J Neurophysiol 118: 2181–2193, 2017.
First published July 12, 2017; doi:10.1152/jn.00283.2017.

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

Measuring and quantifying skin sympathetic nervous system activity in humans

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Submitted 14 April 2017; accepted in final form 10 July 2017

Greaney JL, Kenney WL. Measuring and quantifying skin sympathetic nervous system activity in humans. *J Neurophysiol* 118: 2181–2193, 2017. First published July 12, 2017; doi:10.1152/jn.00283.2017.—Development of the technique of microneurography has substantially increased our understanding of the function of the sympathetic nervous system (SNS) in health and in disease. The ability to directly record signals from peripheral autonomic nerves in conscious humans allows for qualitative and quantitative characterization of SNS responses to specific stimuli and over time. Furthermore, distinct neural outflow to muscle (MSNA) and skin (SSNA) can be delineated. However, there are limitations and caveats to the use of microneurography, measurement criteria, and signal analysis and interpretation. MSNA recordings have a longer history and are considered relatively more straightforward from a measurement and analysis perspective. This brief review provides an overview of the development of the technique as used to measure SSNA. The focus is on the utility of measuring sympathetic activity directed to the skin, the unique issues related to analyzing and quantifying multiunit SSNA, and the challenges related to its interpretation.

microneurography; SSNA; MSNA; cutaneous blood flow; thermoregulation; vasodilation; vasoconstriction

THE SYMPATHETIC NERVOUS SYSTEM stimulates, integrates, and regulates myriad physiological functions; thus systematic investigation of human sympathetic nerve activity continues to be an intense area of active research. The development of the technique of microneurography (Vallbo et al. 2004), which allows for direct recordings from peripheral nerves in conscious humans, has been critical to expanding our understanding of sympathetic function in human health and in disease. The goal of this brief review is to provide an overview of the development of the technique as used to measure skin sympathetic nervous system activity (SSNA). The focus is primarily on the utility of measuring multiunit SSNA, especially in thermoregulatory physiology, unique issues related to analyzing and quantifying SSNA signals, and challenges related to its interpretation. Research topics for which microneurography is commonly utilized to measure muscle sympathetic nerve activity (MSNA), including arterial blood pressure regulation and motor control, are not discussed, except to contrast the two techniques. The importance of, and methodology for, analyzing single-unit postganglionic sympathetic neurons is also briefly discussed. The interested reader is directed to several excellent reviews on these topics (Charkoudian and Wallin 2014; Hagbarth 1993; Hart et al. 2017; Macefield et al. 2002; Mano et al. 2006).

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Historical Overview: Development of Microneurography

Before the development of the technique of microneurography, autonomic activity in humans was commonly estimated from measurement of *1*) effector responses (e.g., heart rate, blood pressure, muscle/skin conductance, sweating) or *2*) neurotransmitter concentrations in the blood. In the mid 1960s, Hagbarth and Vallbo developed the technique of microneurography in the department of clinical neurophysiology at the Academic Hospital in Uppsala, Sweden (Vallbo and Hagbarth 1967; Vallbo et al. 2004). Although the original focus of microneurographic recording in humans was to examine the muscle spindle and its role in motor control, the method also proved capable of recording afferent multiunit nerve activity from skin and muscle as it related to mechanical skin stimuli and voluntary movements (Vallbo and Hagbarth 1967). In one of the first published microneurographic recordings (Vallbo and Hagbarth 1967), the microelectrode was inserted into a cutaneous nerve bundle in the popliteal fossa and multiunit afferent cutaneous impulses were recorded in response to

tapping and more sustained pressure in the area of innervation. From these early experimental revelations and successes, it quickly became apparent that microneurography could be used as a tool to directly measure neural activity from unmyelinated nerve fibers, including efferent postganglionic sympathetic nerves. This realization, that bursts of efferent postganglionic sympathetic nervous system activity could be recorded in a minimally invasive manner from awake humans, spurred hundreds of subsequent studies utilizing the technique of microneurography.

Methodological Approach to Recording SSNA

In microneurography, as applied to awake humans, a tungsten microelectrode, typically with a shaft diameter of 200 μm and a rounded tip of ~5 μm in diameter, is inserted percutaneously in unanesthetized skin and carefully positioned into an underlying peripheral nerve. A second subdermal microelectrode is inserted ~1 cm away from the active recording electrode to serve as a reference electrode. Primarily for practical reasons, the most commonly utilized peripheral nerves are the peroneal, popliteal, radial, median, and ulnar nerves. The electrodes are attached to a grounding unit and preamplifier, with further amplification and filtering occurring in the main amplifier. An analog or digital bandpass filter is applied, and the filtered neural signal is rectified, integrated, and displayed as a mean voltage neurogram. The Nerve Traffic Analyzer from Absolute Design (Solon, IA; formerly Iowa Biosystems) and the NeuroAmp EX from AD Instruments (New South Wales, Australia) are two widely used commercially available systems.

As mentioned earlier, several distinct anatomical features make microneurographic recordings from the aforementioned peripheral nerves possible (Charkoudian and Wallin 2014; Delius et al. 1972a; Vallbo et al. 2004). First, peripheral nerves contain several fascicles surrounded by the perineurium, which largely prevents crosstalk between adjacent fascicles. Second, with some exceptions, notably the fascicle innervating the extensor halluces longus, all nerve fibers in a fascicle are directed to the same tissue (i.e., muscle or skin), although fascicles may be mixed in more proximal segments of a nerve. Third, sympathetic fibers lie in bundles (Tompkins et al. 2013), restricting the spread of the electrical current. These anatomical distinctions allow for single-unit recordings from one fiber or multiunit recordings from several fibers simultaneously. There are several excellent reviews available that discuss firing properties, methodological considerations, and analysis techniques related to single-unit postganglionic recordings in more detail (Macefield 2013; Macefield et al. 2002). The emphasis herein is on multiunit recordings of efferent SSNA.

Distinguishing SSNA from MSNA

Multiunit sympathetic activity occurs as "bursts" of impulses separated by silent periods of varying duration. Depending on whether the active recording microelectrode is positioned into a muscle or skin nerve fascicle, the bursts have differing morphologies and temporal patterns and can be elicited by distinct stimuli (Table 1) (Delius et al. 1972a, 1972b). These differential characteristics allow the microneurographer to reliably identify recordings as either efferent MSNA or SSNA (Fig. 1). Multiunit bursts of MSNA are short lasting and display pulse synchronicity (Fig. 1*A*). Furthermore, rhythmic MSNA bursts occur during spontaneous reductions in blood pressure (Delius et al. 1972a). Increases in MSNA can be evoked by a breath hold or Valsalva maneuver, but not in response to startle stimuli (Delius et al. 1972a, 1972b; Hagbarth and Vallbo 1968; Vallbo et al. 1979). Verification that the recording is from a muscle fascicle can also be confirmed by stimulating muscle afferents. Nerve discharge during percussion over tendons or the muscle belly, stretch of the muscle by appropriate movements (for example, flexing the foot), and the lack of discharge with light touching of the skin on the foot or leg all verify muscle afferent innervation.

Table 1. *Comparison of muscle and skin sympathetic nerve activity responsiveness to different stimuli*

Stimulus	MSNA	SSNA
Respiratory		
Valsalva maneuver (during)	↑	↔
Valsalva maneuver (after)	↓	↔
Apnea (end-inspiratory)	↑	?
Apnea (end-expiratory)	↑	↔
Deep breath	↔	↑
Hyperventilation	↔	↑
Cardiovascular		
Head-up tilt	↑	↔
Lower body negative pressure	↑	↔
Head-down neck flexion	↑	↔
Isometric exercise	↑	↑
Dynamic exercise	↑	↑
Post-exercise ischemia	↑	↔
Thermoregulatory		
Whole body heating (mild)	↑	↓
Whole body heating (moderate/severe)	↑	↑
Whole body cooling (mild)	?	↑
Whole body cooling (moderate/severe)	↑	↑
Other		
Arousal	↔	↑
Mental stress	↑, ↔	↑
Smoking	↓	↑

MSNA, muscle sympathetic nerve activity; SSNA, skin sympathetic nerve activity. [Adapted from Delius et al. (1972c); compiled from Carter and Ray (2008); Charkoudian and Wallin (2014); Delius et al. (1972a, 1972b); Fatouleh and Macefield (2013); Greaney et al. (2016); Hagbarth et al. (1972); Hart et al. (2017); Narkiewicz et al. (1998); Ray et al. (1997); Saito et al. (1990); Silber et al. (1998); Vallbo et al. (2004); and Wilson et al. (2006).]

In contrast, multiunit bursts of SSNA have highly variable shapes and longer durations than MSNA bursts and display less cardiac cycle rhythmicity (Fig. 1*B*) (Delius et al. 1972b; Fatouleh and Macefield 2013). The first multiunit recording of SSNA was obtained in a hypertensive woman and was confirmed by several distinct characteristics (Delius et al. 1972b): *1*) individual bursts were followed by effector responses (i.e., increased skin electrical resistance and vasoconstriction) within the area of neural innervation, *2*) spontaneous volleys of bursts occurred independently of fluctuations in blood pressure, and *3*) ganglionic blockade via intravenous infusion of trimethaphan eliminated the nerve signal. To further distinguish them from MSNA bursts, marked alterations in SSNA also occur in response to arousal stimuli, deep inspiratory breaths, and changes in skin temperature, but not during breath holds or Valsalva maneuvers (Delius et al. 1972a, 1972b). As with MSNA recordings, afferent SSNA discharges can be elicited and used to confirm a recording. In practice, this is

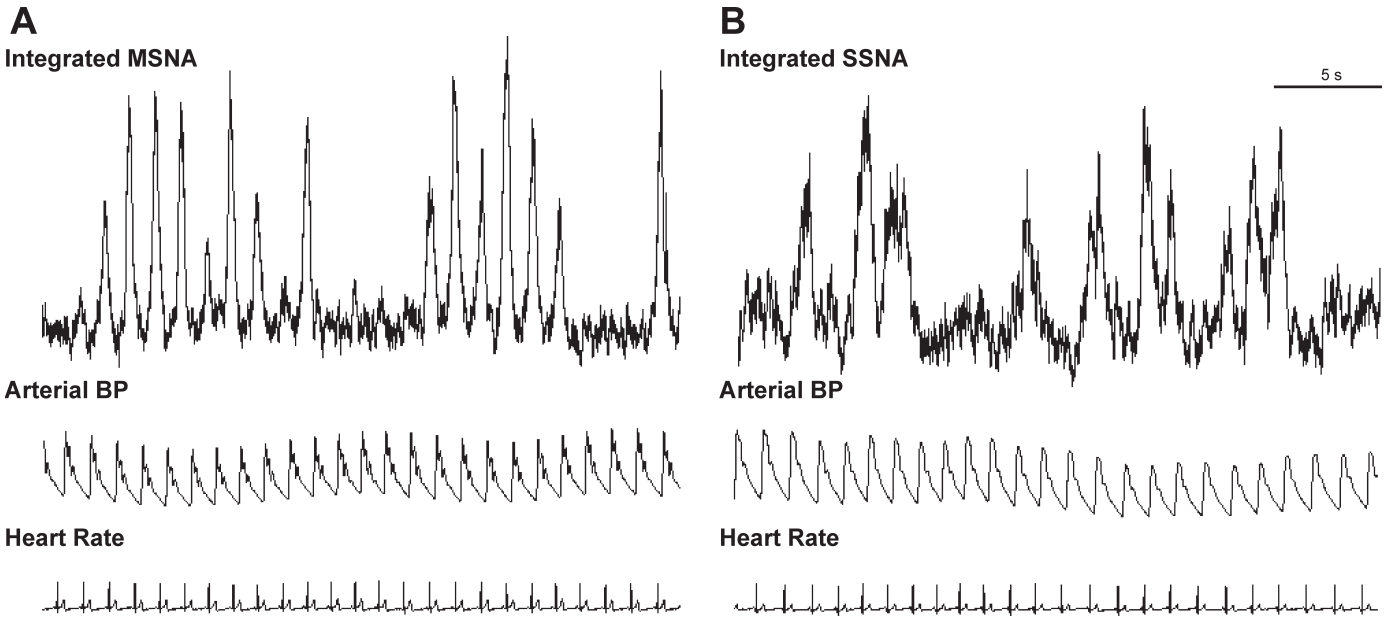

Fig. 1. Original recordings of muscle sympathetic nerve activity (MSNA; *A*) and skin sympathetic nerve activity (SSNA; *B*) depicting the distinct burst morphology and temporal patterns used by microneurographers to distinguish between multiunit efferent MSNA and SSNA recordings. Arterial blood pressure (BP; Finometer) and heart rate (electrocardiogram) are shown for reference.

commonly done by light touching or stroking of the skin on the dorsum of the foot or lateral aspect of the lower leg.

Because neurons innervating muscle and skin traverse the same nerve, it is common to receive interference from one or the other while performing the technique. Recordings of such "mixed" sites are one of the many technical challenges associated with microneurography. Nonetheless, the differing characteristics of MSNA and SSNA recordings and their responsiveness to discrete stimuli not only illustrate that the sympathetic nervous system is highly differentiated but also enable investigations targeting skin-specific reflexes and control mechanisms.

Multiunit vs. Single-Unit Recordings

Most sympathetic fibers display intermittent spontaneous discharges, and because activity in neighboring fibers is generally synchronized, multiunit sympathetic activity occurs as distinct bursts separated by quiescent periods (Delius et al. 1972b, 1972c). Multiunit activity is typically presented as an integrated mean voltage neurogram, in which the neural signal is passed through a resistance-capacitance circuit with a time constant of 0.1 s. From this smoothed signal, burst frequency (number of bursts), burst incidence (number of bursts normalized to 100 heartbeats), and total neural activity (product of burst frequency and burst area/amplitude) can be obtained. Such multiunit recordings from multiple postganglionic axons have become the standard technique to record and quantify efferent sympathetic activity (White et al. 2015).

However, in multiunit recordings, the absolute strength (intensity) of the recorded activity cannot be precisely determined because of the critical dependence on the proximity of the electrode tip to the active postganglionic sympathetic neurons being recorded. This limitation makes the comparison of burst intensity between individuals challenging. In the early years of microneurographic recordings of multiunit sympathetic efferent activity, investigators observed larger and very short-lasting deflections, suggestive of single-unit impulses (Vallbo et al. 2004). This concept was verified by Torebjörk and Hallin (1974), who recorded single-unit afferent and efferent activity from unmyelinated nerve fibers; yet, the low signal-to-noise ratio and lack of adequate technology to inspect the spike waveform made it difficult to prove their single-unit nature.

In the 1990s, Vaughn Macefield undertook a series of studies designed to characterize the firing properties of cutaneous neurons utilizing a single-unit approach (Macefield et al. 1994, 2002; Macefield and Wallin 1999a, 1999b). With the use of a higher impedance microelectrode, the area over which neural activity is detected can be delimited. In these studies, the position of the electrode was adjusted until individual spikes from a putative single fiber were isolated from the underlying noise. With this procedure, the morphology of every spike of candidate units is assessed, and spike superimposition is used to ultimately determine whether a recording is from a single nerve fiber (Macefield et al. 2002). Because postganglionic sympathetic axons travel within the nerve as a group, it is technically very difficult to record from individual axons. The technical challenges associated with recording from individual postganglionic axons, coupled with the time-consuming analysis, have limited the widespread adoption of single-unit recordings. Nonetheless, the single-unit approach has been used to characterize the firing properties of cutaneous vasoconstrictor and sudomotor neurons (Macefield and Wallin 1999a, 1999b), and although the present focus is on multiunit recordings of SSNA, these studies are briefly discussed below.

Quantifying Multiunit SSNA: Analytical Approaches and Challenges

Whereas MSNA is dominated by sympathetic vasoconstrictor impulses that yield a burst pattern that is pulse synchronous (due to tight regulation by the arterial baroreflex; Delius et al. 1972a, 1972b), the activity of multiunit SSNA recordings

comprises vasoconstrictor, sudomotor, piloerector, and perhaps vasodilator fibers. The anatomical structure and impulses associated with these fibers may or may not be distinct (Bini et al. 1980b; Delius et al. 1972a, 1972b). Thus alterations in efferent SSNA may involve both the number of spontaneous bursts and the relative contribution of varying impulse types. Because multiunit recordings of SSNA contain a mixture of various fiber types, it is highly unlikely that there is a specific intrafasicular site from which the activity of only one unique fiber type can be isolated and recorded using microneurography (Young et al. 2009b). Indeed, histological evidence suggests that along unmyelinated nerve fibers, Schwann cells form a complicated network with multiple processes interchanged among fiber axons (Aguayo et al. 1976; Eames and Gamble 1970; Ochoa 1976). The inability to account for the fiber composition comprising a given multiunit SSNA recording, coupled with the inability to determine the location of the microelectrode tip relative to the active neurons being recorded, makes the comparison of basal or baseline SSNA activity between subjects, or within the same subject on different experimental visits, challenging, if not impossible. Hence, longitudinal changes in an SSNA recording within an experiment (i.e., the response to the imposition of varying stimuli or changes in a response over time) are more commonly assessed.

Typically, multiunit microneurography recordings are quantified by burst frequency and/or burst size (e.g., burst strength). In contrast to the analysis of MSNA recordings, which is largely standardized (Hart et al. 2017; White et al. 2015), because SSNA bursts occur *1*) with no apparent rhythmicity and *2*) as bursts of irregular shape and duration, the analysis and quantification of multiunit SSNA present several unique challenges. Given the morphological variability that characterizes SSNA bursts—for example, single wide bursts with multiple peaks commonly occur (Fig. 1*B*)—quantification of SSNA burst number is difficult. In this regard, when simply counting SSNA burst frequency, one often cannot delineate distinct, nonoverlapping bursts or distinguish whether individual bursts are wide or narrow. Although comparisons of SSNA burst frequency between individuals and between subject groups appear in the literature (Grassi et al. 1998, 2014; Middlekauff et al. 1994; Park et al. 2008; Pozzi et al. 2001), caution should be used in interpreting such findings given the potential for multiple fiber types and the irregular nature of SSNA bursts. This is especially so if only SSNA burst frequency was reported.

A common alternative approach to measuring SSNA burst frequency is the quantification of SSNA total activity over a given time period (i.e., the sum of the total area under all bursts detected during that time epoch). The strength of a burst of SSNA is governed largely by the recruitment of additional neurons, whereas increases in the burst firing frequency contributed by each unit are of lesser importance (Macefield and Wallin 1999b), further reinforcing the adoption of analytical approaches that measure total integrated SSNA from multiunit recordings of postganglionic axons. In multiunit recordings, it is not possible to determine whether an increase in burst strength is reflective of an increase in firing incidence of a single unit or the recruitment of additional units, or both, which remains a limitation to the quantification of multiunit SSNA. However, because SSNA bursts are not constrained by the cardiac cycle, it is likely that the recruitment of additional neurons contributes to the characteristic wider and more irregular morphology of SSNA bursts, particularly during increased sympathetic activation (Delius et al. 1972b; Macefield and Wallin 1999b; Vallbo et al. 1979), providing yet another reason to examine total activity for multiunit recordings of SSNA. However, it is important to note that total integrated activity is greatly influenced by the precise position of the microelectrode within the nerve fascicle, which cannot be determined. For this reason (and unlike MSNA), direct between-subject comparisons of baseline SSNA are not feasible (Greaney et al. 2015b, 2017; Young et al. 2009b).

To circumvent these issues, total integrated SSNA can be normalized to a baseline value, a procedure that allows for the within-subject assessment of SSNA responsiveness relative to a given stimulus (Gagnon et al. 2016; Greaney et al. 2015b, 2017; Low et al. 2011; Stanhewicz et al. 2016; Strom et al. 2011; Young et al. 2009a, 2009b). This approach also appropriately accounts for differences in microelectrode position within the nerve fascicle between experimental trials. When total integrated SSNA is being analyzed, a variety of standardization approaches may be applied (Fig. 2), all of which, despite subtle differences, allow for a valid estimation of SSNA responsiveness within a given subject relative to baseline. As such, analysis of multiunit recordings of SSNA depends critically on identifying, and then calculating the total activity (i.e., "area under the curve") of, an appropriate baseline recording segment. Once the total SSNA during a baseline time period of interest is determined (i.e., the mean strength of the signal), this baseline segment of SSNA is often assigned a value of 100 (or 1,000) arbitrary units. SSNA recorded during a subsequent sympathetic provocation is then analyzed as a relative change from that normalized baseline value (Gagnon et al. 2016; Greaney et al. 2015b, 2017; Muller et al. 2013a, 2013b; Stanhewicz et al. 2016; Vissing et al. 1991; Wilson et al. 2001, 2004, 2006; Young et al. 2009a).

Although less commonly used, an alternate approach is to calibrate the full range of the SSNA signal on a scale of 0 to 1,000 arbitrary units, where 0 represents a region with an absence of nerve activity and 1,000 represents the tallest spontaneous burst occurring without any provocation or stimulus (Charkoudian and Wallin 2014). Total SSNA is then calculated as the integral of the recording above zero (Charkoudian and Wallin 2014). This approach has been proffered as an attempt to control for differences in signal quality between subjects. A third approach involves identifying a baseline segment that contains a long nonbursting period and calculating the mean voltage of that quiescent period, essentially setting a baseline in the absence of SSNA bursts to 0 arbitrary units (Low et al. 2011). The area of the integrated bursts above this baseline is then measured during a sympathetic stimulus (Low et al. 2011). This approach provides utility in instances when a sympathetic stimulus elicits such a large increase in SSNA that bursts do not consistently return to baseline, preventing the identification and calculation of the area of individual bursts (e.g., whole body heating-induced increases in core temperature of $\geq$1.5°C; Low et al. 2011). Each of these normalization techniques, depicted in Fig. 2, assumes that the increase in burst intensity/frequency of multiunit SSNA in response to a stimulus represents an increase in efferent sympathetic outflow directed to the effector organ(s). Collectively,

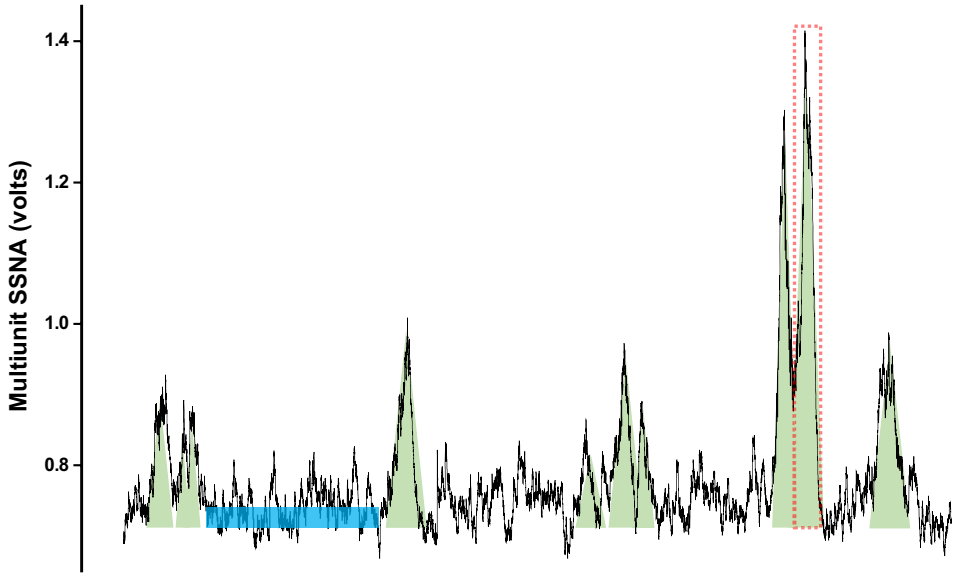

Fig. 2. Representation of the various techniques employed for the normalization of baseline SSNA in multiunit recordings. Normalization to the mean strength of spontaneous activity at rest, in the absence of any stimulus, involves assigning an arbitrary value to a baseline segment of SSNA, represented by setting the integration of the green-shaded area to 100. Normalization to the highest burst of spontaneous activity at rest is represented by the hashed red box, in which the largest burst is assigned a value of 100. In the last approach, a baseline segment containing a long nonbursting period is set to 0 arbitrary units, depicted by the blue box. Each of these normalization procedures allows for the within-subject assessment of SSNA responsiveness relative to a given stimulus.

judicious utilization of these analytical approaches has allowed investigators to appropriately quantify SSNA reactivity to a wide variety of stimuli, advancing our understanding of the regulation of sympathetic neural outflow directed to the cutaneous vasculature.

To better understand the central mechanisms governing SSNA waveform variability and to glean insights as to whether changes in SSNA occur via an increase in burst amplitude, an increase in burst frequency, or both, investigators have also used histogram and spectral analyses. Delius et al. (1972b) first reported a coupling between spontaneous SSNA and resting respiratory rhythm; this coupling was later verified in both integrated SSNA (Bini et al. 1980b) and in single vasoconstrictor and sudomotor neurons (Fatouleh and Macefield 2013; Macefield and Wallin 1999b) using an interval histogram analysis method. Power spectral analysis of integrated SSNA revealed both low- and high-frequency oscillatory components in the variability of bursts of SSNA (Cogliati et al. 2000), similar to those observed in MSNA variability; however, this is not a universal finding (Cui et al. 2006). Although power spectral distribution of the integrated signal, and other mathematical approaches such as wavelet analyses (Bernjak et al. 2012), may provide an alternative quantitative assessment of SSNA, these analytical tools have been used in relatively few investigations incorporating SSNA. Because varied mathematical methodologies can be employed (including the use of autoregressive parametric algorithms and the Welch method based on fast-Fourier transform algorithms), results from such analyses are difficult to compare across studies.

The lack of clear consensus regarding calibrating and normalizing a mean voltage neurogram contributes to the analytical and interpretive complexity associated with quantifying multiunit SSNA. The development of standardized methodology to analyze multiunit SSNA is important and would ultimately improve the ability to compare data among studies. We posit that normalizing SSNA to a baseline value (i.e., setting the mean strength of spontaneous activity at rest, in the absence of any stimulus, to 100 arbitrary units) is the most appropriate normalization technique (Gagnon et al. 2016; Greaney et al. 2015b, 2017; Muller et al. 2013a, 2013b; Stanhewicz et al. 2016; Vissing et al. 1991; Wilson et al. 2001, 2004, 2006; Young et al. 2009a). Changes in SSNA can then be analyzed as a relative change from that normalized baseline. In our opinion, normalization by assigning a maximum burst height to a nonstimulus baseline burst can be misleading. That method assumes that the investigator has obtained a "perfect" multiunit recording. That is, the recording is essentially detecting every spontaneous burst in that fiber, and regardless of how long the baseline data collection continues, that maximum burst is the largest burst that will ever be detected from that recording. This is problematic because SSNA at rest is influenced by multiple external factors (e.g., temperature, arousal, etc.) and likely comprises multiple types (e.g., sudomotor, vasoconstrictor, etc.), which cannot be determined in a multiunit recording. For example, if primarily sudomotor fibers are recorded at baseline and a stimulus is applied that selectively activates vasoconstrictor activity, the analysis resulting from a maximum burst height normalization would not be representative of the actual vasoconstrictor response. For these reasons, it is our opinion that normalization relative to baseline activity in the absence of any stimulus is the most appropriate analytical technique for the analysis and interpretation of multiunit SSNA recordings.

Utility of Assessing and Quantifying SSNA

Despite the technical issues, analytical complexities, and interpretation challenges associated with obtaining and quantifying a SSNA recording using microneurography, many laboratories, including our own, have successfully utilized this technique to advance our understanding of autonomic control of the cutaneous circulation in response to a wide variety of stimuli and environmental perturbations.

Thermoregulory control. Because the skin is the site of thermoregulatory effector function, the majority of investigations utilizing microneurographic recordings of SSNA have examined the neural reflex mechanisms subserving thermoregulation. Indeed, sympathetic activity directed to the skin largely determines and regulates thermoregulatory control of cutaneous vasomotor tone and sweating; this is illustrated schematically in Fig. 3. As discussed above, multiunit record-

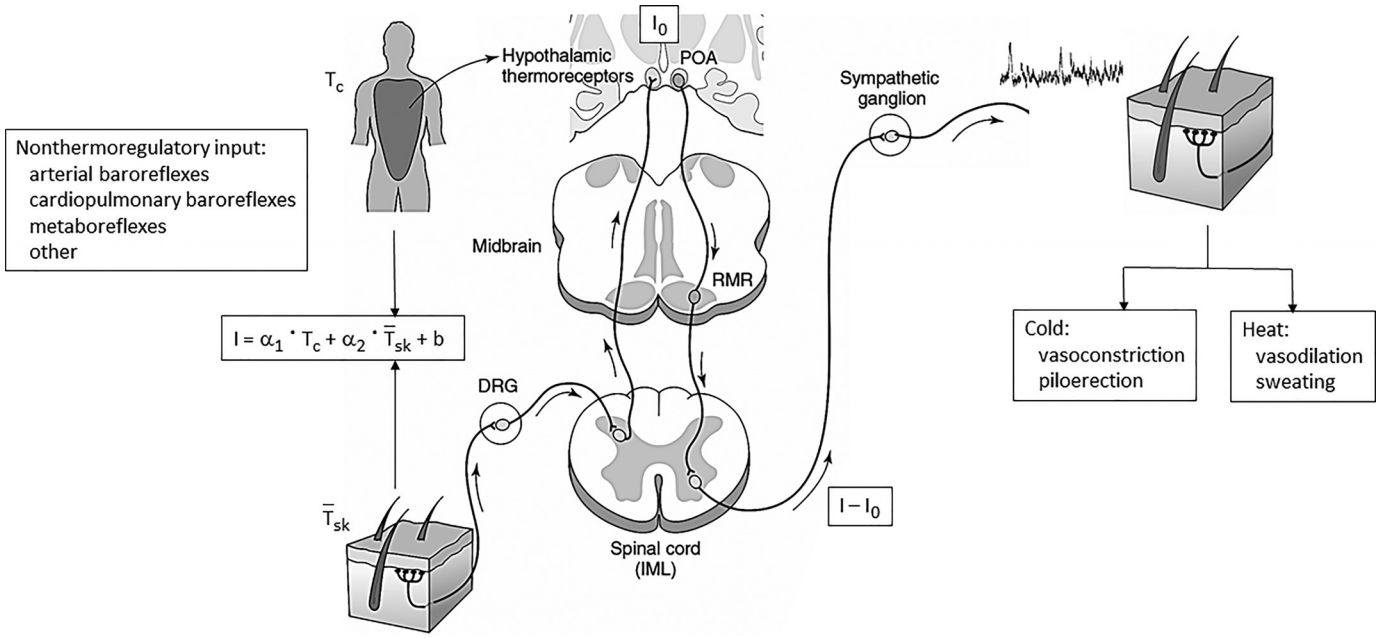

Fig. 3. Schematic diagram depicting thermoregulatory control of skin sympathetic nerve activity (SSNA) to skin effectors. The thermal input signal (I) is a weighted function of core (T_c) and mean skin (T_{sk}) temperatures (i.e., $I = \alpha_1 \cdot T_c + \alpha_2 \cdot T_{sk} + b$), where the ratio of α_1:α_2 is ~9:1 for whole body heating and 7:3 for whole body cooling. Signal input is also modified by local skin temperature and rate of change parameters (not shown), as well as nonthermoregulatory inputs. The input signal I is compared with a "set point" (I_0) in the preoptic area of the hypothalamus (POA), and the error signal ($I - I_0$) determines the output efferent SSNA. Note that set point is conceptual only and not an actual temperature. SSNA determines the onset and gain characteristics of the skin thermoregulatory effector organs, controlling vasoconstriction and piloerection in the cold and vasodilation and sweating in the heat. DRG, dorsal root ganglion; IML, intermediolateral nucleus; RMR, rostral medullary raphe.

ings of integrated SSNA contain bursts of activity that comprise a mixture of sudomotor, vasoconstrictor, vasodilator, and piloerector impulses (Delius et al. 1972b). Differentiation of these impulses was originally determined by simultaneously recording electrodermal and plethysmographic responses during thermal stimuli (Delius et al. 1972a). During body cooling, bursts of multiunit SSNA consisted of primarily vasoconstrictor impulses (Delius et al. 1972a). During mild body heating, vasoconstrictor impulses were reduced; more intense warming elicited increases in sudomotor and, potentially, active vasodilator impulse activity (Delius et al. 1972a). These observations were confirmed and extended by Bini et al. (1980a, 1980b), who demonstrated a striking similarity in vasoconstrictor and sudomotor bursts recorded from different anatomical regions.

SSNA burst appearance is morphologically different during cold vs. heat stress. At thermoneutrality and during whole body cooling, the predominant vasoconstrictor bursts are wider and have a longer duration (Fig. 4*A*; unpublished data) (Bini et al. 1980b; Greaney et al. 2015b). In contrast, in a hot environment when skin vasodilation and sweating are evoked, both SSNA burst duration and the interval between bursts are shorter than what is observed in normothermic conditions (Bini et al. 1980b; Gagnon et al. 2016). In our hands, during whole body passive heating (i.e., clamping high skin temperature and increasing core temperature by 1.0°C using a water-perfused suit), bursts of SSNA actually appear morphologically similar to recordings of MSNA (Fig. 4*B*; unpublished data). This observation is consistent with published original SSNA recordings from multiple laboratory groups that have used whole body heating paradigms (Bini et al. 1980b; Cui et al. 2004, 2013; Gagnon et al. 2016).

Large increases in SSNA in response to whole body cooling have been recorded in multiple studies to examine reflex cutaneous vasoconstriction during cold stress (Cui et al. 2006; Grassi et al. 2003; Greaney et al. 2015b, 2017; Sawasaki et al. 2001; Strom et al. 2011). In healthy young adults, a cold-induced increase in SSNA has been documented during rapid (~3–4 min) (Strom et al. 2011) and prolonged (~30 min) (Cui et al. 2006; Grassi et al. 2003; Greaney et al. 2015b) experimental cooling paradigms, in response to reductions in ambient temperature (Grassi et al. 2003), and with reductions in mean skin temperature elicited by the use of a whole body water-perfused suit (Cui et al. 2006; Greaney et al. 2015b; Strom et al. 2011). The firing properties of individual vasoconstrictor neurons during cooling have also been described during single-unit recordings (Macefield and Wallin 1999b). In every study, cooling-induced increases in total SSNA are readily apparent regardless of the analytical methodologies that have been employed (Cui et al. 2006; Grassi et al. 2003; Greaney et al. 2015b; Strom et al. 2011). In a study recently published by our laboratory (Greaney et al. 2015b), increases in total SSNA during reflex cooling-induced reductions in mean skin temperature were tightly correlated with reductions in cutaneous vascular conductance within the area of neural innervation (Fig. 5*A*), confirming that adrenergic vasoconstrictor impulses mediate reflex cutaneous vasoconstriction during a cold stimulus and providing evidence for a quantitative coupling of the nerve signal and the effector response (Bini et al. 1980b).

Reflex cutaneous vasoconstriction is markedly impaired with aging, even in the absence of apparent diseases or risk factors for disease (Greaney et al. 2015b; Holowatz and Kenney 2010; Stanhewicz et al. 2013). Although compromised thermoregulatory vasoconstriction results from functional def-

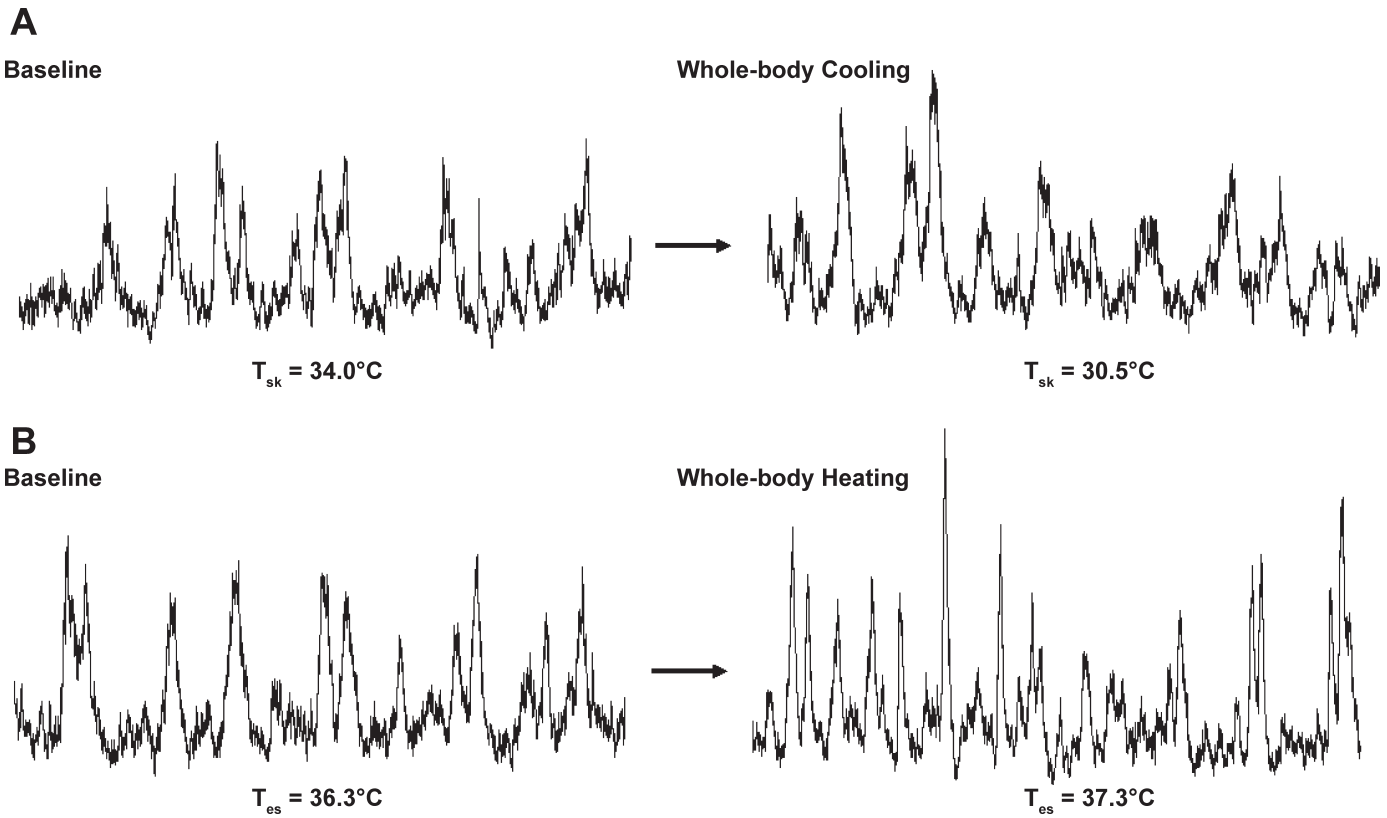

Fig. 4. Original recording of multiunit skin sympathetic nerve activity (SSNA) at baseline and during whole body cooling (*A*). During cooling, vasoconstrictor bursts are wider and have a longer duration. For comparison, an original recording of multiunit SSNA at thermoneutrality and during whole body heating is also presented (*B*). Bursts of SSNA become more narrow and begin to appear morphologically similar to bursts of muscle sympathetic nerve activity. T_{sk}, skin temperature; T_{es}, esophageal temperature.

icits at multiple points along the efferent reflex axis (Greaney et al. 2015a), emerging evidence implicates age-related reductions in SSNA responsiveness to whole body cold stress as a significant contributing factor (Grassi et al. 2003; Greaney et al. 2015b). In a recent study (Greaney et al. 2015b), we demonstrated that blunted efferent sympathetic outflow to the cutaneous vasculature was linearly related to age-related impairments in reflex cutaneous vasoconstriction (Fig. 5*A*). Interestingly, the slope of the relation between SSNA and cutaneous vascular conductance during whole body cooling was not different between young and healthy older adults (Greaney et al. 2015b). These data suggest that the relative inability of older adults to adequately constrict the cutaneous vasculature is not reflective of diminished sensitivity of the end-organ response, but is instead indicative of age-related reductions in the range of efferent SSNA responsiveness to cooling.

We recently extended these findings to middle-aged adults with primary hypertension (Greaney et al. 2017). Using a similar experimental paradigm, we demonstrated that whole body cooling elicited markedly greater increases in multiunit efferent SSNA and, relatedly, augmented cutaneous vasoconstriction in hypertensive adults compared with their normotensive counterparts (Greaney et al. 2017). As in healthy aging, the sensitivity of the reflex response was not different between normotensive and hypertensive adults; however, the response range of the ΔSSNA:ΔCVC relation was extended with hypertension. Collectively, this series of studies examining integrated SSNA during whole body cold stress provides insight into sympathetic control of reflex cutaneous vasoconstriction in health and disease (Cui et al. 2006; Grassi et al. 2003; Greaney et al. 2015b, 2017; Sawasaki et al. 2001; Strom et al. 2011).

Passive whole body heat stress is likewise a robust sympathoexcitatory stimulus (Rowell 1990), evoking substantial increases in both MSNA and SSNA in healthy adults (Gagnon et al. 2015, 2016; Greaney et al. 2015b, 2016; Keller et al. 2006; Low et al. 2011; Stanhewicz et al. 2016). At the onset of heating, reductions in multiunit SSNA have been observed in some studies (Bini et al. 1980b; Cui et al. 2006; Grassi et al. 2003), a finding that is consistent with the concept of the early withdrawal of tonic vasoconstrictor activity to increase skin blood flow (Kellogg et al. 1990). During mild ambient heat exposure (e.g., air temperature increase; increased mean skin temperature of ~3°C), Grassi et al. (2003) reported gradual reductions in SSNA in a group of young adults throughout the heating protocol. Contrary to those data, when core temperature is increased during more pronounced and sustained passive heating, robust increases in SSNA total activity occur (Bini et al. 1980b; Cui et al. 2006; Gagnon et al. 2016; Low et al. 2011; Stanhewicz et al. 2016; Wilson et al. 2001). This increase in multiunit SSNA likely reflects an increase in both sudomotor and vasodilator activity, resulting in eccrine sweat production and cutaneous vasodilation, respectively. Blumberg and Wallin (1987) first demonstrated that the peroneal nerve contains vasodilator fibers, showing that proximal local anesthesia of the stimulated nerve abolished vasodilation in the area of neural innervation. A distinct vasodilator component of SSNA has also been documented in mildly heated adults (Sugenoya et al. 1998). In that study, multiunit bursts of SSNA

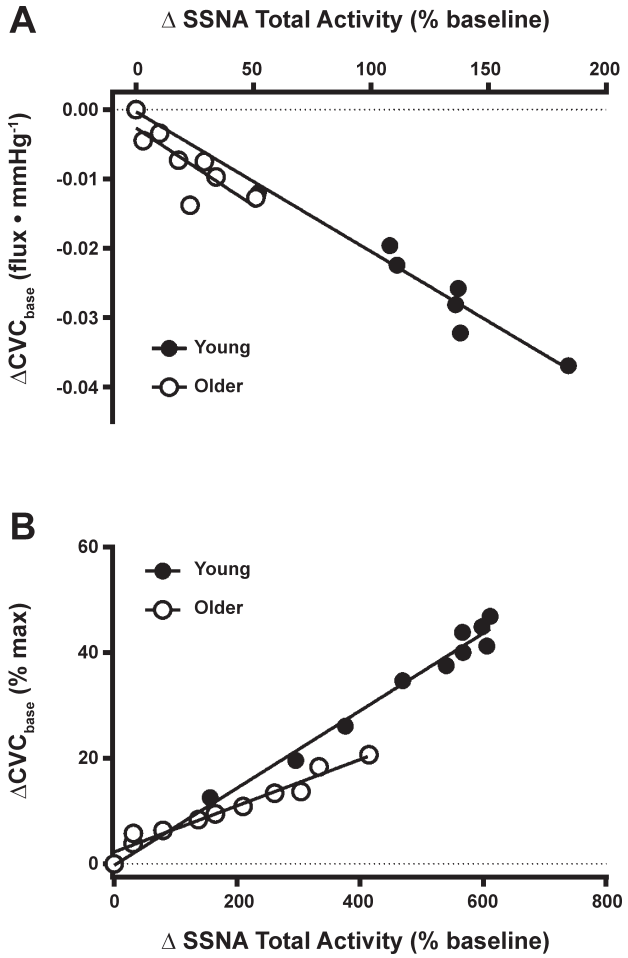

Fig. 5. There was a significant relation between the increase in skin sympathetic nervous system activity (SSNA) and the reduction in cutaneous vascular conductance (CVC) during whole body cooling (i.e., reduction in mean skin temperature from 34.0° to 30.5°C; *A*) and whole body heating (i.e., increase in core temperature of 1.0°C; *B*) in both young (●) and healthy older adults (○). In healthy older adults, blunted cooling-induced increases in SSNA were linearly related to impaired reflex cutaneous vasoconstriction. The slope of the ΔSSNA:ΔCVC relation was not different between groups, suggesting that the relative inability of older adults to decrease skin blood flow during whole body cooling is not reflective of diminished sensitivity of the neural reflex response but is instead indicative of age-related reductions in the range of efferent SSNA responsiveness to cooling. Similarly, blunted heating-induced increases in SSNA were linearly related to impaired reflex cutaneous vasodilation in healthy older adults. However, in contrast to cooling, the slope of the ΔSSNA:ΔCVC relation during heating was also reduced in healthy older adults, suggesting that healthy aging is characterized by a reduced reflex vasodilatory response to increased SSNA. [Adapted from Greaney et al. (2015b) and Stanhewicz et al. (2016).]

were classified according to the effector response (e.g., sweat and cutaneous blood flow). The vast majority of bursts were associated with sweat expulsion and vasodilation, whereas only 10% of bursts were followed by sweat expulsion alone and only 1% of bursts were followed by vasodilation alone (Sugenoya et al. 1998).

Despite validation that multiunit SSNA recordings contain vasodilatory nerve activity (Sugenoya et al. 1998), it remains unresolved whether the vasodilator fibers are activated simultaneously with sudomotor fibers or whether activation of sudomotor fibers alone cause vasodilation by secreting vasodilating molecules. It is generally assumed that sudomotor and vasomotor impulses are controlled by distinct fiber populations; however, microneurographic recordings of multiunit SSNA do not allow for the isolated recording of activity from only one type of fiber (Young et al. 2009b). To our knowledge, only one study has recorded single-unit activity of a sudomotor neuron during heat-induced sweating (Macefield and Wallin 1996), revealing an increased firing rate during moderate sweating. No attempts have been made to record single-unit vasodilator activity during passive heating. Thus the question persists as to whether heating-induced cutaneous vasodilation is mediated by the release of acetylcholine and purported peptidergic cotransmitters from a distinct vasodilator nerve fiber or from sudomotor neurons (Johnson and Proppe 2011).

Alterations in SSNA responsiveness to passive heat stress have functional implications for impaired thermoregulation, especially so for some clinical populations. Increases in skin blood flow and sweating are substantially blunted in apparently healthy older adults during whole body heating (Holowatz and Kenney 2010; Kenney and Fowler 1988; Smith et al. 2013; Stanhewicz et al. 2015). Our laboratory recently completed a series of studies in which we examined the potential for age-related reductions in efferent SSNA responsiveness to contribute to impaired reflex cutaneous vasodilation (Stanhewicz et al. 2015, 2016). Consistent with previous reports (Cui et al. 2006; Gagnon et al. 2016; Low et al. 2011), we observed large increases in multiunit SSNA in young adults during passive heating sufficient to elicit a 1.0°C increase in esophageal temperature (Stanhewicz et al. 2016). Similar to the attenuated SSNA response noted during whole body cooling (Greaney et al. 2015b), healthy older adults demonstrated a smaller increase in SSNA during passive heating (Stanhewicz et al. 2016). Because sweating is also reduced during heat stress in healthy older adults (Kenney and Fowler 1988; Smith et al. 2013), it is possible that age-related reductions in sudomotor activity also contribute to the blunted increase in total SSNA; this warrants future investigation. Importantly, however, the increase in SSNA within an individual was tightly correlated with the increase in cutaneous vascular conductance during heating in both young and older adults (Fig. 5*B*), suggesting that the blunted SSNA responsiveness during heat stress in older adults contributes, at least in part, to age-associated impairments in reflex cutaneous vasodilation (Stanhewicz et al. 2016, 2017). Moreover, the slope of the linear relation between multiunit SSNA and cutaneous vascular conductance during heating was reduced in the older adults (Fig. 5*B*), providing evidence that, in addition to a reduced range of responsiveness for neural outflow, the sensitivity of the reflex response is also diminished with healthy aging (Stanhewicz et al. 2016). To date, only one other study investigated age-related alterations in the SSNA response to mild heat exposure. The authors reported a decrease in SSNA during exposure to a mild increase in ambient temperature (~8°C), a stimulus more likely to elicit the withdrawal of vasoconstrictor tone rather than cause active cutaneous vasodilation. Although not presented quantitatively, the SSNA response was blunted in healthy older adults (Grassi et al. 2003). Taken together, these studies provide evidence that age-related alterations in the efferent SSNA response to heat exposure contribute to impaired thermoregulatory control of cutaneous vasomotor tone.

Impairments in the reflex cutaneous vasodilatory response to heat stress are also evident in adults with hypertension (Holowatz and Kenney 2007a, 2007b; Kenney et al. 1984). Grassi et al. (1998) report that basal SSNA at thermoneutrality is not different between normotensive and hypertensive adults. However, those authors only assessed SSNA burst frequency, which, for reasons outlined above, makes interpretation of their data challenging. To our knowledge, no studies have examined the efferent SSNA response to passive heating in hypertensive adults. However, the SSNA response to passive heat stress has been investigated in heart failure, a common pathophysiological consequence of chronic hypertension. Multiple studies have demonstrated attenuated active cutaneous vasodilation during whole body heating in adults with heart failure (Cui et al. 2005, 2013; Green et al. 2006), which has been attributed, at least in part, to reductions in nitric oxide bioavailability coupled with compromised cardiac function (Green et al. 2006). Perhaps surprisingly, the increase in SSNA during heating in heart failure patients was not different from the response in age-matched healthy adults (Cui et al. 2013), suggesting that blunted increases in efferent sympathetic outflow directed to the cutaneous vasculature does not contribute to impaired reflex vasodilation in heart failure. The sweating response to passive heating was also preserved in heart failure patients (Cui et al. 2005, 2013; Green et al. 2006), providing additional support for the concept that the impairments in the vasodilatory response to passive heating in heart failure are not a result of dysregulated sympathetic outflow but likely reflect altered peripheral vascular and central cardiac function.

Very limited research has been aimed at understanding the role and function of afferent cutaneous fibers activated by thermal stimuli. Recently, Campero et al. (2009) measured SSNA during cooling and menthol (TRPM8 agonist) application in an attempt to determine the specific class of C fiber activated by each stimulus. They reported that the only plausible candidates activated by cooling and menthol are the C2 fibers, although the authors clarified that some of their interpretations are necessarily speculative because of the limited number of afferent fibers sampled and the uncontrolled selectivity of the method (Campero et al. 2009). Interestingly, a recent study employing a novel quantitative sensory test quantified afferent thermosensory function during heat stress (Filingeri et al. 2017), although additional studies are necessary to determine the underlying physiological mechanisms.

Emotion/psychological stress. Acute psychological stress sometimes triggers adverse cardiovascular events, and chronic or repeated psychological stress has been implicated in the development of chronic cardiovascular disease (Chida and Steptoe 2010; Timio et al. 1997). Although the specific mechanisms remain unclear, chronic and/or repeated sympathetic activation is likely involved in the link between stressful emotional stimuli and disease pathology (Lambert and Lambert 2011). Early microneurographic recordings demonstrated that emotional arousal, elicited by unexpected sensory (visual, auditory, or somatosensory) stimuli consistently elicited a distinct, isolated burst of SSNA (Delius et al. 1972c; Hagbarth et al. 1972). Typically, these isolated sensory-stimulated bursts of SSNA are stronger than those occurring spontaneously (Hagbarth et al. 1972). Because these bursts occur in fibers innervating the skin but not muscle, responsiveness to arousal stimuli (sudden noise, unexpected hand clap, etc.) is a common criterion used by microneurographers to distinguish between SSNA and MSNA recordings.

Several investigators have quantified SSNA in response to stimuli that cause persistent stress over short periods of time (e.g., 15 s to 1 min), such as mental arithmetic or the viewing of emotionally charged images. Cognitive (mental arithmetic) and emotional (positive or negative emotionally charged images) stress elicits sustained and reproducible increases in multiunit SSNA (Brown et al. 2012; Brown and Macefield 2014; Greaney et al. 2015b; Henderson et al. 2012; Ito et al. 1996; James et al. 2013; Kuwahara et al. 2015; Muller et al. 2013a, 2013b; Stanhewicz et al. 2016). Increases in SSNA during such stressful stimuli are typically greater at the onset of the stimulus (i.e., within the first 10 s; Greaney et al. 2015b; Muller et al. 2013b), consistent with the concept that SSNA responsiveness to arousal stimuli may abate over time (i.e., habituation following an initial "novelty" effect; Delius et al. 1972c; Hagbarth et al. 1972). For a given individual, the pattern of SSNA responsiveness to a cognitive stimulus appears to be similar during successive trials within an experimental protocol, as well as between trials conducted during separate experiments (Muller et al. 2013b). Moreover, recent investigations have coupled the measurement of multiunit SSNA with brain functional magnetic resonance imaging during the viewing of emotionally charged images in an attempt to discern the cortical and subcortical sites responsible for eliciting changes in SSNA (Henderson et al. 2012; James et al. 2013). Our laboratory has utilized imposition of cognitive stress on thermal stresses in order to more specifically interrogate SSNA responsiveness. In both young and older subjects, we observed further increases in SSNA in response to mental arithmetic at thermoneutrality as well as when superimposed on the background of an already elevated SSNA during cold- or heat-stress conditions (Greaney et al. 2015b; Stanhewicz et al. 2016). This preserved SSNA response to mental stress during whole body temperature changes demonstrates a maintenance of a central ability to elicit further increases in SSNA. Collectively, these studies demonstrate reflex increases in multiunit SSNA in response to cognitive and emotional stimuli; these autonomic responses to stress may have implications for long-term cardiovascular health.

Surprisingly few studies have examined SSNA during psychological stress in clinical pathologies. The increase in SSNA during mental stress is exaggerated in adults with Guillain-Barre syndrome (Yamamoto et al. 1997), those with primary palmoplantar hyperhidrosis (Iwase et al. 1997), and in adults with rosacea (Metzler-Wilson et al. 2015). However, these diseases are relatively uncommon and it remains unclear if SSNA hyperresponsiveness is a general characteristic of cardiovascular disease. In our hands, the increase in SSNA in response to mental arithmetic in hypertensive adults was not different from the response in their normotensive counterparts, either at thermoneutrality or during whole body cooling (Greaney et al. 2017). Nevertheless, it has been suggested that repeated and sustained episodic surges in sympathetic outflow characteristic of generalized sympathoexcitation, and the resultant detrimental vascular sequelae, could potentially increase cardiovascular risk, especially in "at-risk" populations (Lambert et al. 2008; Lambert and Lambert 2011; Mancia et al. 2001). Future studies aimed at delineating the physiological

underpinnings of the sympathetic responses to emotional and psychological stress are warranted.

Cardiovascular regulation. The ability to finely control and modulate cutaneous blood flow is critical for thermoregulation. However, in addition to being responsive to thermoregulatory reflexes, cutaneous vasomotor tone is also controlled by nonthermoregulatory reflex mechanisms necessary for blood pressure homeostasis (Crandall et al. 1996; Kellogg et al. 1990; Rowell et al. 1973). During simulated orthostasis using lower body negative pressure (LBNP) at thermoneutral temperatures, reductions in cutaneous vascular conductance are mediated by enhanced vasoconstrictor activity (Kellogg et al. 1990). In contrast, against the backdrop of hyperthermia, LBNP-induced reductions in cutaneous vascular conductance are primarily the result of withdrawal of cutaneous active vasodilator activity (Kellogg et al. 1990), with recent studies suggesting that the SSNA component that is synchronized with the cardiac cycle is also involved in suppressing vasodilation during postural changes during hyperthermia (Kamijo et al. 2011; Ogawa et al. 2017). This net reflex reduction in cutaneous blood flow contributes to the maintenance of blood pressure and is likely important for acute blood pressure regulation and the prevention of syncope during heat stress (Brothers et al. 2009).

In line with this, it is logical to assume that baroreflex-mediated alterations in SSNA mechanistically contribute to the aforementioned cutaneous circulatory adjustments during simulated orthostasis; however, this notion has been challenged. For example, several studies have convincingly demonstrated that the baroreceptors do not modulate SSNA during upright posture or LBNP in normothermia (Vissing et al. 1994, 1997; Wilson et al. 2001). Furthermore, during supine heat stress, multiunit SSNA was unaffected by pronounced changes in arterial blood pressure, as demonstrated using the modified Oxford technique (Wilson et al. 2001). Investigations of the effects of heat stress on baroreflex control of SSNA during orthostatic stress have yielded equivocal results (Cui et al. 2004; Dodt et al. 1995; Vissing et al. 1994, 1997). For example, Dodt et al. (1995) reported reductions in multiunit SSNA during LBNP and upright tilt in mildly heated adults. In contrast, a series of studies by Vissing et al. (1994, 1997) demonstrated no effect of either −15 mmHg (unloading only cardiopulmonary baroreceptors) or −50 mmHg (unloading both cardiopulmonary and arterial baroreceptors) LBNP on SSNA during hyperthermia, refuting the notion that the mechanism mediating cutaneous vasoconstriction during upright posture in the heat involves baroreflexes. This finding was more recently corroborated during a more severe heat stress protocol (Cui et al. 2004). It is somewhat difficult to reconcile the findings of these studies given that the efferent SSNA response is inconsistent with the effector response (i.e., reductions in skin blood flow). However, because multiunit recordings of SSNA contain multiple types of fibers, it is plausible that during simulated orthostasis in the heat, vasoconstrictor impulses are increased and vasodilator impulses are decreased (or, conversely, sudomotor impulses are increased and vasodilator impulses are decreased), the net effect of which is no change in total integrated SSNA. This possibility requires additional investigation.

More recently, measurement and quantification of SSNA has provided insight into the modulation of central sympathetic outflow as it pertains to blood pressure regulation (Young et al. 2009a). In humans, pharmacologically induced hypertension (e.g., systemic infusion of nitric oxide synthase inhibitors) appears to be partially mediated by the sympathetic nervous system (Lepori et al. 1998; Sander et al. 1999). However, that conclusion was based on studies using either indirect indexes of neural activity or direct measurement of MSNA, which are confounded by the arterial baroreflex. In an attempt to circumvent these issues, Young et al. (2009a) measured SSNA during systemic infusion of a nitric oxide synthase inhibitor. The rationale was that SSNA is not modulated by the baroreflex at thermoneutrality (Cui et al. 2004; Delius et al. 1972c; Wilson et al. 2001) but is highly responsive to maneuvers eliciting alterations in central sympathetic outflow (Cogliati et al. 2000; Vissing et al. 1991; Vongpatanasin et al. 1999). Their findings that pharmacological inhibition of nitric oxide synthase caused a robust and sustained increase in multiunit SSNA provide direct evidence of a role for nitric oxide in the tonic restraint of central sympathetic outflow (Young et al. 2009a) and have important implications for the pathogenesis of hypertension.

Additional Considerations and Future Directions

Microneurography, as with all experimental techniques, is not without its limitations. Experiments using microneurography are time-consuming and demanding for both the researcher and the participant. Prolonged periods searching for the nerve type of interest are not uncommon, during which the microneurographer has to make minute manual adjustments to the positioning of the electrode while paying constant attention to visual and auditory cues signaling a successful nerve recording. The microneurography technique does not allow for large movements by the research subject, and even subtle shifts in body position can impact the quality of the nerve recording, making long experiments physically taxing and limiting its utility during whole body perturbations (e.g., exercise).

Because the microelectrode is inserted into a nerve fascicle, safety concerns must also be addressed. In a prospective study of symptoms after microneurography, ~9% of participants reported lingering signs of local neuropathy after the experiment. However, among those participants, symptoms lasted longer than 2 wk in only one person (Eckberg et al. 1989). Generally, persistent symptoms tended to occur when the period of time spent probing and making adjustments to the microelectrode to obtain a suitable nerve recording was relatively long (>45 min). For that reason, most microneurographers, including those in our laboratory, strictly limit the time spent searching for the nerve to less than 45 min and avoid prolonged probing at the same intraneural location. Recently, some investigators have adopted the use of ultrasound-guided microneurography to locate peripheral nerves and to guide the placement of microelectrodes into the nerve, allowing for greater accuracy (Curry and Charkoudian 2011; Gagnon et al. 2016). This adjunct procedure may be especially useful in populations in which locating nerves using conventional microneurography is more challenging (e.g., obese individuals). However, it is important to note that the ability to use ultrasound as an aid to find the peripheral nerve does not replace the need for basic training in the microneurographic technique itself.

Despite the technical limitations of the technique itself and the challenges related to analyzing and interpreting multiunit

SSNA recordings, research over the past three decades has substantially advanced our understanding of autonomic control of the cutaneous circulation. Direct recordings of SSNA in awake humans have arguably been most important in furthering our understanding of thermoregulatory function. Given that sympathetic activity directed to the skin largely determines and subserves thermoregulatory control of cutaneous vasomotor tone and sweating, this is perhaps not surprising. However, the majority of studies utilizing microneurographic recordings of SSNA to examine thermoregulatory reflex control have been conducted in young healthy adults. Only recently have investigators applied this technique to further understand impaired reflex cutaneous vascular responsiveness to thermoregulatory challenges in aging and in various pathophysiological conditions, including hypertension (Grassi et al. 2003; Greaney et al. 2015b, 2017; Stanhewicz et al. 2016, 2017). This remains an area of active inquiry. Studies designed to further understand the integration of SSNA with respect to blood pressure regulation in health and in disease are also an important area of current and future research.

ACKNOWLEDGMENTS

We acknowledge the contributions of colleagues whose research contributed to this synthesis. We also acknowledge time and effort expended by all the volunteer subjects who participated in research in our laboratories. We also gratefully acknowledge research support for these studies from the National Institutes of Health and American College of Sports Medicine.

GRANTS

The primary research funding from the authors' laboratories that contributed to this synthesis were National Institutes of Health Grants HL120471, AG007004, and HL093238 and an American College of Sports Medicine Research Endowment.

DISCLOSURES

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

AUTHOR CONTRIBUTIONS

J.L.G. and W.L.K. conceived and designed research; J.L.G. performed experiments; J.L.G. analyzed data; J.L.G. and W.L.K. interpreted results of experiments; J.L.G. and W.L.K. prepared figures; J.L.G. drafted manuscript; J.L.G. and W.L.K. edited and revised manuscript; J.L.G. and W.L.K. approved final version of manuscript.

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