EFFECTS OF SEX ON BLOOD PRESSURE RESPONSES TO SLOW BREATHING IN YOUNG HEALTHY INDIVIDUALS

Tessa Evangeline Adler

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Department of Kinesiology and Physical Education

McGill University, Montreal

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PREFACE

Abstract

Slow breathing (SLOWB) is recommended for use as an adjuvant treatment for hypertension. However, the extent to which blood pressure (BP) responses to SLOWB differ between men and women are not well-established. Therefore, we tested the hypothesis that an acute bout of SLOWB would induce larger decreases in BP in males than females, given that males typically have higher resting BP. We also examined autonomic contributors to reduced BP during SLOWB, that is, muscle sympathetic nerve activity (MSNA), and spontaneous cardiovagal (sequence method) and sympathetic baroreflex sensitivity (BRS). We tested normotensive females (n=10, age: 22±2y, BMI: 22±2kg/m^2) and males (n=12, 23±3y, 26±4kg/m^2). Subjects were tested at baseline and during the last 5-min of a 15-min RESPeRATE-guided SLOWB session. Overall, SLOWB reduced systolic BP (SBP) by 3.2±0.8 mmHg (main effect, p<.01). Females had lower SBP (main effect, p=.02); we observed no interaction between sex and SLOWB. SLOWB also reduced MSNA burst incidence by -5.0±1.4 bursts/100hb (main effect, p<.01). Although females tended to have lower burst incidence (main effect, p=.1), there was no interaction between sex and SLOWB. Cardiovagal BRS improved during SLOWB (21.0 vs 36.0 ms/mmHg, p=.03) with no effect of sex. Despite lower overall BP in females, our data support a lack of basement effect on SLOWB-induced reductions in BP, as SLOWB was equally effective in reducing BP in males and females. Our findings support the efficacy of the RESPeRATE device for reducing BP in both sexes, even in young, normotensive individuals.

Abstraite

La respiration lente (SLOWB) est recommandée comme traitement adjuvant de l'hypertension. Cependant, la mesure dans laquelle les réponses de la pression artérielle (PA) à SLOWB diffèrent entre les hommes et les femmes n'est pas bien établie. Par conséquent, nous avons testé l'hypothèse qu'une poussée aiguë de SLOWB induirait des baisses plus importantes de la pression artérielle chez les hommes que chez les femmes, étant donné que les hommes ont généralement une pression artérielle au repos plus élevée. Nous avons également examiné les facteurs contribuant à l'autonomie réduite de la pression artérielle au cours de la SLOWB, c'est-à-dire l'activité nerveuse sympathique du muscle (ANSI) et la sensibilité cardiovagale spontanée (méthode de la séquence) et la sensibilité du baroréflexe sympathique (BRS). Nous avons testé des femmes normotendues (n = 10, âge: 22 ± 2 ans, IMC: 22 ± 2kg / m2) et des hommes (n = 12, 23 ± 3 ans, 26 ± 4kg / m2). Les sujets ont été testés au début et au cours des 5 dernières minutes d'une session de SLOWB guidée par RESPeRATE de 15 minutes. Globalement, SLOWB a réduit la pression systolique (PAS) de 3,2 ± 0,8 mmHg (effet principal, p <0,01). Les femmes avaient une PAS inférieure (effet principal, p = 0,02); nous n'avons observé aucune interaction entre le sexe et SLOWB. SLOWB a également réduit l'incidence des salves MSNA de -5,0 ± 1,4 rafales / 100hb (effet principal, p <0,01). Bien que l'incidence chez les femmes ait tendance à être plus faible (effet principal, p = 0,1), il n'y a pas eu d'interaction entre le sexe et le SLOWB. La BRS cardiovagale s'est améliorée pendant le SLOWB (21,0 vs 36,0 ms / mmHg, p = 0,03) sans effet du sexe. Malgré une TA globale inférieure chez les femmes, nos données corroborent l'absence d'effet de sous-sol sur les réductions de TA induites par SLOWB, ce dernier étant tout aussi efficace pour réduire la TA chez les hommes et les femmes. Nos résultats confirment l'efficacité du dispositif RESPeRATE pour réduire la pression artérielle chez les deux sexes, même chez les personnes jeunes et normotendues.

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Contribution of Authors

All chapters were written by Tessa E. Adler. Drs. Charlotte Usselman, Jenna Gibbs, and Dennis Jensen contributed advisory feedback for all chapters.

CHAPTER 1: LITERATURE REVIEW

Clinical Relevance of Blood Pressure Regulation

Blood pressure (BP) is one of the strongest predictors for cardiovascular disease, the leading cause of death for people in the United States (128). Even compared to people with only moderately high BP (systolic BP: 130-139 mmHg or diastolic BP: 85-89 mm Hg), people with optimal BP are 20% less likely to experience an adverse cardiovascular event within 10 years (121). Unfortunately, hypertension is exceedingly prevalent. In the United States, it is estimated that 46% of adults are above the threshold for Stage 1 Hypertension (systolic BP: 130-139 mmHg or diastolic BP: 80-89 mm Hg), as defined by the 2017 joint recommendations from the American College of Cardiology and American Heart Association (128). With the goal of reducing the incidence of hypertension, it is important to understand why hypertension occurs and how it might be prevented. From a mechanistic perspective, high BP is often associated with dysfunction in the reflexes or control systems that govern BP regulation. It is therefore critically important to understand how these mechanisms function and how they might be optimized to maintain BPs below the threshold for elevated cardiovascular risk.

Acute Regulation of Blood Pressure

Blood pressure in the human body is in constant flux. It is this dynamism that enables human beings to survive severe hemorrhage, perform prolonged exercise, and tolerate orthostatic challenges. Given the wide variety of circumstances to which BP can adapt, it is perhaps unsurprising that the physiological mechanisms responsible for the regulation of BP are complex and multifactorial. As a complex, self-regulating system, BP regulation is comprised of many individual processes that are collectively both synergistic and redundant (90). Despite the significant mechanistic diversity of specific reflexes and controls that govern BP regulation, these feedback loops generally share a common pattern. First, changes in BP are detected by receptors sensitive to chemical or mechanical stress. These signals are integrated centrally in the brainstem and hypothalamus. Efferent signals travel through the two branches of the autonomic nervous system (sympathetic and parasympathetic) towards the three end-organs that can alter BP: the heart, the vasculature, and the kidneys. Selected feedback loops that govern acute BP regulation will be described in the following sections, with a close focus on differences between the sexes.

AUTONOMIC CONTROL OF BLOOD PRESSURE

The capacity of the autonomic nervous system to mediate changes in blood pressure evolved over 300 million years ago, which underscores its fundamental role in maintaining homeostasis across a wide diversity of species (125). The autonomic nervous system is split into two branches, the parasympathetic (PNS) and sympathetic nervous systems (SNS). The PNS is primarily characterized by cholinergic neurotransmission, in which post-ganglionic release of

acetylcholine stimulates post-synaptic muscarinic acetylcholine receptors located in various effector organs (34). The SNS acts by noradrenergic neurotransmission, in which post-ganglionic release of catecholamines bind to post-synaptic adrenergic receptors located in target organs (38). The PNS and SNS maintain appropriate BP for a given circumstance by altering vascular resistance and cardiac output, the determinants of BP. Vascular resistance can be modulated by inducing vaso-constriction or dilation, and cardiac output can be changed by modifying heart rate and contractility, among other factors. Autonomic balance refers to the relative activity of the PNS and SNS, which generally induce opposing effects on BP, although recent findings have complicated this picture (44, 45). The paramount importance of the autonomic nervous system for short-term BP regulation has long been established (27), while more recent discoveries have demonstrated the significance of the autonomic nervous system as a determinant of long-term BP regulation as well (58).

The PNS and SNS both innervate the heart, where they induce opposing effects (Figure 1). Both are tonically active, though parasympathetic activity predominates at rest (63). Sympathetic neurons stimulate β_1-adrenoreceptors on both cardiac myocytes and pacemaker cells, resulting in a faster and stronger beat. This increases both heart rate and stroke volume, which increases cardiac output and therefore BP (assuming constant vascular conductance). Parasympathetic innervation mainly targets the muscarinic receptors on modified cardiac myocytes of the sinoatrial and atrioventricular nodes, with some innervation of the atria and ventricles (26). For this reason, PNS stimulation of the heart results in a slower beat with minimal impact on contractility (39). PNS-driven decreases in heart rate reduce cardiac output and therefore, BP.

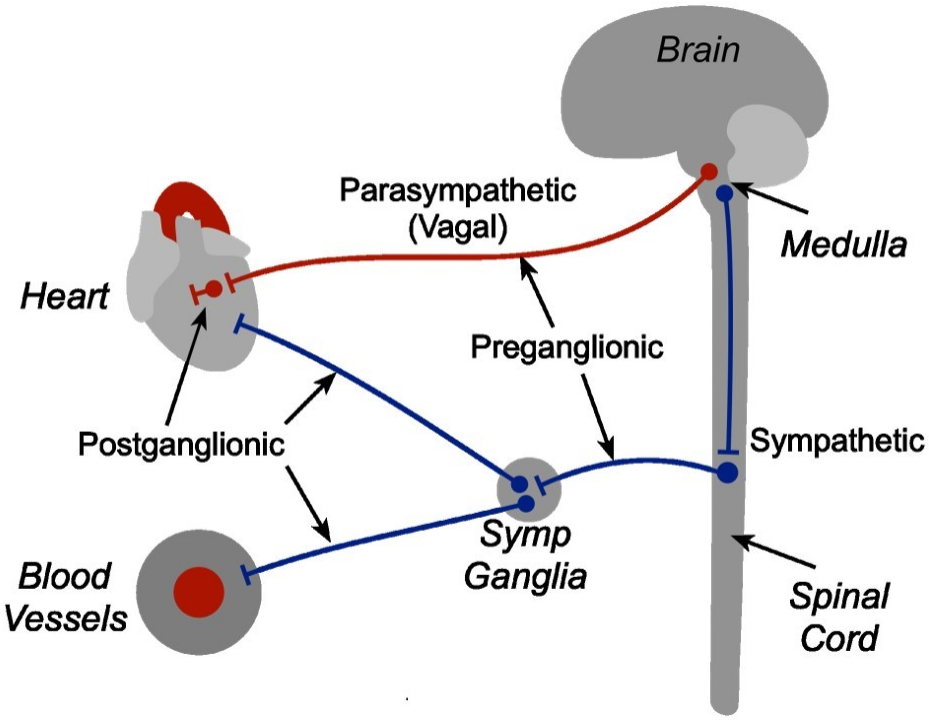


Figure 1: Autonomic innervation of the heart and vasculature. Diagram from Klabunde, R.E. Cardiovascular Physiology Concepts (2016).

While the effects of the PNS on BP are limited to the heart, the SNS also regulates BP by modulating resistance in the peripheral vasculature (Figure 1). Broadly, sympathetic activation at the vasculature induces vasoconstriction, thereby increasing total peripheral resistance and BP (if cardiac output remains constant). Upon activation, post-ganglionic sympathetic fibers release quanta of norepinephrine (NE) into the synaptic cleft where NE binds to receptors on vascular smooth muscle cells that surround the walls of the vasculature (76) (Figure 2). Alpha-adrenoreceptors on vascular smooth muscle cells respond to NE by contracting, thus inducing vasoconstriction (82). By Poiseuille's Law, decreasing radii in the vasculature equates to a rise in total peripheral resistance, which leads to a proportional rise in BP (Figure 3). However, as a complicating factor, β_2-adrenoreceptors are also found on the vascular smooth muscle, and these respond to NE by inducing vasodilation (105). Therefore, the balance of α- and β_2-adrenoreceptors determines the degree to which the vascular smooth muscle cells will constrict in response to a given level of sympathetic activation.

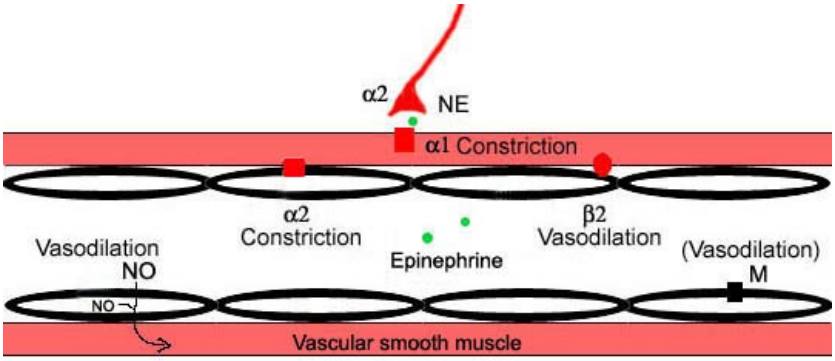


Figure 2: Adrenoreceptors in the vascular smooth muscle. Diagram from the University of California San Francisco, iROCKET learning module. (2007)

$$Q = \frac{\pi P r^4}{8\eta l}$$

Figure 3: Pouiselle's Law. The equation relates flow (Q), pressure (P), vessel radius (r), fluid viscosity (η), and the length of the tube (l).

Sex differences:

In the past twenty years, it has come to light that autonomic regulation of BP differs greatly between males and females (16, 59, 79). Compared to age-matched males, young females have lower resting BP (25, 130) and lower sympathetic activation of the peripheral vasculature, as quantified through measures of muscle sympathetic nerve activity (MSNA) (49, 86). Sex steroids have been implicated as a major contributor to these sex differences (79, 119).

Within the family of sex steroids, the cardiovascular effects of estrogens are the most established, and estradiol is widely acknowledged to be a primary contributing factor to the "cardioprotection" observed in young females (119). A primary mechanism by which estradiol acts on the vasculature is through nitric oxide (NO)-dependent vasodilation, which occurs via the upregulation of both acute production (22) and long-term genomic expression (126) of endothelial NO synthase (eNOS). By upregulating NO, a potent vasodilator, estradiol counteracts sympathetically-mediated vasoconstriction with corresponding vasodilation to maintain BP. The impact of progesterone remains poorly-understood, as it can induce either vasoconstriction or -dilation depending on the hormone's concentration and the location of the target blood vessel (reviewed in (127)). Testosterone exerts divergent effects on BP between the sexes (79). In males, the relationship between testosterone and hypertension remains unclear (79) given the discrepancy between animal studies that indicate a positive relationship between androgens and hypertension (reviewed in (79)), and longitudinal studies demonstrating an inverse relationship between endogenous testosterone production and systolic BP (65). In females, androgen exposure increases both BP (79) and MSNA (107). BP in females is also affected by menstrual cycle phase (29) and aging, with females experiencing a more rapid increase in BP during the menopausal transition than males of similar age (85). Similarly, MSNA in females is affected by menstrual cycle phase (21, 81) and rapidly increases after menopause (85), though it bears

mentioning that these findings regarding the effects of aging on MSNA was a cross-sectional rather than longitudinal study (85). Collectively, these studies point to the major impact of sex steroids on BP regulation in both males and females.

Neurovascular transduction, the translation of MSNA to constriction in the vasculature, is quite distinct between young males and females. In young males, MSNA correlates with total peripheral resistance (TPR) (24), while in young females, these two variables are unrelated (Figure 4) (45). These findings indicate that major sex differences exist in neurovascular transduction. Subsequently, hypotheses for the mechanistic explanation for these sex differences have pointed to the balance of α- and β_2- adrenergic receptors in the vasculature, with respect to either the relative population of these receptors or their sensitivity (44, 53, 59). It was observed that infusing NE in the brachial artery induced brisk and potent vasoconstriction in young males, but not young females; however, these sex differences were eradicated when β_2- adrenergic receptors were blocked with propranolol in the females (44). It has therefore been proposed that sympathetically-mediated vasoconstriction is offset in young females by the vasodilatory effects of β_2- adrenergic receptors (16, 57, 59). Mechanistically, β_2- adrenergic vasodilation occurs via an NO-dependent pathway, so it has been hypothesized that estrogen-associated upregulation of NO may also play a role in determining these sex differences (16).

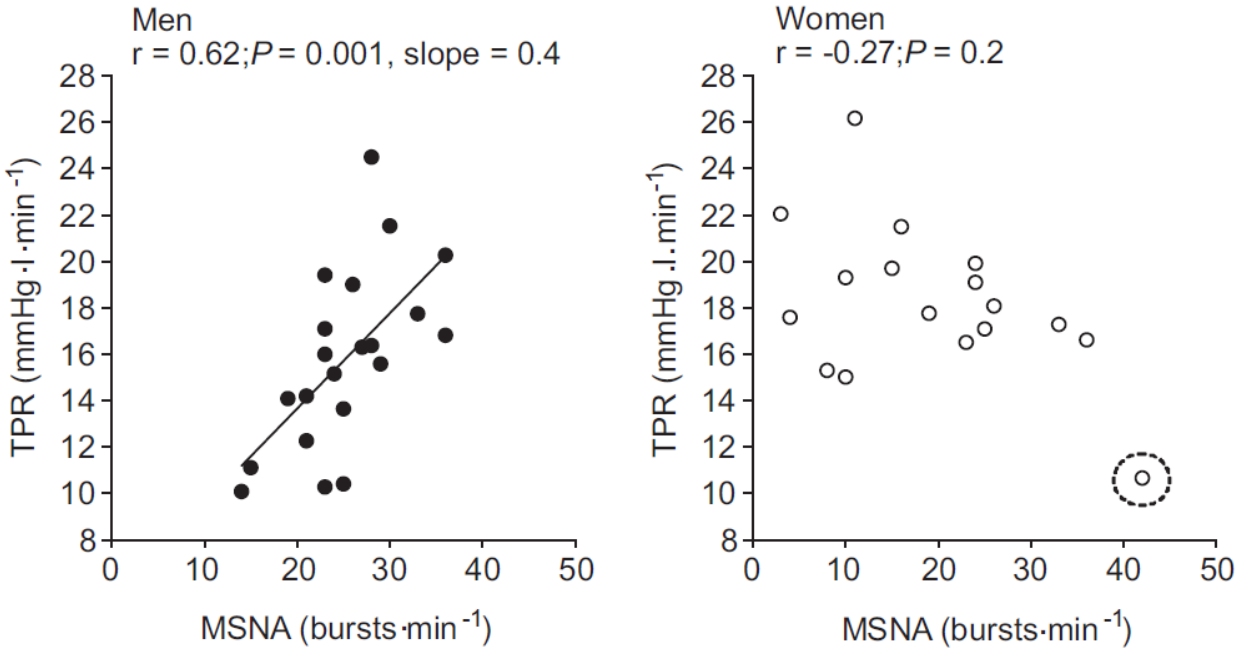


Figure 4: Relationship between total peripheral resistance (TPR) and muscle sympathetic nerve activity (MSNA) in young healthy males and females. In males, there is a positive correlation between MSNA and TPR, while in young females, there is no relationship between these two variables. Adapted from Hart et al. 2009 (45)

MECHANISMS OF ACUTE BLOOD PRESSURE REGULATION

The Baroreflex

The baroreflex is the primary physiological mechanism responsible for regulating acute changes in BP. Baroreceptors are spray-type, mechano-sensitive nerve endings located in the walls of large arteries in the neck and chest, predominantly the carotid sinus and the aortic arch. When BP rises, baroreceptors are stimulated by the corresponding stretch of the arterial walls. The afferent signals from aortic and carotid baroreceptors travel via the vagal and glossopharangeal nerves, respectively, to the medulla oblongata. Within the nucleus tractus solitarius, the baroreceptor input is organized into two distinct efferent arms. In one efferent pathway, inhibition of the sympathetic vasoconstrictor center decreases outflow of MSNA; this results in vasodilation of the peripheral vasculature and decreased total peripheral resistance. In parallel, the excitation of the parasympathetic center results in vagal release of acetylcholine at the sinoatrial and atrioventricular nodes of the heart, thereby decreasing heart rate. The two efferent arms of the baroreflex act in concert to decrease BP by decreasing both total peripheral resistance and cardiac output (via decreased heart rate). The baroreflex feedback loop is summarized in Figure 5.

Baroreflex sensitivity (BRS) refers to the capacity of the baroreflex to buffer changes in BP (Figure 6). Due to the fact that the baroreflex decreases BP by two distinct efferent arms, cardiovagal and sympathetic BRS can be assessed separately. Cardiovagal BRS is the relationship between systolic BP and heart rate (reflecting the cardiac effect of the PNS) while sympathetic BRS is the relationship between diastolic BP and muscle sympathetic nerve activity (reflecting the vasoconstrictor effects of the SNS).

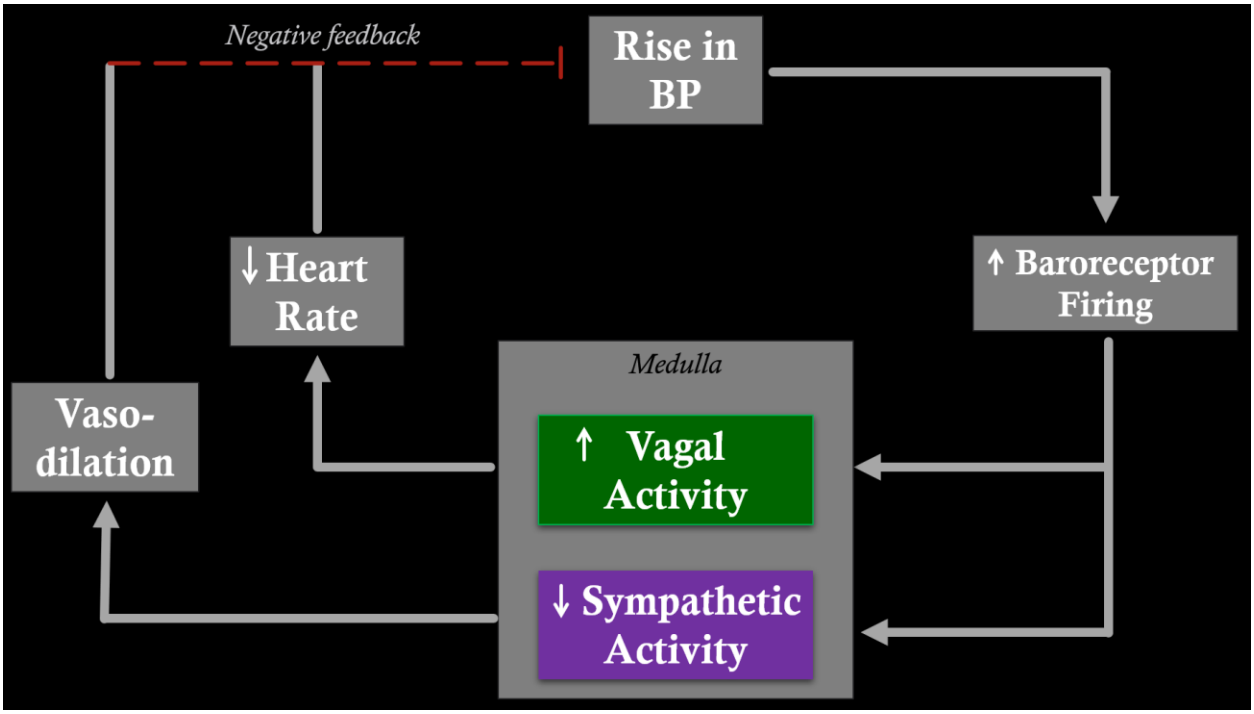


Figure 5: Diagram of the baroreflex negative feedback loop. Following a rise in BP, baroreceptors are stimulated by mechanical stretch in the carotid sinus and aortic arch. Increased baroceptor firing rate is integrated at the medulla, inhibiting the sympathetic vasoconstrictor center and increasing vagal outflow. This results in increased vasodilation and decreased heart rate, with a net effect of reducing BP.

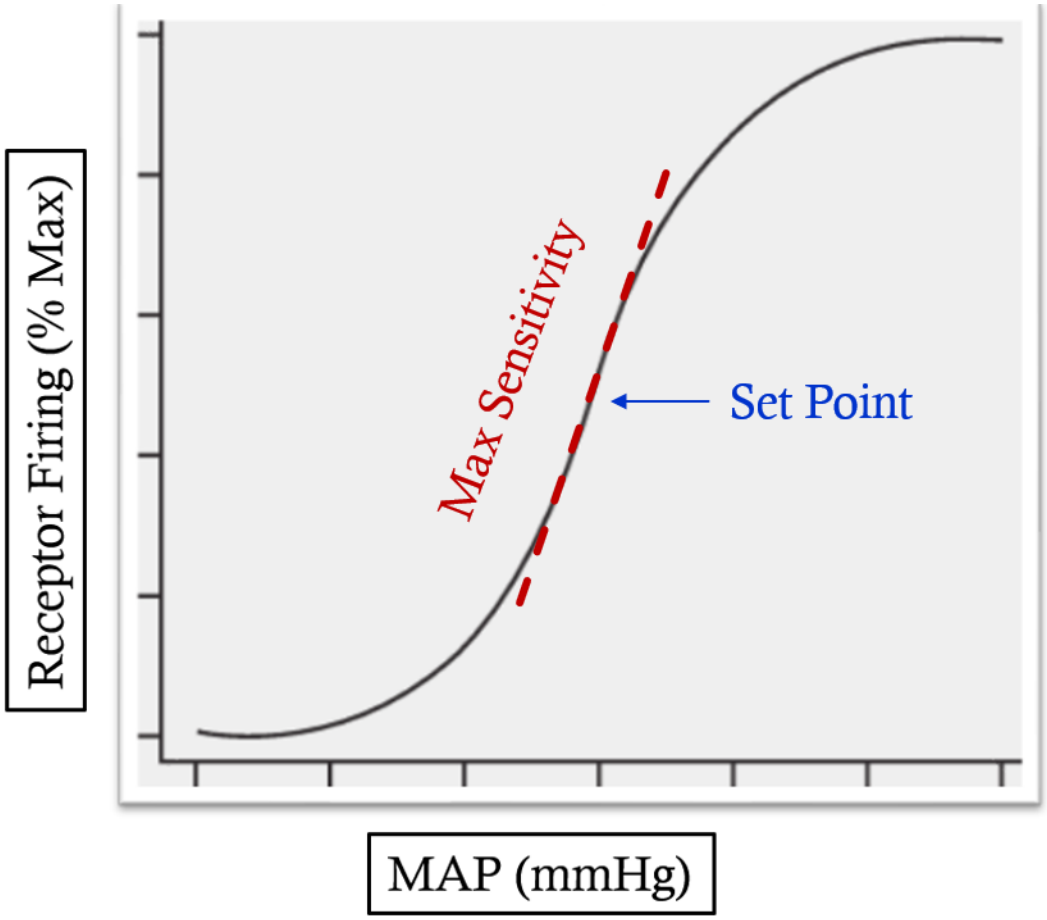


Figure 6: Sigmoidal relationship between mean arterial pressure (MAP) and baroreceptor firing rate. Above a threshold, baroreceptor firing increases in frequency as mean arterial pressure increases, until a saturation point is reached. Two aspects of this curve are notable for their important implications for cardiovascular health. The gain refers to the maximum slope of the response curve; this indicates the sensitivity of the baroreceptors to changes in arterial pressure (i.e. maximum sensitivity). The set point is the arterial pressure that the reflex maintains.

Sex differences:

In assessments of resting baroreflex function in young healthy males and females, some groups have reported no differences between the sexes with respect to either cardiovagal or sympathetic baroreflex sensitivity (30, 110, 111). Other studies have indicated that females have reduced baroreflex buffering of BP (25, 100) and reduced cardiovagal baroreflex gain (12). One recent study has shown that females may rely more on cardiovagal baroreflex control of BP, while males rely more on the sympathetic arm of the reflex (67). While further research is needed to clarify differences in baroreflex function between the sexes, these studies indicate that there may be sex differences in functional reliance on different efferent arms of the baroreflex to achieve BP outcomes. Additionally, recent studies have shown that cardiovagal and sympathetic baroreflex sensitivity are uncorrelated in young people (30, 111), underscoring that the ability to regulate BP via each pathway is quite distinct. Given these findings, investigations into baroreflex function are incomplete without information regarding both efferent arms of baroreflex function.

THE CHEMOREFLEX

The chemoreflex is a feedback loop that acts to maintain appropriate blood chemistry (i.e., pH) and oxygen levels by inducing both respiratory and cardiovascular changes. Activation of the chemoreflex induces both stimulation of ventilation and cardiovascular changes in response to changes in arterial blood chemistry (62). Chemoreceptors detect chemical changes in the concentration of hydrogen ions in the bloodstream, which reflects the partial pressures of carbon dioxide and oxygen in the bloodstream. Peripheral chemoreceptors located in the carotid bodies are primarily sensitive to depressed oxygen concentrations in the bloodstream (hypoxia),

whereas central chemoreceptors, located in the brainstem, are primarily sensitive to elevated concentrations of carbon dioxide (hypercapnia) (62). In response to either hypoxia or hypercapnia, the chemoreflex stimulates the vasomotor center of the brainstem, eliciting increased sympathetic nerve activity and thereby increasing BP (62). Under resting normal physiological conditions in healthy humans, the changes in arterial oxygen concentrations with respiration are insufficient to elicit a peripheral chemoreflex response that would affect BP or heart rate (15). Further, activation of the baroreflex (in response to high BP) directly inhibits both the respiratory and autonomic effects of the chemoreflex (reviewed in (62)). Given these factors, the effects of chemoreflex activation on BP are more significant at lower levels of mean arterial pressure (43), when the baroreflex is not simultaneously activated.

Sex differences:

The effects of sex on chemoreflex-driven sympathetic excitation remain poorly understood (120). In one investigation, hypoxic breathing induced the same degree of sympathetic excitation in young healthy males and females, though activation of MSNA peaked earlier in females than males (54). This study did not control for phase of menstrual cycle (54). In another study, sympatho-excitation in response to severe chemoreflex stress (i.e., a maximal voluntary end-inspiratory apnea after re-breathing) was similar between young healthy males and females only during the mid-luteal phase, when endogenous hormone production is high (118). During the early follicular phase, when endogenous hormone production is low, sympatho-excitation in females exceeded that of males (118). Though data is limited, these two studies indicate that chemoreflex activation of the SNS may be exaggerated in young females compared to age-matched males, depending on menstrual cycle phase (120). It is currently unknown

whether these sex differences are primarily linked to central or peripheral chemoreflex activation.

RESPIRATORY SINUS ARRYTHMIA

Heart rate variability (HRV), an important clinical marker of cardiovascular health, refers to the dynamic changes in heart rate that occur on a beat-to-beat basis in healthy humans. Reduced HRV is strongly associated with both modifiable and non-modifiable cardiovascular risk factors, and factors that increase (improve) HRV are associated with reduced cardiovascular risk (see (113) for a recent review). Respiratory sinus arrythmia (RSA), which specifically refers to the cyclic fluctuations in heart rate that correspond to breathing frequency, is the primary determinant of HRV in the high-frequency band of oscillations (0.15 to 0.40 Hz) (13). In the 19th century, Carl Ludwig first described the tendency of the heart rate to increase during inspiration and decrease during expiration (78). RSA results in periodic oscillations in BP, with an increase in MAP at the beginning of expiration, while MAP is slightly decreased during the rest of the respiratory cycle (43, 52). Both the mechanisms that generate RSA and its potential physiological role remain controversial (70).

The most well-established theory for the mechanism generating RSA involves the interaction of the respiratory and circulatory centers in the brainstem (32). The impact of respiration rhythm on autonomic responsiveness has been termed “respiratory gating” (32, 77). During inspiration, it has been shown that chemoreceptor activation of the respiratory center of the medulla “spills over” into the vasomotor center, hyperpolarizing the cardiac vagal neurons and rendering them briefly unresponsive to baroreceptor input, which increases heart rate (37). A second central mechanism for RSA involves the activation of slowly adapting pulmonary stretch

receptors during inspiration (108). This afferent feedback from the lungs induces vagal withdrawal, which induces tachycardia (5, 108). In double-lung transplant patients with intact hearts, RSA is present but blunted (53% of that in healthy subjects), indicating that pulmonary stretch receptor feedback via the vagal nerve plays a necessary role in generating RSA (108).

Respiratory changes in intrathoracic pressure may also play a role in inducing RSA (5, 10, 114). During inspiration, the diaphragm muscles generate negative pressure in the thoracic cavity (10). The corresponding drop in transmural pressure between the pulmonary vasculature and the thoracic cavity induces passive vasodilation in the pulmonary veins, which reduces the flow of oxygenated blood to the left atrium (43). This directly reduces stroke volume and cardiac output (114). It has been theorized that the corresponding drop in BP would trigger an inhibition of baroreceptor afferent activity, subsequently mediating a reflex increase in heart rate and BP (5, 114). However, the contribution of the baroreflex to RSA remains controversial (70, 131), with some suggesting that the time latency associated with the baroreflex (~1.5 seconds) renders this explanation implausible (31). Further, in heart transplant patients, RSA is blunted but present, indicating a necessary role for non-neural factors in generating RSA (10, 101). The prevailing hypothesis for a non-neural mechanism is that increased intrathoracic pressure during inspiration induces mechanical stretching of the sinoatrial node, and this results in a slight cardiac acceleration during inspiration (70, 101).

Sex differences:

Young healthy females have higher RSA than young males, as measured by spectral analysis of the high-frequency range of HRV (55) and mean difference in inter-beat interval during inspiration and expiration (103). Further, a recent meta-analysis of over 60,000 young adult participants illustrated major differences in HRV between the sexes (68). The ratio of low-

frequency to high-frequency HRV is lower in young females than males (68), indicating higher RSA and potentially, greater parasympathetic than sympathetic control of heart rate in females (68), although it remains controversial whether PNS and SNS influences on heart rate can be distinguished by these frequency components (113). Sex differences in RSA disappear after the age of 50 (117). Given the endocrinological changes associated with the menopausal transition (115), this observation suggests a potential role for sex steroid hormones in mediating differences in RSA among young healthy adults. One potential explanation for these observed sex differences is the presence of estrogen receptors in the medulla (96). It is therefore possible that estrogen levels in premenopausal females may modulate the "respiratory gating" of autonomic outflow, which could alter the magnitude of RSA.

MAYER WAVES

Mayer waves are spontaneous fluctuations in BP that oscillate at a frequency slower than respiration (~0.1 Hz) (60). These waves have also been termed "vasomotor waves" because these waveforms cohere most strongly with oscillations in sympathetic nerve activity, and their amplitude increases during sympathetic activation (see (60) for review). It is most likely that Mayer waves owe their existence to the baroreflex, which displays the intrinsic resonance of any closed negative feedback loop (60). As described above, a rise in BP triggers baroreceptor excitation, which inhibits sympathetic outflow and therefore reduces BP. This drop in BP would then reduce baroreceptor firing, thereby disinhibiting sympathetic outflow and increasing BP. These fluctuations in BP are therefore both spontaneous and self-sustaining (60). On the basis of this model, it has been proposed that Mayer waves serve no particular physiological purpose and are a secondary consequence arising from the intrinsic resonant properties of the baroreflex as a negative feedback loop (23).

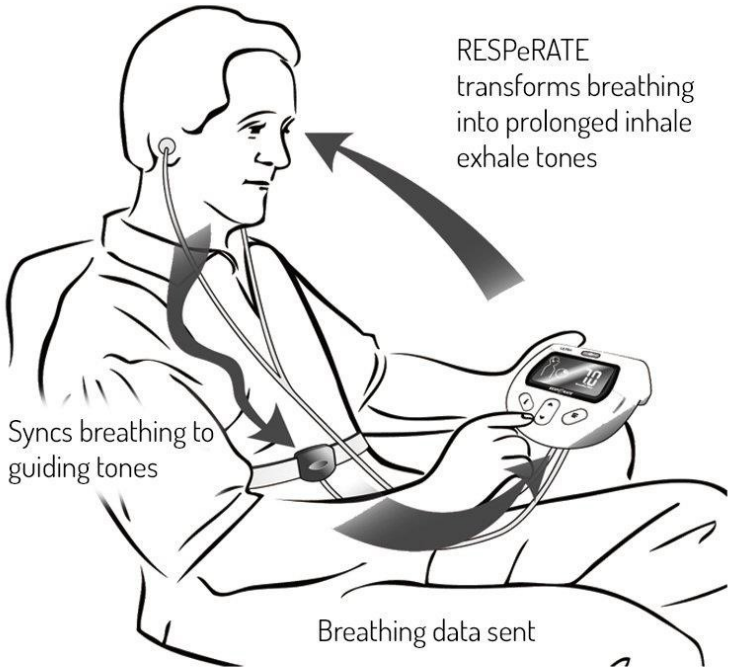
Sex differences:

In the most recent review of Mayer waves, it was stated that no sex differences exist with respect to either the frequency or amplitude of these oscillations (60). However, it must be noted that the only study to directly compare these variables between young healthy males and females did not control for the effects of hormonal status or menstrual cycle (112). This is a serious limitation given known effects of sex hormones on BP regulation (127). Therefore, it would be imprudent at present to rule out the possibility that sex may modulate the frequency or amplitude of Mayer waves.

Slow Breathing and Blood Pressure Regulation

WHAT IS SLOW BREATHING?

Slow breathing, defined in a recent review as a respiration rate of 4-10 breaths per minute (95), induces various effects on the respiratory, cardiovascular, and autonomic nervous systems. The practice of slow breathing was developed by Eastern cultures; pranayama yoga breathing has been practiced for thousands of years as both a spiritual and health-enhancing technique (18). Since the mid-20th century, the scientific community has documented the physiological effects and potential health benefits of slow breathing, including reduced BP, increased respiratory efficiency, and a shift towards parasympathetic rather than sympathetic dominance (95). This increase in scientific attention has culminated in clinical recognition, with the American Heart Association's 2013 recommendation of slow breathing as an effective adjunctive treatment for reducing BP in people with hypertension (17). Specifically, the AHA evaluated evidence associated with use of the RESPeRATE (17), (Intercure Ltd., Israel), an FDA-approved device that plays interactive guiding tones to coach an individual to slowly reduce their respiration rate to a targeted "therapeutic breathing zone" (5-10 breaths/minute) over the course of a session (between 10-60 minutes) (Figure 7).



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436 **Figure 7: Diagram illustrating proper use of the RESPeRATE device for slow breathing.**

437 Image from Intercure Ltd., Israel.

EFFECTS OF SLOW BREATHING ON BLOOD PRESSURE

Slow breathing has been shown to reduce BP, both in the short- and long-term. In laboratory settings, slow breathing has been shown to acutely reduce BP in young, healthy individuals (28, 83) and in various clinical populations, including people with type 1 diabetes (9) and hypertension (56). With respect to chronic changes, a recent meta-analysis of thirteen clinical trials (608 individuals in the pooled sample population) found that, on average, eight weeks of daily slow breathing with the RESPeRATE device reduces BP by 4/3 mmHg (systolic/ diastolic BP), after accounting for the placebo effect in control groups (17). A threshold effect has been observed, in that participants who achieved at least 23 min of slow breathing per week experienced clinically meaningful (>3 mmHg) reductions in BP (33). On the basis of this meta-analysis, the AHA indicated Level of Evidence B (moderate quality of evidence) and Class of Recommendation IIA (moderate recommendation) for the efficacy of the RESPeRATE device in reducing BP in people with hypertension (17).

PROPOSED MECHANISMS OF ACTION

The mechanism by which slow breathing reduces BP remains incompletely understood (17), although there is evidence for the involvement of multiple physiological pathways (see (95) for a recent review; Figure 8). It has been suggested that slow breathing induces an autonomic balance that is favorable for cardiovascular health (95). Indeed, slow breathing with the RESPeRATE device has been shown to reduce MSNA, compared to baseline (7, 48, 99) and compared to listening to relaxing music (88). Slow breathing has also been shown to acutely improve cardiovagal baroreflex sensitivity (9, 56, 91, 92) and sympathetic baroreflex sensitivity (91), although it must be noted that this measure of sympathetic baroreflex sensitivity relied on

462 indirect measures rather than the direct method of microneurography. There is also evidence for
463 the relevance of chemoreflex sensitivity in achieving reduced BP with slow breathing (8). In
464 young healthy individuals, it was shown that reducing the breathing rate from 15 to 6 breaths per
465 minute reduced the chemoreflex response to both hypercapnia and hypoxia (8). One potential
466 mechanistic explanation for this finding is that baroreflex activation during slow breathing may
467 inhibit activation of the chemoreflex (62).

468 Slow breathing may enhance phasic modulation of sympathetic activity by entraining the
469 baroreflex (50, 95). Slow breathing increases RSA (50, 91, 122). Further, when slow breathing is
470 performed at 0.1 Hz (equivalent to 6 breaths per minute), the oscillations in BP due to RSA
471 synchronize with the frequency of Mayer wave oscillations (50). This synchronization amplifies
472 HRV and may entrain the baroreflex, thereby improving the capacity of the baroreflex to buffer
473 changes in BP (95).

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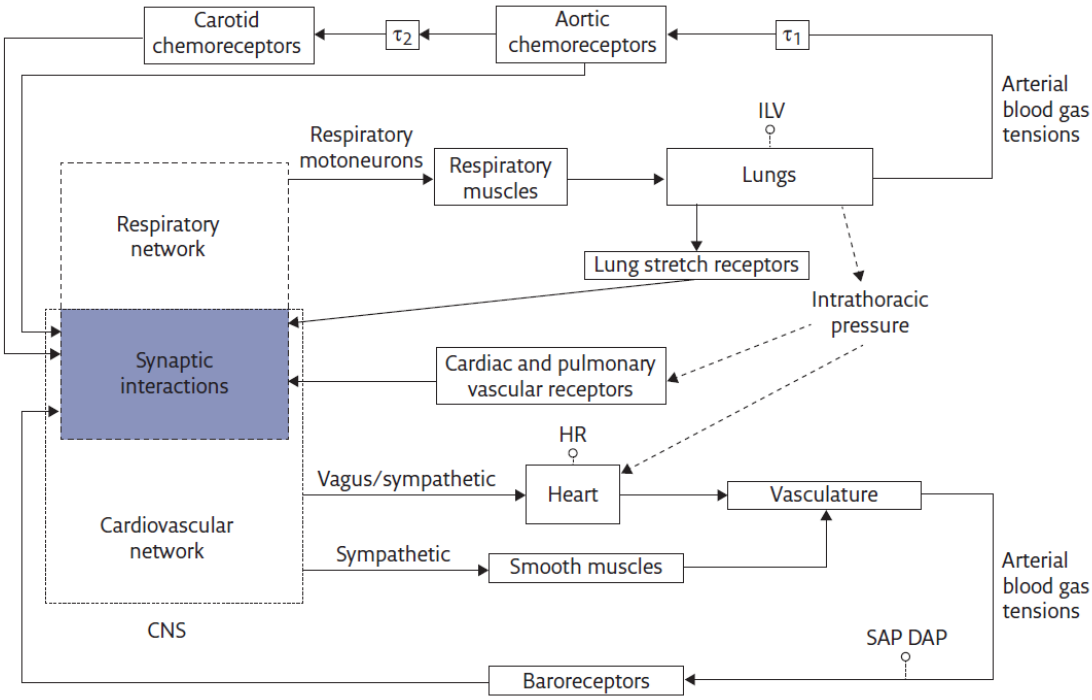

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476 **Figure 8: Overview of respiratory and cardiovascular regulation: aspects relevant to slow**

477 **breathing.** Figure from Russo et al. 2017 (95).

478

Sex Differences

The effect of sex on BP and autonomic responses to slow breathing in young healthy individuals remains to be fully elucidated. Most studies investigating the effects of slow breathing on BP have focused on hypertensive and older (i.e., post-menopausal) individuals (3, 4, 11, 20, 33, 40, 56, 74, 88, 93, 97, 98, 123, 124). Some studies of the long-term effects of slow breathing on BP in older individuals included sex as a covariate, among multiple other variables, and found that it did not affect BP outcomes (20, 97, 98). In one investigation, device-guided slow breathing had a significant effect on daytime BP in post-menopausal females only, compared to age-matched males (4). This finding led these authors to suggest that slow breathing may be more tightly linked to changes in autonomic arousal in females than males (4), though it must be noted that this assertion can only be applied to post-menopausal females, given the sample population. The American Heart Association's 2013 recommendations regarding slow breathing makes no mention of potential sex differences, and none of the thirteen studies included in the corresponding meta-analysis included young females in their sample (17).

To date, no study has been designed to investigate whether sex affects the autonomic mechanisms associated with slow breathing. Many investigations into physiological mechanisms associated with slow breathing have focused exclusively on males (7, 28, 48, 50, 56). Among those that included females, none have examined potential sex differences within the sample nor properly accounted for hormonal status by current standards (i.e., menstrual cycle phase, menopausal status, hormonal contraceptive use) (9, 56, 73, 92).

Methods to Assess Autonomic Function

MICRONEUROGRAPHY

As action potentials travel through sympathetic neurons from the brainstem to the rest of the body, the corresponding electrical activity can be measured in fully conscious human subjects. This technique, called microneurography, was first achieved in 1965 by Hagbarth and Vallbo at the Uppsala Academic Hospital in Sweden (42). Microneurography can be performed in a variety of nerves to suit the experimental aims. The deep branch of the peroneal nerve typically facilitates other aspects of experimentation and is most commonly recorded by microneurographers (129). The peroneal nerve of the lower leg, accessed dorsally to the fibular head, is relatively easy to immobilize for long periods of time in various postures for experimentation (from supine to seated-upright) (129). In microneurography, as depicted in Figure 9, a small recording electrode is inserted transcutaneously into a nerve and positioned within recording range of post-ganglionic sympathetic axons (42). A second electrode is embedded in the nearby skin and serves as a reference by which changes in the electrical potential of the recording electrode can be detected (42). Electrodes are typically made of tungsten, a highly conductive but biologically inert metal (129).

In the decades since the invention of microneurography, standards of data collection have been established (129). Within the nerve bundle, activity from sympathetic neurons that innervate both the skin and muscle may be detected, and this interference threatens the quality of subsequent data analysis (129). Fortunately, there are established techniques to verify whether the electrode is positioned in recording range of muscle sympathetic neurons, skin sympathetic neurons, or a mixture of the two. Muscle sympathetic neurons burst with greater frequency

526 during an apnea and fire in a pulse-synchronous fashion, whereas skin sympathetic neurons are
527 responsive to startle stimuli (i.e., loud noise) and not pulse-synchronized (41). It is also
528 imperative to avoid recording activity from motor units, which result from muscle tension and
529 can be prevented by ensuring participant comfort and properly immobilizing their lower limb
530 throughout the experiment (129). Generally, a high-quality microneurographic recording has a
531 signal-to-noise ratio of 3:1 (129). After an adequate raw signal is acquired, it is amplified,
532 filtered, rectified, and integrated. Acquisition of a high-quality MSNA recording facilitates
533 subsequent data analysis.
534

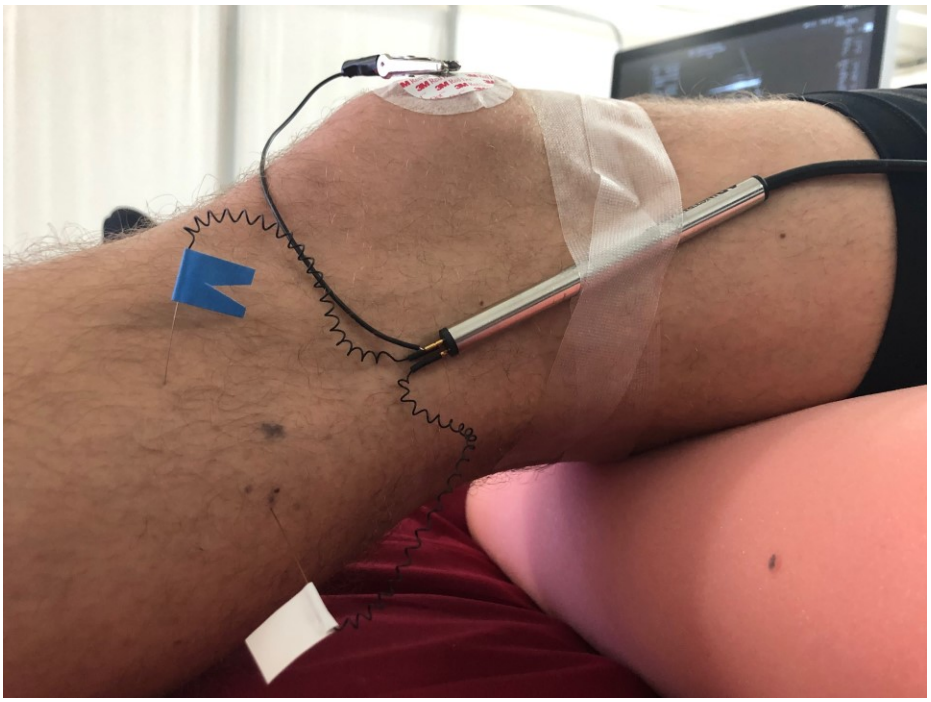


Figure 9: Microneurography experimental set-up. The recording electrode, indicated by the white flag, is inserted transcutaneously into the peroneal nerve. The reference electrode, indicated by the blue flag, is embedded in subcutaneous tissue located near the site.

Analytical Techniques

Proper identification of sympathetic bursts requires training and adherence to established, physiologically-based standards. First, due to the delay in signal conduction from the brainstem to recording site, MSNA data must be time-shifted backwards by ~1-1.4 seconds. Second, as previously mentioned, muscle sympathetic bursts are pulse-synchronous and only occur once per cardiac cycle during diastole. Therefore, each presumed burst in the time-shifted MSNA signal must be verified by ensuring that the initiation of the burst occurs in coordination with both diastole and the R-wave of an ECG signal, and that no two bursts occur within the same cardiac cycle.

Quantification of MSNA can be performed by a variety of approaches. Given the utility and limitations of each approach, it is currently recommended to report MSNA as quantified by the three most common methods: burst frequency, burst incidence, and total MSNA.

Burst frequency, the first established method for MSNA quantification, is simply the number of identified bursts per minute. This approach is intuitive and useful, but its utility for between-subject comparisons is limited because muscle sympathetic bursts can only occur once per cardiac cycle. Therefore, subjects with a faster heart rate have more “opportunities” for sympathetic bursts to occur. A normalization technique was developed to account for this variation: Burst incidence is calculated as the number of bursts per hundred heart beats. Finally, total MSNA accounts for the amplitude of each burst in addition to the rate of burst firing. Typically, burst amplitude is calculated by assigning a relative value of 100 to the tallest burst that occurred during quiet rest, and a relative value of 0 to the mean non-bursting voltage (i.e., background noise). All identified bursts are assigned values in arbitrary units respective to this

scale. Total MSNA for a given epoch is then calculated by multiplying the mean burst height throughout the epoch by either burst frequency or burst incidence.

BAROREFLEX SENSITIVITY

Historically, baroreflex sensitivity (BRS) has been investigated in human subjects by measuring pressor responses to pharmacological interventions (102) or physical stimulation of the carotid baroreceptors (104). In the modified Oxford technique, human participants are injected with a potent vasodilator (sodium nitroprusside) followed in quick succession by a vasoconstrictor (phenylepinephrine hydrochloride) via the brachial artery (94). This 2-minute sequence of infusions induces a wide range of BPs (varying by about 30 mmHg), which enables visualization of the entire sigmoidal BRS curve (36). The response of the baroreceptors to these drastic changes can be measured in both efferent arms of the reflex (cardiovagal and sympathetic).

While the modified Oxford technique has often been described as the “gold standard” for measuring BRS, researchers have recently pointed out that there are serious limitations for the utility of this approach to measure sympathetic BRS (111). Particularly in young people, the high BPs associated with the administration of phenylepinephrine hydrochloride result in near-complete inhibition of sympathetic nerve activity (30), which precludes the measurement of sympathetic baroreflex gain. Therefore, non-invasive techniques to assess spontaneous BRS may yield a more comprehensive picture of both efferent arms of the baroreflex in young healthy subjects (111).

It is now known that investigations into overall BRS are incomplete without information about the function of both efferent arms of the reflex. Recent studies have indicated that cardiovagal and sympathetic indices of BRS are not correlated in young healthy individuals (30,

111). When groups were stratified by sex, it revealed that cardiac BRS might predict some of the variation in sympathetic BRS among young females, but not young males (30, 111). These researchers have therefore concluded that indices of cardiovagal and sympathetic BRS cannot stand on their own as a complete picture of baroreflex function; studies must investigate both arms of the reflex (30, 111).

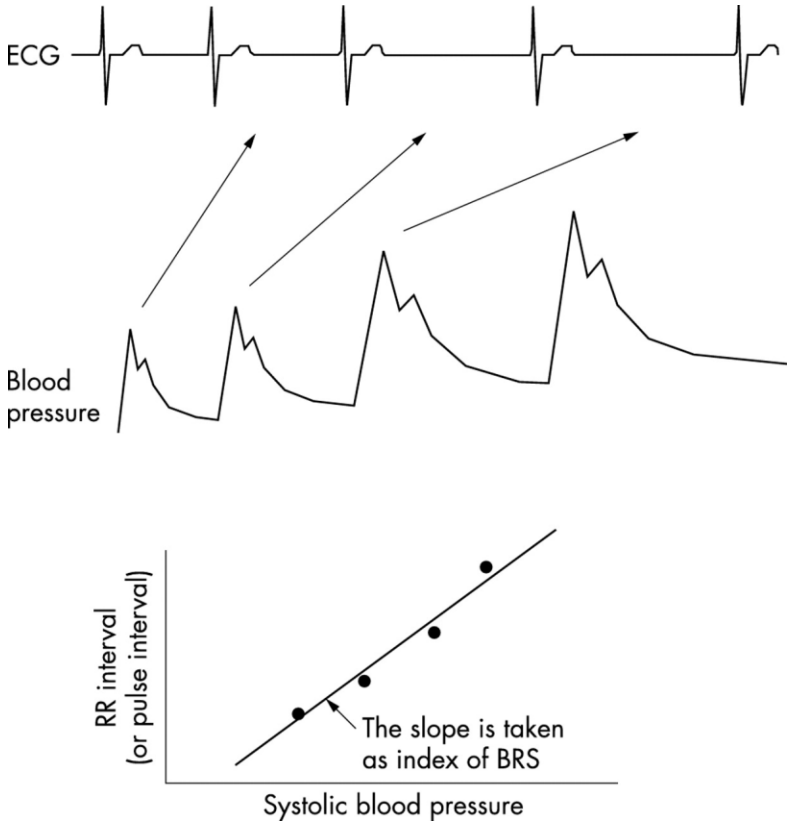
CARDIOVAGAL BAROREFLEX SENSITIVITY

Upwards of twenty distinct methodological approaches have been developed to estimate spontaneous cardiovagal BRS (71). Most of these approaches rely on one of two major techniques: spectral analysis or the sequence method. The spectral analysis method involves computation of the *alpha*-coefficient in either the low- or high-frequency bands of HRV (71). In the sequence method, the slope between changes in heart rate and changes in BP is reported as the index of BRS (71).

In the sequence method (Figure 10), automated analysis detects sequences in which a given number of consecutive cardiac cycles exhibit synchronous increases or decreases in BP and heart rate. Specific approaches using the sequence method differ in two main ways: first, the specific measures of BP and heart beat rhythm used; and, second, the threshold criteria used to validate a given sequence. The standardized values for BRS parameters are systolic BP and R-R interval. While alternative measures of BP (i.e., mean BP) and heart beat rhythm (i.e., pulse interval) have been used, these alternative measures result in poor concordance with other indices of BRS such as the spectral method and Z-method (71).

Various threshold criteria have been developed with the intention of reducing artifacts and verifying whether a given sequence is truly a spontaneous baroreflex response, with the goal of increasing the reliability of the BRS index. These thresholds include: the number of

610 consecutive heartbeats required per sequence, the correlation coefficient between changes in BP
611 and R-R interval in each sequence, and the number of sequences that determine a BRS estimate
612 (71). However, as threshold requirements become more stringent, they can limit the ability of the
613 procedure to estimate BRS if few sequences meet the criteria. In a validation study that included
614 subjects with known baroreflex impairment (i.e., heart failure and diabetes mellitus with known
615 neuropathy), approaches with higher thresholds for sequence inclusion were completely unable
616 to report a value for BRS in these individuals (71).
617



618

619

620 **Figure 10: Sequence method for assessing cardiovagal baroreflex sensitivity.** Concurrent

621 increases or decreases in both R-R interval and SBP represent individual sequences. The mean

622 slope of all individual sequences within a dataset is used as an index of cardiovagal BRS.

623

SYMPATHETIC BAROREFLEX SENSITIVITY

While assessment of cardiovagal BRS has been performed since the 1960s, a methodological approach for assessing sympathetic BRS was established more recently (66). In this approach, a measure of sympathetic activity is plotted against diastolic BP, because baseline sympathetic nerve activity is most tightly linked to diastolic BP (46, 66). Recently, it has been recommended to quantify sympathetic BRS as a function of both MSNA burst incidence and total MSNA, as the two measures can yield distinct information (129). Compared to other indices of sympathetic activity, burst incidence has been shown to yield a greater number of significant baroreflex slopes for analysis (66), but total MSNA may provide important insights into the functional impact of sympathetic activity on the vasculature (30, 64).

Similar to assessment of cardiovagal BRS, sympathetic BRS requires at least 5 minutes of data collected during quiet rest (46). First, MSNA burst incidence and total MSNA must be quantified for the duration of the protocol. Next, MSNA data for each cardiac cycle is binned by diastolic BP. Usually, these bins are 3-mmHg to account for variation in diastolic BP due to factors outside of the baroreflex, such as RSA (46). The number of bursts per hundred heart beats in each 3-mmHg bin is then plotted against diastolic BP, and linear regression analysis is performed. The slope of the line is taken to represent sympathetic BRS. For total MSNA, the total MSNA per 3-mmHg bin is plotted against diastolic BP and again, the slope is reported as an index of sympathetic BRS. Some groups incorporate a threshold criteria of a minimum correlation coefficient of 0.5 to report the corresponding slope (47, 111). However, it has been noted that this may exclude individuals with low sympathetic BRS (111).

CHAPTER 2: MANUSCRIPT

Introduction

Based on the 2017 AHA guidelines for blood pressure (BP) management, 46% of adults in the United States have hypertension (128). These numbers are equally concerning for women as for men— 43% of adult women are hypertensive (128). Given that even slightly elevated BP is associated with increased risk of adverse cardiovascular events (121), and that approximately 13-16% of adults with high BP have drug-resistant hypertension (1), the evaluation and establishment of non-pharmacological treatments is clearly a public health priority.

Slow breathing (SLOWB; <10 breaths/min) has a variety of favorable effects on the cardiovascular system, including the clinically relevant effect of reducing BP (95). Even in normotensive middle-aged women and men, a single 2-minute bout of SLOWB has been shown to reduce systolic BP by 3.4 to 3.9 mmHg (83). Persistent reductions in 24-hour ambulatory BP have been observed after as little as 1-2 weeks of daily SLOWB sessions (93). As a result, in 2013 the American Heart Association (AHA) issued a Class IIA recommendation for SLOWB as an effective adjuvant treatment to lower BP in hypertensive individuals (17). However, investigations into the effects of device-guided SLOWB on the cardiovascular system have largely focused on older, hypertensive individuals (17). Interestingly, it has been shown that *pranayama* breathing, a slow breathing technique practiced in yoga, can acutely reduce BP in young normotensive adolescents of both sexes (69). As BP in adolescence is strongly predictive of BP in middle age, these data present the intriguing possibility that SLOWB could be usefully employed as a preventative therapy against future development of hypertension in normotensive individuals (61).

Given the potential therapeutic benefits associated with SLOWB, it is imperative to understand whether this BP-lowering effect is sex-dependent. Moreover, while the precise mechanisms by which SLOWB reduces BP have yet to be fully elucidated (95), several of the key mechanisms which are thought to govern the BP-lowering effects of SLOWB are known to differ between the sexes. From a mechanistic perspective, previous investigations into the role of the autonomic nervous system in mediating the BP response to SLOWB either focused exclusively on men (7, 28, 48, 56) or did not address potential sex differences within the sample (9, 56, 73, 92). Potentiation of the baroreflex is thought to play a major role in the BP response to SLOWB (91, 95), as SLOWB acutely increases cardiovagal baroreflex sensitivity (9, 56, 91, 92) and reduces sympathetic nerve activity (7, 48, 88, 99). With respect to potential sex differences in these mechanisms, it has been established that basal sympathetic outflow is higher in young men relative to age-matched women (49, 86). Likewise, young women exhibit blunted cardiovagal and sympathetic baroreflex responsiveness to decreases in BP relative to similarly-aged men (6, 25, 100). Further, cardiovagal and sympathetic baroreflex sensitivity are slightly correlated in young healthy women, while these measures are completely unrelated in men (30, 111). Due to these sex differences and mounting evidence that autonomic regulation is fundamentally different between the sexes (59), we therefore posit that sex may exert an effect on the neural and cardiovascular outcomes of SLOWB. In other words, there exists a strong possibility that device-guided SLOWB may not be an equally effective BP-lowering adjunctive therapy in women and men.

The purpose of this study was to determine whether there is an effect of sex on BP and associated autonomic responses to an acute 15-min bout of device-guided SLOWB among young healthy individuals. We hypothesized that SLOWB-induced reductions in systolic BP would be

693 greater among healthy young normotensive men than women, because young men have higher
694 resting systolic BP than similarly-aged women (35). In order to investigate the role of the
695 autonomic nervous system in mediating these responses, we compared SLOWB-induced changes
696 in sympathetic and cardiovagal baroreflex sensitivity between women and men.

697

Methods

Participants: We recruited 22 healthy participants aged 18-35, grouped by sex assigned at birth (10 female; 12 male). As the present study was designed to examine differences on the basis of sex, not gender, groups are divided into male and female individuals rather than men and women. Participants were physically active, non-obese, non-smokers with no known endocrinological, respiratory, and cardiovascular diseases. Participants were normotensive (<130/80 mmHg) and were not taking any medications. We recruited potential participants via posters in the community who participated after providing written, informed consent. This study conformed to guidelines in the *Declaration of Helsinki* and was approved by the Faculty of Medicine Institutional Review Board at McGill University (IRB Study Number A05-M14-18A).

Experimental procedures: On a separate day before testing, participants were familiarized to non-invasive aspects of instrumentation and trained to perform SLOWB using the RESPeRATE device (Intercure Ltd., Israel). Briefly, the RESPeRATE device detects the user's baseline respiration rate with a belt transducer, then plays auditory musical cues that coach the user to progressively reduce their respiratory rate from baseline levels (40). For 12h prior to testing, participants were asked to fast and abstain from caffeine, alcohol, and strenuous exercise. All experiments were performed in the morning (08h00-12h00) and female individuals were tested during the early follicular phase of the menstrual cycle or placebo phase of oral contraceptive use when both estrogen and progesterone concentrations are low. Nine female participants were not taking hormonal contraceptives and one was taking an oral contraceptive (desogestrel and ethinyl estradiol); all were eumenorrheic (cycle length: 22-30 days). Testing took place in a dimly lit room at an ambient air temperature of 22-25°C. Upon arrival at the laboratory, participants were asked to void their bladders. Participants lay supine on a padded

table for instrumentation. Following instrumentation, baseline data was recorded during 10-min of quiet rest. Each participant then performed a 15-min SLOWB session, following auditory cues from the RESPeRATE device.

Instrumentation: Subjects were instrumented for beat-to-beat BP (Finometer MIDI, Finapres, Amsterdam, The Netherlands), heart rate (standard 5-lead ECG), and respiration rate (RESP; respiratory belt transducer, ADInstruments, Bella Vista, NSW, Australia). Beat-by-beat BP values were calibrated to the mean of three resting BPs (manual sphygmomanometry). All cardiovascular variables were acquired at a frequency of 1.0 kHz and saved for offline analysis (PowerLab and LabChart, ADInstruments).

Postganglionic multi-unit muscle sympathetic nerve activity (MSNA) was recorded from the common peroneal nerve via the microneurographic technique, as previously described (NeuroAmp EX, ADInstruments, New South Wales, Australia) (42, 129). A tungsten microelectrode (35 mm in length, 200 μm in diameter, epoxy insulated impedance of 2 ± 0.4 M-Ohms with an uninsulated 1-5 μm tip) was inserted transcutaneously into the peroneal nerve and positioned within recording distance of sympathetic neurons innervating the vasculature of the muscle. MSNA recordings were verified by pulse-synchronized bursts of activity that increased in firing frequency during an end-expiratory apnea, but were unaffected by arousal to a loud noise (41). A reference electrode was positioned subcutaneously 1-3 cm from the recording site. Multi-unit neural activity was amplified 100 times by a head stage before bandpass filtration (0.7-2 kHz) and integration (absolute value, time constant 0.1 s). The MSNA signal was acquired at a sampling frequency of 10.0 kHz (PowerLab and LabChart, ADInstruments).

742 *Data analysis:* A single trained observer (YC) identified bursts of sympathetic activity
743 via semiautomated peak detection. MSNA was quantified as burst frequency (bursts/min) and
744 burst incidence (bursts/100 heartbeats).

745 Systolic BP (SBP), diastolic BP (DBP), and mean arterial BP (MAP) were obtained from
746 the beat-to-beat BP waveforms. Cardiac output (Q; L/min) was calculated offline using the Non-
747 Invasive Cardiac Output algorithm (three-element Windkessel model; ADInstruments). Cardiac
748 index was calculated as Q/ Body-surface area, with body-surface area estimated using the
749 Mostellar formula (84). Total peripheral resistance (TPR; L/min/mmHg) was calculated as
750 MAP/Q and total peripheral resistance index was calculated as TPR/ Body-surface area.

751 Spontaneous cardiovagal baroreflex sensitivity was assessed via the sequence method
752 (14). Sequences of three or more consecutive heartbeats in which R-R interval and SBP
753 concurrently increased or decreased were identified using Ensemble (Elucimed Ltd., Wellington,
754 New Zealand). A minimal coefficient of correlation between changes in SBP and changes in R-R
755 interval was required to validate a sequence ($r^2 > 0.8$). Given the relatively slow heart rate of
756 participants during quiet rest and SLOWB, we expected that the effects of the baroreflex on the
757 R-R interval would be observed within the same cardiac cycle; we therefore did not apply a lag
758 of one beat to the dataset (14). Of all identified sequences, the mean slope between SBP and R-R
759 interval was taken as the index of baroreflex sensitivity (BRS). We also calculated BRS
760 separately for up sequences (in which SBP and R-R interval were both increasing) and down
761 sequences (in which SBP and R-R interval were both decreasing) while the set point for
762 cardiovagal BRS was computed as the prevailing R-R interval (ms) divided by the prevailing
763 SBP (mmHg) (80).

764 Spontaneous sympathetic baroreflex sensitivity was determined by calculating the slopes
765 of the relationships between DBP and MSNA burst incidence (106). Sympathetic data were
766 shifted backward to align the peak of each sympathetic burst with the diastolic period that
767 initiated it. DBP data was averaged into 2-mmHg bins, and the percent occurrence of a
768 sympathetic burst (ranging from 0 to 100%) within each DBP bin was taken as sympathetic burst
769 incidence. We also calculated the set point for sympathetic BRS as mean burst incidence divided
770 by the prevailing DBP.

771 *Statistical analysis:* All data for baseline and SLOWB were compiled into 5-min time
772 bins, and all comparisons were made between the 5-min baseline period and the final 5-min of
773 SLOWB. Two-factor repeated measures ANOVAs were used to assess main effects of time
774 (repeated measures within-subjects, baseline vs. final 5 min of SLOWB) and sex (2 levels: male,
775 female) on all outcome variables (GraphPad Prism 8, La Jolla, California). This study's primary
776 outcome variable was SBP because this metric of BP has been shown to be most affected by
777 SLOWB in a past meta-analysis (17). Our secondary outcome variable was MSNA burst
778 incidence and frequency, and our tertiary variables were cardiovagal and sympathetic BRS. We
779 also quantified DBP due to its correlation with MSNA (46, 66), and MAP due to its ubiquity in
780 the literature. If a statistically significant interaction between time and sex was observed, we
781 performed Tukey's *post hoc* pairwise comparisons to detect differences. Values are reported as
782 means ± SD for all analyses except where we observed a main effect in an ANOVA; these
783 results were reported as the difference between means ± SEM to reflect the overall main effect of
784 condition. Alpha was set to 0.05.

785

Results

Participants: Baseline characteristics, including hemodynamics, are presented in Table 1. All participants identified as cis-gender, as self-reported by questionnaire. Race was also self-reported by questionnaire; within the male group, 10 participants were white, one was Chinese, and one was black. Within the female group, three participants were white, three were Chinese, two were South Asian, one was Arab, and one abstained from answering. Body mass index (BMI) was higher in males than females (Table 1). We did not exclude 7 male and 2 female subjects with relatively high BMI (25-34 kg/m^2) because these subjects were not overweight/ obese; via our health history questionnaire, we determined that these subjects were extremely physically active with a high level of resistance training, resulting in high muscle mass which elevated BMI (19). This group of participants was physically active, engaging in regular physical activity, 3-5 times per week. BPs were similar between the sexes at baseline (Table 1). Sympathetic nerve recordings were obtained in 18 of 22 participants (8 female and 10 male individuals). Sympathetic burst frequency and burst incidence were not different between the sexes at baseline (Table 1). There were no sex differences in baseline cardiovagal or sympathetic BRS (Table 1).

Respiration rate did not differ between the sexes at baseline, and significantly decreased during SLOWB (-7.0 ± 0.9 breaths/min; Table 2).

During SLOWB, we observed reductions in both SBP (-3.2 ± 0.8 mmHg; Fig. 1A) and DBP (-1.3 ± 0.4 mmHg; Fig. 1B). Although SBP and DBP were consistently lower in female individuals, we observed no interactions between the effects of SLOWB and sex on BP (Fig. 1A, B).

808 SLOWB reduced MSNA burst frequency (-2.7 ± 1.0 bursts/ min; Fig. 2A) and burst
809 incidence (-5.0 ± 1.4 bursts/ 100hb; Fig. 2B). We observed non-significant trends towards lower
810 MSNA burst frequency and incidence in females compared to males and observed no
811 interactions between the effects of SLOWB and sex (Fig. 2A, 2B).

812 There was no effect of SLOWB on cardiovagal BRS for down sequences ($P = 0.99$; Fig.
813 3A). SLOWB increased cardiovagal BRS for up sequences (18.9 *vs* 23.7 ms/ mmHg; mean ± SE
814 of difference: 4.7 ± 2.2 ms/mmHg; main effect of SLOWB, $P < 0.01$; Fig. 3B). No sex
815 differences were observed in any measure of cardiovagal BRS. The set point for cardiovagal
816 BRS did not differ between the sexes ($P = 0.8$) and was unaffected by SLOWB (8 ± 2 *vs* 8 ± 1
817 ms/mmHg; $P = 0.2$).

818 The slope of sympathetic BRS became steeper during SLOWB (-3.3 ± 1.3 *vs* -4.2 ± 1.7
819 bursts·100 heart beats-^{1}·mmHg^{-1} ; $P = 0.04$; Fig. 4). There was no effect of sex ($P = 0.77$) and no
820 interaction between the effects of sex and SLOWB ($P = 0.98$). The set point for sympathetic
821 BRS did not differ between the sexes and was unaffected by SLOWB (0.2 ± 0.1 *vs* 0.2 ± 0.1
822 bursts/100hb/mmHg; $P = 0.2$). Three individuals (2 female; 1 male) were excluded from this
823 analysis because the correlation coefficient for the relationship between DBP and MSNA burst
824 incidence did not meet the threshold criteria defined *a priori* (i.e., $R^2>0.8$).

825

Discussion

Contrary to our hypothesis, we found that BP responses to SLOWB were not sex-dependent in our population of young, normotensive individuals. Rather, device-guided SLOWB using RESPeRATE induced similar decreases in BP in individuals of both sexes, even though females had lower BP overall. Mechanistically, we observed no sex differences in the autonomic responses to slow breathing. Namely, despite a trend towards lower MSNA burst frequency and incidence in females, SLOWB-induced reductions in MSNA burst frequency and incidence, increases in sympathetic BRS and sensitization of cardiovagal BRS were similar between male and female participants. SLOWB also increased sympathetic BRS in both sexes. Finally, we found that cardiovagal BRS was sensitized in response to increases in SBP (i.e., up sequences) but not decreases in SBP (i.e. down sequences); again, this improvement did not differ between sexes. Taken together, our study suggests that BP reductions are achieved equally in young healthy individuals of both sexes during an acute bout of SLOWB, and that these reductions are mediated autonomically by a combination of sympathetic withdrawal and parasympathetic activation.

Our finding that SLOWB acutely reduces systolic BP is in alignment with past research (reviewed in (17)), but we are the first to demonstrate that these trends are also observed in young female individuals. The acute decreases that we observed in systolic BP were modest yet consistent with past investigations; the meta-analysis conducted by the AHA reported an average reduction in SBP of 4 mmHg (17) and we report a reduction of 3 mmHg, which falls within the range of a clinically meaningful reduction (89). It is notable that even in young normotensive individuals, SLOWB acutely achieved this magnitude of BP reduction. In other words, even though young healthy females have low baseline BP, we did not observe a “basement effect:”

849 Device-guided SLOWB effectively induced a further, clinically-relevant decrease in BP in these
850 individuals (89).

851 To the best of our knowledge, we are the first to investigate whether the effects of
852 SLOWB on a direct measure of sympathetic activity (i.e. MSNA) are sex-dependent. Although
853 we did not find a statistically significant difference in baseline burst frequency and incidence
854 between the sexes, the values we report are similar to the ranges of previous studies, especially
855 given that MSNA tends to be lower in young healthy individuals (49, 86). Overall, when taking
856 into account the time periods of baseline and SLOWB, female individuals tended to have lower
857 MSNA burst frequency ($P = 0.11$) and incidence than males ($P = 0.09$). Despite these relatively
858 low values of baseline MSNA burst frequency and incidence, SLOWB induced equivalent
859 reductions in both measures of MSNA in both sexes. Our observation that SLOWB acutely
860 reduces MSNA aligns with past research studies (7, 48, 88, 99), but we make the important
861 contribution of extending these findings to include individuals of both sexes.

862 In alignment with past research (9, 56, 91, 92), we found evidence that the SLOWB-
863 induced reduction in BP is mediated by sensitization of the baroreflex, via both efferent arms of
864 the reflex. In both sexes, SLOWB increased the steepness of the slope in sympathetic BRS; that
865 is, during SLOWB, a similar change in diastolic BP was associated with a greater change in burst
866 incidence. This finding aligns with previous research (91), although we are the first to examine
867 sympathetic BRS during SLOWB by the direct measure of MSNA. Concurrently, we also
868 observed exaggerated parasympathetic activation in response to transient increases in systolic BP
869 during SLOWB. However, the parasympathetic response to transient decreases in BP was
870 unaffected by SLOWB. In sum, we found no uniform effect of SLOWB on cardiovagal
871 baroreflex sensitivity; rather, baroreceptor unloading and baroreflex activation were

differentially affected by SLOWB. In a sample of young normotensive men, Tzeng et al. observed the same differential effects of SLOWB on cardiovagal BRS (116). These authors concluded that if the cardiac baroreflex were the sole mediator of the SLOWB-induced BP reduction, a definitive response would have been observed during both increases and decreases in BP (116). Therefore, other mechanisms must be at play, and indeed, our finding that the sympathetic efferent arm of the baroreflex is also sensitized contributes importantly to our understanding of the fundamental mechanisms by which SLOWB reduces BP.

Taken together, these data provide compelling preliminary support for the use of SLOWB as a preventative therapy in normotensive individuals who may possess cardiovascular risk factors, such as a family history of cardiovascular disease (51). Our study contributes to the clinically-relevant question of whether the AHA's 2017 recommendation of SLOWB as an anti-hypertensive approach may be applicable to young female individuals, as this population was under-represented in the pooled study population (17). While young, healthy individuals might not be currently at risk of severe hypertension, it remains important to investigate approaches to maintain healthy BP in this population. With the aim of reducing the incidence of hypertension and cardiovascular disease, an intriguing option is to preemptively target BP earlier in the lifespan. Adolescent BP strongly predicts BP in middle age (132), and concerningly, early signs of endothelial dysfunction are observed even in otherwise healthy young men (61) and women with mild hypertension (2). Therefore, by aiming to reduce BP in young, apparently healthy people, perhaps these lower BPs can be maintained throughout the lifespan and reduce the incidence of cardiovascular disease later in life. While our study provides preliminary support for the use of the RESPeRATE device to reduce BP in young female individuals, a double-blinded randomized control trial is necessary to evaluate the long-term efficacy of this potential anti-

hypertensive therapeutic option for this population (i.e., assessing 24h ambulatory BP after 8 weeks of use).

Methodological Considerations

In our SLOWB protocol, participants followed the respiratory pace set by the RESPeRATE device, but we did not measure or control for other important aspects of respiration. During deep inspiration, slowly-adapting pulmonary stretch receptors are activated (108), stimulating vagal withdrawal and tachycardia (5, 108). Therefore, differences in tidal volume during SLOWB may have affected the extent of vagal withdrawal due to pulmonary stretch receptor activation. This may explain some of the variation that we observed in the effects of SLOWB on cardiovagal baroreflex sensitivity. Given known sex differences in airway size and lung volume regulation (75), it is possible that our SLOWB protocol may have affected respiratory influences on autonomic regulation differently between the sexes. Additionally, we did not measure the partial pressures of oxygen or carbon dioxide (as in (87)). In two participants (1 male; 1 female), we observed small net *increases* in MSNA burst frequency and incidence during SLOWB. We speculate that chemoreflex activation may have played a role in mediating these increases in MSNA. Given that females have greater chemoreflex activation of the sympathetic nervous system compared to males (118), it is possible that SLOWB may have differentially affected chemoreflex activation between the sexes. Measuring PO_2 and PCO_2 along with MSNA would enable a more complete understanding of the potential sex-dependent interactions between activation of the baro- and chemoreflex during SLOWB.

An unforeseen issue with RESPeRATE-guided SLOWB was that many participants began to fall asleep while using the device. This occurred despite the fact that we actively monitored use of the device, instructed participants to open their eyes and gave regular prompts

to follow the breathing patterns. This raises concerns as to whether the device could be correctly employed in private use. Further, this may constitute a confounding variable, given that our findings mirror known effects of light sleep on cardiovascular and autonomic outcomes. During stage 1 non-REM sleep, BP and MSNA are known to decrease (109), while cardiovagal baroreflex gain increases in response to hypertensive stimuli (72). Therefore, we cannot conclude with absolute certainty that the acute effects we observed on BP, MSNA, and baroreflex sensitivity were due to the SLOWB rather than the effect of falling asleep. For future investigations, we recommend that future researchers compare the effects of device-guided breathing to a control protocol, such as a device playing quiet music (as in (3)). Including a control protocol would also reduce the potential role of the placebo effect.

Conclusions

Our study provides preliminary and novel support for the efficacy of the RESPeRATE device to acutely reduce blood pressure (BP) in young normotensive females. Moreover, we also demonstrate that slow breathing appears to reduce BP by similar mechanisms between the sexes. In both female and male individuals, slow breathing reduced muscle sympathetic nerve activity and sensitized the cardiovagal efferent arm of the baroreflex (in response to increasing SBP only). Given our finding that device-guided slow breathing was able to induce acute reductions in BP even in young, healthy, normotensive individuals, we propose that slow breathing may be an effective preventative therapy against development of hypertension. In the future, applying this therapy in normotensive individuals with a family history of hypertension, both acutely and long-term, would provide support for this hypothesis.

941 **Table 1. Baseline subject characteristics, grouped by sex.** BMI, body mass index; BSA, body
942 surface area; MAP, mean arterial pressure; SBP, systolic BP; DBP, diastolic blood pressure; PP,
943 pulse pressure; HR, heart rate; CO, cardiac output; CO_i, cardiac index; RESP, respiration rate,
944 TPR, total peripheral resistance.

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	Females Mean ± SD	**Males Mean ± SD**	***p*-value**
n	10	12	n/a
Age (yr)	22 ± 2	23 ± 3	0.4
BMI (kg/m^2)	22 ± 2	26 ± 4	0.02
BSA (m^2)	1.67 ± 0.12	2.04 ± 0.14	<0.01
MAP (mmHg)	83.3 ± 11.8	90.2 ± 6.6	0.10
SBP (mmHg)	113.4 ± 10.7	121.0 ± 8.5	0.08
DBP (mmHg)	67.7 ± 10.6	72.7 ± 7.0	0.20
PP (mmHg)	45.7 ± 6.8	48.2 ± 7.9	0.44
HR (bpm)	60.2 ± 9.4	57.5 ± 5.6	0.41
CO (L/min)	4.6 ± 1.1	5.5 ± 1.0	0.05
COi ($L/min/m^2$)	2.8 ± 0.8	2.7 ± 0.5	0.76
TPR (mmHg/L/min)	19.3 ± 5.4	17.1 ± 3.6	0.27
TPRi ($mmHg/L/min/m^2$)	11.5 ± 2.9	8.5 ± 1.8	0.01
RESP (breaths/min)	13.4 ± 4.7	12.7 ± 3.5	0.70
MSNA burst frequency (bursts/min)	9 ±5	10 ± 6	0.52
MSNA burst incidence (bursts/100hb)	14 ± 8	18 ± 9	0.37
Cardiovagal baroreflex sensitivity (ms/mmHg)	23.5 ± 9.6	21.9 ± 10.9	0.25
Sympathetic baroreflex sensitivity (bursts·100 heart $beats^{-1}$·$mmHg^{-1}$)	-3.4 ± 1.6	-3.2 ± 1.2	0.8

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948 **Table 2. Hemodynamics during baseline and slow breathing.** Values are presented as means
949 ± SD. BSL, baseline; SLOWB, last 5-min of slow breathing; RESP, respiration rate; HR, heart
950 rate; CO, cardiac output; CI, cardiac index; TPR, total peripheral resistance; TPR_i, total
951 peripheral resistance index.

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	Women (*n* = 10)		**Men (*n* = 12)**				
	BSL	**SLOWB**	**BSL**	**SLOWB**	Effect of Sex *p*-value	Effect of Time *p*-value	Sex x Time *p*-value
RESP (breaths/min)	13.6 ± 4.5	5.5 ± 0.8	11.2 ± 4.0	5.1 ± 0.8	0.16	<0.01	0.28
HR (bpm)	61.3 ± 11.2	60.8 ± 10.2	58.0 ± 6.0	59.5 ±7.5	0.55	0.64	0.13
CO (L/min)	4.86 ± 1.10	4.98 ± 1.12	5.71 ± 1.15	5.97 ± 1.29	0.07	0.12	0.53
CO_i (L/min/m^2)	2.95 ± 0.81	3.01 ± 0.78	2.82 ± 0.60	2.95 ± 0.69	0.73	0.11	0.43
TPR (mmHg/L/min)	17.7 ± 4.0	17.0 ± 3.9	16.5 ± 4.0	15.6 ± 4.3	0.45	0.02	0.81
TPRi (mmHg/L/min/m^2)	10.6 ± 2.1	10.2 ± 2.1	8.1 ± 2.0	7.7 ± 2.1	0.01	0.03	0.91

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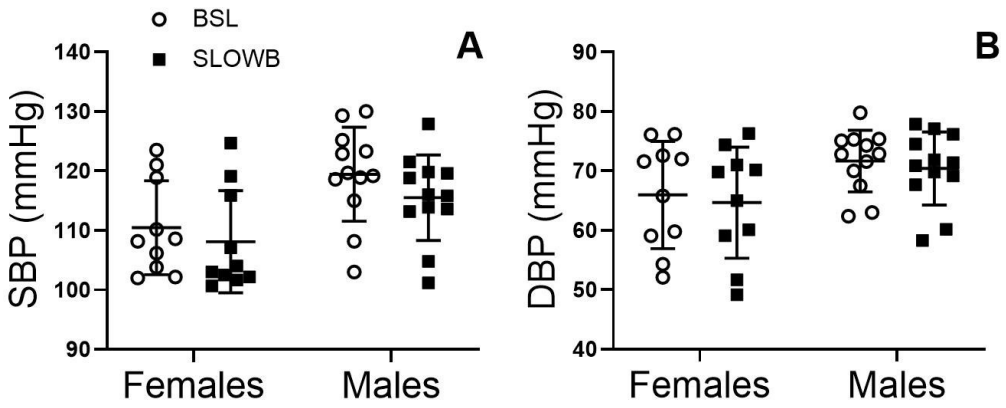


Figure 11: Effect of slow breathing on blood pressure. (A) SBP decreased in both females and males during SLOWB. Effect of SLOWB, $P < 0.01$; effect of sex, $P = 0.02$; SLOWB x sex, $P = 0.33$. (B) DBP decreased in females and males during SLOWB. Effect of SLOWB, $P < 0.01$; effect of sex, $P = 0.09$; SLOWB x sex, $P = 0.99$. Data are presented as group means ± SD. Groups: females, n=10; males, n=12.

BSL, baseline; SLOWB, last 5 minutes of slow breathing; SBP, systolic blood pressure; DBP, diastolic blood pressure.

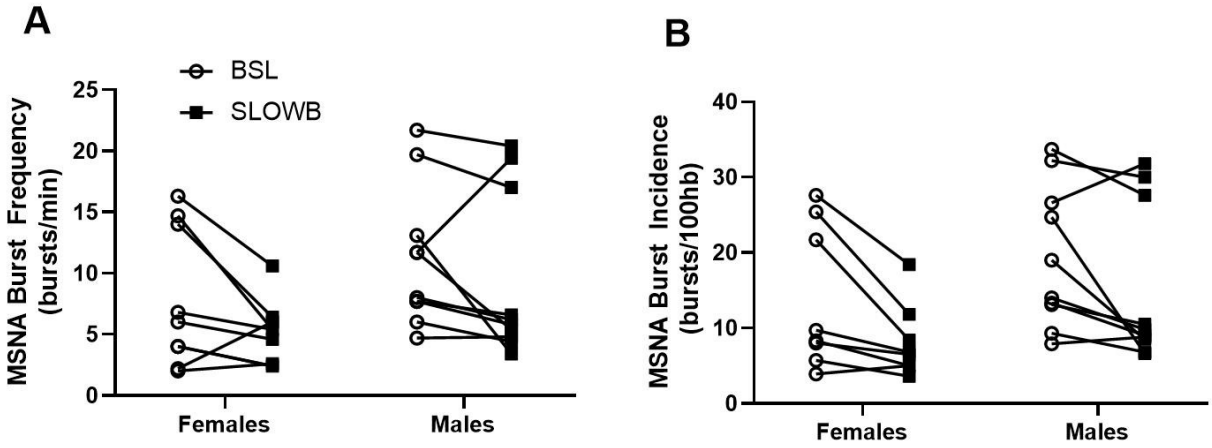
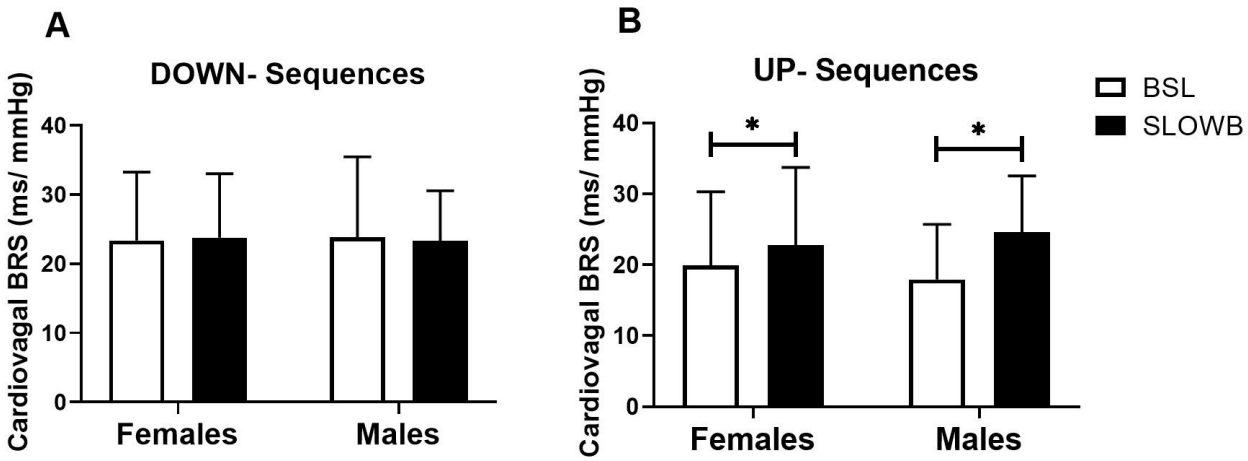


Figure 12: Effect of slow breathing on muscle sympathetic nerve activity. (A) MSNA burst frequency decreased in both females and males during SLOWB ($P = 0.02$); individual data shown. Burst frequency tended to be lower in females than males ($P = 0.16$); no SLOWB x sex interaction ($P = 0.41$). (B) MSNA burst incidence decreased during SLOWB ($P < 0.01$); individual data shown. Females tended to have lower burst incidence ($P = 0.14$); no interaction between SLOWB and sex ($P = 0.69$). Groups: females, n=8; males, n=10.

MSNA, muscle sympathetic nerve activity; BSL, baseline; SLOWB, last 5 minutes of slow breathing; SBP, systolic blood pressure; DBP, diastolic blood pressure.



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980 **Figure 13: Effect of slow breathing on cardiovagal baroreflex sensitivity.** (A) Cardiovagal
981 BRS in response to hypotensive stimuli (DOWN-sequences) was unchanged with SLOWB in
982 both female and male individuals. (B) Cardiovagal BRS in response to hypertensive stimuli (UP-
983 sequences) increased during SLOWB in both females and males. Groups: females, n=10; males,
984 n=12.

985 BRS, baroreflex sensitivity; BSL, baseline; SLOWB, last 5 minutes of slow breathing; SBP,
986 systolic blood pressure; DBP, diastolic blood pressure.

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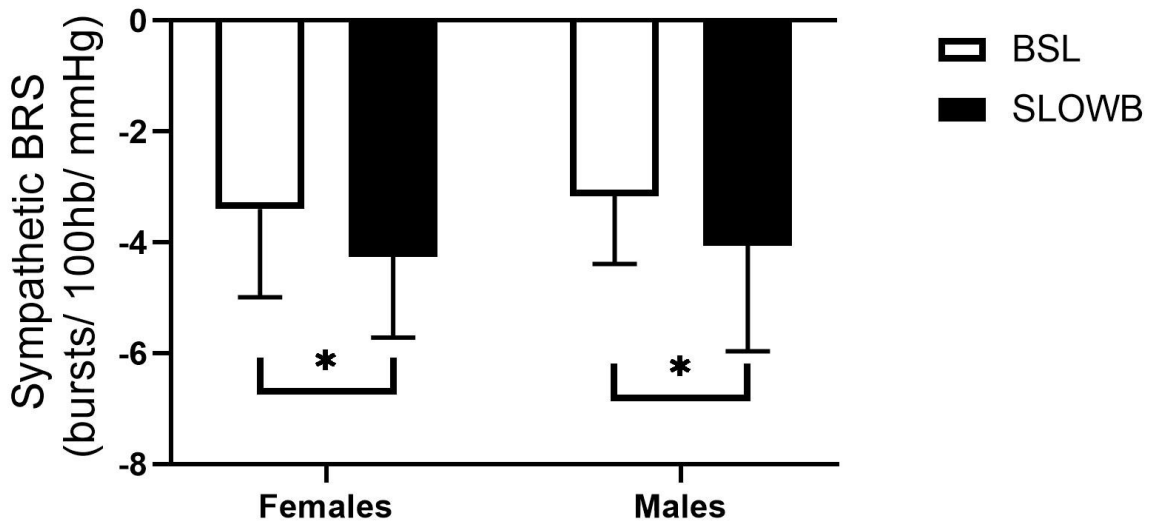

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991 **Figure 14: Effect of slow breathing on sympathetic baroreflex sensitivity.** Sympathetic BRS
992 decreased in response to the SLOWB stimuli in both females and males. Groups: females, n = 6,
993 males, n = 9.

994 BRS, baroreflex sensitivity; BSL, baseline; SLOWB, last 5 minutes of slow breathing.

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