.

microneurography; muscle sympathetic nerve activity; neural control of the circulatoin; recruitment strategies; reflex cardiovascular control

OVERVIEW

Direct neurophysiological measures form an important component of understanding sympathetic nerve activity (SNA) and how it relates to stimuli that induce neural changes, as well as to end organ function. Neurophysiological recordings of mammalian postganglionic sympathetic nerves were reported in anesthetized rabbits and cats early in the 20th century (Adrian et al. 1932; Bronk et al. 1936). These recordings highlighted the fundamental properties of postganglionic sympathetic nerve recordings that include a neurogram with poor signal-to-noise and a characteristic bursting pattern of synchronized neuronal activity that normally is entrained at various frequencies to the cardiac cycle, to respiratory oscillations, and to blood pressure oscillations. However, translation of these details to humans was limited by the anesthesia and invasive nature of these preparations performed in lower animals. Thus, development of the microneurographic method for postganglionic SNA recordings (Hagbarth and Vallbo 1968) represented a major leap forward in autonomic neuroscience because it provided, for the first time, access to the actual neural signal in conscious humans. Similar to the first recordings of postganglionic SNA in anesthetized lower animals, a notable feature of the first observations in humans was the low signal-to-noise neurogram with bursts of synchronous neural activity (but with lower frequency than lower animals) that were entrained to various oscillating patterns associated with cardiac, respiratory and blood pressure cycles. The nature of this burstiness and its conservation across species has been reviewed in detail (McAllen and Malpas 1997). Importantly, the SNA bursts are comprised of individual action potentials (APs), a feature that

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offers potential to study coding (communicating) information. Thus, in the past 50 years, the scientific knowledge in SNA enabled by microneurography revolves around its ability to record the raw SNA activity of humans under various states from which the patterns of APs can be studied at multiple levels, such as compound APs in the integrated neurogram, to single APs.

This review highlights the impact of microneurography for understanding SNA from the perspective of neurological coding or communication strategies that can be derived from the neurogram provided by this technique. Readers interested in the methodological details and discussions of the technique, its history, reproducibility, standards of practice, utility across many physiological and clinical states, and safety, are directed to these reviews and reports (Hagbarth and Vallbo 1968; Hart et al. 2017; Kimmerly et al. 2004; Mano 1997; Mano et al. 2006; Mitchell 1990; Vallbo et al. 2004; Wallin 2004; White et al. 2015). In this document, methodological details are provided only when they are relevant to the technical issues used to study individual APs. It is understood that SNA represents efferent C-fiber activity, accessible in peripheral nerves, that involves two distinct populations: one that targets skeletal muscle vasculature, referred to as muscle sympathetic nerve activity (MSNA), and a second that targets skin hair piloerector cells, sweat glands, and vascular targets, collectively referred to as skin sympathetic nerve activity. This paper focuses on the outcomes gleaned from measurements of MSNA, the signal that forms the common neural component of reflex cardiovascular regulation and blood pressure control (Delius et al. 1972a). Also, this review focuses on human neural recordings. However, data from studies performed in lower animals will be integrated as appropriate to develop the broader understanding of how such human recordings represent fundamental homeostatic neural adjustments.

Within these parameters, this review addresses the following topics: *1*) A brief historical perspective, *2*) The anatomical basis of postganglionic sympathetic axons, *3*) The validity and reliability of postganglionic SNA to reflect preganglionic activity, *4*) Sympathetic discharge patterns and recruitment in muscle sympathetic nerve activity, *5*) Microneurographic data to interpret neurovascular coupling, *6*) Clinical implications, and *7*) Neuromodulation of SNA.

A BRIEF HISTORICAL PERSPECTIVE

Borrowing from the Aristotelian philosophical idea of "sympathy" (suffering together), Galen (130–216 AD) speculated that the ganglionated nerves, which he was exposing in his dissections, enabled "functional unity, or physiological sympathy," among the internal organs [as reviewed in Finger (1994)]. Experiments performed by Claude Bernard (1813–1878 AD) and Brown-Seguard (1817–1894 AD) on the impact of severed nerves on blood flow provided the first evidence of active neurogenic control over cardiovascular function [as cited in Cooper (2008) and Bing et al. (1982)]. Early in the 20th century, Walter B. Cannon (1871–1945) stressed the fundamental role of the autonomic nervous system in his overarching hypothesis of "homeostasis" (Cannon 1932), arguing that the ability to sustain physiological values within a narrow range of life-supporting values required important concepts such as variability, reactivity, and control. Such homeostatic control requires a message, methods to receive and respond to that message, as well as feedback regarding the success of that response. Thus appropriate homeostatic control incorporated principles of feedback control (Wiener 1948) where physiological sensors produce information that enables the efferent autonomic response to communicate and enact homeostatic adjustments to the varied number and magnitudes of stressors that are integrated on short- and long-term scales. But what exactly is meant by "communication" or "message" within the sympathetic nervous system? The sections below attempt to address this question by highlighting the data made available by microneurographic methods.

THE ANATOMICAL BASIS OF POSTGANGLIONIC SYMPATHETIC AXONS

The fundamental sympathetic communication pathways, illustrated in Fig. 1 (adapted from Jänig and Häbler 2003), include the supramedullary neural structures that determine the stress, the intermediary neural pathways and modulatory interconnecting pathways (e.g., Llewellyn-Smith 2009), the neural-end organ interface, and the end organ response. Information regarding the type and magnitude of stress is relayed to the brainstem nuclei from peripheral sensors that provide information regarding blood pressure (baroreceptors), central blood volume (cardiopulmonary baroreceptors), blood gas levels (chemoreceptors), lung stretch receptors, muscle metaboreceptors, other visceral sources, as well as supramedullary sites. These reflex and higher cortical inputs are reviewed extensively elsewhere (Beissner et al. 2013; Cechetto and Shoemaker 2009; Charkoudian and Wallin 2014; Critchley et al. 2000; Eckberg 2003; Fadel 2013, 2015; Fadel et al. 2001; Gianaros and Sheu 2009; Halliwill et al. 2003; Thayer et al. 2012; Williamson et al. 2006).

The final neural segments involved in carrying the intended central message to the effector organ are the postganglionic neurons and these provide the site of access for microneurographic recordings in humans. Successful recordings of these postganglionic APs can be difficult, in part due to anatomical features. As illustrated in Fig. 2, immunohistochemical studies in cadaveric peroneal/fibular nerves indicate that tyrosine hydroxylase-containing axons are organized as bundles of 1 to ~45 axons (median size of ~5 axons) within most (but not all) fascicles (Tompkins et al. 2013). The terminology is notable

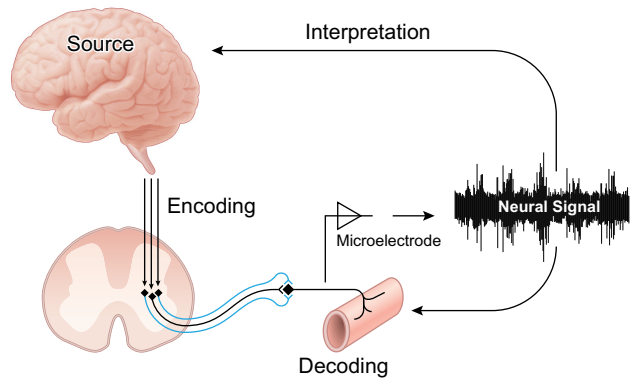

Fig. 1. Major anatomical segments contributing to discharge patterns in the postganglionic sympathetic neural signal and their interpretation. Adapted by permission from Jänig and Häbler (2003).

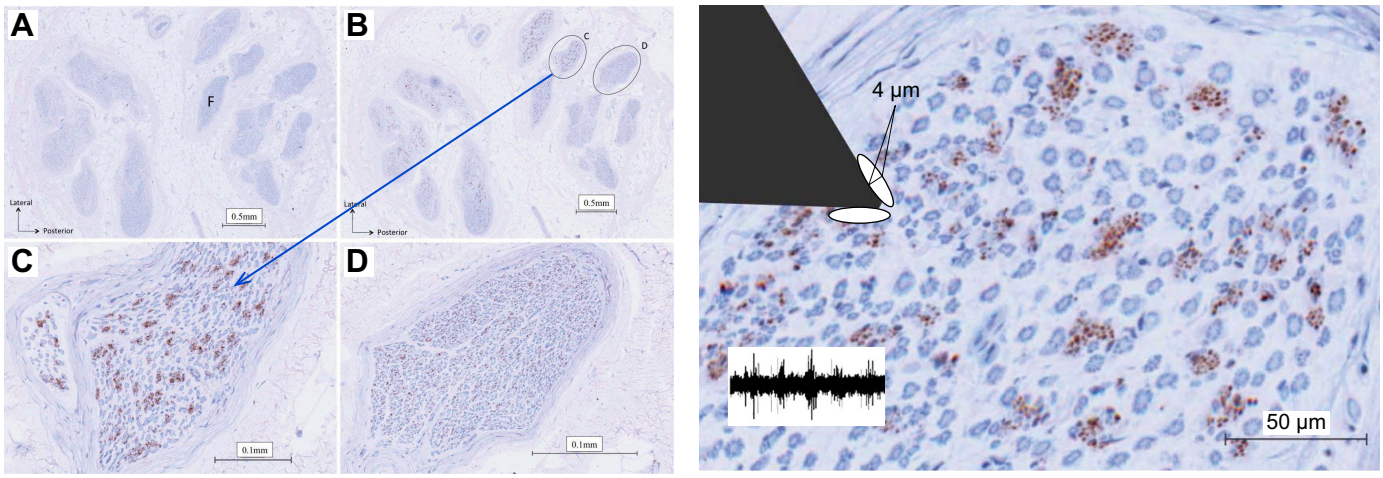

Fig. 2. Cross section of right human common peroneal nerve. *A*: nerve section used as a negative control. *B*: positively stained nerve section. Sympathetic axons are represented by brown regions within the nerve fascicles. Myelinated fibers are represented by the blue ovals in the fascicle. *C* and *D*: enlargements from *B* of a positively stained nerve fascicles demonstrating the differences in arrangement between fascicles. *Right*: enlargement of segment from *C* with the tip of a microelectrode overlaid identifying the small (calculated) region of action potential detection at the electrode's tip. From Tompkins et al. (2013).

here because the tyrosine-expressing fibers do not form their own fascicle. Rather, the bundles coexist with other myelinated axons. Morphologically, the sympathetic axons range from 0.05 to 0.2 μm. These measures concur with those observed in the renal nerve (Sato et al. 2006) and cervical sympathetic trunk (Aguayo et al. 1973) of mature rats. The variations in axonal diameter suggest the possibility of variations in AP conduction velocity, a feature that will be described in detail below (SYMPAPTHETIC DISCHARGE PATTERNS AND RECRUITMENT IN MUSCLE SYMPATHETIC NERVE ACTIVITY). Of potential interest, the microelectrode shaft often is 200 μm in diameter, that has been beveled to a 3- to 5-μm tip. Thus, positioning the relatively large tip into a bundle of muscle SNA axons (i.e., of sufficient number of axons to assess synchronized activity that forms a "burst") highlights the need for technical skill and patience in the practice of microneurography.

THE VALIDITY AND RELIABILITY OF POSTGANGLIONIC SNA TO REFLECT PREGANGLIONIC ACTIVITY

The reliability of the postganglionic MSNA signal in terms of its faithful reproduction of the central message forms an integral premise of microneurography. A key to the transfer of information from the central nervous system to peripheral SNA resides within the paravertebral ganglia. The ganglia are proposed to exhibit the ability to amplify the preganglionic signal by distributing axons that produce suprathreshold (i.e., "strong") excitatory postsynaptic potentials from one preganglionic to multiple (e.g., 2–15) postganglionic neurons (Bahr et al. 1986; Jänig and Häbler 2000; McLachlan 2003; Purves et al. 1986; Wang et al. 1995). Furthermore, computational and advanced dynamic clamping methods (Horn and Kullmann 2007; Rimmer and Horn 2010; Springer et al. 2015) suggest the ability to enhance the firing probabilities of any postganglionic neuron through convergent synapses from multiple preganglionic neurons that individually produce subthreshold excitatory postsynaptic potentials but, depending on their timing, could summate to cause a postganglionic discharge. How these concepts apply to different ganglia or postganglionic axons of varying size and/or varying targets, or in human ganglia, remain to be studied. These functional and anatomical considerations raise the question about the faithfulness of transmission from pre- to postganglionic axons from which microneurographic recordings are made. However, several lines of evidence support the concept that postganglionic nerve activity represents preganglionic content.

First, despite the above limitations and options for complex recruitment patterns, Birks and colleagues (Birks 1978; Birks and Isacoff 1988; Birks et al. 1981) reported a predictable change in amplitude of the postganglionic compound AP following graded stimulation of the preganglionic neurons. Furthermore, delicate studies by John Horn's group in the bullfrog provided evidence that the aorta vasoconstrictor response scaled to the preganglionic sympathetic stimulation, and this vasoconstriction was minimized by both inhibition of postganglionic neurotransmitter release (guanethidine) and reduction in ganglionic transmission through postjunctional antagonism with tubocurarine (Thorne and Horn 1997). Therefore, postganglionic recordings of MSNA appear to reflect the fundamental message emanating from the central nervous system (i.e., the preganglionic signal) on a relative, but perhaps not absolute, scale.

Second, strong correlations exist between graded stimuli that evoke a systemic reflex sympathetic response, and the postganglionic output. For example, the linear relationship between baroreflex unloading and MSNA burst frequency (Burke et al. 1977; Kimmerly and Shoemaker 2002; Mano 1998; Ogoh et al. 2003) support the general idea that postganglionic recordings do reflect patterns of change in central autonomic stimulation.

Additional evidence relating MSNA patterns to central sources comes from studies of the mechanisms determining cardiac-synchronous pulses of MSNA. Specifically, data from cats and humans indicate that the cardiac-related rhythm in SNA reflects baroreceptor-induced entrainment of a nonlinear oscillator by pulse-synchronous baroreceptor nerve activity. For example, after arterial baroreceptor denervation in anesthetized cats, oscillations in SNA persist with a frequency near that of the heartbeat (Gebber 1980; Gebber and Barman 1980). Likewise, in both cats and humans, power in the cardiac-related band of SNA remained after partialization of the SNA autospectrum with arterial pulse or heart rate (Barman et al.

2003). These findings support the concept that the cardiac-related rhythm in MSNA is due to baroreceptor-induced forcing of a central sympathetic oscillator whose frequency is close to the heart rate. Likewise, Cogliati et al. (2000) demonstrated similar low- and high-frequency rhythms in skin SNA and MSNA, suggesting the presence of a common central mechanism that regulated efferent SNA. As well, K-complexes measured in the electroencephalogram during sleep, reflecting arousal states, associate with larger integrated bursts in the absence of baroreflex stimuli (Hornyak et al. 1991; Tank et al. 2003). Finally, activation patterns of regions in the forebrain and midbrain correlate with MSNA burst activity, measured concurrently in real time (Henderson et al. 2012). Thus, postganglionic SNA reflects patterns of neural activity in supramedullary sites.

The above observations support the idea that preganglionic mechanisms contribute directly to, and are faithfully transmitted in the postganglionic efferent sympathetic signal. Thus, microneurographic recordings of postganglionic SNA are interpreted to reflect a direct representation of central sympathetic information. However, the histochemical data provided above (Fig. 2) illustrate an additional question regarding reliability of the MSNA signal in reflecting a generalized sympathetic response. Here, we see that the microneurography electrode likely interrogates only one bundle of sympathetic axons among many such bundles; therefore, it detects only a small sample of the entire postganglionic sympathetic outflow. To what extent does the activity in one bundle reflect the nature in all bundles? This question can be addressed using concurrent microneurographic recordings in bilateral limbs. Specifically, bilateral microneurographic recordings illustrate the conservation of MSNA burst patterns in each leg (Diedrich et al. 2009), even though the intrafascicular C-fiber bundles being interrogated are of unknown location or size when measured at baseline (but they certainly are different bundles). These combined observations provide confidence that postganglionic recordings of MSNA reflect a consistent outflow regardless of fascicular bundle, or limb.

Nonetheless, one must be careful with the interpretation of microneurographic MSNA signals. For example, the selection of sympathetic axonal bundles from which microneurographic recordings are made are essentially "random" and different bundles contain varying numbers of axons from which to record. Therefore, absolute sizes of the integrated burst, variability in the number of detectable APs (see SYMPATHETIC DISCHARGE PATTERNS AND RECRUITMENT IN MUSCLE SYMPATHETIC NERVE ACTIVITY) within each burst, and concurrent interpretations of changes in these variables, must be constrained to within-test patterns. Also, well-documented differences exist between MSNA to skeletal muscle and SNA to the skin (Vissing et al. 1994). Furthermore, different subtypes of SNA reside in the rat renal nerve (DiBona et al. 1996) and rabbit ear (Riedel and Peter 1977) and not all are directed toward, or cause constriction of, vascular beds. Additionally, the MSNA signal measured in peripheral nerves and directed to vasculature of the muscle may not represent sympathetic drive to other organs. In humans, regional variations in sympathetic nerve activity exist, as measured by norepinephrine spillover in deep target organs, unavailable for microneurographic interrogation (Esler 2011). As well, direct nerve recordings (Anderson et al. 1987; Vallbo et al. 1979) and measurements of regional norepinephrine spillover (Esler et al. 1984a, 1984b) suggest that different sympathetic subdivisions may be activated to different degrees and in different combinations depending on the functional demand. This phenomenon appears to stretch to upper vs. lower limb differences. For example, mental stress induced increased MSNA in the leg but not in the arm (Anderson et al. 1987). Also, evidence for lateralization of burst size in the right and left legs, despite highly coupled burst expression, also suggest some role of central processes in mediating leg-to-leg variations (Diedrich et al. 2009). Nonetheless, forearm venous plasma concentration and total body spillover of norepinephrine correlate positively with the strength of MSNA measured in the legs (Wallin et al. 1981) as do interstitial and plasma norepinephrine concentrations during graded lower body suction (Khan et al. 2002). Moreover, concurrent studies with norepinephrine spillover and microneurography (Esler et al. 1988) indicate strong correlations between patterns of reflexive changes in sympathetic outflow in the leg with the heart (Kingwell et al. 1994; Lambert et al. 2011; Thompson et al. 1998) and kidney (Wallin et al. 1996). Therefore, regional variations exist in sympathetic patterns of reactivity and efferent neural control although the distribution to cardiovascular organs, and reflexive responses, appear to exhibit similar underlying patterns. Overall, the faithful transmission of sympathetic outflow through the ganglia, MSNA levels that scale with reflex sensory inputs, and the relationship between MSNA and norepinephrine spillover (at least in skeletal muscle and heart), suggest that microneurographic recordings of efferent sympathetic nerve activity provide a faithful representation of central and peripheral components of the sympathetic pathway that affects cardiovascular control.

SYMPATHETIC DISCHARGE PATTERNS AND RECRUITMENT IN MUSCLE SYMPATHETIC NERVE ACTIVITY

While MSNA scales with reflex stimuli that are integrated in the central nervous system, questions remain regarding the manner through which the central nervous system adjusts and communicates specific stress responses within the sympathetic nervous system. As a neural system, MSNA may contain structured patterns that link the type and magnitude of stress to the desired response from the target organ. Certainly, sensory neural systems (Arnal et al. 2015; Joos et al. 2014; Plack et al. 2014) and neuromuscular control (Henneman et al. 1965) express coded information. The ability to record directly from SNA represents a key impact of microneurographic techniques as they have led to a growing understanding that complex layers of efferent activity exist. The following list includes information from various sources that points to the existence of ordered recruitment strategies within SNA:

1) The specific and goal-oriented sympathetic responses to stress that normally achieve their goal (Janig 2006) infers an accurate and highly integrated communication strategy.
2) Somata of sympathetic neurons projecting to cutaneous vs. striated muscle vessels in the rabbit ear can be distinguished by their location, size, and/or immunohistochemical profile of neurotransmitter content (Morris et al. 1999), suggesting that neurovascular regulation involves coordination between neuronal pathways contain-

ing neurochemically and morphologically distinct populations of sympathetic neurons.

3) The presence of varying sizes of postganglionic neurons where larger neurons innervate greater target organ fields (Gibbins et al. 1998).

4) The presence of varying burst size in postganglionic compound actions potentials (rodent and human), reflecting variations in the number of APs synchronized within that cardiac cycle (Ninomiya et al. 1993).

5) Sympathetic cotransmitters at the neurovascular junction (adenosine triphosphate, norepinephrine, and neuropeptide Y, for example) whose release patterns appear to be dependent upon the frequency pattern of the discharging axons (Burnstock 1985; Pernow et al. 1989; Wahlestedt et al. 1990; Wier et al. 2009; Zukowska-Grojec et al. 1998b).

6) Observations that larger bursts exhibit shorter conduction transit times to the recording electrode with the major difference observed in the leading edge of the integrated neurogram (Wallin et al. 1994). From this pattern, Wallin and colleagues postulated the existence of two primary recruitment strategies: *1*) Variations in synaptic delays that could lead to temporal coding of active neurons, and *2*) Recruitment of a latent subpopulation of fast-conducting axons in some bursts, an example of population coding. Additionally, a rate-coding mechanism may occur with greater firing probability of single neurons. Differentiation between these potential mechanisms requires an understanding of the patterns among all APs in the multiunit recording, an emerging area of investigation.

7) The strong correlation between spontaneous variations in SNA burst size and the vasoconstrictor response in humans (Fairfax et al. 2013b) (see MICRONEUROGRAPHIC DATA TO INTERPRET NEUROVASCULAR COUPLING).

Exploring AP patterns in MSNA. Whether studying signal "intensity" in time-domain analyses focusing on burst frequency and size, or rhythmic oscillations under steady-state conditions using spectral analysis models (see Montano et al. 2009), the fundamental aspect of the MSNA signal resides in the underlying patterns of synchronized AP number and their respective size. Therefore, a fundamental question for exploration of SNA recruitment strategies becomes "what AP patterns exist within the sympathetic neurogram"? Using microneurographic data, the problem of AP behavior within the raw MSNA signal has been addressed from two perspectives. The first was introduced by Macefield and Wallin (Macefield et al. 1994), who studied single axons over time using a modification of the microneurography technique: they used a higher impedance active electrode (e.g., 10 vs. 3 MΩ). The electrode's impedance affects its "field" of AP detection and the signal-to-noise character. Thus, a higher impedance electrode was used to isolate one (or a few) dominant APs closest to the exposed electrode tip. Since every axon produces a characteristic AP morphology, template-based matching of the APs supports the viewpoint that, if only one dominant AP is highlighted then its behavior can be studied over time, as confirmed by its morphological similarity to other APs. This technique has been reviewed recently (Macefield and Wallin 2018). The possibility of multiple axons with the same proximity to the electrode tip producing the same AP size remains untested, as does the possibility of AP summation from two concurrently firing but smaller APs, although these possibilities are expected to express very small probability. This single unit technique enabled the study of AP probabilities under many conditions (Elam and Macefield 2001; Lambert et al. 2011, 2013; Macefield 2012; Macefield and Elam 2003; Schlaich et al. 2004). Some uses of this approach have also studied the behavior of two to three dominant axons simultaneously (Millar et al. 2013). For the purposes of this review, the main observation was that, in healthy individuals, a single sympathetic axon has a low firing probability (usually just once per burst) and expresses varying within-burst latencies (Macefield and Elam 2003). Importantly, increased probability of a single unit firing within a given burst could not account for the increase in integrated burst size during an apnea. Therefore, this group speculated that latent axons existed that become recruited at times, contributing to the integration of a larger burst (Macefield and Wallin 1999).

Addressing the possibility of neuronal recruitment in SNA required a new approach that could interrogate multiunit AP patterns and detect recruited axons that are not firing under baseline conditions (or fire with very low probability). Employing developments in signal processing, two fundamental approaches are reported that address this problem. These approaches include wavelet denoising (Brychta et al. 2006; Diedrich et al. 2003; Salmanpour et al. 2010; Steinback et al. 2010) and a mixed separation template matching model (Tan et al. 2009). Arguably, the mixed-separation approach may produce greater reliability in discovering small APs within the background noise. However, to date, the only data available regarding MSNA coding strategies from multiunit recordings have used the wavelet model, and these studies form the bulk of our discussion on sympathetic AP recruitment. Figure 3 illustrates the analytical processes used to isolate integrated bursts of MSNA (left series of data) as well as to extract individual AP data from the raw MSNA neurogram using the wavelet denoising approach (right series of data).

Using the "Symlet 7" wavelet provided by MATLAB, Diedrich and colleagues (Diedrich et al. 2003) pioneered the approach of multiunit AP detection. They reported the existence of four distinct AP shapes of varying peak-to-peak amplitude in MSNA. However, Symlet 7 wavelet shares some spectral characteristics that mimic the background noise in MSNA recordings (Zhang et al. 2007). To overcome this challenge, and reduce the problem of false positives, Salmanpour et al. (2008a, 2008b, 2010) developed a new wavelet modeled from human APs to increase detection resolution. Using a modified continuous transform, the exact location of each AP was identified and extracted. Additional modifications included clustering of similarly shaped APs (based on a 32-point K-means method) (Fig. 3, *right*), and quantifying the timing of the AP within the burst relative to the R-wave preceding the systolic pulse that terminated the burst through the baroreflex mechanism. Thus, the occurrence, recruitment, and timing of APs as a function of their shape could be quantified. Of note, this method uses a lower impedance electrode than those interested in recording single units, with a larger recording field. Therefore, this AP detection is from a multiunit recording and a cluster of APs of similar shape does not infer that these are the same unit. However, assuming only one or two occurrences of any AP in a burst (Macefield et al.

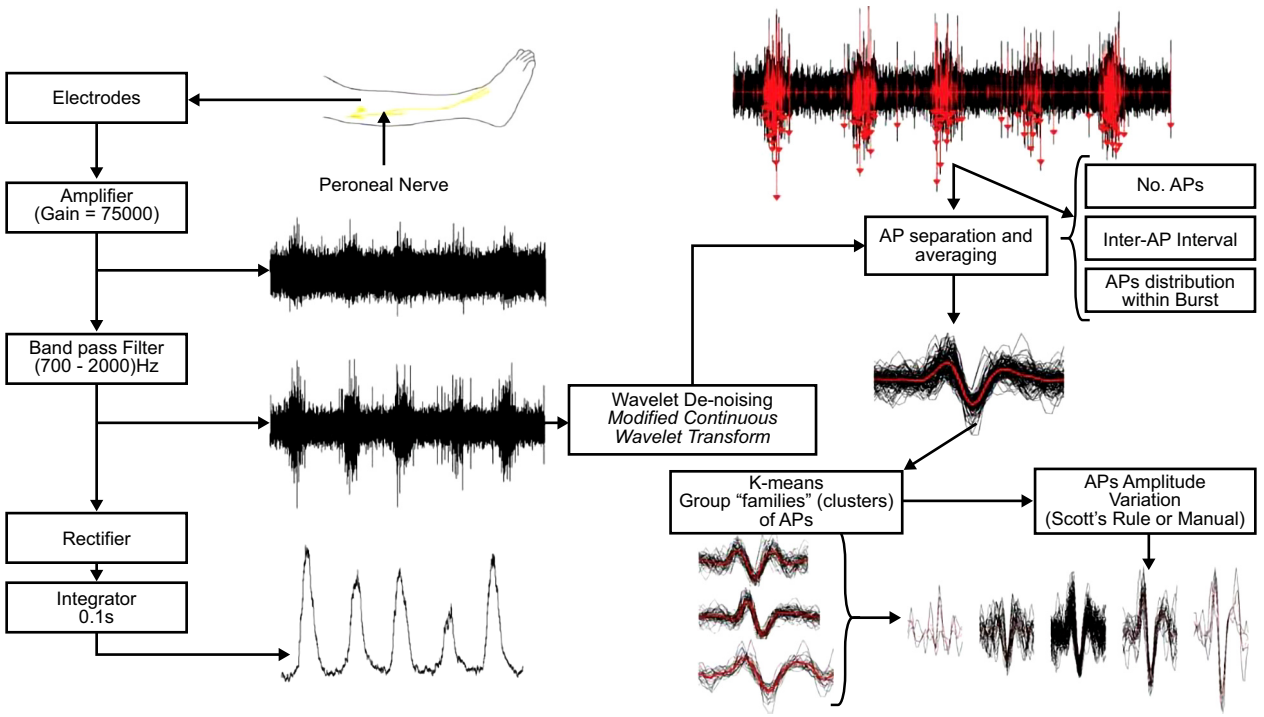

Fig. 3. Processing stages for common detection of sympathetic nerve activity to achieve the integrated neurogram (*left*) highlight bursts of neural activity, and the process of locating, extracting, and binning postganglionic sympathetic action potentials (APs) (*right*). The primary processing of the integrated neurogram involves band-pass filtering (~700–2,000 Hz), full-wave rectification, and integration with a 0.1-s time constant (some use root-mean square processing (Delius et al. 1972a; Hagbarth and Vallbo 1968; Vallbo et al. 1979; Vallbo et al. 2004). This processing sequence exposes the variations in interburst period and burst size (*left*). A modified continuous wavelet transform is an example of an approach to locate the exact position of each AP for extraction, clustering, and binning. See Salmanpour et al. (2010) for details.

1994) any additional occurrences of APs of similar shape and size can be interpreted within the context of recruitment vs. rate coding patterns. Also, as this approach does not depend upon the proximity of the electrode position to a specific axon (but just electrode stability), the arrival and/or disappearance of any new AP clusters from burst to burst or over time can be quantified and then interpreted within the context of recruitment patterns. Stability of the electrode within its bundle of axons represents the underlying assumption of this approach, normally inferred subjectively based on the expertise of the microneurographer, and on the stability of the neurogram's signal-to-noise aspect throughout a segment of data. Indeed, all recordings should be monitored continuously during data acquisition as well as scanned in their entirety afterward for noticeable baseline shifts, subsequent changes in the height of the MSNA bursts, and signal-to-noise variations of APs and their surrounding noise. Notably, the technique requires training to gain the required knowledge on what constitutes an acceptable MSNA signal. It is highly recommended that one trains in a laboratory that is led by an experienced microneurographer and performs the technique regularly. For continued advancement of our understanding of sympathetic regulation in humans, high-quality MSNA signals are obligatory and a trained microneurographer recognizes that a quality signal cannot be obtained in some individuals.

With the continuous wavelet transform approach, AP patterns have been studied under many circumstances. Figure 4 provides an example of AP patterns observed under baseline conditions followed by a prolonged apnea performed at total lung capacity (Steinback et al. 2010). Similar patterns were observed during sustained isometric handgrip to fatigue (Badrov et al. 2016b) and severe lower body negative pressure (LBNP) (Badrov et al. 2015; Salmanpour et al. 2011a). One consistent pattern that emerged from these studies of MSNA was an array of AP probabilities that varied with AP size. Specifically, even under baseline conditions, small APs were expressed with high within-burst probability, and larger APs expressed a lower within-burst probability. However, during a reflex state, the low probability APs observed at baseline increased their frequency. Concurrently, new clusters of larger APs emerged, representing a recruitment response. A second pattern observed was that the recruitment of new large APs progressed with the severity of the stress, be it apneas (Badrov et al. 2017), isometric exercise (Badrov et al. 2016b), or baroreceptor unloading (Badrov et al. 2015; Salmanpour et al. 2011a). Importantly, the appearance of larger APs observed during fatiguing handgrip persisted during a period of postexercise forearm ischemia highlighting the important role of peripheral muscle metaboreceptors in the recruitment of the latent AP pool. We interpret the finding of recruitable larger APs during various reflexes as evidence in support of Wallin's (Wallin et al. 1994) original conjecture of a latent population of larger, higher threshold, and fast-conducting APs. The data also suggest that a range of such subpopulations exist, with some reserved for very high stress.

In contrast to the prolonged apnea and fatiguing handgrip models, baroreflex-mediated recruitment appears to operate on a scale of reduced sensitivity. During graded LBNP, the low-threshold, high-probability APs present under baseline conditions increase in probability. Recruitment of latent neuronal

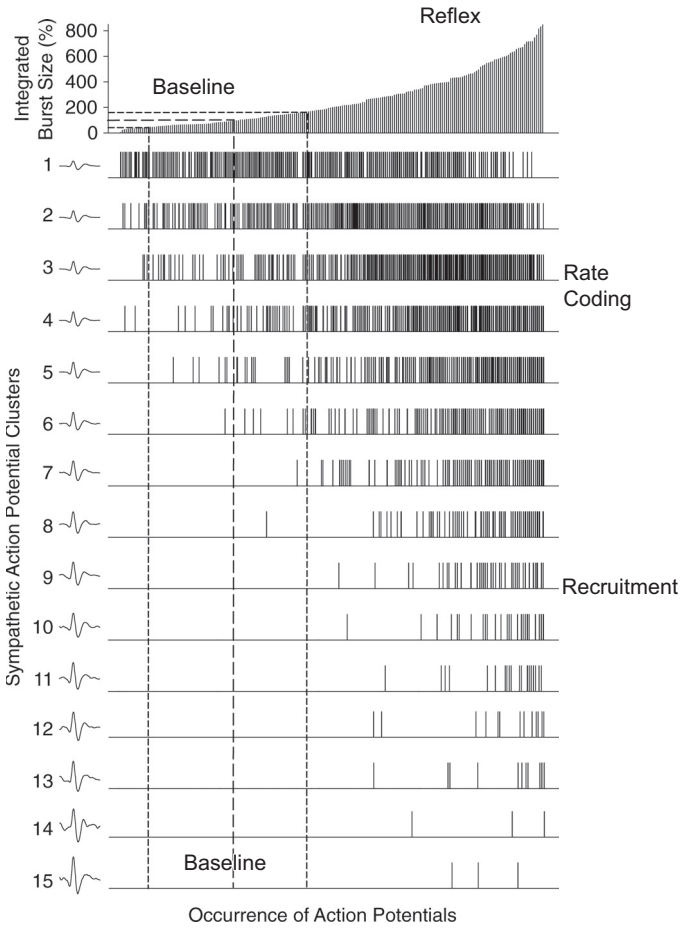

Fig. 4. Representative data from a single individual illustrating the pattern of action potential (AP) occurrence within each burst (organized from smallest to largest integrated burst size in the top panel) as a function of their cluster (illustrated along the left). Cluster refers to all APs of similar morphology (see Fig. 3 legend). Data were collected in a healthy individual on going from baseline to the break point of a maximal lung volume apnea (performed by elite free diver). The range of integrated sympathetic bursts are ordered by burst size as a percentage of baseline. Dashed lines represent the means and standard deviations of integrated burst sizes at baseline. Each vertical line (i.e., spike) represents the occurrence of postganglionic sympathetic APs as a function of integrated burst in which they occurred. Clusters of larger APs are predominately recruited at higher levels of sympathetic activation. The APs present at baseline (clusters 1–8) generally express an increase in firing probability (per burst) as the reflex state endures toward the apnea break point, an example of rate coding. However, the emergence of new APs (clusters 9–15) during the reflex state illustrate recruitment of larger APs that were not firing at baseline. From Steinback et al. (2010).

subpopulations during LBNP appears to require severe levels of suction between −60 to −80 mmHg, with some interindividual variation (Badrov et al. 2015; Salmanpour et al. 2011a). Whether this recruitment level reflects an individual's orthostatic tolerance has not been studied. An additional unstudied observation is the apparent reduction in probability of small APs during LBNP. This observation may reflect the activity of special subpopulations that exhibit paradoxical reductions in firing probability under conditions of mild decreases in cardiac filling pressure as well as increases in firing of some single axons under conditions of both mild lower body negative and positive pressure (Millar et al. 2013, 2015).

AP latency shifts. The mere emergence of larger APs during reflexive states, outlined above, could simply reflect a shift in electrode position relative to axons within interrogated bundle. However, on the basis that larger axons should conduct the AP at a faster rate, the latency of each AP from its corresponding cardiac cycle may provide additional insight into the concept of AP recruitment. Action potential latency reflects the time it takes for the neural signal generated in the brain stem to arrive at the postganglionic recording site, a period that includes axonal conduction speeds and synaptic delays along the series of segments outlined in Fig. 1. This delay typically is quantified by calculating the time between the R-wave of the electrocardiogram associated with the burst and its detection at the peripheral recording site. The same approach can be used for each AP within any given burst. Therefore, displaying AP latency as a function of AP cluster size indicates the shorter latency of larger AP clusters (Steinback et al. 2010). Of note, repeated observations that the larger APs also express faster conduction velocity (Badrov et al. 2015, 2017; Steinback et al. 2010) discount the potential problem that they simply represent axons that lie closer to the recording electrode.

The AP latency appears to be a modifiable feature of SNA outflow. For example, average AP latency was increased both at baseline and during an apnea following 60 days of head-down bedrest (Klassen et al. 2017). Also, an unexpected observation of the early studies on AP recruitment during acute stressors was a shift in the latency of all APs when the task involved volitional effort to sustain the reflex stimulus (Fig. 5) (Badrov et al. 2015). It may be considered that the cause of the AP shift in this case is chemoreflex stress. However, the potential effect of perceptual stress associated with volitional effort must also be considered, an option related to the concept of central command (Williamson et al. 2006). For example, the actual chemoreflex stress induced by a 20- to 30-s end-expiratory apnea is not severe, but the perceptual effort involved in sustaining the maneuver grows with time. Also, the same shift toward reduced AP latencies can be observed during short apneas where the chemoreflex stress is unremarkable (Badrov

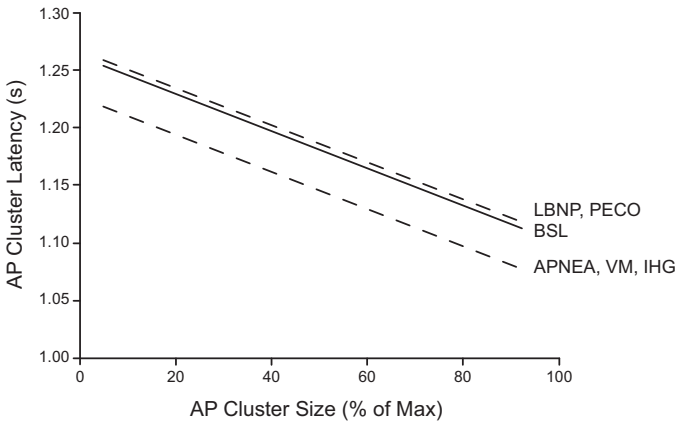

Fig. 5. A schematic summary of data from various studies outlining the relationship between action potential (AP) size and its conduction latency, and how this fundamental pattern shifts to faster conduction during volitional apneas, Valsalva maneuvers (VM), and fatiguing isometric handgrip (IHG) but not necessarily during lower body negative pressure (LBNP) or a period of post exercise circulatory occlusion (PECO) following fatiguing IHG. Cluster latency is determined as the time from the R-wave of the electrocardiogram for the heart beat immediately following the generation of the burst. The latency for all APs in each cluster within each burst can be quantified and averaged to determine the mean cluster latency. Data from Badrov et al. (2015, 2016b); Salmanpour et al. (2011a, 2011b).

et al. 2017), a 15- to 20-s Valsalva maneuver (Salmanpour et al. 2011b), and with sustained fatiguing isometric handgrip (Badrov et al. 2016b) where there is no apnea. In contrast, baroreflex unloading with LBNP in the absence of volitional straining effort pressure caused either little change, or a lengthening of the AP latency profile (in severe LBNP) (Badrov et al. 2015; Salmanpour et al. 2011a). Furthermore, reductions in latency occur in Valsalva and apnea maneuvers despite diverging heart rate responses, and latency shifts occur in a Valsalva maneuver but not during LBNP despite both eliciting a baroreflex-mediated response and change in heart rate. These data suggest the hypothesis that perceptual effort affects synaptic latencies associated with efferent MSNA.

The factors determining the timing of each AP cluster in our studies, and even the timing of the AP from the same axon in Macefield's reports (Macefield et al. 1994), remain a puzzle. On one hand, variations in neuronal conduction could simply be noise (Stein et al. 2005) reflecting true variations in conduction velocity and/or interspike intervals. On the other, the reproducible decline in conduction latency with some reflex-specific patterns suggest that this feature also expresses a reflection of central intentions, where variations in preganglionic firing latencies are observed (McAllen and Trevaks 2003). While measuring integrated burst latency changes as a function of burst size, Wallin and colleagues (Wallin et al. 1994) wondered about the possibility of modifiable synaptic latencies but no mechanisms have been explored. One possible determinant of these observations may include variations in the number and impact of converging primary and secondary synapses with postganglionic neurons (Bratton et al. 2010; Horn and Kullmann 2007; Schobesberger et al. 2000) that could affect the timing of postganglionic AP generation. Finally, the relevance of such latency changes remains unknown either from their central generation or in terms of potential variations in end organ control.

MICRONEUROGRAPHIC DATA TO INTERPRET NEUROVASCULAR COUPLING

Quantifying AP patterns in MSNA suggests the presence of a deterministic message from the central nervous system to the muscle's vasculature. However, the impact of MSNA discharge patterns on burst-by-burst vascular control remains a key question. On the basis of phase-shifted oscillations in blood pressure, and vascular resistance, the MSNA neurogram has been interpreted to be vasoconstrictor in nature (Delius et al. 1972b). Thus, establishing the relationship between MSNA and cardiovascular oscillations seems like a logical starting point in decoding the MSNA message, providing deeper insight into central messages. Transfer function approaches applied to steady-state conditions have outlined distinct associations between MSNA rhythms and corresponding blood pressure oscillations that vary in an expected manner with different populations (Briant et al. 2016; Montano et al. 2009). These models provide information regarding the timing between MSNA outflow and the integrated cardiovascular response under steady-state conditions. However, the fundamental concept of associating MSNA to blood pressure is debated (Taylor et al. 1998). At issue is the problem that blood pressure oscillations provide poor specificity when attempting to isolate a role of MSNA patterns. For example, as illustrated within the context of the sympathetic contributions to Mayer waves, blood pressure is a complex signal and can be linked to contributions from heart rate, cardiac output, myogenic vascular reactivity, and MSNA, each expressing a different time course of action either directly on blood pressure and/or indirectly through changes in vascular resistance (Zamir et al. 2011): these variables are difficult to dissociate in the intact and conscious human. Furthermore, difficulties arise in determining whether oscillatory patterns in systolic, diastolic, or mean arterial pressure (and their timing) provide the key stimulus for changes in myogenic and neurogenic contribution to blood pressure. Therefore, the details of sympathetic neurovascular coupling remain difficult to conceptualize and to quantify in the integrated system.

Nonetheless, specific features of the MSNA signal point to its discharge properties as being fundamental to its role in regulating vasomotor behavior. As noted above, the baroreflex pathways entrain MSNA bursts to the cardiac cycle (Fagius et al. 1985), resulting in the "bursty" pattern. Uniquely, this burstiness appears to be a fundamental aspect of the relationship between MSNA and neurovascular function. Specifically, in a feline model, greater vasoconstriction was observed when the cut end of postganglionic lumbar sympathetic nerves was stimulated in bursts that mimic in vivo patterns rather than continuous stimulation (Ninomiya et al. 1993). This study exhibited the importance of noncontinuous efferent drive in goal-directed messaging to the vascular end organ. Likewise, the pattern of sympathetic impulses has a major impact on the degree of vasoconstriction elicited. Indeed, random patterns of sympathetic nerve stimulation elicit greater contractile responses in comparison to regularly delivered stimuli (Nilsson et al. 1985). How these latter findings relate specifically to spontaneous MSNA patterns is difficult to say because electrical stimulation engages all axons in the sympathetic chain, an unlikely scenario under normal intact conditions.

Recall, furthermore, that the MSNA pattern is one of cardiac-gated bursts that vary in both frequency and size. One debate in the use of microneurographic MSNA as an "input" signal for vasoconstriction lies within the quantification of burst frequency with, or without, concurrent regard for burst size. The burst frequency aspect of the signal expresses strong within-individual reproducibility (Fagius and Wallin 1993; Kimmerly et al. 2004) and resistance to change despite small changes in the electrode position. Also, burst frequency is a volatile aspect of MSNA and, therefore, can represent the bulk of changes in total MSNA. Therefore, the use of burst frequency as the primary metric of total sympathetic outflow has been promoted (Hart et al. 2017), acknowledging the practice that has persisted since the inception of the technique for human studies. On the other hand, as outlined above, the size of a burst provides distinct information reflecting complex patterns of AP recruitment. However, routine use of burst size as an quantifiable and functionally relevant component of MSNA has been discounted due to uncertainties regarding stability of electrode position within the human sympathetic nerve bundles. Also, the lack of correlation between blood pressure and burst size indicates poor baroreflex control over this feature. Nonetheless, the experienced investigator recognizes changes in electrode position. Furthermore, normalizing the burst size in the integrated neurogram overcomes this problem to some extent (Sverrisdóttir et al. 1998) and when this is done, the distribu-

tion of burst size exhibits strong within-individual reproducibility in studies separated by about 4 weeks (Kimmerly et al. 2004). As an aside, baroreflex mechanisms appear to apply to the appearance of small APs only, but not the larger AP clusters (Salmanpour and Shoemaker 2012), providing a mechanistic link between earlier conjecture that larger bursts are regulated by different central features (Kienbaum et al. 2001; Malpas and Ninomiya 1992).

Acknowledging these considerations, MSNA burst size provides important insight into sympathetic neurovascular coupling in humans. Specifically, individual MSNA burst heights were related to the magnitude of the vasoconstrictor responses in the leg from which MSNA bursts were recorded (Fairfax et al. 2013b) (Fig. 6). Furthermore, the total summed height of MSNA bursts occurring consecutively in groups provide a graded vasoconstriction and pressor response. Interestingly, the graded effect of burst height on the degree of vasoconstriction appears more prominent in the leg than in the forearm (Fairfax et al. 2013a), perhaps due to greater alpha-adrenergic sensitivity in the leg compared with the arm (Pawelczyk and Levine 2002). While these data may be challenged because of their reliance on a low sympathetic burst rate, and of the baseline conditions of the recordings, they provide unique insight into the burst-by-burst variations exerted by the sympathetic nervous system in vasomotor control. These data also infer an important role in vasoconstriction for low-probability, large APs that occur in larger bursts. Relating recruitment and timing of AP clusters to vasomotor control remains a key area for future studies.

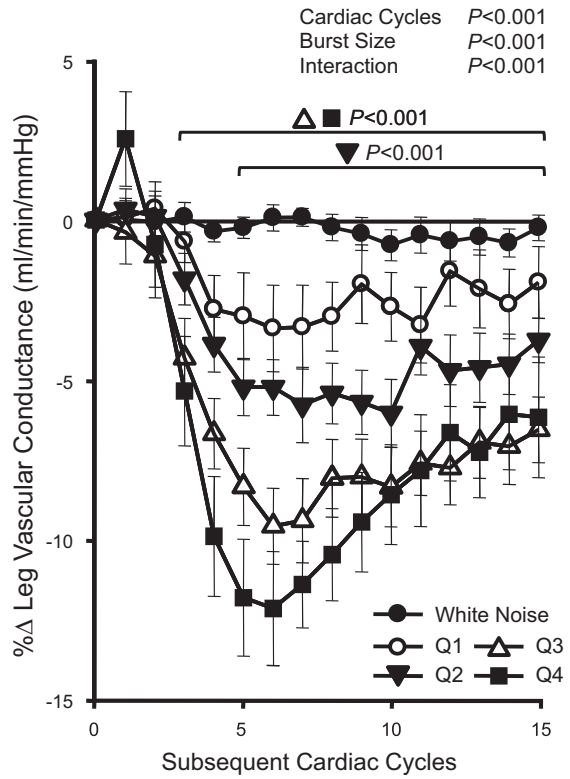

Fig. 6. Summary data showing beat-by-beat percent changes in leg vascular conductance following MSNA bursts of varying height grouped into quartiles from smallest to largest (Q1–Q4). Brackets denote significant difference from percent changes in white noise. Values are means ± SE. From Fairfax et al. (2013b).

CLINICAL IMPLICATIONS

Microneurography has become a fundamental tool in understanding sympathetic aberrations in several pathologies including congestive heart failure (Azevedo et al. 2000), ischemic heart disease (Badrov et al. 2016a; Malliani and Montano 2004), hypertension (Grassi 1998), Tako Tsubo disorder (Vaccaro et al. 2014), sleep apnea (Floras 2016), and chronic kidney disease (Kaur et al. 2017), as well as in obesity (Esler et al. 2001; Lambert et al. 2014), metabolic syndrome (Grassi 2006), and diabetes (Coats and Cruickshank 2014; Holwerda et al. 2016). Agreeing with measures of circulating catecholamines and norepinephrine turnover (Esler 1995, 1997, 2001, 2003), baseline MSNA burst frequency tends to increase with age (Grassi et al. 1995; Hogikyan and Supiano 1994; Iwase et al. 1991) and particularly in heart failure with reduced ejection fraction (Floras 2009; Zucker et al. 1995). The potentially damaging effect to tissues by excessive and prolonged adrenergic (Izzo 1989; Zhang and Faber 2001a, 2001b) and neuropeptide Y (Zukowska-Grojec et al. 1998a) stimulation creates considerable interest in understanding the mechanisms through which aberrant neural traffic develops, as well as in the development of effective treatments.

While mechanisms determining aberrant sympathetic outflow in many patient populations remain unclear, modifications in sympathetic recruitment patterns may provide some insight. Using the single-unit method, different groups demonstrate heightened firing probability of axons at baseline in clinical states such as obesity, heart failure, sleep apnea, and hypertension (Elam and Macefield 2001; Elam et al. 2003; Lambert et al. 2013; Macefield and Wallin 1999; Schlaich et al. 2004, 2009). In the multiunit AP detection approach, patients with chronic heart failure exhibited greater burst frequency as well as APs/burst compared with similarly aged controls (Maslov et al. 2012). Thus, both approaches converge on the conclusion that low threshold axons observable under baseline conditions are recruited more frequently in at least some disease states. This pattern is different that than observed with ischemic heart disease with clinically normal ejection fraction where increases in baseline MSNA burst frequency occur but with little change in the number of APs per burst (Badrov et al. 2016a). Therefore, the modified recruitment threshold of axons available under baseline conditions may be related to cardiac function and, by inference, cardiac sensory input to central autonomic control (Millar et al. 2015).

In addition to, or perhaps because of, changes in baseline control outlined in the above paragraph, age and cardiovascular disorders may affect reflexive AP recruitment. Specifically, in contrast to similarly aged adults, patients with ischemic heart disease (but normal ejection fraction) expressed a near-absent ability to recruit larger APs during apneas of similar length (Badrov et al. 2016a). Similarly, heart failure patients with reduced ejection fraction expressed diminished ability to increase the APs/burst despite the large stress imposed by a preventricular contraction (Maslov et al. 2012), yet recruitment of new APs was normal. Furthermore, using the single-axon recording method, Elam and colleagues (Elam et al. 2003) observed recruitment of new axons during PVCs in both chronic heart failure and sleep apnea patient groups. Thus, human heart failure appears to minimize, but not abolish, the ability to recruit larger APs during the condition of a preven-

tricular contraction. The available data are difficult to interpret because, to date, no studies have reported MSNA and AP recruitment patterns using direct comparisons of patients with normal and abnormal ejection fraction, or the presence of congestive conditions, under the same reflex conditions.

NEUROMODULATION OF SNA

In concert with its use in establishing sympathetic changes in disease states, the microneurographic technique also has provided evidence regarding the ability of therapeutic strategies to modulate sympathetic outflow. Whereas many pharmacological approaches are used to manipulate the end organ's response in, for example, hypertension (Chobanian 2009), recent efforts have focused on targeting the central origins of sympathetic outflow through nonpharmacological means. In the context of this review, neuromodulation refers to nonpharmacological techniques or bioelectric devices that manipulate the activity of discrete cortical or subcortical brain centers (Rossi et al. 2016) governing sympathetic outflow to the heart and vasculature.

Behavioral modifications that regulate sympathetic outflow have focused on physical activity and diet in populations where sympathetic outflow is high due to metabolic and age-related disorders. For example, microneurography methods detected an ~30% reduction in sympathetic outflow (as assessed by burst frequency) following diet-induced weight loss (10% loss) in obese participants (Lambert et al. 2014). Although the ability of exercise to modify baseline MSNA in young healthy individuals is weak, recent collective evidence (Carter and Ray 2015) supports the role of the ability for endurance training to reduce MSNA burst frequency in individuals with heightened cardiovascular risk. New studies are needed to study whether recruitment patterns are restored with exercise training in patient populations (i.e., ischemic heart disease patients outlined above). Of note, physical deconditioning performed by young healthy individuals in the head-down position has little impact on baseline MSNA, although this intervention did increase the magnitude of change in AP frequency and recruitment during apneic stress (Klassen et al. 2017). Therefore, behavioral modifications do affect MSNA but these effects depend on the population, the specific metric of sympathetic activity, and the state of the individual. The mechanisms regulating these outcomes are not known. These findings also highlight the utility of AP detection along with multiunit burst frequency measures to better interrogate and understand sympathetic control of the circulation in health and disease.

In addition to behavioral methods, bioelectric methods are also being studied in the regulation of the sympathetic nervous system. Electrical nerve stimulation represents an emerging area of research involving surgical and noninvasive approaches. While direct manipulation of midbrain activity via implantation of electrodes was traditionally employed to alleviate neurological pain or tremor, targeted deep brain stimulation also demonstrates potential as a method for sympathetic nervous system neuromodulation. For example, a case-series study by Sverisdottir and colleagues (Sverrisdóttir et al. 2014) illustrated the sympathoinhibitory effect of electrical stimulation of the dorsal subthalamic nucleus and ventrolateral periaqueductal gray regions in patients with Parkinson's disease and chronic neuropathic pain (Sverrisdóttir et al. 2014). Whether this approach altered neuronal signals emerging from these specific nuclei, or by modifying activity of fibers-of-passage from higher cortical sites remains to be determined. Additionally, chronic stimulation of carotid sinus baroreceptors (Heusser et al. 2010, 2016) evoked long-term reductions (3+ years) in MSNA burst frequency of heart failure patients with impaired ejection fraction (Dell'Oro et al. 2017). Therefore, electrical stimulation of central and baroreflex pathways by embedded electrodes exert important effects in clinical states.

Noninvasive methods of nerve stimulation provide additional evidence for sympathetic neuromodulation. For example, transcutaneous electrical stimulation of the auricular branch of the vagus nerve distributed to the skin of the ear (200 μs, 30 Hz) reduced MSNA burst frequency and incidence in healthy volunteers (Clancy et al. 2014). Furthermore, submotor electrical stimulation (100 Hz, 50 μs) of forearm muscle somatosensory nerves (likely Type I and II proprioceptor afferents) through anesthetized skin did not modulate sympathetic outflow during supine rest or an end-inspiratory apnea yet attenuated the rise in sympathetic burst frequency (8 vs. 12 bursts/min) during baroreflex unloading with −30 mmHg lower body suction (Goswami et al. 2012). Moreover, in patients with class III heart failure, Labrunée et al. (2013) found electrical stimulation (80 Hz, 3 s on–3 s off pattern, 200-μs pulse width) of the quadriceps both above and below the motor threshold reduced MSNA in supine participants following the treatment. Confirmatory and mechanistic studies are required to understand how transcutaneous stimulation of sensory pathways affect MSNA. Nonetheless, the sensitivity of MSNA to various methods of neuromodulation may present an opportunity to study neurorecruitment strategies as well as neurovascular transduction.

SUMMARY

Development of microneurographic methods to obtain direct recordings of sympathetic nerve activity in humans has enabled a new era of investigation into, and understanding of, the mechanisms by which this nervous system communicates among organs to facilitate homeostatic adjustments across a wide range of physiological and psychological states and disease. Considering MSNA, this technique has exposed the primary findings of synchronized groups of APs entrained to arterial blood pressure, with options to recruit latent subpopulations of faster-conducting axons and/or modify synaptic latency periods. New studies are needed to expose the mechanistic basis of these recruitment strategies and their impact on end-organ vascular control. More recent efforts using both invasive and noninvasive sympathetic neuromodulation suggest a more profound interplay between sensory inputs that modify efferent sympathetic outflow, reaching sites of processing within the higher cortical pathways. In these ways, microneurography has become a fundamental tool in the study of the sympathetic nervous system that communicates complex but seemingly ordered instructions for homeostatic adjustments.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS

J.K.S. conceived and designed research; J.K.S., M.B.B., and P.J.F. prepared figures; J.K.S., S.A.K., and P.J.F. drafted manuscript; J.K.S., M.B.B., S.A.K., and P.J.F. edited and revised manuscript; J.K.S., M.B.B., S.A.K., and P.J.F. approved final version of manuscript.

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