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NEUROVASCULAR RESPONSES TO MELATONIN IN HUMANS

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by

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ABSTRACT

Melatonin is synthesized and secreted by the pineal gland in a circadian rhythm. As the master circadian regulator in the body, melatonin has many physiological functions depending on its site of action. Pharmacological levels of melatonin have been reported to decrease nerve activity of medial vestibular nuclei in the rat and are associated with attenuated muscle sympathetic nerve activity (MSNA) responses to baroreceptor unloading in humans. Additionally, melatonin has been demonstrated to differentially alter blood flow to assorted vascular beds by MT_1 vs. MT_2 melatonin receptor activation. Therefore, the overall objective of this dissertation project was to determine the neurovascular changes elicited by exogenous and endogenous plasma melatonin levels at rest and during vestibular stimulation.

The purpose of Aim 1 and 2 (Chapter 3) was to determine if melatonin alters the vestibulosympathetic reflex (VSR) and vestibulocollic reflex (VCR) in humans. In Aim 1, MSNA, arterial blood pressure, and heart rate were measured in 12 healthy subjects (28±1 yr; 6 male, 6 female) during head-down rotation (HDR) before and 45 min after ingestion of either melatonin (3 mg) or placebo (sucrose). Subjects returned at least 2 days later at the same time of day to repeat the trial after ingesting the opposite drug. Melatonin increased MSNA during baseline as compared to placebo (11±2 vs. 9±1 bursts/min; $p<0.05$). However, melatonin significantly attenuated MSNA responses during HDR as compared to placebo (burst frequency: Δ4±1 vs. Δ7±1 bursts/min and total MSNA: Δ51±20 and Δ96±15%, respectively; $p<0.02$). In Aim 2, vestibular evoked myogenic potentials (VEMP) were measured in 10 healthy subjects (26±1 yr; 4 male and 6 female) before and after ingestion of 3 mg melatonin. Melatonin did not alter the timing of the p13 and n23 peaks (pre-melatonin: 13.2±0.4 and 21.3±0.6 msec vs. post-melatonin: 13.5±0.4 and 21.4±0.7 msec, respectively) or the p13-n23 inter-peak amplitudes (pre-melatonin: 22.5±4.6 a.u. and post-melatonin: 22.7±4.6 a.u.). In summary, melatonin attenuates

the VSR does not alter the VCR in humans suggesting melatonin's effect on the VSR might be mediated by the utricles.

The purpose of Aim 3 (Chapter 4) was to determine the effect of melatonin on blood flow to various vascular beds in humans. Renal (Doppler ultrasound), forearm (venous occlusion plethysmography), calf (venous occlusion plethysmography) and cerebral blood flows (transcranial Doppler), arterial blood pressure, and heart rate were measured in 10 healthy subjects (29 ± 1 yr; 5 male, 5 female) while lying on a table for 3 minutes. The protocol began 45 min after the ingestion of either melatonin (3 mg) or placebo (sucrose). Subjects returned at least 2 days later at the same time of day to repeat the trial after ingesting the opposite drug. Melatonin did not alter heart rate and mean arterial pressure. Renal blood flow velocity (RBFV) and renal vascular conductance (RVC) were lower during the melatonin trial as compared to placebo (RBFV: 40 ± 3 vs. 45 ± 2 $cm \cdot s^{-1}$; RVC: 0.47 ± 0.02 vs. 0.54 ± 0.01 $cm \cdot s^{-1} \cdot mmHg^{-1}$, respectively). In contrast, forearm blood flow (FBF) and forearm vascular conductance (FVC) were greater with melatonin compared to placebo (FBF: 2.4 ± 0.2 vs. 1.9 ± 0.1 $ml \cdot 100ml^{-1} \cdot min^{-1}$; FVC: 0.029 ± 0.003 vs. 0.023 ± 0.002 a.u., respectively). Melatonin did not alter calf or cerebral blood flow measurements compared to placebo. In summary, exogenous melatonin differentially alters vascular blood flow in humans.

Increases in sympathetic nerve activity are hypothesized to contribute to the observed circadian rhythm of adverse cardiac events. Pharmacological levels of melatonin increase MSNA at rest and attenuate reflexes involved in blood pressure regulation such as the vestibulosympathetic reflex (Aim 1). Melatonin typically increases 8-10 times over daytime concentrations during the late evening hours. The purpose of Aim 4 (Chapter 5) was to determine if MSNA at rest and the VSR follow a circadian rhythm in humans in relation to the endogenous melatonin circadian rhythm. Arterial blood pressure, heart rate, calf blood flow and

MSNA were measured in 9 healthy subjects (28±1 yr; 5 male, 4 female) at rest and during head-down rotation. Each subject was tested around noon and 10-12 hr later that evening (day: 11:34±13 min, night: 22:10±5 min). MSNA at rest was significantly lower at night compared to day (8±1 vs. 11±2 bursts/min, respectively; $P<0.05$). Heart rate and arterial blood pressure were significantly increased at night compared to day (heart rate: 70±4 vs. 66±4 beats/min, arterial blood pressure: 91±2 vs. 87±1 mmHg, respectively). MSNA and cardiovascular responses to head-down rotation were not significantly altered at night compared to day (Δ3±1 bursts/min, Δ25±6% for MSNA and calf blood flow, respectively). The data indicate that MSNA at rest is lower during the late evening hours and exhibits a circadian rhythm whereas the vestibulosympathetic reflex is not altered by endogenous changes in melatonin. A circadian rhythm of MSNA in humans may be important for understanding the increase of adverse cardiac events in the morning hours.

The current project demonstrated that 3 mg melatonin ingestion attenuates the vestibulosympathetic reflex, increases MSNA at rest and differentially alters vascular blood flow. Additionally, we observed lower MSNA at rest at night compared to daytime. Together, these data demonstrate melatonin's multiple effects on neurovascular control in humans.

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Chapter 1

Introduction

Bors & Ralston (1951) observed that a mystery pineal gland lightened the skin color of tadpoles, frogs, toads, and fish. The pineal gland extract was determined to cause melanin granules to amass in melanocytes resulting in the lightened skin color. In 1958, Aaron Lerner first isolated and characterized this substance from bovine pineal gland extracts (Lerner *et al.*, 1958). He "suggested that this substance be called *melatonin*" (Lerner *et al.*, 1958). It was not until nine years later that a possible role for melatonin in blood pressure regulation was discovered. Zanoboni and Zanoboni-Muciaccia (1967) observed increases of ~30 mmHg in systolic blood pressure in pinealectomized rats. Holmes and Sugden (1976) further demonstrated the importance of melatonin in blood pressure regulation by administering high doses of melatonin in the drinking water of pinealectomized rats. They observed that melatonin prevents an increase in blood pressure in pinealectomized rats and actually decreases blood pressure in these rats below that of blood pressure in vehicle-treated control rats. When melatonin was removed from the drinking water, blood pressure proceeded to increase to hypertensive levels (Holmes & Sugden, 1976). These findings demonstrated the importance of melatonin in blood pressure regulation, thus, initiating the line of research that continues with the current project.

1.1 Synthesis and Regulation of Melatonin

Melatonin, (N-acetyl-5-methoxytryptamine), the main hormone synthesized by the pineal gland, exhibits a circadian rhythm increasing shortly after darkness and peaking between 2:00 and 4:00 AM. Melatonin levels gradually decrease during the second half of the night and remain at

negligibly low levels during daylight hours. Melatonin synthesis is regulated by the suprachiasmatic nucleus (SCN), which receives light signals from photoreceptive cells in the retina via the retinohypothalamic tract (Pandi-Perumal *et al.*, 2006). During darkness, the overall sympathetic signal originating from the SCN is increased thereby stimulating protein synthesis in the pinealocytes, most importantly arylakylamine *N*-acetyltransferase (AA-NAT). AA-NAT is the rate-limiting step in the process of converting serotonin to melatonin (Figure 1-1). From the pinealocytes, melatonin enters the circulation by simple diffusion and is ultimately converted to 6-hydroxymelatonin in the liver and excreted in the urine. Because melatonin half-life is ~30 min, plasma melatonin levels are directly related to melatonin synthesis in the pineal gland (Cardinali & Pevet, 1998). Additionally, endogenous and exogenous melatonin readily cross the blood-brain barrier (Le Bars *et al.*, 1991).

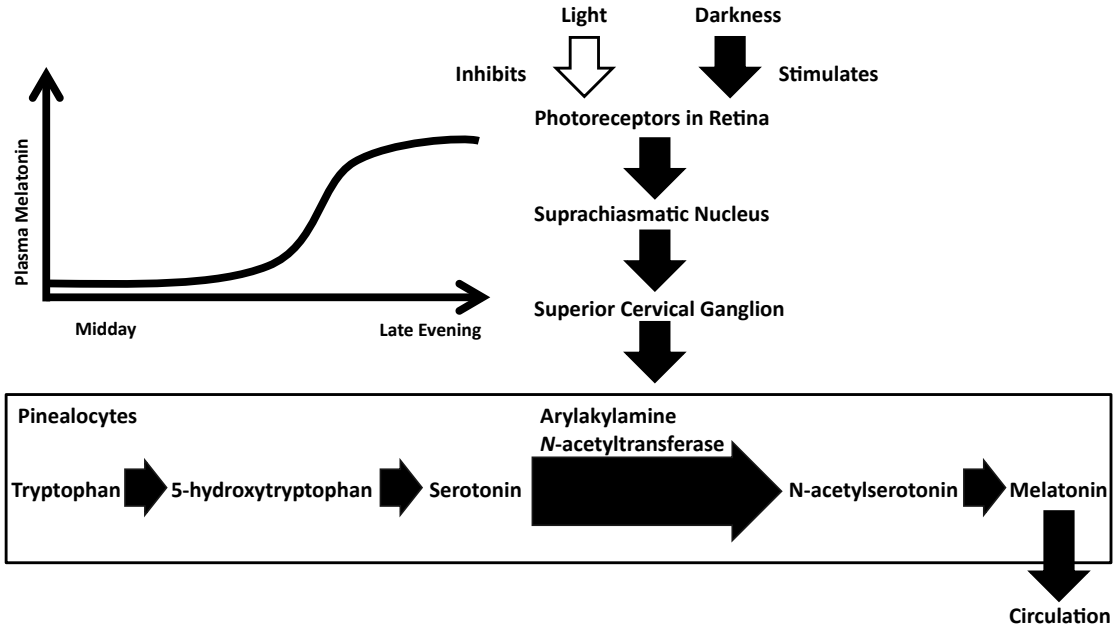

Figure **1-1**: Summary illustration of melatonin regulation by light and synthesis by pinealocytes in the pineal gland.

When naturally occurring in the body, melatonin has been demonstrated to impact sleep quality, body temperature, sexual maturation, and to entrain peripheral clock genes (Brzezinski, 1997; Pandi-Perumal *et al.*, 2006). The circadian rhythm of melatonin is also hypothesized to be important for the diurnal variation in blood pressure because populations with non-dipping blood pressure at night have decreased peak melatonin secretion at night (Jonas *et al.*, 2003; Zeman *et al.*, 2005). Melatonin, ingested as a supplement, is taken commonly by the general population as an over-the-counter sleep aid (Bliwise & Ansari, 2007).

1.2 Mechanisms of Melatonin's Action

Melatonin predominantly acts through two membrane bound melatonin receptors (MT_1 and MT_2). Depending on the location in the body, these receptors have been demonstrated to have different effects. Functioning in the SCN to further regulate melatonin signaling, MT_2 receptors induce phase shifts in the circadian rhythm while MT_1 receptors decrease nerve firing within the SCN (Pandi-Perumal *et al.*, 2006). The differential expression of MT_1 and MT_2 receptors has not been determined in the vasculature of humans. However, melatonin receptor function has been characterized in the caudal artery of the rat. MT_1 receptors are expressed in the vascular smooth muscle of the rat caudal artery (Masana *et al.*, 2002). Functioning as a G-protein coupled receptor, MT_1 receptor activation by melatonin results in the inhibition of calcium-activated potassium channels (BK_{Ca}), thus, eliciting the observed potentiated adrenergic vasoconstriction (Geary *et al.*, 1998; Vandeputte *et al.*, 2001). MT_2 receptors are evenly expressed throughout the caudal artery (Masana *et al.*, 2002) and cause a decrease in vascular tone by possibly increasing NO-synthase activity (Geary *et al.*, 1998; Paulis & Simko, 2007).

Additionally, melatonin binds to MT_3, serotonin 5-HT_{1A}, and dopamine D_1 receptors and functions as an antioxidant. The MT_3 receptor is also known as quinone reductase 2 (QR2). QR2

expression has been observed in human skeletal muscle, liver, kidney, heart, and, minimally, in brain and pancreas (Nosjean *et al.*, 2001). There is an ongoing debate as to whether QR2 is detoxifying (by association to QR1) or promotes a toxic cellular environment (Vella *et al.*, 2005). Research suggests that melatonin has a very low affinity for QR2 (micromolar range compared to nanomolar for MT_1 and MT_2) and inhibits QR2 (Nosjean *et al.*, 2001). If QR2 promotes a toxic cellular environment, melatonin's inhibition of QR2 could partially explain melatonin's function as an antioxidant at pharmacological levels.

By binding to the serotonin 5-HT_{1A} receptor, melatonin has been demonstrated to decrease body temperature and decrease serotonin levels in the hypothalamus in the rat (Lin & Chuang, 2002). The decrease in body temperature was attributed to a decrease in metabolic heat production and an increase in heat loss due to cutaneous vasodilation (Lin & Chuang, 2002). This finding demonstrates melatonin's role in body temperature regulation, which is hypothesized to partially cause the drowsiness effect of melatonin. However, the mechanism for melatonin altering cutaneous vasodilation was not determined in the study.

Melatonin's binding to the dopamine D_1 receptor is hypothesized to cause an antidepressant-like effect in the mouse (Binfare *et al.*, 2010). Additionally, renal dopamine D1 receptor activation is hypothesized to play a role in blood pressure regulation by inhibiting sodium reabsorption (Banday & Lokhandwala, 2008). Therefore, melatonin may be involved in long-term blood pressure regulation via a similar mechanism. However, in patients with essential hypertension, the chronic ingestion of melatonin before bedtime only lowered nighttime blood pressure and not daytime blood pressure limiting the potential use of melatonin for long-term blood pressure regulation in humans (Scheer *et al.*, 2004; Cagnacci *et al.*, 2005).

Melatonin also functions as a potent antioxidant for free radicals such as superoxide and hydroxyl, especially at pharmacological levels (Paulis & Simko, 2007). This response would appear to be beneficial, especially for blood pressure regulation because antioxidant

supplementation has been demonstrated to lower blood pressure in hypertensive populations (Ceriello *et al.*, 1991; Duffy *et al.*, 1999). However, melatonin also scavenges for the NO free radical, which, depending on the source (eNOS vs. iNOS) could be detrimental or beneficial for blood pressure regulation (Paulis & Simko, 2007). Ramelteon is a MT_1 and MT_2 melatonin receptor agonist but lacks melatonin's antioxidant properties. Thus, knowing the effect of Ramelteon on blood pressure in humans would further our understanding of the mechanisms by which melatonin functions in humans. In a review of the literature, a laboratory-controlled study examining the acute effects of Ramelteon on blood pressure in humans could not be found. A clinical assessment report issued by the European Medicines Agency found changes in systolic and diastolic blood pressure in 1% of subjects using Ramelteon compared to placebo (van Zwieten-Boot & Sampaio, 2008). However, the specific conditions for Ramelteon ingestion were not described in the report. Although these antioxidant interactions may alter melatonin's hypotensive response, their role in blood pressure regulation has not been categorically tested.

1.3 Acute Melatonin Ingestion Decreases Blood Pressure in Humans

As previously mentioned, Zanoboni and Zanoboni-Muciaccia (1967) and Holmes and Sugden (1976) pioneered the research of melatonin and blood pressure regulation in the rat. However, over twenty years passed before for the examination of acute ingestion of melatonin on blood pressure in humans occurred. In a series of studies, Cagnacci and colleagues (Cagnacci *et al.*, 1998; Arangino *et al.*, 1999) measured changes in resting arterial blood pressure and norepinephrine levels in humans during supine and standing conditions after ingesting 1 mg of melatonin. In healthy men and women, they observed a decrease in mean arterial blood pressure (MAP) while the subjects were supine 45 minutes after melatonin ingestion. Additionally, they observed an attenuated increase in norepinephrine after standing for 5 min with melatonin

ingestion compared to placebo in both sexes. Interestingly, melatonin decreased norepinephrine levels at rest compared to placebo in men but not in women in a supine position. Thus, Cagnacci and colleagues suggested melatonin may lower blood pressure by inhibiting central sympathetic outflow. These findings supported previous research in the rat by Chuang et al. (1993), who suggested that melatonin's hypotensive response is centrally mediated.

1.4 Components of Blood Pressure Regulation

In recent years, extensive research has examined melatonin as a possible antihypertensive agent (Girouard *et al.*, 2001; Jonas *et al.*, 2003; Nava *et al.*, 2003; Scheer *et al.*, 2004; Cagnacci *et al.*, 2005; Zeman *et al.*, 2005; Grossman *et al.*, 2006). Although melatonin may have an important role in aiding patients with hypertension, the extensive use of melatonin by the general population might cause unwanted hypotension and syncope. From the 2002 National Health Interview Survey, 5.2% of the United States population uses melatonin as a sleep aid and 54% of these users do so without medical consultation (Bliwise & Ansari, 2007). Understanding the mechanisms of melatonin's action is important for maximizing its beneficial effects and negating its untoward effects. The following sections will discuss the components of blood pressure regulation that have been demonstrated to be affected by melatonin (i.e., baroreflex) and those that will be further discussed in this project (i.e., vestibulosympathetic reflex, peripheral vascular resistance).

1.4.1 Baroreflex

The canonical pathway for blood pressure regulation is the baroreflex. Its activation leads to an increase in muscle sympathetic nerve activity and subsequent modification of vascular

resistance (Sundlof & Wallin, 1978; Davy *et al.*, 1998; Ray, 2003). The baroreflex functions via negative feedback to alter the autonomic nervous system. When blood pressure is elevated, the baroreceptors are activated and send an excitatory signal to the nucleus of the solitary tract, which in turn sends a stimulatory signal to the caudal ventrolateral medulla. The activated caudal ventrolateral medulla then inhibits firing of the rostral ventrolateral medulla, the primary regulator of muscle sympathetic nerve activity. A drop in blood pressure would remove the inhibitory effect of the caudal ventrolateral medulla on the rostral ventrolateral medulla, thereby, increasing muscle sympathetic nerve activity (MSNA).

Previous research in humans indicates that 1 mg of melatonin decreases plasma levels of norepinephrine during standing (Cagnacci *et al.*, 1998; Arangino *et al.*, 1999). Standing is a nonspecific stimulus of the baroreflex. Therefore, Ray (2003) hypothesized that melatonin would attenuate MSNA responses during lower body negative pressure (LBNP), a baroreflex specific stimuli. MSNA was measured during two levels of LBNP (-10 and -40 mmHg) after 3 mg melatonin ingestion. LBNP augmented MSNA responses; however, melatonin ingestion attenuated the increase in MSNA during LBNP when compared to the placebo trial. In contrast, MSNA responses to isometric handgrip and a cold pressor test were not altered by melatonin. These results suggest that melatonin's MSNA suppressor effect was specific to the baroreflex.

1.4.2 Vestibulosympathetic Reflex

Doba and Reis (Doba & Reis, 1974) demonstrated that the vestibular system is important in postural blood pressure regulation in animals by demonstrating a drop in blood pressure during tilt in cats with transected vestibular nerves. The vestibular apparatus is part of the fluid-filled membranous labyrinth located in the inner ear and consists of two major parts: the semicircular canals and the otolith organs. The semicircular canals are sensitive to angular acceleration

whereas the otolith organs are sensitive to both gravity and linear acceleration. Further research in animals developed the hypothesis that the otolith organs activate the vestibulosympathetic reflex (VSR) as a feed forward mechanism for maintaining blood pressure during an orthostatic challenge (Yates & Miller, 1994; Jian *et al.*, 1999). Kerman and Yates (Kerman & Yates, 1998) demonstrated that the VSR differentially augments sympathetic activity and Wilson et al. (Wilson *et al.*, 2006) demonstrated that the VSR differentially alters limb blood flow in cats, thereby providing additional support that the vestibular apparatus plays a significant role in postural blood pressure control. In humans, Shortt and Ray (Shortt & Ray, 1997) demonstrated that head down rotation (activator of the otolith organs) elicits an increase in MSNA and an increase in calf vascular resistance. Additionally, the VSR is able to increase MSNA during baroreceptor unloading suggesting that the VSR might contribute importantly to maintaining orthostasis in humans (Ray, 2000; Dyckman *et al.*, 2007).

Afferent nerves from the otolith organs (Cranial Nerve VIII) synapse at the vestibular nuclei (Yates & Miller, 1994; Wilson *et al.*, 1995). In the cat, VSR activation has been demonstrated to include the medial and inferior vestibular nuclei (Yates & Miller, 1994). Using an *in vitro* model, Podda et al. (2003) demonstrated that melatonin attenuates nerve firing of the medial vestibular nuclei in the rat. Thus, we hypothesized that melatonin will attenuate the VSR in humans. This hypothesis will be discussed in Chapters 3 and 5.

1.4.3 Peripheral Vascular Resistance

Blood pressure is the product of cardiac output (heart rate x stroke volume) and total peripheral resistance. Because activation of melatonin receptors in the vasculature results in vasoconstriction and vasodilation (Doolen *et al.*, 1998) and previous research in humans demonstrated that acute melatonin ingestion (<3 mg) does not alter heart rate (Cagnacci *et al.*,

1997; Cagnacci *et al.*, 1998; Arangino *et al.*, 1999; Ray, 2003), these data suggest that melatonin might alter blood pressure by modulating total peripheral resistance. Change in vessel diameter has a potent effect on vascular resistance because vessel resistance is inversely proportional to the radius to the fourth power. Because previous research on melatonin receptors suggests the predominant function in the vasculature is via sympathetic-potentiated vasoconstriction (Geary *et al.*, 1998) and NO mediated vasodilation (Geary *et al.*, 1998; Paulis & Simko, 2007), this section will focus on these two mechanisms.

Vasoconstriction of the vasculature is from vascular smooth muscle cell contraction. Sympathetic nerve fibers terminate at vascular smooth muscle cells in the arteries and arterioles. Norepinephrine is released from postganglionic sympathetic nerve fibers, which then binds to α_1-adrenergic receptors on vascular smooth muscle cells. Activation of the α_1-receptor results in increased intracellular Ca^{2+} and subsequent vasoconstriction of the vascular smooth muscle cells. Melatonin mediates vasoconstriction by activating MT_1 receptors, which inhibit calcium-activated potassium channels (BK_{Ca}) (Geary *et al.*, 1998; Vandeputte *et al.*, 2001). In addition to altering vasoconstriction at the vascular smooth muscle cells, melatonin may modulate vasoconstriction by altering sympathetic activity. First, pharmacological levels of melatonin have been observed to increase the efferent activity of the adrenal nerve in the rat (Niijima *et al.*, 1998). Melatonin may alter central MSNA outflow via a similar mechanism in humans. Second, melatonin ingestion has been demonstrated to decrease circulating norepinephrine but not epinephrine levels in supine male subjects (Arangino *et al.*, 1999). The origin for this change in circulating norepinephrine was not determined.

Vasodilation of the vasculature is a result of the relaxation of vascular smooth muscle cells in the arteries and arterioles by either a decrease in sympathetic nerve activity or by changes in shear stress. Shear stress applied to the endothelial surface causes an increase in NO synthase activity via Ca^{2+} dependent and independent mechanisms, thus, increasing endothelial cell NO

output. NO diffuses to nearby vascular smooth muscle cells and binds to the NO receptor resulting in dephosphorylation of myosin and decreases in intracellular Ca^{2+}, thereby relaxing the vascular smooth muscle cells (Busse & Fleming, 2006). It is hypothesized that melatonin functions by increasing NO synthase activity via a MT_2 receptor Ca^{2+} dependent mechanism (Simko & Paulis, 2007).

In the rat, melatonin has been demonstrated to vasoconstrict the tail and cerebral arteries (Capsoni *et al.*, 1995; Doolen *et al.*, 1998; Vandeputte *et al.*, 2001; Ersahin *et al.*, 2002; Masana *et al.*, 2002). In contrast, melatonin mediated vasodilator responses have been reported in rat and rabbit aorta, iliac, and renal arteries (Satake *et al.*, 1986; Shibata *et al.*, 1989; Satake *et al.*, 1991a; Satake *et al.*, 1991b). The different vascular effects observed with melatonin are attributed to the relative distribution of MT_1 and MT_2 melatonin receptors. The distribution of melatonin receptors is determined from ^{125}I-melatonin binding assays and functionally from serial experiments with the MT_1 and MT_2 specific antagonist, Luzindole, and the MT_2 specific antagonist, 4P-PDOT. However, these methods are not available for human studies, thus, the relative expression levels of MT_1 and MT_2 receptors in the various tissues in humans are unknown.

The overall effect of melatonin supplementation on vascular resistance/neurovascular control of blood flow is important for understanding how melatonin alters blood pressure and ultimately affects orthostatic homeostasis. The relative expression levels of MT_1 and MT_2 receptors in the various tissues of the body are unknown, and MT_3 receptors are not implicated in blood flow regulation. Because melatonin receptor activation results in either vasoconstriction or vasodilation, we hypothesized that melatonin ingestion will differentially alter vascular resistance in humans. These topics will be further discussed in Chapter 4.

1.5 Specific Aims and Hypotheses

Regulation of blood pressure and the redirecting of blood blow during gravitational stressors is an important component of orthostatic tolerance. This project will further our understanding of how melatonin modulates blood pressure homeostasis in humans during resting conditions. The specific aims of this project are:

Aim #1 To determine sympathetic responses during otolithic activation before and after acute melatonin ingestion in healthy subjects.

Hypothesis #1 Acute melatonin ingestion will attenuate MSNA responses during head-down rotation (i.e., VSR).

Aim #2 To determine myogenic potentials during vestibular stimulation before and after acute melatonin ingestion in healthy subjects.

Hypothesis #2 Acute melatonin ingestion will attenuate vestibular evoked myogenic potentials (VEMP) during an auditory stimulus.

Aim #3 To determine the effects of acute melatonin ingestion on regional blood flow at rest in healthy subjects.

Hypothesis #3 Acute melatonin ingestion will differentially alter vascular conductance in renal, forearm, calf, and cerebral vascular beds.

Aim #4 To determine if MSNA at rest and the vestibulosympathetic reflex have a circadian rhythm.

Hypothesis #4 MSNA at rest will be decreased and the vestibulosympathetic reflex will be attenuated in the late evening hours compared to midday.

Chapter 2

Common Methods

In this section, specific protocols to address the hypotheses of this project are described. The experimental designs and statistical analyses used to test each hypothesis are described in the subsequent chapters.

2.1 Microneurography

Multifiber recordings of MSNA were obtained from a tungsten microelectrode inserted in the peroneal nerve behind or lateral to the knee, as previously described (Ray, 2000). A reference electrode was placed subcutaneously 2-3 cm from the recording electrode. The criteria for an adequate MSNA signal included: 1) tapping of the muscles or tendons innervated by the nerve produced afferent mechanoreceptor discharges; 2) apnea produced an increase in sympathetic nerve activity; 3) stroking of the skin did not produce any afferent activity; and 4) sudden, unexpected arousal stimulus (shout or clap) did not produce any increases in sympathetic activity (Vallbo *et al.*, 1979). The nerve signal was amplified (20,000 – 50,000 times), fed through a bandpass filter with a band width of 700-2,000 Hz, integrated using a 0.1 s time constant (University of Iowa Bioengineering, Iowa City, IA), and recorded digitally (16SP Powerlab, ADInstruments, New Castle, Australia). The mean voltage neurogram was routed to a computer screen and a loudspeaker for monitoring during the study. Sympathetic recordings that demonstrated possible electrode site shifts, altered respiratory patterns (e.g., breath holding, inspiratory gasp, and hyperventilation), or electromyographic artifact during experimental intervention were excluded from analysis.

2.2 Vestibular Evoked Myogenic Potentials (VEMP)

Subjects were in the supine position for the duration of the experimental protocol. VEMP testing was performed with the Interacoustics Eclipse EP25 (Interacoustics, Denmark). Surface EMG was recorded bilaterally using surface electrodes placed at half the distance between the mastoid tip and sternal notch on the muscle belly of the sternocleidomastoid muscle and a reference and ground electrode were placed on the forehead. The subject's nose was centered 3-4 cm under a stationary band suspended from the ceiling. During the VEMP trials, subjects were instructed to lift and turn their head to the left so that their cheek touched the band. Controlling the degree of head movement limited the variation in neck tension between trials. VEMP were evoked by a tone burst stimulus transmitted by an insert earphone to the ear ipsilateral of muscle recording (right side). The stimulus rate was 5.3 Hz at 100 db nHL and 150 sweeps were averaged per trial. Each trial lasted ~30 sec. In order to minimize startle from the tone burst onset, the noise level was ramped up to maximum intensity. The latency from stimulus (p13 and n23) and inter-peak (p13-n23) amplitude were measured. Four trials were performed to ensure reproducibility of VEMP measurements. Subjects rested 1-2 min between trials to minimize fatigue.

2.3 Doppler Ultrasound

Doppler ultrasound (HDI 5000, ATL Ultrasound, Bothell, WA) was used to measure renal blood flow velocity (RBFV). The renal artery was scanned by the anterior abdominal approach with a curved-array transducer (2–5 MHz) with a 2.5-MHz pulsed Doppler frequency. The probe insonation angle to the artery was $\leq 60°$. The focal zone was set at the depth of the artery. The transducer was held in the same place to record velocity tracings during each trial, and

the data were obtained in the same phase of the respiratory cycle. Continuous measurements of RBFV were taken during each trial. A renal vascular conductance index (RVC) was calculated by dividing RBFV by MAP.

2.4 Transcranial Doppler

Transcranial Doppler was used to measure cerebral blood flow. A 500M Transcranial Doppler System (Multigon Industries, Inc., Yonkers, NY) with a 2 MHz pulsed wave probe was used. The probe was centered over the middle cerebral artery using the transtemporal position. Cerebral blood flow was expressed as mean blood velocity (MCA-BV) in cm/sec. A cerebrovascular conductance index (MCA-VC) was calculated by dividing MCA-BV by MAP.

2.5 Venous Occlusion Plethysmography

Venous occlusion plethysmography was used to measure calf and forearm blood flow. Room temperature was monitored before each session to minimize temperature related fluctuations in skin blood flow. A Hokanson EC-4 system (Bellevue, WA) with mercury-in-silastic strain gauges was used. Strain gauges were positioned around the maximal circumference of the left calf and forearm. Ankle and wrist cuffs were inflated to 220 mmHg to arrest circulation to the foot and hand, respectively. Calf and forearm blood flow was recorded every 15 s during the trials. During blood flow measurements, a venous collecting cuff was inflated proximal to the knee and elbow to a pressure of 50 mmHg. Calf and forearm vascular conductance (CVC and FVC, respectively) were calculated by dividing calf or forearm blood flow by MAP.

2.6 Arterial Blood Pressure

Arterial blood pressure was continuously recorded using a Finometer (FMS, Amsterdam, the Netherlands). Before each trial, brachial artery blood pressure was measured by an automated sphygmomanometer (Dinamap, General Electric, Waukesha, WI).

2.7 Heart Rate

Heart rate was continuously recorded using an electrocardiogram and Finometer (FMS, Amsterdam, the Netherlands).

2.8 Venous Blood Draw

An arm vein catheter was used to obtain venous blood samples from 8 subjects in Chapter 3 for measurement of plasma melatonin. The blood sample was stored on ice and subsequently spun to separate the plasma. Plasma melatonin was measured in duplicate by radioimmunoassay (Buhlmann Laboratories, Germany). All samples were analyzed together after the completion of all tests.

Chapter 3

Melatonin attenuates the vestibulosympathetic but not vestibulocollic reflexes in humans: selective impairment of the utricles

3.1 Introduction

Melatonin, which is synthesized in a circadian rhythm, increases during the night and decreases throughout the day (Reiter, 1991). Endogenous melatonin has been demonstrated to impact sleep quality, sexual maturation, and tumor suppression (Brzezinski, 1997). As a supplement, melatonin is commonly ingested by the general population as an over-the-counter sleep aid (Bonn, 1996). Pharmacological levels of melatonin have been demonstrated to lower blood pressure, function as an antioxidant, and improve jet lag symptoms (Kennaway & Wright, 2002; Simko & Paulis, 2007; Peyrot & Ducrocq, 2008). Cagnacci et al. (1998) and Arangino et al. (1999) observed a reduction in arterial blood pressure at rest with acute melatonin ingestion in men and women. Additionally, Ray (2003) demonstrated that melatonin attenuates muscle sympathetic nerve activity (MSNA) in response to lower body negative pressure in humans. It was concluded that melatonin attenuates baroreflex-mediated increases in MSNA. The reduction in arterial blood pressure and decrease in MSNA response to orthostatic stress suggest that melatonin might affect orthostatic tolerance in humans. However, the effect of melatonin on other mechanisms affecting postural blood pressure is equivocal.

Hypothesized as an orthostasis feed-forward mechanism, the vestibulosympathetic reflex (VSR) has been demonstrated to affect blood pressure regulation in animals (Doba & Reis, 1974; Jian *et al.*, 1999; Holmes *et al.*, 2002). Ray and colleagues (Shortt & Ray, 1997; Ray & Hume, 1998; Ray *et al.*, 1998; Ray, 2000; Monahan & Ray, 2002b, a; Dyckman *et al.*, 2007; Sauder *et*

al., 2008) demonstrated that head-down rotation (HDR), which activates the VSR, elicits increases in MSNA and calf and renal vascular resistance in humans. Importantly, the VSR is able to increase MSNA during baroreceptor unloading (Ray, 2000; Dyckman *et al.*, 2007). These findings suggest that the VSR contributes to orthostasis in humans.

The vestibulocollic reflex (VCR) functions as a head posture regulator (Boyle, 2001) and is measured in part by vestibular evoked myogenic potentials (VEMP) (Colebatch *et al.*, 1994; Wilson *et al.*, 1995). VEMP have been used clinically to test for vestibular disorders such as superior semicircular canal dehiscence (Brantberg *et al.*, 1999) and Ménière's disease (Rauch *et al.*, 2004). The tone bursts that elicit VEMP stimulate only the saccule thereby representing a specific marker of otolithic function (Colebatch *et al.*, 1994).

Afferent nerves from the otolith organs, which trigger the VSR and VCR, synapse at the vestibular nuclei (Yates & Miller, 1994; Wilson *et al.*, 1995). Using an *in vitro* model, Podda et al. (2003) demonstrated that melatonin attenuates nerve firing of the medial vestibular nuclei in the rat. Thus, reductions in neuronal activity through the medial vestibular nuclei by melatonin could attenuate the VSR and VCR in humans.

The purpose of the present studies was to determine the effect of melatonin on the vestibulosympathetic and vestibulocollic reflexes. Study 1 investigated the effects of exogenous melatonin on the VSR in humans. Study 2 determined if the VCR is affected by melatonin and specifically addresses the effect of melatonin on the saccule. Based on previous studies, it was hypothesized that increases in melatonin would attenuate MSNA responses during HDR and attenuate VEMP responses to auditory stimuli. Results from Study 1 support the concept that exogenous melatonin attenuates sympathetic nerve responses during activation of the VSR. The data in Study 2 suggest that melatonin does not alter the saccules function in humans. This finding indicates that melatonin's effect on the VSR is via the utricles.

3.2 Methods

3.2.1 Subjects

In Study 1, 12 healthy subjects (6 male and 6 female; age: 28 ± 1 yr; height: 174 ± 3 cm; weight: 71 ± 5 kg) were tested. In Study 2, 10 healthy subjects (4 male and 6 female; age: 26 ± 1 yrs; height: 174 ± 3 cm; weight: 70 ± 5 kg) were in the experimental group. Nine of these subjects participated in Study 1. Additionally, 8 healthy subjects (1 male and 7 female; age: 25 ± 1 yrs; height: 167 ± 2 cm; weight: 62 ± 3 kg) were in the time control group for Study 2. All subjects were normotensive, nonsmokers, nonobese, and not taking any medications that would interfere with the measurements of the protocol. All subjects received a physical examination before participation. Written informed consent was obtained from all subjects after verbal explanation of the experimental protocol. The Institutional Review Board of The Pennsylvania State University College of Medicine approved this study.

3.2.2 Experimental Protocol

3.2.2.1 Study 1

Each subject randomly ingested in a double-blind manner either melatonin (3 mg; Major Pharmaceuticals, Livonia, MI) or placebo (sucrose; Forest Pharmaceuticals, Inc., St. Louis, MO). Melatonin and placebo were purchased from the Milton S. Hershey Medical Center pharmacy. The dose of 3 mg melatonin was used in the current study for two reasons: 1) it is a common over-the-counter dose used in the population; and 2) the dose has been previously demonstrated by our laboratory to attenuate the baroreflexes (Ray, 2003). Each subject and the investigator performing the microneurography measurements and MSNA analyses were blinded to the drug

condition. The experimental protocol began 45 min after ingestion of either melatonin or placebo. 45 minutes is the time required for plasma melatonin levels to elevate and plateau after 3 mg melatonin ingestion (Ray, 2003). Venous blood samples were obtained from 8 subjects prior to ingestion and following the experimental protocol. Subjects returned at least 2 days later at the same time of day to repeat the trial after ingesting the opposite drug. MSNA, heart rate, and mean arterial blood pressure (MAP) were measured continuously in the prone position during 3 min of baseline, 3 min of HDR, and 3 min of recovery.

During baseline, the subject's neck was extended with the chin supported to bring the head upright as close to the vertical plane as possible. This position approximates the gravitational orientation of the head when an individual is in the upright posture (Shortt & Ray, 1997). For HDR of both trials, the head was maximally lowered in the vertical plane over the edge of the table. An investigator moved the head by supporting the forehead and chin, thus producing a passive head movement. Once the head became stationary, only afferent inputs from the otolith organs and not the semicircular canals were engaged. Subjects remained in the prone position throughout the trials.

3.2.2.2 Study 2

Subjects in the experimental group first performed a control bout of VEMP testing (no drug), ingested 3 mg of melatonin (Major Pharmaceuticals, Livonia, MI), waited 45 minutes, than performed a second bout of VEMP testing. 45 minutes is the time required for plasma melatonin levels to elevate and plateau after 3 mg melatonin ingestion (Ray, 2003). Each bout included 4 separate trials of VEMP testing to ensure reproducibility. The time control subjects performed the same protocol but ingested a placebo (sucrose; Forest Pharmaceuticals, Inc., St. Louis, MO) instead of melatonin.

3.2.3 Data Analysis

3.2.3.1 Study 1

Sympathetic bursts were identified from individual inspection of the mean voltage neurograms and with computer assistance. Signal-to-noise ratio of 2:1 and a latency period of ~1.3 seconds from the R wave of the EKG was required. MSNA was expressed as burst frequency (bursts/min) and total MSNA (i.e., sum of bursts area). The area of the bursts was measured by a computer program (Peaks; ADInstruments). Absolute changes from baseline are reported for burst frequency. Relative changes (%) from baseline are reported for total MSNA.

For all trials, the 3 min of baseline were averaged together and reported as the baseline value for the respective trial. Because MSNA has been demonstrated to significantly increase during the first minute of HDR and not change during prolonged HDR (Shortt & Ray, 1997), only the first minute of HDR is reported and used for statistical tests for the various measurements. Statistical analyses of the data during HDR trials were performed with a two-within factor [drug X intervention (HDR)] repeated measures ANOVA (n = 12). Plasma melatonin levels were analyzed with a two-within factor (drug X time) repeated measures ANOVA (n = 8). A paired t-test was performed to compare hemodynamic values at baseline between the two trials. Significance was set at $p < 0.05$. All data are presented as mean ± SE.

3.2.3.2 Study 2

The four VEMP trials were averaged together to represent the predrug and postdrug interventions. Latency from stimulus (p13 and n23) and inter-peak amplitude (absolute value between p13 and n23 peaks) were calculated. p13 and n23 refer to the first nadir and peak,

respectively, after stimulus onset. Statistical analyses of the data during VEMP trials were performed with a one-within (time), one-between (group) ANOVA (n = 18; 10 experimental and 8 control subjects). Significance was set at $p < 0.05$. All data are presented as mean ± SE.

3.3 Results

3.3.1 Study 1 – Vestibulosympathetic Reflex (VSR)

Ingestion of melatonin significantly increased plasma melatonin compared to placebo (865 ± 28 vs. 6 ± 1 $pg \cdot ml^{-1}$; $p < 0.05$; Table 3-1). Plasma melatonin did not differ before melatonin or placebo ingestion (6 ± 1 and 9 ± 3 $pg \cdot ml^{-1}$, respectively).

MAP and heart rate were not different between the two drug trials during baseline (Table 1). MSNA burst frequency was significantly higher during the melatonin than the placebo trial (11 ± 2 and 9 ± 1 bursts/min, respectively; $p < 0.05$; Table 3-1).

Table **3-1**: Baseline values for Study 1.

	Study 1	
	Placebo	Melatonin
Plasma melatonin ($pg \cdot ml^{-1}$)	6 ± 1	865 ± 28 *
Mean arterial pressure (mmHg)	85 ± 1	86 ± 2
Heart rate ($beats \cdot min^{-1}$)	60 ± 3	61 ± 3
MSNA (bursts/min)	9 ± 1	11 ± 2 *

MSNA, muscle sympathetic nerve activity. Values are expressed as mean ± SE. * Significantly different from placebo; $p < 0.05$. (n = 8 for plasma melatonin, n = 12 for MSNA, mean arterial pressure, and heart rate)

A representative neurogram of MSNA from one subject is presented in Figure 3-1. Melatonin significantly attenuated MSNA responses during HDR as compared to placebo (burst

frequency: Δ 4 ± 1 vs. Δ 7 ± 1 bursts/min and total MSNA: Δ 51 ± 20 and Δ 96 ± 15%, respectively; $p < 0.02$; Figure 3-2). Heart rate and MAP responses were not significantly altered by melatonin during HDR (Δ 2 ± 1 beats/min and Δ -1 ± 1 mmHg, respectively).

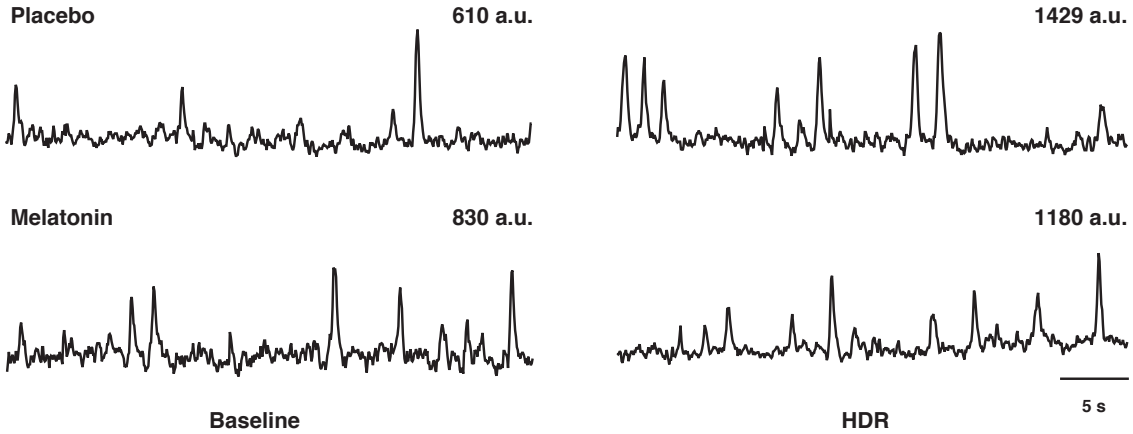

Figure **3-1**: Representative neurogram from one subject during baseline and head-down rotation (HDR) after the ingestion of placebo or melatonin. MSNA total activity is indicated above each recording.

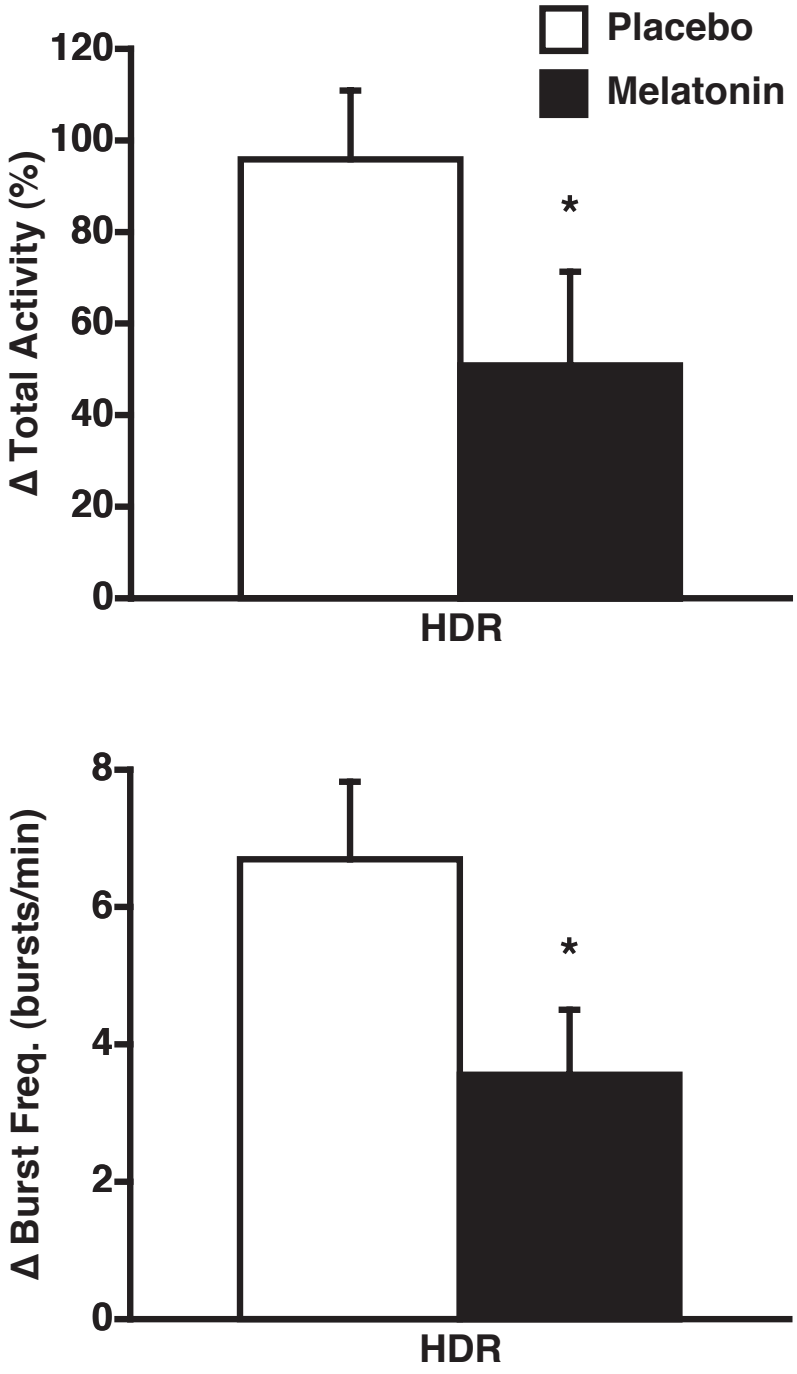

Figure **3-2**: Muscle sympathetic nerve activity (MSNA) responses to head-down rotation (HDR) with and without melatonin ingestion. MSNA responses to HDR were significantly attenuated with melatonin (n = 12). * Significantly different from placebo; $p < 0.05$. ΔTotal Activity: $P = 0.02$; ΔBurst Frequency: $P = 0.018$.

3.3.2 Study 2 – Vestibular Evoked Myogenic Potentials (VEMP)

A representative averaged VEMP waveform from one subject is presented in Figure 3-3. Melatonin did not alter the timing of the first VEMP peak (p13) nor the second VEMP peak (n23) (Table 3-2). VEMP p13-n23 inter-peak amplitude was not altered by melatonin (pre-melatonin: 22.5 ± 4.6 a.u. and post-melatonin: 22.7 ± 4.6 a.u.; Figure 3-4). Comparable results for the p13-n23 inter-peak amplitude were observed in the control group (bout 1: 26.1 ± 5.6 a.u. and bout 2: 26.1 ± 6.9 a.u.; Figure 3-4). Increases in inter-peak amplitude were observed in one subject after increases in neck tension (unpublished observation). This observation serves as a positive control to demonstrate our ability to modulate inter-peak amplitude.

Table **3-2**: Vestibular evoked myogenic potential (VEMP) peak latencies from tone burst onset.

	Experimental		Time Control	
	Pre	Post	Pre	Post
p13 (msec)	13.2 ± 0.4	13.5 ± 0.4	13.2 ± 0.3	13.3 ± 0.3
n23 (msec)	21.3 ± 0.6	21.4 ± 0.7	20.2 ± 0.6	20.5 ± 0.6

Subjects in the experimental group (n = 10) received melatonin and subjects in the time control group (n = 8) received placebo. Melatonin did not alter the p13 and n23 peak latencies. p13 and n23 refer to the first and second VEMP peaks, respectively, after stimulus onset. Values are expressed as mean ± SE.

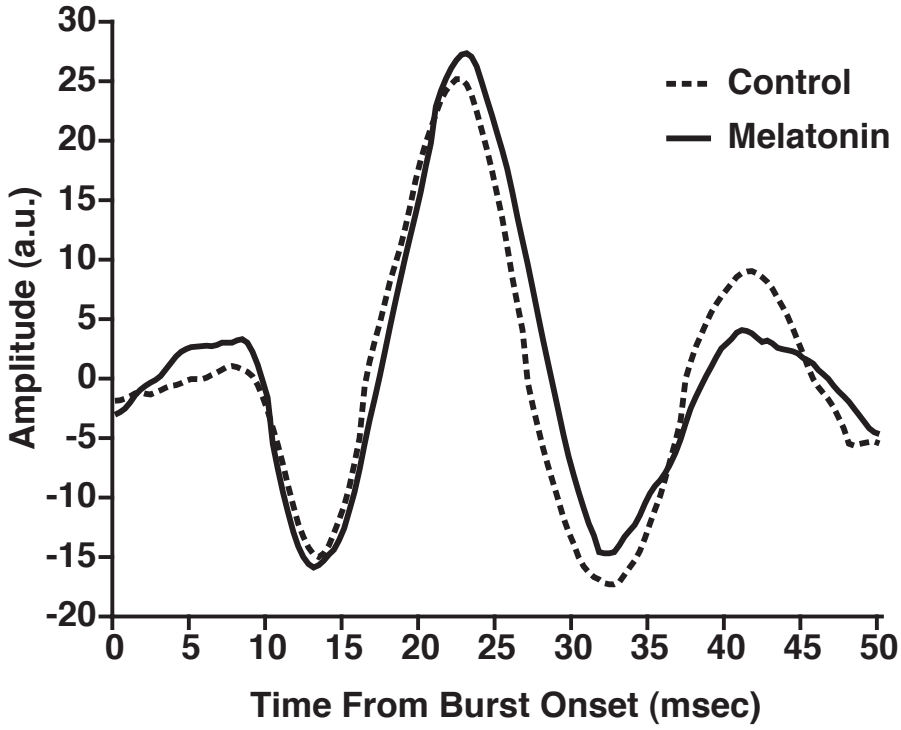

Figure **3-3**: Representative averaged vestibular evoked myogenic potential (VEMP) waveforms from one subject before and after the ingestion of melatonin.

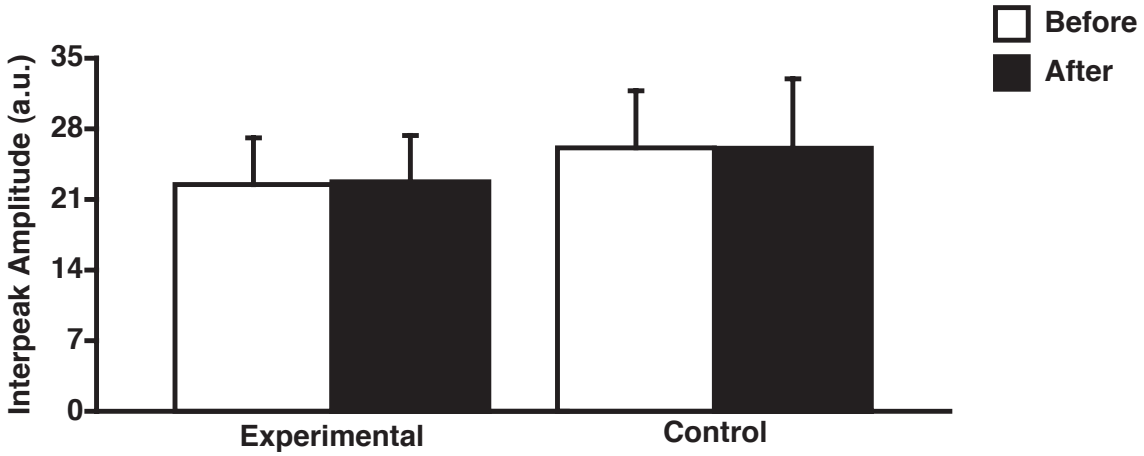

Figure **3-4**: p13-n23 inter-peak amplitudes for experimental (melatonin, n = 10) and time control groups (n = 8). Melatonin did not alter the inter-peak amplitudes. The inter-peak amplitudes measured from the time control group were comparable to the experimental trial (time X group interaction: P = 0.91).

3.4 Discussion

Three novel findings from this study are: 1) exogenous melatonin attenuates MSNA increases during otolith organ activation; 2) exogenous melatonin increases MSNA at rest; and 3) exogenous melatonin does not alter the VEMP response to saccule activation in humans suggesting melatonin's effect on the VSR is mediated by the utricles. These results support the concept that exogenous melatonin contributes to reduced orthostatic tolerance in humans.

In animal models, melatonin has been associated with reduced neuronal activity in several areas of the brain (Stehle *et al.*, 1989; Hogan *et al.*, 2001; Podda *et al.*, 2003). Importantly, Podda et al. (2003) demonstrated that melatonin decreases neuronal activity of the medial vestibular nuclei. Therefore, it is possible that the decrease in the VSR observed in our study by exogenous melatonin is mediated by this mechanism. Additionally, decreases in vestibular nuclei activity by melatonin could alter the baroreflex. Neural interactions of vestibular and baroreceptor afferents have been demonstrated to occur in the nucleus tractus solitarius (NTS) (Donoghue *et al.*, 1982; Donoghue *et al.*, 1984; Balaban & Beryozkin, 1994; Yates *et al.*, 1994; Ruggiero *et al.*, 1996; Xue *et al.*, 2004). Importantly, Xue et al. (2004) demonstrated a decreased baroreflex response during orthostatic stress in otoconia-deficient mice, suggesting a lack of otolith organ input alters the baroreflex. Furthermore, we demonstrated that the baroreceptors can alter the VSR in humans (Dyckman *et al.*, 2007). Together, these findings support the concept that melatonin attenuates both the VSR and baroreflex, which would negatively impact blood pressure regulation.

Exogenous melatonin causes an increase in MSNA at rest. Previous research from our laboratory observed a similar, yet not statistically significant increase in MSNA at rest with melatonin (Ray, 2003). The increase in MSNA at rest observed in the present study is not very large. In comparison, we observed an increase of 2 bursts/min at rest after melatonin ingestion in

the present study whereas aging (old vs. young) increases MSNA at rest by 16 bursts/min (Ray & Monahan, 2002). One possible explanation is that ingesting 3 mg of melatonin causes peripheral vasodilation (Doolen *et al.*, 1998) and activates the baroreflex. Melatonin ingestion has been demonstrated to decrease blood pressure in humans (Cagnacci *et al.*, 1998; Arangino *et al.*, 1999), possibly triggering the baroreflex and increasing MSNA. However, in the current study MAP was not altered with melatonin at rest. Additionally, we did not observe and subjects did not describe a flushing effect due to increased blood flow to the skin vascular beds after melatonin ingestion. But, the lack of decrease in MAP with melatonin at rest could be masked by baroreflex-mediated increases of MSNA. A second explanation is that melatonin may stimulate neural centers of the brain that increase MSNA. Exogenous melatonin has been demonstrated to increase adrenal nerve activity by acting on the suprachiasmatic nucleus (SCN) (Niijima *et al.*, 1998). A similar mechanism might increase MSNA with melatonin ingestion in humans.

In contrast to the attenuated MSNA responses during HDR in Study 1, decreases in VEMP inter-peak amplitude were not observed in Study 2. In air-conducted sound VEMP, as used in the current study, the tone burst stimulates the saccules (Colebatch *et al.*, 1994; Schlindwein *et al.*, 2008). HDR is a general stimulus of the otolith organs. These data suggest melatonin does not alter the saccules and attenuates the VSR via the utricles.

The degree of contractile force in the sternocleidomastoid muscle positively relates to increases in p13-n23 inter-peak amplitude (Colebatch *et al.*, 1994). Therefore, neck tension needs to remain relatively constant between trials and bouts if an accurate comparison of the reflex is to be made. In the current study, a constant degree of neck flexion was maintained for each trial to minimize changes in neck tension between trials and bouts. Additionally, the effect of fatigue on VEMP activation was minimized in Study 2 because no change was observed in p13-n23 inter-peak amplitude between bout 1 and bout 2 in the time control group.

What is the physiological implication of exogenous melatonin modulating the VSR? Upon movement from a supine to standing position, blood pooling in the lower limbs occurs resulting in a blood volume shift of 25-30% (Grubb, 2005). To maintain blood perfusion to the brain, the VSR is hypothesized to function as a feed-forward mechanism and has been demonstrated to increase sympathetic outflow to vascular beds and increase peripheral vascular resistance (Yates & Miller, 1994; Ray *et al.*, 1997; Shortt & Ray, 1997; Kerman & Yates, 1998; Hume & Ray, 1999; Monahan & Ray, 2002a; Wilson *et al.*, 2006; Sauder *et al.*, 2008). VSR mediated sympathetic outflow could be attenuated by exogenous melatonin reducing peripheral vascular resistance during orthostasis and decreasing blood perfusion to the brain contributing to syncopal symptoms.

Certain subsets of the population, such as older adults and astronauts (Buckey *et al.*, 1996; Grubb *et al.*, 2008), are more prone to orthostatic intolerance than others. Because melatonin secretion decreases with age, elderly populations commonly take melatonin to aid with sleep (Bonn, 1996). Likewise, astronauts commonly take melatonin as a sleep aid due to the disruption of their circadian rhythm during spaceflight (Dijk *et al.*, 2001). What are the implications of using melatonin as a sleep aid in these populations? If melatonin does attenuate the VSR in humans, then older adults and astronauts taking melatonin could have an even greater risk of orthostatic intolerance. This is particularly significant with aging because aging alone attenuates the VSR (Monahan & Ray, 2002b; Ray & Monahan, 2002; Sauder *et al.*, 2008).

The plasma melatonin levels induced by ingestion of the supplement were significantly greater (865 $pg \cdot ml^{-1}$) than the expected peak physiological levels during evening hours (~60 $pg \cdot ml^{-1}$) (Brzezinski, 1997). However, 3 mg of melatonin is commonly used by the general population (Bonn, 1996) and by astronauts during spaceflight to improve sleep (Dijk *et al.*, 2001). Therefore, the plasma melatonin levels observed and the attenuated increase in MSNA during HDR after melatonin ingestion have physiological relevance.

In summary, melatonin attenuates the VSR and augments MSNA at rest. However, melatonin does not alter the VCR during VEMP stimulation of the saccules in humans suggesting melatonin's effect on the VSR is mediated by the utricles. The attenuation of MSNA responses during HDR with melatonin ingestion supports the concept that exogenous melatonin negatively affects orthostatic tolerance in the general population and could be deleterious to older adults and astronauts who are susceptible to orthostatic intolerance.

Chapter 4

Melatonin differentially affects vascular blood flow in supine resting humans

4.1 Introduction

Melatonin is synthesized and released into the circulation by the pineal gland in a circadian rhythm. The rise in melatonin at night has been demonstrated to promote sleep and decrease body temperature (Brzezinski, 1997). As an over-the-counter supplement, melatonin is commonly ingested as a sleep aid and to overcome jetlag in the general population (Brzezinski, 1997). Melatonin predominantly acts through two membrane bound receptors (MT_1 and MT_2). In the vasculature, opposite effects have been demonstrated for the two receptors, activation of MT_1 receptors cause constriction and activation of MT_2 receptors cause dilation (Doolen *et al.*, 1998). In the rat, melatonin has been demonstrated to constrict the coronary artery (Weekley, 1993) and decrease in vivo cerebral blood flow (Capsoni *et al.*, 1995) while dilating the pulmonary artery (Weekley, 1993). When ingested as a supplement in humans, melatonin enhances the cutaneous vasodilating response during heating (Aoki *et al.*, 2006) and blunts the cutaneous vasoconstrictor response during cooling (Aoki *et al.*, 2008). The different vascular effects observed with melatonin are attributed to the relative distribution of MT_1 and MT_2 melatonin receptors. The relative expression levels of MT_1 and MT_2 receptors in the various tissues of the body are unknown. To date, very little information exists regarding the impact that melatonin has on blood flow in different vascular beds in humans. This information is important because over 15.5 million of Americans have been reported to use melatonin as a supplement (Bliwise & Ansari, 2007). Therefore, the purpose of the present study was to determine the effect of melatonin on renal, forearm, calf, and cerebral blood flow in humans. Because melatonin has been

demonstrated to elicit differential vascular responses in animals, we hypothesized that increases in melatonin would elicit differential effects on the vasculature in humans.

4.2 Methods

4.2.1 Subjects

Ten healthy subjects (5 male and 5 female; age: 29 ± 1 yrs; height: 169 ± 3 cm; weight: 62 ± 4 kg) were tested in the experimental group. An additional group of 7 healthy subjects (2 male and 5 female; age: 29 ± 1 yrs; height: 165 ± 4 cm; weight: 59 ± 4 kg) were tested as time controls to determine reproducibility of the measurements. All subjects were normotensive, nonsmokers, nonobese, and not taking any medications that would interfere with the measurements of blood flow. Day in menstrual cycle and usage of birth control was recorded for all female subjects. All subjects received a physical examination before participation. Written informed consent was obtained from all subjects after verbal explanation of the experimental protocol. The Institutional Review Board of The Pennsylvania State University College of Medicine approved this study.

4.2.2 Experimental Protocol

Each subject randomly ingested a tablet in a double-blind manner containing either melatonin (3 mg; Major Pharmaceuticals, Livonia, MI) or sucrose (sucrose; Forest Pharmaceuticals, Inc., St. Louis, MO). Melatonin and placebo were purchased from the Milton S. Hershey Medical Center pharmacy. Subject's waited 45 min after ingestion before the experimental protocol began. Subjects returned no sooner than 2 days later at the same time of

day to repeat the other trial. Renal, cerebral, forearm, and calf blood flow, heart rate, and mean arterial blood pressure (MAP) were measured continuously while lying on a table for 3 min. Time control subjects performed the same protocol but ingested sucrose for both trials.

4.2.3 Data Analysis

Statistical analyses of the data were performed by paired t-test. Significance was set at $p < 0.05$. All data are presented as mean ± SE.

4.3 Results

MAP and heart rate were not different between the two drug trials (Table 4-1).

Table **4-1**: Hemodynamic values for placebo and melatonin trials.

	Placebo	Melatonin
Mean arterial pressure (mmHg)	84 ± 1	85 ± 2
Heart rate (beats·min^{-1})	63 ± 3	60 ± 3

Values are expressed as mean ± SE. (n = 10)

4.3.1 Vascular Responses

Renal blood flow velocity and renal vascular conductance were significantly decreased by melatonin compared to placebo (renal blood flow velocity: 40 ± 3 vs. 45 ± 2 $cm \cdot s^{-1}$; renal vascular conductance: 0.47 ± 0.02 vs. 0.54 ± 0.01 $cm \cdot s^{-1} \cdot mmHg^{-1}$, respectively; Figure 4-1).

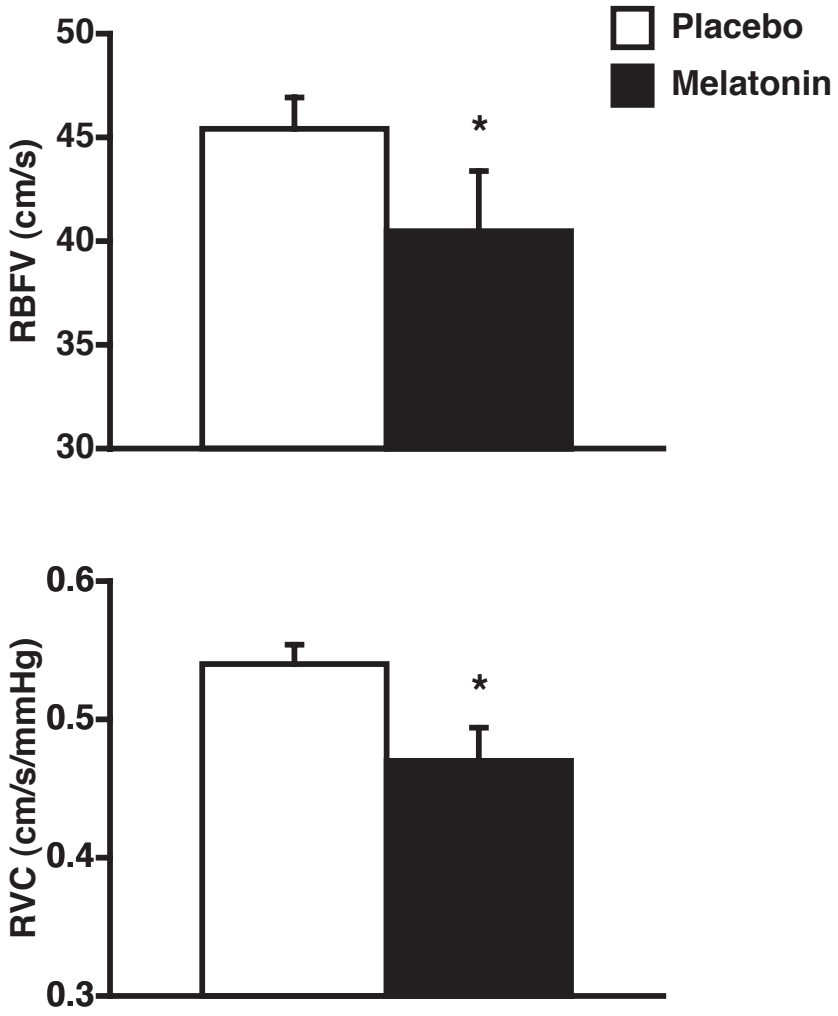

Figure **4-1**: Renal blood flow velocity (RBFV) and renal vascular conductance (RVC) after placebo or melatonin ingestion. Melatonin ingestion significantly decreased RBFV and RVC (n = 10). Values are means ± SE. ***** Significantly different from placebo; $p < 0.05$. RBFV: P = 0.047; RVC: P = 0.024.

Forearm blood flow and forearm vascular conductance were significantly increased by melatonin compared to placebo (forearm blood flow: 2.4 ± 0.2 vs. 1.9 ± 0.1 ml·100ml^{-1}·min^{-1}; forearm vascular conductance: 0.029 ± 0.003 vs. 0.023 ± 0.002 a.u., respectively; Figure 4-2).

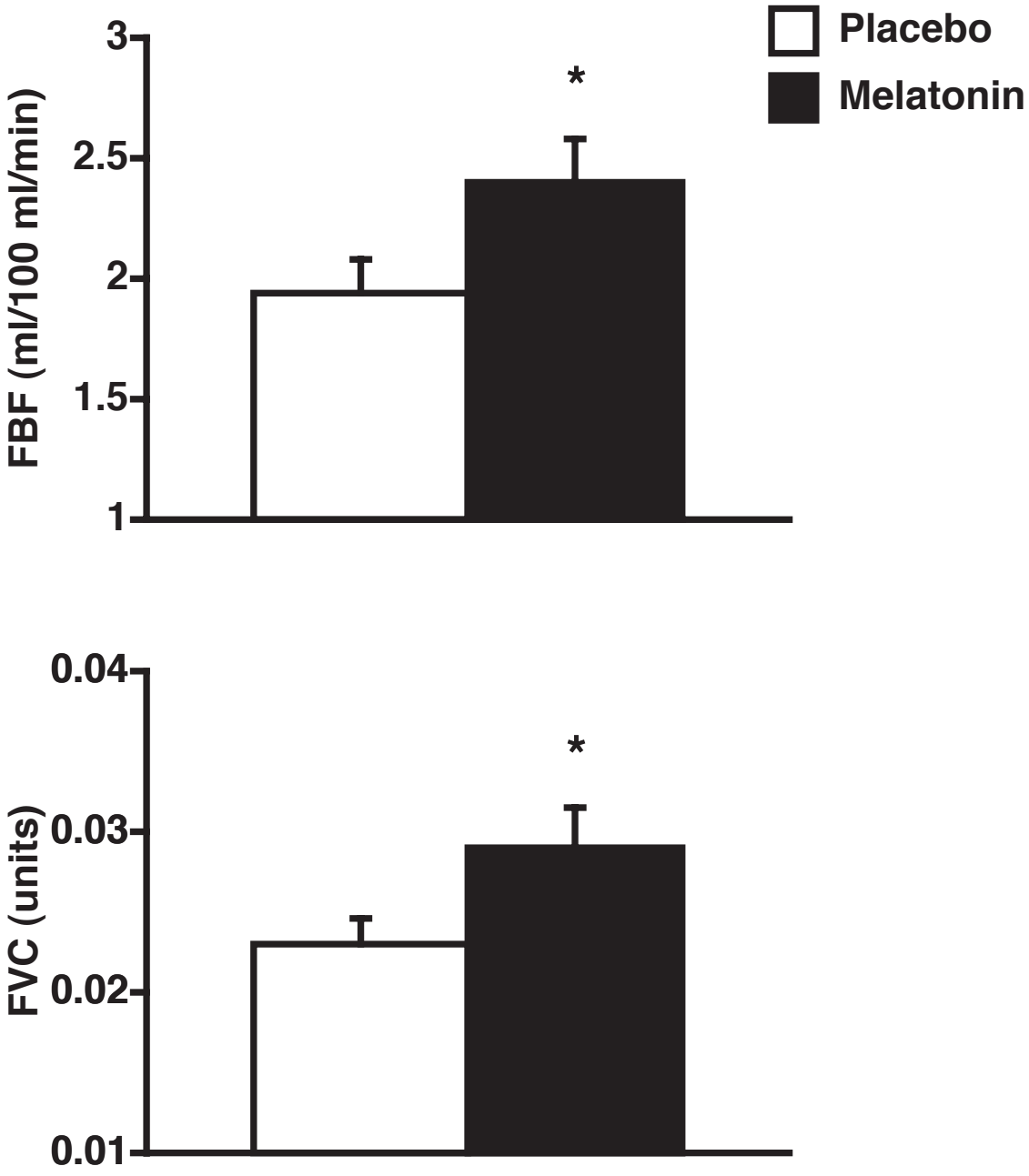

Figure **4-2**: Forearm blood flow (FBF) and forearm vascular conductance (FVC) after placebo or melatonin ingestion. Melatonin ingestion significantly increased FBF and FVC (n = 10). Values are means ± SE. * Significantly different from placebo; $p < 0.05$. FBF: $P = 0.002$; FVC: $P = 0.007$.

Calf blood flow and calf vascular conductance were not significantly different between placebo and melatonin trials (calf blood flow: 2.3 ± 0.2 vs. 2.1 ± 0.2 ml·100ml^{-1}·min^{-1}; calf vascular conductance: 0.028 ± 0.003 vs. 0.025 ± 0.003 a.u., respectively; Figure 4-3).

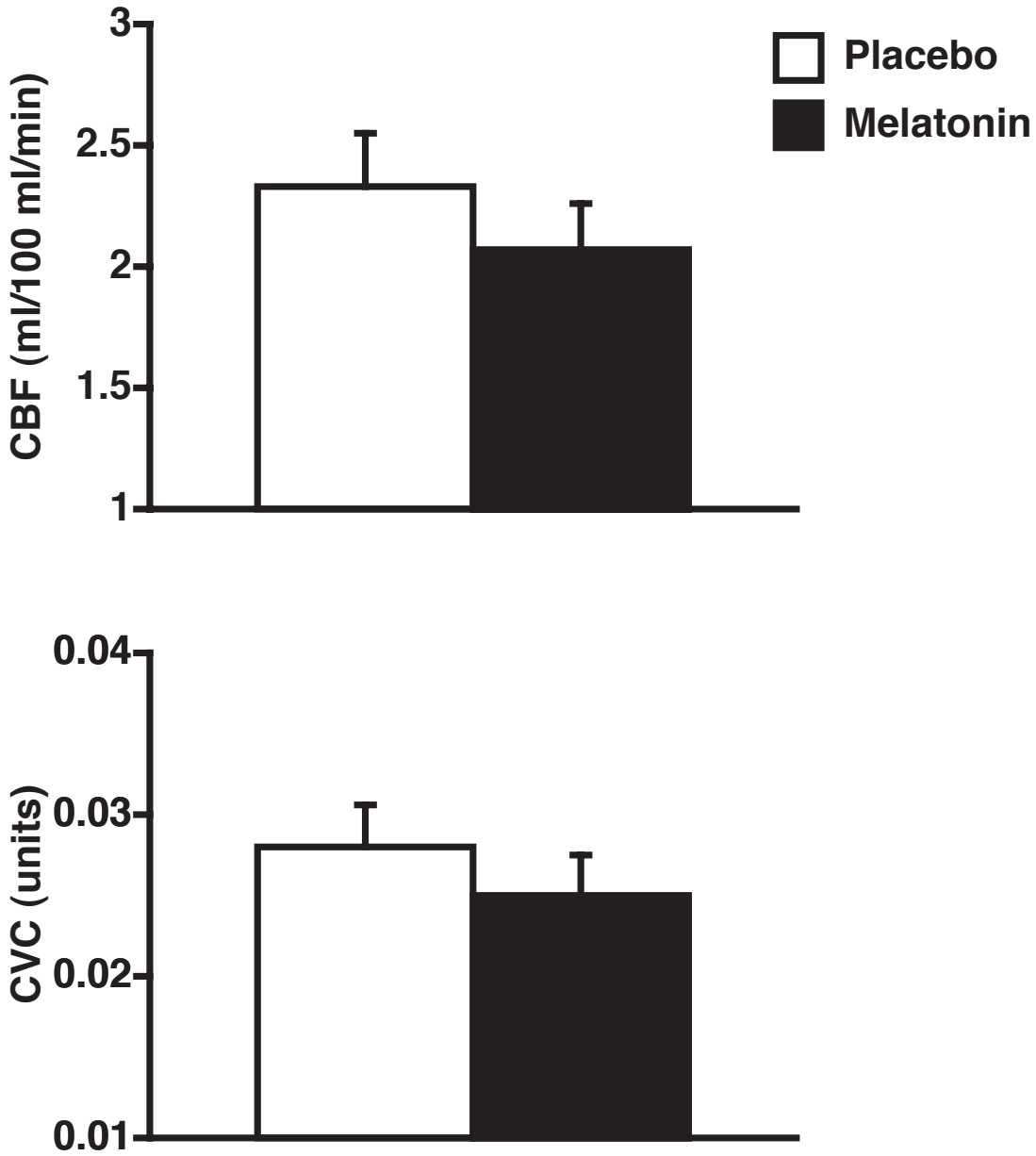

Figure **4-3**: Calf blood flow (CBF) and calf vascular conductance (CVC) after placebo or melatonin ingestion. Melatonin ingestion did not alter CBF or CVC (n = 10). Values are means ± SE. CBF: P = 0.21; CVC: P = 0.22.

Cerebral blood flow and cerebrovascular conductance were not significantly different between placebo and melatonin trials (cerebral blood flow: 55 ± 4 vs. 56 ± 4 $cm \cdot s^{-1}$; cerebrovascular conductance: 0.66 ± 0.05 vs. 0.66 ± 0.05 $cm \cdot s^{-1} \cdot mmHg^{-1}$, respectively; Figure 4-4).

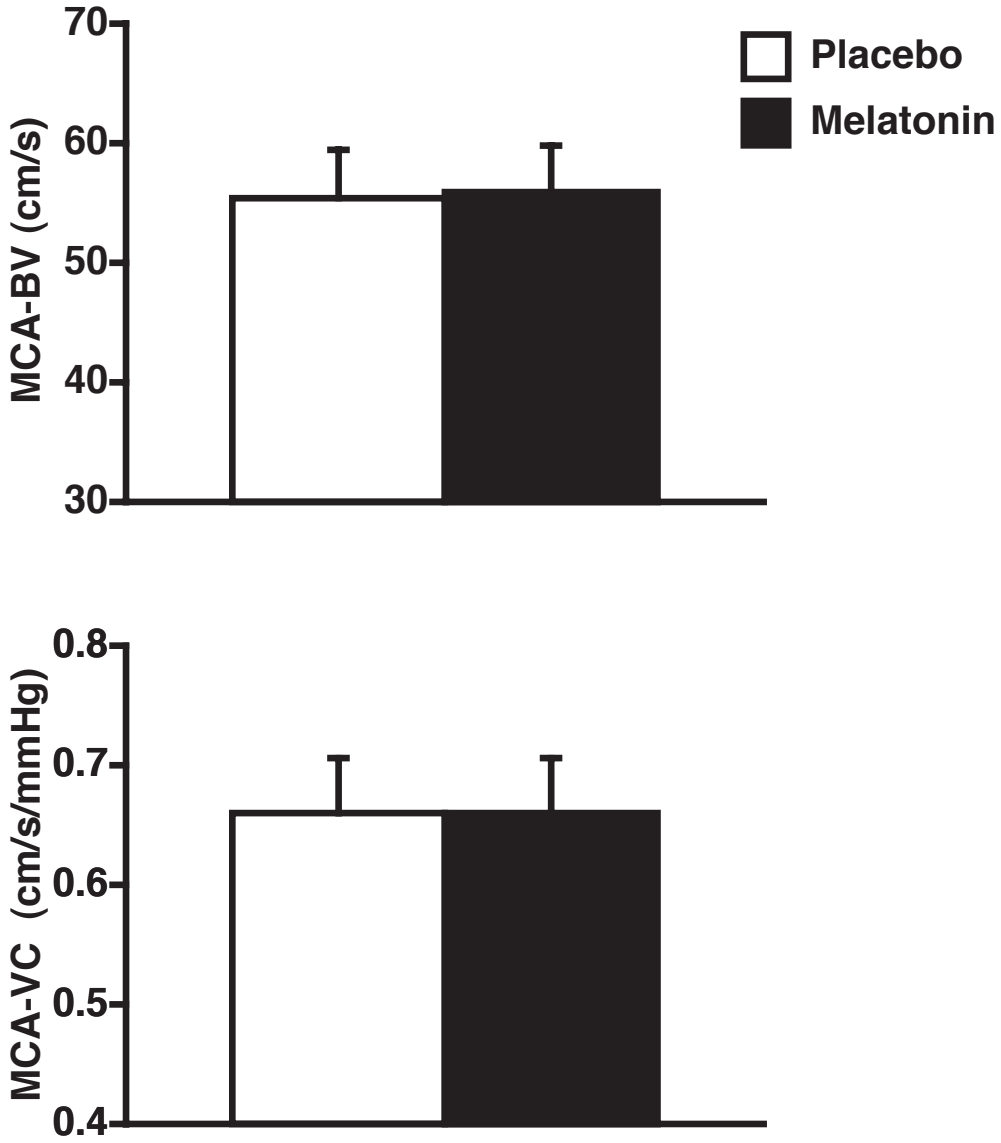

Figure **4-4**: Cerebral blood flow velocity (MCA-BV) and cerebrovascular conductance (MCA-VC) after placebo or melatonin ingestion. Melatonin ingestion did not alter MCA-BV or MCA-VC (n = 10). Values are means ± SE. MVC-BV: P = 0.88; MVC-VC: P = 0.97.

4.3.2 Time Control

Heart rate and mean arterial blood pressure were not different between time control trials (heart rate: 70 ± 4 vs. 68 ± 3 beats/min and mean arterial pressure: 87 ± 2 vs. 86 ± 2 mmHg). Renal, forearm, calf, and cerebral blood flows and vascular conductances were not different between time control trials. The differences between trials for renal, forearm, calf, and cerebral conductances were: renal vascular conductance: Δ -0.03 ± 0.06 $cm \cdot s^{-1} \cdot mmHg^{-1}$; forearm vascular conductance: Δ -0.002 ± 0.005 a.u.; calf vascular conductance: Δ -0.002 ± 0.004 a.u.; cerebrovascular conductance: Δ 0.04 ± 0.05 $cm \cdot s^{-1} \cdot mmHg^{-1}$, respectively.

4.4 Discussion

The novel finding of the present study was that melatonin differentially alters blood flow in vascular beds in humans. Renal blood flow was significantly decreased and forearm blood flow was significantly increased after melatonin ingestion. However, calf and cerebral blood flows were not altered after melatonin ingestion.

Previous research in animal models suggests melatonin may have a differential effect on the vasculature. In the rat, melatonin has been demonstrated to vasoconstrict the tail and cerebral arteries (Capsoni *et al.*, 1995; Doolen *et al.*, 1998; Vandeputte *et al.*, 2001; Ersahin *et al.*, 2002; Masana *et al.*, 2002). Additionally, vasoconstrictive responses have been observed with melatonin in porcine coronary arteries (Weekley, 1993). In contrast, melatonin mediated vasodilator responses have been reported in rat and rabbit aorta, iliac, and renal arteries (Satake *et al.*, 1986; Shibata *et al.*, 1989; Satake *et al.*, 1991a; Satake *et al.*, 1991b). The current study is the first to demonstrate melatonin's differential effect of blood flow in multiple vascular beds in humans.

Mechanisms for melatonin's action on the vasculature have been demonstrated to include direct melatonin receptor activation (Weekley, 1993; Masana *et al.*, 2002) and through intercellular pathways (Viswanathan *et al.*, 1990; Satake *et al.*, 1991b; Ting *et al.*, 2000). Melatonin binding to MT_1 receptors on the vascular smooth muscle cells has been demonstrated to cause vasoconstriction by potentiating norepinephrine signaling (Viswanathan *et al.*, 1990; Weekley, 1993; Geary *et al.*, 1997; Doolen *et al.*, 1998; Vandeputte *et al.*, 2001; Masana *et al.*, 2002). By activating MT_1 receptors, melatonin inhibits calcium-activated potassium channels (BK_{Ca}) on vascular smooth muscle cells (Geary *et al.*, 1998; Vandeputte *et al.*, 2001). Through a series of experiments using 4P-PDOT, an MT_2 selective antagonist, on rat caudal arteries, Doolen et al. (1998) demonstrated that MT_2 receptors mediate vasodilation. Masana et al. (2002) confirmed the vasodilation function of MT_2 receptors. It is hypothesized that MT_2 receptors activation by melatonin increases NO synthase activity in endothelial cells (Simko & Paulis, 2007). In addition to receptor-mediated vasodilation, previous research has demonstrated a vasodilation effect without specific melatonin binding. Satake et al. (1991b) observed a dilation response in rat aorta with melatonin administration although Viswanathan et al. (1990) demonstrated no specific ^{125}I-melatonin binding in rat aorta. Similar non-receptor mediated vasodilation responses to melatonin were observed in porcine vascular smooth muscle, (Ting *et al.*, 2000). Previous research from our laboratory suggests exogenous melatonin may alter vascular blood flow by another mechanism in humans. We observed an increase in muscle sympathetic nerve activity with 3 mg melatonin ingestion (Chapter 3 paper). This increase in muscle sympathetic nerve activity might cause a greater vasoconstriction in the periphery. Together, these data suggest melatonin alters vascular blood flow through a combination of mechanisms.

^{125}I-melatonin binding is commonly used to determine the presence of melatonin receptors in various vascular beds (Viswanathan *et al.*, 1990; Morgan *et al.*, 1994). In the rat,

specific ^{125}I-melatonin binding was not observed in the renal artery suggesting melatonin receptors are not located in the renal artery (Viswanathan *et al.*, 1990). Therefore, the decrease in renal vascular conductance observed in the present study could be from two possible sources. First, there may be species related differences in melatonin receptor distribution with MT_1 receptors located in human renal arteries but not in rat renal arteries. Second, exogenous melatonin could stimulate neural centers of the brain that increase renal sympathetic nerve activity. Exogenous melatonin has been demonstrated to increase adrenal nerve activity via the suprachiasmatic nucleus (Niijima *et al.*, 1998) and muscle sympathetic nerve activity (Chapter 3 paper). Renal norepinephrine spillover has been demonstrated to positively correlate with muscle sympathetic nerve activity at rest in humans (Wallin *et al.*, 1996). Thus, the increase in muscle sympathetic nerve activity observed with melatonin may reflect increases in renal sympathetic nerve activity.

Forearm blood flow increased with melatonin in the present study. In contrast, calf blood flow was not altered by melatonin in the current study. Previous research by Aoki et al. demonstrated melatonin's cutaneous vasodilator response in the forearm (Aoki *et al.*, 2006; Aoki *et al.*, 2008). Additionally, an increase in skin peripheral blood flow by intravenous melatonin was observed with a distal to proximal skin temperature gradient and finger pulse volume (van der Helm-van Mil *et al.*, 2003). However, a distinction between arm and leg skin blood flow was not made in the study (van der Helm-van Mil *et al.*, 2003). Because MT_1 receptor activation causes vasoconstriction and MT_2 receptor activation causes vasodilation (Doolen *et al.*, 1998), these studies suggest that the net effect of melatonin on the vasculature is related to the distribution of MT_1 and MT_2 receptors. Unfortunately, specific receptor blockers in humans are not currently available, which would specifically delineate MT_1 vs. MT_2 changes in vascular blood flow. Additionally, an increase in muscle sympathetic nerve activity could counteract the receptor mediated vasodilator effect of melatonin in the calf vascular bed resulting in observable

no change in calf vascular conductance with melatonin in the present study. A greater response to α_1-adrenergic receptor stimulation has been observed in the calf vs. forearm vascular beds in humans as measured by strain-gauge plethysmography (Pawelczyk & Levine, 2002). These data further suggest that an increase in muscle sympathetic nerve activity could mask melatonin's vasodilatory response in the calf vascular bed.

Previous research demonstrated that melatonin decreases regional cerebral blood flow in the rat (Capsoni *et al.*, 1995). However, we did not observe a change in middle cerebral artery blood flow velocity with melatonin ingestion in the present study as measured by transcranial Doppler. Additionally, van der Helm-van Mil et al. (2003) did not observe a change in basilar artery blood flow as measured by magnetic resonance imaging with a single pulse of intravenous melatonin in humans. If melatonin alters regional cerebral blood flow in humans as in the rat (Capsoni *et al.*, 1995), our data in combination with other's results suggest that melatonin must alter blood flow in other cerebral arteries involved in brain perfusion in humans.

In contrast to previous research in humans at rest in the supine position (Cagnacci *et al.*, 1998; Arangino *et al.*, 1999), blood pressure did not change in the present study after ingesting melatonin. Cagnacci and Arangino used 1 mg of melatonin to decrease mean arterial blood pressure in men and women (Cagnacci *et al.*, 1998; Arangino *et al.*, 1999). However, in the current study, 3 mg of melatonin was ingested. Previous research from our laboratory suggests 3 mg of melatonin does not alter blood pressure (Ray, 2003). Therefore, the effect of exogenous melatonin on blood pressure appears to be dose related. Research in animals confirms a dose response to melatonin concentration in relation to vascular changes (Doolen *et al.*, 1998; Ting *et al.*, 2000), adrenal nerve activity (Niijima *et al.*, 1998), and hormonal secretion responses (Forsling *et al.*, 1999). These data suggest melatonin functions differently within the body depending on the ingested dose and might explain the different observed responses in blood

pressure with acute melatonin supplementation (Cagnacci *et al.*, 1998; Arangino *et al.*, 1999; Ray, 2003).

Plasma melatonin levels were not measured in the present study. Previous research from our laboratory clearly demonstrates that ingesting 3 mg of melatonin increases plasma melatonin >100 fold than endogenous daytime plasma melatonin levels and that the time course to reach near maximal plasma melatonin levels in young, healthy subjects is ~45 min (Ray, 2003) (Chapter 3 paper). Because young, healthy subjects were also used for the present study, a deviation from our laboratory's previous observations of plasma melatonin levels is not expected.

In summary, melatonin differentially alters vascular blood flow in humans. The different vascular effects observed with melatonin are likely attributable to increased sympathetic nerve activity and the relative distribution of MT_1 and MT_2 melatonin receptors. These findings add to the knowledge of melatonin's effect on the vasculature in humans and are important for understanding the effects of melatonin on blood pressure regulation in humans.

Chapter 5

Circadian rhythm of muscle sympathetic nerve activity: implications for melatonin

5.1 Introduction

The frequency of adverse cardiac events increases in the morning hours following a decrease in the events at night (Muller *et al.*, 1985; Muller *et al.*, 1987; Willich *et al.*, 1987; Maron *et al.*, 1994). An increase in sympathetic nerve activity, due to circadian rhythm (Krantz *et al.*, 1996) or an increase in morning physical activity (Parker *et al.*, 1994; Krantz *et al.*, 1996), has been hypothesized to increase the occurrence of early morning cardiac events. Previous research with patients that have an increased susceptibility to cardiovascular incidents (e.g. hypertensive, coronary heart disease) demonstrated an altered circadian rhythm of heart variability (Guzzetti *et al.*, 1991; Nakano *et al.*, 2001), vasodilatory response (Shaw *et al.*, 2001), and plasma melatonin level (Brugger *et al.*, 1995). Therefore, it is probable that the cyclic variability of cardiac events is in part caused by the body's circadian rhythm.

The suprachiasmatic nucleus (SCN) is the master circadian regulator in humans and modulates melatonin synthesis in the pineal gland. Melatonin levels increase at night and decrease during the day in a circadian rhythm. In the rat, melatonin has been demonstrated to alter adrenal sympathetic nerve activity via the SCN. The administration of peak endogenous melatonin decreases adrenal nerve activity while the administration of pharmacological melatonin increases adrenal nerve activity (Niijima *et al.*, 1998). In humans, previous research from our laboratory demonstrated that the ingestion of pharmacological levels of melatonin increases muscle sympathetic nerve activity (MSNA) at rest (Chapter 3 paper). Additionally,

pharmacological levels of melatonin have been demonstrated to attenuate reflexes involved in blood pressure regulation such as the baroreflex (Ray, 2003) and the vestibulosympathetic reflex (VSR) (Chapter 3 paper). Therefore, the purpose of the present study was to determine if MSNA at rest and the VSR follow a circadian rhythm in humans. Based on previous research and because melatonin is elevated at night, we hypothesized that MSNA at rest will be decreased and the VSR will be attenuated in the late evening hours compared to midday.

5.2 Methods

5.2.1 Subjects

Nine healthy subjects (5 male and 4 female; age: 28 ± 1 yr; height: 174 ± 3 cm; weight: 71 ± 6 kg) were tested. All subjects were normotensive, nonsmokers, nonobese, and not taking any medications that would interfere with the measurements of the protocol. All subjects received a physical examination before participation. Written informed consent was obtained from all subjects after verbal explanation of the experimental protocol. The Institutional Review Board of The Pennsylvania State University College of Medicine approved this study.

5.2.2 Experimental Protocol

Eight subjects started the first trial at midday (11:34 ± 13 min) when physiological melatonin levels are lowest (Brzezinski, 1997). Subjects returned 10-12 hours after the completion of the midday trial in the evening (22:10 ± 5 min), to repeat the procedures when physiological melatonin levels are near their peak (Brzezinski, 1997). Subjects were instructed not to sleep between the two testing sessions and not to exercise 12 hours before the first session

and not between the two testing sessions. Additionally, subjects fasted at least 4 hours before each testing session. One subject performed the night trial first and the midday trial second. MSNA, calf blood flow, heart rate, and mean arterial blood pressure (MAP) were measured continuously in the prone position during 10 min of baseline, 3 min of head-down rotation (HDR), and 3 min of recovery. MSNA recordings were made in the same leg for both the day and night trials. MSNA recordings at rest were repeated in seven subjects on two separate days at midday to determine reliability of measurement.

During baseline of both trials, the subject's neck was extended with the chin supported to bring the head upright as close to the vertical plane as possible. This position approximates the gravitational orientation of the head when an individual is in the upright posture (Shortt & Ray, 1997). For HDR, the head was maximally lowered in the vertical plane over the edge of the table. An investigator moved the head by supporting the forehead and chin, thus producing a passive head movement. Once the head became stationary, only afferent inputs from the otolith organs and not the semicircular canals were engaged.

5.2.3 Data Analysis

Sympathetic bursts were identified from individual inspection of the mean voltage neurograms and with computer assistance. Signal-to-noise ratio of 2:1 and a latency period of ~1.3 seconds from the R wave of the EKG was required. MSNA was expressed as burst frequency (bursts/min) and total MSNA (i.e., sum of bursts area). The area of the bursts was measured by a computer program (Peaks; ADInstruments). Absolute changes from baseline are reported for burst frequency. Relative changes (%) from baseline are reported for total MSNA.

For all trials, the 10 min of baseline were averaged together and reported as the baseline value for the respective trial. During HDR only the first minute is reported and used for statistical

tests for the various measurements. Statistical analyses of the data during HDR trials were performed with a two-within factor [time of day X intervention (HDR)] repeated measures ANOVA (n = 9). A paired t-test was performed for hemodynamic and MSNA values at baseline. Pearson's product correlation between the changes during night and day of MAP and MSNA during baseline was performed to determine association. Significance was set at $p < 0.05$. All data are presented as mean ± SE.

Using estimates of changes in MSNA during HDR in previous studies (Shortt & Ray, 1997; Ray & Hume, 1998; Hume & Ray, 1999; Ray, 2000), changes in MSNA during HDR with melatonin (Chapter 3), and changes in MSNA during LBNP with melatonin (Ray, 2003), a power analysis was run to determine how many subjects would need to be studied to detect a statistically significant effect of the intervention and time of day on MSNA responses (Effect size ≥ 3 bursts/min; SD = 2.5 bursts/min; Power ≥ 0.8, alpha < 0.05, two-sample two-tailed t-test).

5.3 Results

5.3.1 Rest

The left panel of Figure 5-1 displays a representative neurogram from one subject during baseline of the day and night conditions. MSNA burst frequency was significantly lower at night compared to day (9 ± 1 vs. 11 ± 2 bursts/min, respectively; $p < 0.05$; Figure 5-2). In comparison, MSNA burst frequency was not different between the two midday trials (n = 7; 12 ± 1 vs. 11 ± 2 bursts/min; P = 0.82). Heart rate and MAP were significantly elevated at night compared to day (70 ± 4 vs. 66 ± 4 beats·min^{-1} and 91 ± 2 vs. 87 ± 1 mmHg respectively; $p < 0.05$). Calf blood flow and calf vascular resistance were not significantly different between night and day at rest (calf blood flow: 3.2 ± 0.3 vs. 2.8 ± 0.5 ml·100ml^{-1}·min^{-1}; calf vascular resistance: 26 ± 4 vs. 29 ±

5 a.u., respectively). The increase in MAP and decrease in MSNA at night during baseline was not significantly correlated ($R^2 = 0.25$).

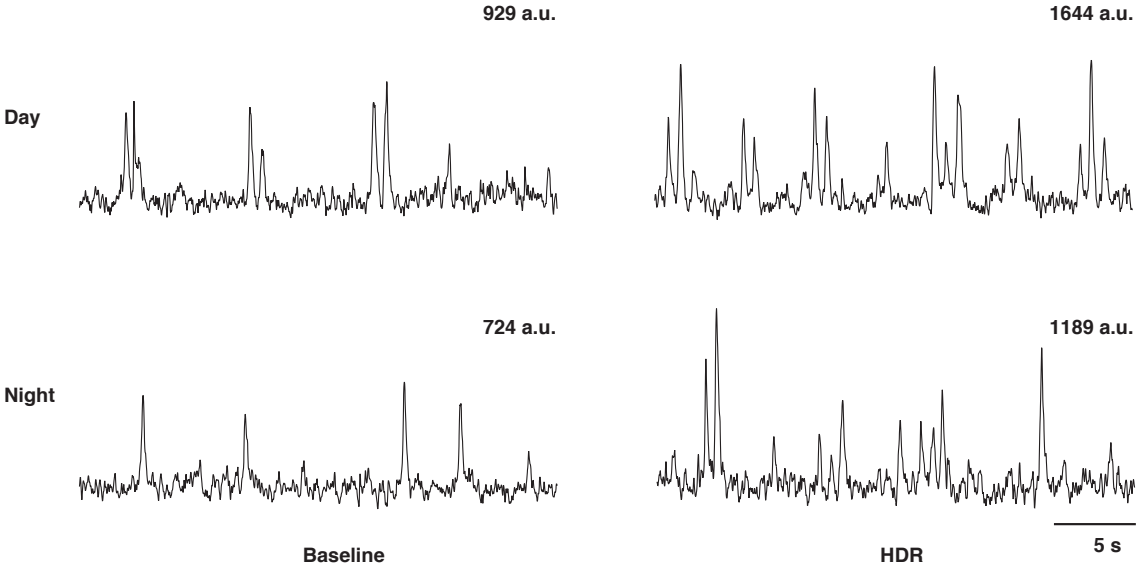

Figure **5-1**: Muscle sympathetic nerve activity recordings (MSNA) from one subject at baseline and during 30 s of head-down rotation (HDR) during the day and night conditions. MSNA total activity is indicated above each recording.

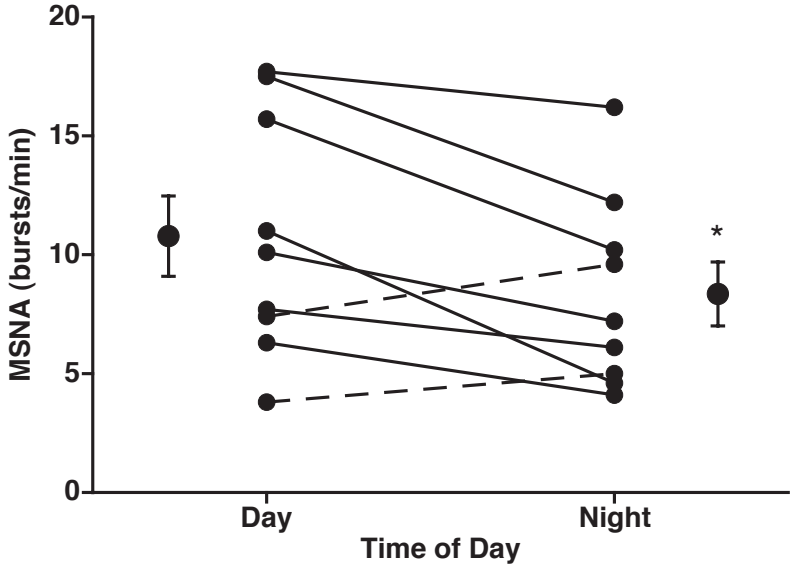

Figure **5-2**: Muscle sympathetic nerve activity (MSNA) at rest during the day and night trials. MSNA decreased at night in seven of the nine subjects. MSNA was significantly decreased at night compared to daytime levels at rest ($P = 0.02$; $n = 9$).

5.3.2 Head-Down Rotation (HDR)

The right panel of Figure 5-1 displays a representative neurogram from one subject during 30 s of HDR during the day and night conditions. HDR significantly increased MSNA responses during the night and day trials ($p < 0.05$). However, time of day had no effect on MSNA responses during HDR (burst frequency: Δ 3 ± 1 vs. Δ 3 ± 1 bursts/min and total MSNA: Δ 41 ± 24 and Δ 68 ± 21%, night and day, respectively; Figure 5-3). Additionally, HDR significantly decreased calf blood flow and increased calf vascular resistance during the night and day ($p < 0.05$). However, the time of day had no affect on calf blood flow or vascular resistance during HDR (calf blood flow: Δ -25 ± 6 vs. Δ -26 ± 7% and calf vascular resistance: Δ 34 ± 10 vs. Δ 41 ± 13%, night vs. day, respectively; Figure 5-4). Heart rate and MAP responses to HDR were not significantly altered between night and day (HR: Δ 0 ± 1 vs. Δ 0 ± 1 beats/min and Δ -3 ± 2 vs. Δ -1 ± 2 mmHg, respectively).

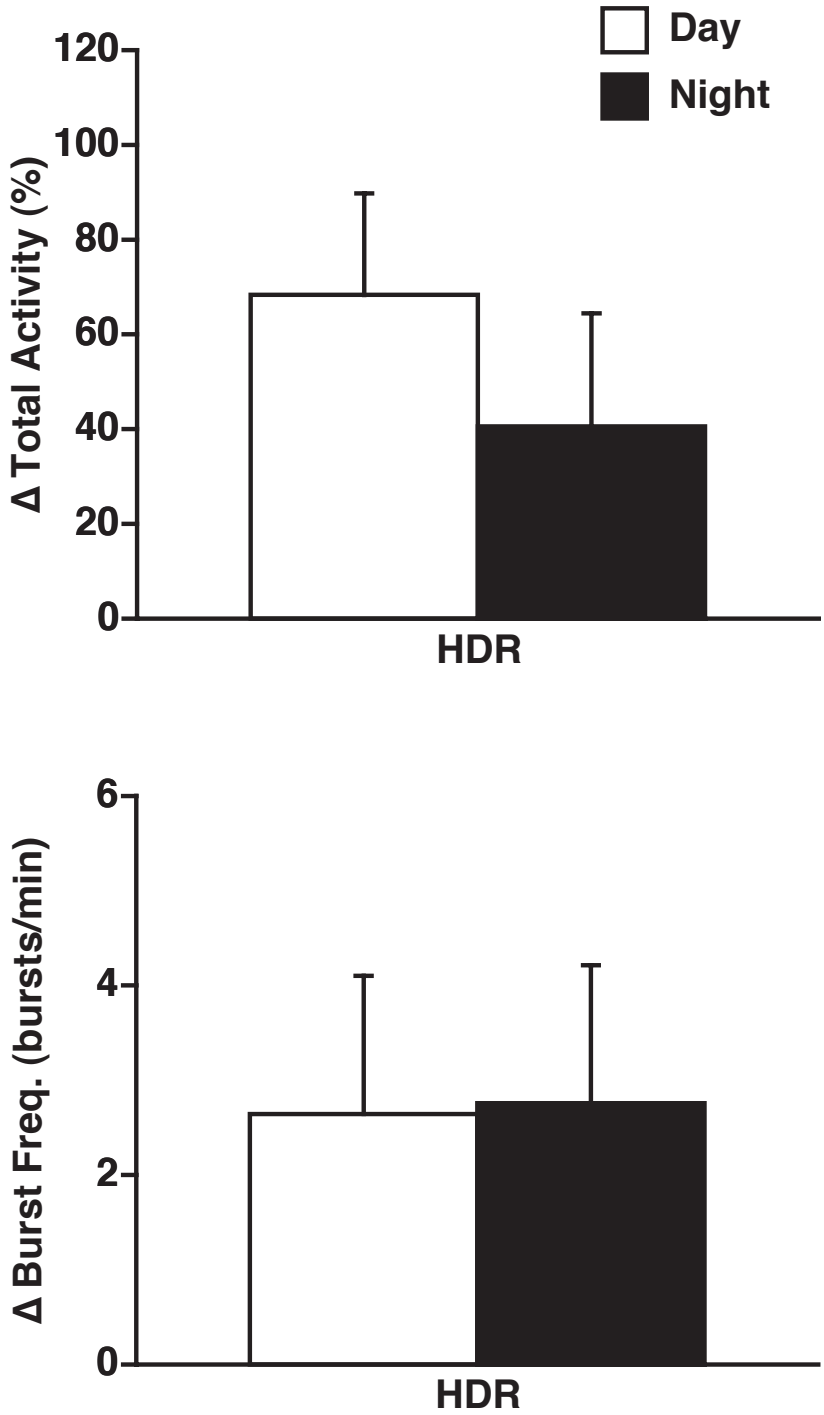

Figure **5-3**: Muscle sympathetic nerve activity (MSNA) responses to head-down rotation (HDR) during the day and night. MSNA responses to HDR were not altered by time of day (n = 9). ΔTotal Activity: P = 0.26; ΔBurst Frequency: P = 0.95.

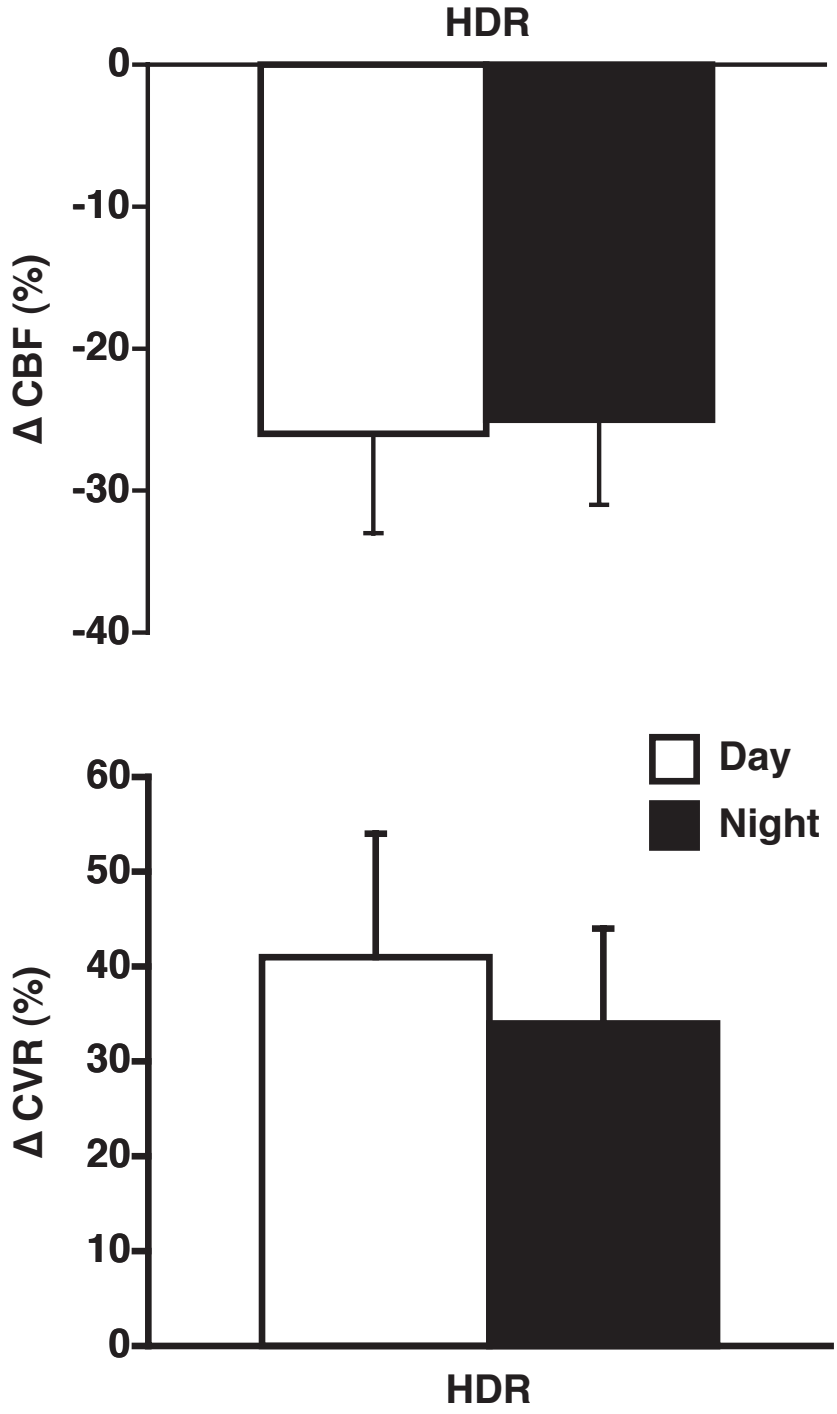

Figure **5-4**: Changes in calf blood flow (CBF) and calf vascular resistance (CVR) to head-down rotation (HDR) during the day and night. CBF and CVR responses to HDR were not altered by time of day (n = 9). ΔCBF: P = 0.72; ΔCVR: P = 0.20.

5.4 Discussion

Two novel findings from the present study are: 1) MSNA at rest is lower at night compared to day, suggesting a diurnal variation in MSNA in humans; and 2) the vestibulosympathetic reflex is not altered by circadian changes in endogenous melatonin.

Previous research in humans suggests the mechanism for sympathetic activity increasing the risk of adverse cardiac events in the morning is either intrinsic or related to increased physical activity after awakening in the morning hours (Parker *et al.*, 1994; Krantz *et al.*, 1996). Because the subjects in the present study were awake for the day and night trials, the observed diurnal variation in MSNA suggests there is an intrinsic circadian rhythm to MSNA at rest in humans.

Increasing and decreasing sympathetic activity has been demonstrated to alter adrenergic responsiveness of the vasculature in humans (Hogikyan & Supiano, 1993; Charkoudian *et al.*, 2006). In the morning hours compared to afternoon, Panza et al. (1991) observed an increase in alpha-sympathetic vasomotor tone. This alteration in alpha-vasoconstrictor activity coincides with the observed decrease in MSNA at rest in the present study. Furthermore, Middlekauff et al. (1995) hypothesized that an increase in tissue responsiveness to norepinephrine increases the incidence of adverse cardiac events.

Why was a circadian rhythm in MSNA at rest observed in the present study and not in the study by Middlekauff et al. (1995)? Middlekauff et al. made their measurements at 6:30 to 8:30 AM and 2:00 to 4:00 PM to coincide with the observed peak and trough of adverse cardiac events, respectively. To confirm a difference between the time points in circadian rhythm, cortisol was measured as their circadian marker. In the present study, time points of 11:30 AM and 10:15 PM were chosen to coincide with known troughs and zenith in plasma melatonin. Plasma melatonin levels would not be expected to be different between the two time points used by Middlekauff et al.. Previous research from our laboratory observed an increase in MSNA with

pharmacological melatonin in humans (Chapter 3 paper). Niijima et al. (1998) observed a similar pattern of melatonin's effect on adrenal sympathetic nerve activity in rats. Endogenous peak melatonin levels decreased and pharmacological melatonin levels increased adrenal sympathetic nerve activity (Niijima *et al.*, 1998). We hypothesize that an increase in melatonin due to its circadian rhythm during the night trial in the current study is important for a decrease in MSNA at rest at night. Therefore, if melatonin is important for the circadian rhythm of MSNA in humans, the results of Middlekauff et al. (1995) would be expected due to their time points tested.

Previous research in humans has demonstrated that heart rate and MAP follows a circadian rhythm decreasing during the night from daytime levels. Why did heart rate and MAP significantly increase during the evening trial in the current study? During the evening hours, light has been demonstrated to increase heart rate (Scheer *et al.*, 1999). Before both trials, subjects started in a lighted environment for equipment setup. During both trials, the lights were turned off to minimize light stimulation. The exposure to light during setup may have caused the increased heart rate and subsequent increase in MAP observed in the night trial of the present study. Baroreflex loading has been demonstrated to decrease MSNA at rest (Dyckman *et al.*, 2007). It is possible that the decrease in MSNA at rest during the night trial is due to the observed elevated MAP. However, this effect is not likely because the change from night to day in MAP and MSNA was not significantly correlated ($r^2 = 0.25$). Therefore, it is probable that baroreflex loading did not affect the results observed in the present study. Additionally, the subjects were not instructed to rest prior to the night study. Because the subjects were active in their daily activities prior to arriving in the laboratory, we would expect their MSNA at rest to be elevated. Thus, the circadian rhythm of MSNA is a powerful observation.

Previous research from our laboratory demonstrated that 3 mg of exogenous melatonin attenuates the VSR in humans (Chapter 3 paper). However, VSR was not altered by time of day in the current study (Figure 3). The expected peak physiological melatonin levels during evening

hours is ~60 $pg \cdot ml^{-1}$, whereas, the peak in plasma melatonin due to 3 mg oral melatonin ingestion was ~865 $pg \cdot ml^{-1}$ (Chapter 3 paper). Therefore, it is plausible that the lower plasma melatonin concentration hindered its attenuation of the VSR in the present study.

Epidemiological data suggest night shift workers have a 40% increased risk of cardiovascular disease (Boggild & Knutsson, 1999). Although the exact cause is not known, the underlying mechanism is hypothesized to be due to changes in circadian rhythm such as changes in melatonin and cortisol (Sack *et al.*, 1992; Weibel & Brandenberger, 1998). Because we observed a circadian rhythm in MSNA at rest in young, healthy subjects in the current study, future research on circadian rhythms of MSNA needs to include populations that exhibit altered circadian rhythms in addition to increased risk for adverse cardiac events.

Additional studies would further our understanding of melatonin's effect on the circadian rhythm of MSNA. First, measuring MSNA at rest during the daytime before and after ingesting 0.5 mg melatonin, a dose that approximates endogenous peak melatonin levels, would elucidate melatonin's role in the circadian rhythm of MSNA. Second, a longer time course overlapping the melatonin transition periods (day to night and night to day) would provide additional time points to more clearly depict the circadian rhythm in MSNA at rest.

In summary, MSNA at rest with subjects in the prone position decreases during the late evening hours compared to midday and exhibits a circadian rhythm whereas the VSR does not. The diurnal variation of MSNA is a novel finding that furthers our understanding of circadian rhythm in humans. Furthermore, a circadian rhythm of MSNA in humans may be important for understanding the cause of increase adverse cardiac events in the morning hours.

Chapter 6

Conclusion

Chapters 3-5 are journal articles that have been accepted or submitted for publication. Appendices D-I are figures representing data not included in the submitted manuscripts (Chapters 3-5). This section will discuss the significance of the findings in Chapters 3-5 and provide future direction for research in the field of neurovascular adaptations by melatonin in humans.

6.1 Summary of Findings

Hypothesis #1 Increases in melatonin will attenuate MSNA responses during head-down rotation (i.e., VSR).

Observation #1 Data from the current study suggest: 1) exogenous melatonin attenuates MSNA responses during head-down rotation; and 2) exogenous melatonin increases MSNA at rest.

Hypothesis #2 Increases in melatonin will attenuate the vestibulocollic reflex as measured by VEMP responses to an auditory stimulus.

Observation #2 Data from the current study suggest melatonin does not alter the vestibulocollic reflex.

Hypothesis #3 Increases in melatonin will elicit differential responses in vascular conductance in renal, forearm, calf, and cerebral vascular beds.

Observation #3 Data from the current study suggest that melatonin differentially alters blood flow in vascular beds in humans. Renal vascular conductance

was significantly decreased and forearm vascular conductance was significantly increased after melatonin ingestion. However, calf and cerebral vascular conductance were not altered after melatonin ingestion.

Hypothesis #4 MSNA at rest will be decreased and the VSR will be attenuated in the late evening hours compared to midday.

Observation #4 Data from the current study suggest: 1) MSNA at rest is lower at night compared to day, suggesting a diurnal variation in MSNA in humans; and 2) the VSR does not exhibit a circadian rhythm in relation to endogenous changes in melatonin.

6.2 Significance of Findings

Melatonin is used by ~15.6 million Americans as a sleep aid, over half of which do not consult their physician before using (Bliwise & Ansari, 2007). Complementary and alternative medicine use has increased from 33.8% to 42.1% of the U.S. population from 1990 to 1997, respectively (Bliwise & Ansari, 2007). Although not directly measured, melatonin use is expected to have increased over this time frame (Bliwise & Ansari, 2007). With a typical dose of 3 mg and recommendation to ingest melatonin 30 min to 2 hours before bedtime (Sanchez-Barcelo *et al.*, 2010), it is important to know how acute melatonin ingestion affects the body. The findings described in Chapters 3 and 4 are 45 min post melatonin ingestion, well within the recommended 2-hour time frame.

Our data clearly demonstrate that depending on concentration (peak endogenous vs. ingestion of 3 mg melatonin), melatonin has various effects within the body: 1) exogenous melatonin attenuates the VSR and endogenous increases in melatonin have no effect on the VSR;

and 2) exogenous melatonin increases MSNA at rest and endogenous increases in melatonin decrease MSNA at rest. Additionally, we did not observe a decrease in MAP after ingestion of 3 mg melatonin although the ingestion of 1 mg of melatonin has been reported to decrease MAP in men and women by 6-9 mmHg (Cagnacci *et al.*, 1997; Cagnacci *et al.*, 1998; Arangino *et al.*, 1999). Why such a difference in observed responses to varying levels of melatonin in humans? One possibility is that melatonin is implicated with several functions/receptor types such as MT_1, MT_2, MT_3, serotonin 5-HT_{1A}, and dopamine D1 receptors, and functioning as an antioxidant. The receptor affinity for melatonin is heterogeneous spanning endogenous and exogenous levels of melatonin. For example, MT_3 receptors have a low affinity for melatonin and may only be activated with higher pharmacological doses of melatonin (>3 mg). The K_i ratios for melatonin binding for MT_3/MT_1 and MT_3/MT_2 are 2,300 and 890, respectively (Nosjean *et al.*, 2001). Melatonin is probably not activating the MT_3 receptors at endogenous melatonin levels, but may activate the receptor with exogenous melatonin ingestion. Therefore, the function of these melatonin-binding sites could cause biological responses and side effects at pharmacological doses of melatonin leading to the divergent responses to melatonin observed in the present studies.

The highest concentrations of melatonin receptors are found in the brain suggesting there might be a central component to melatonin's effect on neurovascular control (Pandi-Perumal *et al.*, 2006). However, minimal research has been performed in this area, and the data do not provide a clear trend to predict the results in humans. In the rat in vivo model, melatonin at peak endogenous levels decreases adrenal nerve activity while melatonin at pharmacological levels increases adrenal nerve activity (Niijima *et al.*, 1998). In contrast, in a rat in vitro model of medial vestibular nuclei neurons, two widely different doses of melatonin produced a graded attenuated response of the number of spontaneous bursts (Podda *et al.*, 2003). These data provide a possible mechanism for the increase and decrease of MSNA with exogenous and peak

endogenous levels of melatonin, respectively, and how melatonin may attenuate the VSR. But what is the ultimate effect of the observed differences in MSNA at rest and during head-down rotation in the present study on vascular blood flow? Interestingly, the observed increase in MSNA in combination with decreased renal blood flow, increased forearm blood flow, and no change in calf blood flow after ingesting 3 mg of melatonin in the current project is the same pattern of responses as observed during mental stress in humans (Carter *et al.*, 2005; Kuipers *et al.*, 2008). These data suggest another layer of complexity exists for neurovascular regulation because MSNA has a differential effect on the vasculature during specific stimuli (i.e., mental stress and melatonin ingestion). Moreover, a difference in MSNA responses to a reflex can have no measureable effect on the vasculature. For example, Appendices D-G demonstrate that ingestion of 3 mg melatonin compared to placebo does not alter renal, forearm, calf, and cerebral vascular responses during 3 min head-down rotation in humans. Additionally, similar responses in MSNA (attenuated) and forearm vascular resistance (no change) were observed between melatonin and placebo trials during lower body negative pressure (Ray, 2003). One possible explanation is that melatonin potentiates norepinephrine-induced vasoconstriction (Vandeputte *et al.*, 2001) counteracting the observed attenuation of the VSR (Chapter 3) and baroreflex (Ray, 2003) responses by melatonin in humans. Although inconclusive to the mechanism of melatonin's action, these data represent a starting point to understanding melatonin's integrative role in neurovascular control.

In the present studies, a difference in blood pressure after ingesting 3 mg melatonin was not observed in subjects in the prone position. In contrast, subjects were oriented in the supine position during blood pressure measurements in prior studies that demonstrated that 1 mg of acute melatonin ingestion decreases blood pressure (Cagnacci *et al.*, 1998; Arangino *et al.*, 1999). Is body position a factor in melatonin's acute effect on resting blood pressure in humans? Previous research from our laboratory suggests no. Subjects were in the supine position during the lower

body negative pressure protocol after ingesting 3 mg of melatonin (Ray, 2003). Dr. Ray did not observe a change in blood pressure at rest due to melatonin ingestion suggesting body position, supine vs. prone, did not cause the observed differences in melatonin's effect on blood pressure at rest.

What are the physiological implications of altered VSR and blood flow by acute melatonin ingestion in humans? The ability of the body to alter blood flow during orthostasis is important to maintain venous return to the heart. Any change in the body's ability to maintain blood pressure via increased vasoconstriction could hinder the orthostatic response. The amount of vasoconstrictor reserve has been demonstrated to alter the control of orthostasis (Fu *et al.*, 2004). In Chapter 3, the VSR is attenuated and in Chapter 4 renal vascular resistance is increased at rest after melatonin ingestion while in the prone position. During orthostasis and VSR activation, renal vascular resistance is increased (Sauder *et al.*, 2008; Conboy *et al.*, 2010). Therefore, melatonin may alter the vasoconstrictor reserve and vasoconstrictor capacity in the renal vascular bed. Additionally, melatonin has been demonstrated to attenuate the baroreflex (Ray, 2003) and plasma norepinephrine during standing (Cagnacci *et al.*, 1998; Arangino *et al.*, 1999), further suggesting melatonin may alter the sympathetic response to orthostasis and may increase the incidence of hypotension during orthostasis. In contrast, Appendices H and I demonstrate that ingestion of 3 mg melatonin compared to placebo does not alter mean arterial blood pressure and MSNA responses, respectively, during 25 min head-up tilt. The lack of melatonin ingestion altering MSNA or mean arterial blood pressure responses during head-up tilt is particularly interesting and unexpected. Therefore, Appendices H and I demonstrate the body's ability to use other mechanisms (e.g. vasomotor, heart rate) to maintain blood pressure during orthostasis in a young healthy population.

It is important to note that the findings in Chapters 3 and 4 are from acute melatonin ingestion. One of the driving forces for the current project was the observation that acute

ingestion of 1 mg of melatonin lowers blood pressure in men and women (Cagnacci *et al.*, 1998; Arangino *et al.*, 1999). We did not observe a decrease in blood pressure with 3 mg of acute melatonin. However, previous research in humans suggests there may be a significant hypotensive response with chronic doses of melatonin greater than 1 mg. Scheer et al. (2004) demonstrated that 2.5 mg is not effective in lowering blood pressure when ingested acutely but successfully lowers nocturnal blood pressure after 3 weeks of chronic supplementation in male patients with essential hypertension. Similar nocturnal hypotensive responses have been observed in female patients with essential hypertension (Cagnacci *et al.*, 2005) and in male patients with nocturnal hypertension (Grossman *et al.*, 2006). These findings highlight a new type of melatonin administration, time-release capsules. The significance of a time-release capsule is that it may mimic the natural rise and plateau in plasma melatonin during evening hours and may better aid populations that have altered circadian rhythms of melatonin.

6.3 Future Direction

Two separate lines of research emerged during this project: 1) effect of exogenous melatonin on blood pressure regulation; and 2) role of circadian rhythm of melatonin in adverse cardiac events. Future research on these two topics is outlined below.

6.3.1 Exogenous Melatonin

1. Common over-the-counter doses of melatonin range from 1-5 mg. Different effects have been observed with various melatonin doses, such as 1 mg melatonin ingestion decreases blood pressure (Cagnacci *et al.*, 1998; Arangino *et al.*, 1999) and 3 mg melatonin ingestion does not alter blood pressure (Ray, 2003) (Chapters 3 and 4). Additionally,

time-release capsules of varying doses have started to be examined (Scheer *et al.*, 2004; Cagnacci *et al.*, 2005). Future research should include investigating the dose response for melatonin and its effects on the VSR, the baroreflex, and blood flow.

2. Previous research in humans suggests chronic melatonin use lowers blood pressure at night (Scheer *et al.*, 2004; Cagnacci *et al.*, 2005). However, the mechanisms of action for melatonin in this observed response are not known. Two possible studies are 1) determine if sympathetic activity at night is altered with chronic melatonin ingestion; and 2) determine if vascular responsiveness is altered with chronic melatonin ingestions.

3. The expression patterns of melatonin receptors in humans needs to be determined to better predict and test the effects of melatonin on blood pressure regulation. Any study should include categories of gender, age, and disease state/risk. This study would be difficult to complete because the possible significance of the findings do not justify performing an invasive technique such as vascular biopsies. Two other options are obtaining tissue samples from cadavers and determining if melatonin has similar effects in non-human primates.

4. Ramelteon is a new melatonin substitute that activates melatonin receptors but does not function as an antioxidant. Repeating the blood flow studies (Chapter 4) with Ramelteon would help delineate melatonin's effects on the receptors versus functioning as an antioxidant. Whether Ramelteon also activates other melatonin binding sites (e.g. MT_3, serotonin 5-HT_{1A}, and dopamine D1 receptors) is equivocal. If Ramelteon does not bind to these sites, the specific effects of MT_1 and MT_2 receptor activation can be determined.

6.3.2 Circadian Rhythm

1. The findings in Chapter 5 demonstrated that young, healthy individuals have a circadian rhythm of MSNA. However, this population is not at risk for adverse cardiac events. Therefore, populations with increased risk of adverse cardiac events and abnormal circadian rhythms of melatonin (e.g., night shift workers and older adults) need to be studied to determine if a circadian rhythm of MSNA is implicated in these conditions.

2. With the development of time-release melatonin capsules, it is important to determine the effect of these capsules on populations with altered circadian rhythms of melatonin. Patients with essential hypertension often have an altered circadian rhythm of melatonin (Jonas *et al.*, 2003; Zeman *et al.*, 2005). With the nightly ingestion of a time-release melatonin capsule, these patients have reduced blood pressure at night (Scheer *et al.*, 2004; Cagnacci *et al.*, 2005). Whether a change in the circadian rhythm of MSNA causes this observed decrease in nightly blood pressure is unknown.

3. In Chapter 5, we demonstrated that MSNA at rest is lower at night compared to daytime in relation to the endogenous circadian rhythm of melatonin. Additional studies would further our understanding of melatonin's effect on the circadian rhythm of MSNA at rest: 1) measuring MSNA at rest during the daytime before and after ingesting 0.5 mg melatonin, a dose that approximates endogenous peak melatonin levels; and 2) a longer time course overlapping the melatonin transition periods (day to night and night to day) would provide additional time points to more clearly depict a circadian rhythm in MSNA at rest.

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Appendix A

Subject Consent Form

This form is not valid unless this box includes an approval stamp by the IRB

CONSENT FOR RESEARCH

Penn State College of Medicine
The Milton S. Hershey Medical Center

Title of Project: The Effects of Melatonin on the Vestibulosympathetic Reflex

Principal Investigator: Chester A. Ray, Ph.D.

Other Investigators: Urs A. Leuenberger, M.D., Cheryl Blaha, R.N., Jessica Mast, R.N., Charity Sauder, M.S., Heather Kemp, B.S., Amber Morgan, B.S., and Jonathan Cook, M.S.

Participant's Printed Name: _____

This is a research study. Research studies include only people who want to take part. This form gives you information about this research, which will be discussed with you. It may contain words or procedures that you don't understand. Please ask questions about anything that is unclear to you. Discuss it with your family and friends and take your time to make your decision.

1. **Purpose of the Research:**
   You are being offered the opportunity to take part in this research because you are a healthy volunteer.

   The purpose of this research is to investigate the effects of a substance that helps with sleeping (melatonin) on the part of the body's nervous system that controls blood pressure and blood flow (sympathetic nervous system) when

your vestibular system (a portion of your inner ear that helps to maintain balance, and regulate blood pressure) is activated. We speculate that melatonin will cause a decrease in muscle sympathetic nerve activity and affect blood flow to various areas of your body when your vestibular system is stimulated. Melatonin is a substance found naturally in your body and is also available as an over the counter supplement that is not regulated by the Food and Drug Administration.

Approximately 48 people will take part in this research at the Hershey Medical Center.

2. Procedures to be Followed:

There will be two parts to the study (Study A and Study B). While both parts utilize the same procedures, they will differ in regards to whether you take a 3 mg melatonin capsule or placebo capsule and the timing of the procedures and blood draws.

You will receive a physical examination by a licensed healthcare worker at the first study session.

If you are a woman of childbearing potential, a urine pregnancy test will be performed before each study session to insure that you are not pregnant.

An intravenous catheter (plastic tube) will be inserted into a vein near your elbow to collect blood. The nurse will examine your arms to determine which vein to insert this catheter in. The blood will be used to measure melatonin concentration. The total amount of blood taken for each study day will be about one and a half tablespoons or about one ounce.

For Study A only, the investigators will randomly select either a 3 mg melatonin capsule or a sugar capsule. You will not know which capsule you are taking. You will swallow either the 3 mg melatonin capsule or a sugar capsule. If you are participating in Study B you will not take either the melatonin or placebo capsule.

Your body temperature will be measured using an ear (tympanic) thermometer.

A small cuff will be placed on one of your fingers to measure your blood pressure. A cuff will also be placed on your upper arm to monitor blood pressure similar to that used in a doctor’s office.

Three, two-inch square adhesive patches will be attached to your chest and will be connected to an electrocardiogram (EKG) machine to continuously monitor your heart rate and the electrical activity of your heart.

A Velcro belt will be placed around your chest to measure your breathing. This device is called a pneumotrace.

Blood pressure cuffs will be placed around your upper arm or thigh and wrist or ankle, while a mercury-filled strain gauge (similar to a rubber band) will be placed around the limb. The wrist cuff will be inflated to above your systolic pressure, (the pressure that is measured while your heart is beating) stopping blood flow, while the upper arm or thigh cuff will be only slightly inflated for short periods of time while measurements are being taken. This test measures the flow of blood in either your arm or calf, and it is called venous occlusion plethysmography. The blood pressure cuffs may be inflated for up to 10 minutes at a time and then released to give you a break.

Doppler ultrasound may be used to measure blood flow through the blood vessels in your arms, legs, or other major organ systems (i.e. the kidney, liver, etc). The Doppler ultrasound technique is similar to the one used to monitor the development of an unborn fetus. A probe will be placed over the arteries in your leg, arm, abdomen, or neck. There may be multiple probes placed on you at a time. The probe is about six inches in length and two inches wide and will be held in place by one of the investigators. Doppler ultrasound will be performed for up to 10 minutes at a time. The machine will be removed or turned off between these measurements.

You will be positioned on a tilt table that can tilt you head upwards to a maximum of 90˚. (Note: 45˚ of tilt is halfway between lying down and standing, and 90˚ is standing upright.) After the tilt you will be returned to the lying (supine) position. This is done to simulate the effects of standing.

You will undergo a procedure called microneurography. This involves introducing two very fine, sterilized nerve wires (microelectrodes), thinner than a sewing needle, through the skin, one of which is inserted further into a superficial nerve of your leg or arm (behind and below the outside of your right knee or on the upper portion or your arm). This procedure is used to measure nerve activity from the brain to the leg or arm. A small pen-like device will be placed on the outside of your skin close to where the nerve is and a small electrical current will be used to stimulate the nerve. This will cause your arm or leg to twitch. Once the nerve is mapped the needles will be inserted through the skin. Locating the nerve with the nerve wires will take a maximum of one hour. During this procedure you may have the electrode readjusted; however, the time used to first find the nerve and adjust the electrodes will not exceed one hour. The electrodes will remain in place for the remainder of the study time (approximately 1-2 hours). If the time for readjusting the electrodes exceeds an hour we will discontinue the protocol. This microneurography procedure may be repeated each day of testing. You may ask the study staff to stop the procedure if you are too uncomfortable.

You will be asked to place your hand into ice water for 2 minutes or less to stimulate your nervous system.

You will be asked to hold your breath for as long as you can. This is called apnea.

At the end of the study procedures all the monitors will be removed and you will be able to leave the laboratory.

You will be asked whether you feel drowsy or sleepy during the study and immediately following procedures. If you do feel drowsy or if symptoms of drowsiness are observed (excessive yawning, falling asleep, etc.) you will be asked to make arrangements to have someone else drive you home or you may rest in the laboratory until you feel more alert.

For Study A only, you will be asked to return to the laboratory 2-4 days later, at the same time of the day, so the same procedures can be repeated taking the opposite capsule (melatonin or placebo).

For Study B only you will be asked to return to the laboratory for a continuation of this study. This will require you to arrive at the laboratory at midday for 2-3 hours and return to the laboratory 10-12 hours later on the same day for 2-3 hours. The same procedures that took place at the midday session will be repeated (with the exception of the blood draw). You will be asked to return at a later date for the blood draw at a time of day similar to when you come for the nighttime data collection session.

You may be asked to return to repeat sections of this research if there are problems with collecting or recording data.

You will be asked to complete a follow-up questionnaire 7-10 days after completion of testing.

3. <u>Discomforts and Risks:</u>

The discomfort associated with inserting the catheter into a vein in your arm is a slight pinch or pinprick when the sterile needle enters the skin. The risks include mild discomfort and/or a black-and-blue mark at the site of puncture. Less common risks include a small blood clot, infection or bleeding at the puncture site, and on rare occasions fainting during the procedure.

Side-effects of melatonin may include drowsiness, a feeling of uneasiness or sadness, giddiness, and nausea.

There is a very small risk of a skin allergic reaction to the electrode adhesive pads used for monitoring heart rate.

There may be some discomfort associated with inflation of the plethysmography cuffs. This discomfort will end once the interventions have been completed.

There is a very small risk of skin burns from the Doppler sound wave signal, which can be avoided by keeping the sound wave intensity at low levels. If you report warmth or discomfort, the Doppler intensity will be readjusted downwards.

The tilt table should cause no discomfort and should not pose any major risk of illness. Blood pressure and heart rate will be closely monitored during the entire procedure. At high levels of tilt (60 to 90 degrees) lightheadedness or fainting may occur which is associated with low blood pressure. If your blood pressure begins to drop or you feel symptoms associated with syncope (fainting) you will be returned to the supine (lying on your back) position immediately.

Stimulation of the arm or leg nerve through the skin will cause twitching in the calf and foot of the leg or forearm and hand of the arm. Placement of the microneurography electrode in the nerve will be associated with a pinprick sensation. It may also cause lightheadedness or mild cramping, twitching, or tingling in the calf/foot or forearm/hand. There may be mild tenderness at the site of the electrode insertion, which may last for a few days. There is a very small risk of bleeding or infection at the electrode insertion site. To minimize the risk of nerve damage with microneurography, the electrode will be manipulated for 1 hour or less. If the electrodes cannot be successfully placed, the rest of the protocol will proceed.

There may be some discomfort associated with placing your hand in ice water. This discomfort will end once the interventions have been completed.

It is important that a fetus (developing unborn baby) not be exposed to any unnecessary risks. If you are a female capable of becoming pregnant, you must not be pregnant at the beginning of this investigation. Pregnancy tests will be performed before the beginning of each testing session during this research study.

There is no risk associated with the sugar pill that will be administered during this study, the ear thermometer, holding your breath for a short period of time, the belt that will monitor respiration, or the finger blood pressure monitoring unit or blood pressure cuff placed on the upper arm (Dinamap).

4. Possible Benefits:

a. Possible benefits to the participant:

You will not benefit from taking part in this research study.

b. Possible benefits to others:

A better understanding of how melatonin affects the nervous system and blood pressure regulation.

5. Other Options that Could be Used Instead of this Research:

You do not have to take part in this research study.

6. Time Duration of the Procedures and Study:

Study A: If you agree to take part in this study, your involvement will last approximately 3-4 hours per study day (6-8 hours total). If you are asked to repeat some of the testing the time commitment will be the same.

Study B: If you agree to take part in this portion of the study, your involvement will last approximately 2-3 hours per session (4-6 hours total). You will also be asked to return at a later date at a time similar to the nighttime session to have blood drawn. This session will last less than one hour.

The questionnaire will take you approximately 5 minutes to complete.

7. Statement of Confidentiality:

a. Privacy and confidentiality measures

Your records that are used in the research at The Milton S. Hershey Medical Center (HMC) and Penn State College of Medicine (PSU) will be labeled with your initials and subject number until the data is analyzed, at which time your initials will be removed. The list that matches your name with the code number will be kept in a locked file in Dr. Ray's office. The research records will be kept in a password-protected computer file in Dr. Ray's laboratory. Your samples collected for research purposes will be labeled with a code number, date, and protocol number and will be stored in a freezer in the Core Endocrine Lab until analysis is completed. Your samples will then be discarded.

In the event of any publication or presentation resulting from the research, no personally identifiable information will be shared.

7b. The use of private health information:

If you give your consent, health information about you will be collected for this research. Health information is protected by law as explained in the HMC Privacy Notice. If you have not received this notice, please request a copy from the researcher. At HMC/PSU your information will only be used or shared as explained in this consent form or when required by law. However, some of the other people/groups who receive your health information may not be required by Federal privacy laws to protect your information and may share it without your permission.

If you do not want us to use your protected health information, you may not participate in this research.

Your permission for the use, storage, and sharing of your identifiable health information will continue indefinitely. Any research information in your medical record will be kept indefinitely.

If you choose to participate, you are free to withdraw your permission for the use and sharing of your health information at any time. You must do this in writing. Write to Dr. Ray and let him know that you are withdrawing from the research study. His mailing address is Penn State College of Medicine, Heart and Vascular Institute, H047, 500 University Dr., Hershey, PA 17033.

If you withdraw your permission:

- We will no longer use or share medical information about you or your samples for this research study, except when the law allows us to do so.
- We are unable to take back anything we have already done or any information we have already shared with your permission.
- We may continue using and sharing the information obtained prior to your withdrawal if it is necessary for the soundness of the overall research.
- We will keep our records of the care that we provided to you as long as the law requires.

The research team may use the following sources of health information.

- Your medical history as it relates to the research study.
- All data collected during the testing period.
- Questionnaire results.

Representatives of the following people/groups <u>within HMC/PSU</u> may use your health information and share it with other specific groups in connection with this research study.

- The principal investigator, Chester A. Ray, Ph.D.
- The HMC/PSU Institutional Review Board
- The HMC/PSU Human Subjects Protection Office
- The research team

- The study coordinators

The above people/groups may share your health information with the following people/groups outside HMC/PSU for their use in connection with this research study. These groups, while monitoring the research study, may also review and/or copy your original PSU/HMC records.

- The Office of Human Research Protections in the U. S. Department of Health and Human Services
- Food and Drug Administration
- The National Institutes of Health

8. Costs for Participation:

a. Costs:

There will be no added cost to you for participating in this research study.

b. Treatment and compensation for injury:

Every effort to prevent injury as a result of your participation will be taken. It is possible, however, that you could develop complications or injuries as a result of participating in this research study. In the event of injury resulting from this research, medical treatment is available but will be provided at the usual charge. It is the policy of this institution to provide neither financial compensation nor free medical treatment for research-related injury.

Costs for the treatment of research-related injuries will be charged to your insurance carrier or to you. Some insurance companies may not cover costs associated with research studies. If for any reason these costs are not covered by your insurance, they will be your responsibility. You will also be responsible for any deductible, co-insurance and/or co-pay. You will not lose any legal rights by signing this form.

9. Compensation for Participation:

You will receive $20 an hour for each study day. If you do not complete the study or you are removed from the study because you do not comply with instructions you will be compensated for what you have completed. Partial compensation for testing days will be $20 per hour. If you are asked to return to the lab to repeat testing you will be receive $20 an hour. You will be asked to provide your address and social security number for tax reporting purposes.

10. Research Funding:

The institution will be reimbursed by the research sponsor The National Institutes of Health (NIH) for use of this site's facilities and for the work the research staff does for this research.

11. Voluntary Participation:

Taking part in this research study is voluntary. If you choose to take part in this research, your major responsibilities will include following the instructions of the investigators during testing sessions. You do not have to participate in this research. If you choose to take part, you have the right to stop at any time. If you decide not to participate or if you decide to stop taking part in the research at a later date, there will be no penalty or loss of benefits to which you are entitled.

Your research doctor may take you out of the research study without your permission. Some possible reasons for this are: You do not adhere to the investigator's instructions or follow the protocol as stated in this consent form.

12. Contact Information for Questions or Concerns:

You have the right to ask any questions you may have about this research. If you have questions, complaints or concerns or believe you may have developed an injury related to this research, Chester A. Ray, Ph.D. at 717-531-3906 or the Cardiology doctor on 24-hour call at 717-531-8521 (have the operator page the doctor).

If you have questions regarding your rights as a research participant or you have concerns or general questions about the research or about your privacy and the use of your personal health information, contact the research protection advocate in the HMC Human Subjects Protection Office at 717-531-5687. You may also call this number if you cannot reach the research team or wish to talk to someone else.

For more information about participation in a research study and about the Institutional Review Board (IRB), a group of people who review the research to protect your rights, please visit the HMC IRB's Web site at http://www.hmc.psu.edu/irb. Included on this web site, under the heading "Participant Info", you can access federal regulations and information about the protection of human research participants. If you do not have access to the internet, copies of these federal regulations are available by calling the HSPO at (717) 531-5687.

Signature and Consent/Permission to be in the Research

Before making the decision regarding enrollment in this research you should have:

- Discussed this study with an investigator,
- Reviewed the information in this form, and

- Had the opportunity to ask any questions you may have.

Your signature below means that you have received this information, have asked the questions you currently have about the research and those questions have been answered. You will receive a copy of the signed and dated form to keep for future reference.

Participant: By signing this consent form, you indicate that you are voluntarily choosing to take part in this research.

_____	_____	_____	_____
Signature of Participant	Date	Time	Printed Name

Person Explaining the Research: Your signature below means that you have explained the research to the participant/participant representative and have answered any questions he/she has about the research.

_____	_____	_____	_____
Signature of person who explained this research	Date	Time	Printed Name

Appendix B

Subject Addendum Consent Form

This form is not valid unless this box includes an approval stamp by the IRB

ADDENDUM TO CONSENT FOR RESEARCH
Penn State College of Medicine
The Milton S. Hershey Medical Center

Title of Project: The Effects of Melatonin on the Vestibulosympathetic Reflex

Principal Investigator: Chester A. Ray, Ph.D.

Other Investigators: Urs A. Leuenberger, M.D., Cheryl Blaha, R.N., Jessica Mast, R.N., Charity Sauder, M.S., Heather Kemp, B.S., Amber Morgan, B.S., and Jonathan Cook, M.S.

Participant's Printed Name _____

This consent form addendum gives you additional information about this research, which will be discussed with you. This addendum may contain words or procedures that you do not understand. You are urged to ask questions about anything that is unclear to you. You will receive a copy of the signed and dated consent form addendum to keep.

You have been asked to repeat the following procedures for this study.

___	A urine pregnancy test will be performed on women of child bearing potential to rule out pregnancy.
___	An intravenous catheter (plastic tube) will be inserted into a vein near your elbow to collect blood. The nurse will examine your arms to determine which vein to insert this catheter in. The blood will be used to measure melatonin concentration. The total amount of blood taken for each study day will be about one and a half tablespoon or about one ounce.
___	You will swallow either a 3 mg melatonin capsule or a sugar capsule.
___	Your body temperature will be measured using an ear (tympanic) thermometer.
___	You will have EKG patches placed on your chest to measure the electrical activity of your heart.
___	A small cuff (Finapres cuff) on your finger and a cuff on your arm will measure your blood pressure.
___	Doppler Ultrasound will be used to measure blood flow through the blood vessels in your arms, legs, or other major organ systems (i.e. the kidney, liver, etc…).
___	You will be tilted head upwards to a maximum of 90˚ for a maximum of 30 minutes.
___	You may have Velcro belt placed around your chest to monitor your breathing.
___	You may have blood pressure cuffs placed around the upper portion of your arm or leg and also around your wrist or ankle. These cuffs will be inflated periodically to varying levels to measure blood flow.
___	You may undergo microneurography to measure nerve activity in your arm or leg.
___	You may be asked to place your hand in a bucket of ice water for 2 minutes.
___	You may be asked to hold your breath for as long as you can.
	You will be asked to return to the laboratory 2-4 days later, at the same

___	time of the day, so the same procedures can be repeated taking the opposite capsule (melatonin or placebo).
___	You will be asked to return 10-12 hours later to repeat the protocol.
___	You will be asked to return to the lab at time similar to the nighttime session to have blood drawn. The amount of blood collected will be the same as previous sessions.
___	You will be asked to complete a follow-up questionnaire 7-10 days after completion of testing.

The other sections of the original consent form you signed are still applicable, including the potential benefits and risks. If you would like, the information in the original consent form may be reviewed with you.

Your continued participation in this research is voluntary. You may refuse to continue in the study, now or any time in the future. If you decide to stop taking part in the research now or at a later date, there will be no penalty or loss of benefits to which you are entitled.

Signature and Consent to be in the Research

Participant: Your signature below means that you have read the above new information about this research study and have asked the questions you currently have about the research. By signing this consent form, you indicate that you are voluntarily choosing to continue taking part in this research and have had the opportunity to ask questions and those questions have been answered.

_____	_____	_____	_____
Signature of Participant	Date	Time	Printed Name

Person explaining research: Your signature below means that you have explained the new information about the research to the participant/participant representative and have answered any questions he/she has about the research.

_____	_____	_____	_____
Signature of person who explained this research	Date	Time	Printed Name

Appendix C

Subject Consent Form - VEMP

CONSENT FOR RESEARCH

Penn State College of Medicine
The Milton S. Hershey Medical Center

This form is not valid unless this box includes an approval stamp by the IRB

Title of Project: The Effects of Melatonin on the Vestibulosympathetic Reflex

Principal Investigator: Chester A. Ray, Ph.D.

Other Investigators: Urs A. Leuenberger, M.D., Cheryl Blaha, R.N., Jessica Mast, R.N., Charity Sauder, M.S., Amber Morgan, B.S., and Jonathan Cook, M.S.

Participant's Printed Name: _____

This is a research study. Research studies include only people who want to take part. This form gives you information about this research, which will be discussed with you. It may contain words or procedures that you don't understand. Please ask questions about anything that is unclear to you. Discuss it with your family and friends and take your time to make your decision.

1. **Purpose of the Research:**
   You are being offered the opportunity to take part in this research because you are a healthy volunteer.

   The purpose of this research is to investigate the effects of a substance that helps with sleeping (melatonin) on the vestibular system (a portion of your inner ear that helps to maintain balance, and regulate blood pressure). We speculate that melatonin will cause a change in the response of your vestibular system to an auditory stimulus (tone). Melatonin is a substance found naturally in your body and is also available as an over the counter supplement that is not regulated by the Food and Drug Administration. The

system used to emit the auditory stimulus and measure your vestibular system's response to it is called a VEMP (vestibular evoked myogenic potential) system and is used in the clinical setting to determine if your inner ear is functioning properly and is approved by the Food and Drug Administration.

Approximately 15 people will take part in this portion of this research at the Hershey Medical Center.

2. Procedures to be Followed:

You will receive a physical examination by a licensed healthcare worker at the first study session.

If you are a woman of childbearing potential, a urine pregnancy test will be performed before each study session to insure that you are not pregnant.

An intravenous catheter (plastic tube) will be inserted into a vein near your elbow to collect blood. The nurse will examine your arms to determine which vein to insert this catheter in. The blood will be used to measure melatonin concentration. The total amount of blood taken for each study day will be about three tablespoons or two ounces (about one and a half tablespoons before you take the melatonin capsule and one and a half tablespoons drawn 45 minutes after you ingest the melatonin capsule).

Your body temperature will be measured using an ear (tympanic) thermometer.

A small cuff will be placed on one of your fingers to measure your blood pressure. A cuff will also be placed on your upper arm to monitor blood pressure similar to that used in a doctor's office.

Three, two-inch square adhesive patches will be attached to your chest and will be connected to an electrocardiogram (EKG) machine to continuously monitor your heart rate and the electrical activity of your heart.

You will be asked to lay on a table to perform the following study.

You will be connected to a VEMP (vestibular evoked myogenic potential) system. This will consist of inserting small headphones into both of your ears. These headphones will emit tones that will stimulate your vestibular system. Two adhesive electrode patches will be placed on the left and right sides of your neck. Additionally, two adhesive electrodes will be placed on your forehead. These electrodes will monitor the muscle contractions of your neck during the playing of the auditory tones. You will perform several trials with the investigators asking you to lift your head off the table to create neck tension and then a series of tones (approximately 150) will sound in about 30

seconds. You will be allowed to rest in between sessions. The trials are repeated for each ear.

After performing the protocol once the investigators will give you a 3 mg melatonin capsule. You will swallow the 3 mg melatonin capsule. You will be asked to wait 45 minutes and the above protocol will be repeated (the blood draw and the VEMP testing).

You will be asked whether you feel drowsy or sleepy during the study and immediately following procedures. If you do feel drowsy or if symptoms of drowsiness are observed (excessive yawning, falling asleep, etc.) you will be asked to make arrangements to have someone else drive you home or you may rest in the laboratory until you feel more alert.

You will be asked to complete a follow-up questionnaire 7-10 days after completion of testing.

3. Discomforts and Risks:

The discomfort associated with inserting the catheter into a vein in your arm is a slight pinch or pinprick when the sterile needle enters the skin. The risks include mild discomfort and/or a black-and-blue mark at the site of puncture. Less common risks include a small blood clot, infection or bleeding at the puncture site, and on rare occasions fainting during the procedure.

Side-effects of melatonin may include drowsiness, a feeling of uneasiness or sadness, giddiness, and nausea.

There is a very small risk of a skin allergic reaction to the electrode adhesive pads used for monitoring heart rate.

It is important that a fetus (developing unborn baby) not be exposed to any unnecessary risks. If you are a female capable of becoming pregnant, you must not be pregnant at the beginning of this investigation. Pregnancy tests will be performed before the beginning of each testing session during this research study.

There is no risk associated the ear thermometer, the finger blood pressure monitoring unit, blood pressure cuff placed on the upper arm (Dinamap), or the VEMP system.

4. Possible Benefits:

a. Possible benefits to the participant:

You will not benefit from taking part in this research study.

b. Possible benefits to others:

A better understanding of how melatonin affects vestibular system.

5. Other Options that Could be Used Instead of this Research:

You do not have to take part in this research study.

6. Time Duration of the Procedures and Study:

If you agree to take part in this study, your involvement will last approximately 3-4 hours.

The questionnaire will take you approximately 5 minutes to complete.

7. Statement of Confidentiality:

a. Privacy and confidentiality measures

Your records that are used in the research at The Milton S. Hershey Medical Center (HMC) and Penn State College of Medicine (PSU) will be labeled with your initials and subject number until the data is analyzed, at which time your initials will be removed. The list that matches your name with the code number will be kept in a locked file in Dr. Ray's office. The research records will be kept in a password-protected computer file in Dr. Ray's laboratory. Your samples collected for research purposes will be labeled with a code number, date, and protocol number and will be stored in a freezer in the Core Endocrine Lab until analysis is completed. Your samples will then be discarded

In the event of any publication or presentation resulting from the research, no personally identifiable information will be shared.

7b. The use of private health information:

If you give your consent, health information about you will be collected for this research. Health information is protected by law as explained in the HMC Privacy Notice. If you have not received this notice, please request a copy from the researcher. At HMC/PSU your information will only be used or shared as explained in this consent form or when required by law. However, some of the other people/groups who receive your health information may not be required by Federal privacy laws to protect your information and may share it without your permission.

If you do not want us to use your protected health information, you may not participate in this research.

Your permission for the use, storage, and sharing of your identifiable health information will continue indefinitely. Any research information in your medical record will be kept indefinitely.

If you choose to participate, you are free to withdraw your permission for the use and sharing of your health information at any time. You must do this in writing. Write to Dr. Ray and let him know that you are withdrawing from the research study. His mailing address is Penn State College of Medicine, Heart and Vascular Institute, H047, 500 University Dr., Hershey, PA 17033.

If you withdraw your permission:

- We will no longer use or share medical information about you or your samples for this research study, except when the law allows us to do so.
- We are unable to take back anything we have already done or any information we have already shared with your permission.
- We may continue using and sharing the information obtained prior to your withdrawal if it is necessary for the soundness of the overall research.
- We will keep our records of the care that we provided to you as long as the law requires.

The research team may use the following sources of health information.

- Your medical history as it relates to the research study.
- All data collected during the testing period.
- Questionnaire results.

Representatives of the following people/groups <u>within HMC/PSU</u> may use your health information and share it with other specific groups in connection with this research study.

- The principal investigator, Chester A. Ray, Ph.D.
- The HMC/PSU Institutional Review Board
- The HMC/PSU Human Subjects Protection Office
- The research team
- The study coordinators

The above people/groups may share your health information with the following people/groups <u>outside HMC/PSU</u> for their use in connection with this research study. These groups, while monitoring the research study, may also review and/or copy your original PSU/HMC records.

- The Office of Human Research Protections in the U. S. Department of Health and Human Services
- Food and Drug Administration
- The National Institutes of Health

8. <u>Costs for Participation:</u>

a. Costs:

There will be no added cost to you for participating in this research study.

b. Treatment and compensation for injury:

Every effort to prevent injury as a result of your participation will be taken. It is possible, however, that you could develop complications or injuries as a result of participating in this research study. In the event of injury resulting from this research, medical treatment is available but will be provided at the usual charge. It is the policy of this institution to provide neither financial compensation nor free medical treatment for research-related injury.

Costs for the treatment of research-related injuries will be charged to your insurance carrier or to you. Some insurance companies may not cover costs associated with research studies. If for any reason these costs are not covered by your insurance, they will be your responsibility. You will also be responsible for any deductible, co-insurance and/or co-pay. You will not lose any legal rights by signing this form.

10. Compensation for Participation:

You will receive $20 an hour for the study day. If you do not complete the study or you are removed from the study because you do not comply with instructions you will be compensated for what you have completed. Partial compensation for testing days will be $20 per hour. You will be asked to provide your address and social security number for tax reporting purposes.

10. Research Funding:

The institution will be reimbursed by the research sponsor The National Institutes of Health (NIH) for use of this site's facilities and for the work the research staff does for this research.

11. Voluntary Participation:

Taking part in this research study is voluntary. If you choose to take part in this research, your major responsibilities will include following the instructions of the investigators during testing sessions. You do not have to participate in this research. If you choose to take part, you have the right to stop at any time. If you decide not to participate or if you decide to stop taking part in the research at a later date, there will be no penalty or loss of benefits to which you are entitled.

Your research doctor may take you out of the research study without your permission. Some possible reasons for this are: You do not adhere to the investigator's instructions or follow the protocol as stated in this consent form.

12. Contact Information for Questions or Concerns:

You have the right to ask any questions you may have about this research. If you have questions, complaints or concerns or believe you may have developed an injury related to this research, Chester A. Ray, Ph.D. at 717-531-3906 or the Cardiology doctor on 24-hour call at 717-531-8521 (have the operator page the doctor).

If you have questions regarding your rights as a research participant or you have concerns or general questions about the research or about your privacy and the use of your personal health information, contact the research protection advocate in the HMC Human Subjects Protection Office at 717-531-5687. You may also call this number if you cannot reach the research team or wish to talk to someone else.

For more information about participation in a research study and about the Institutional Review Board (IRB), a group of people who review the research to protect your rights, please visit the HMC IRB's Web site at http://www.hmc.psu.edu/irb. Included on this web site, under the heading "Participant Info", you can access federal regulations and information about the protection of human research participants. If you do not have access to the internet, copies of these federal regulations are available by calling the HSPO at (717) 531-5687.

Signature and Consent/Permission to be in the Research

Before making the decision regarding enrollment in this research you should have:

- Discussed this study with an investigator,
- Reviewed the information in this form, and
- Had the opportunity to ask any questions you may have.

Your signature below means that you have received this information, have asked the questions you currently have about the research and those questions have been answered. You will receive a copy of the signed and dated form to keep for future reference.

Participant: By signing this consent form, you indicate that you are voluntarily choosing to take part in this research.

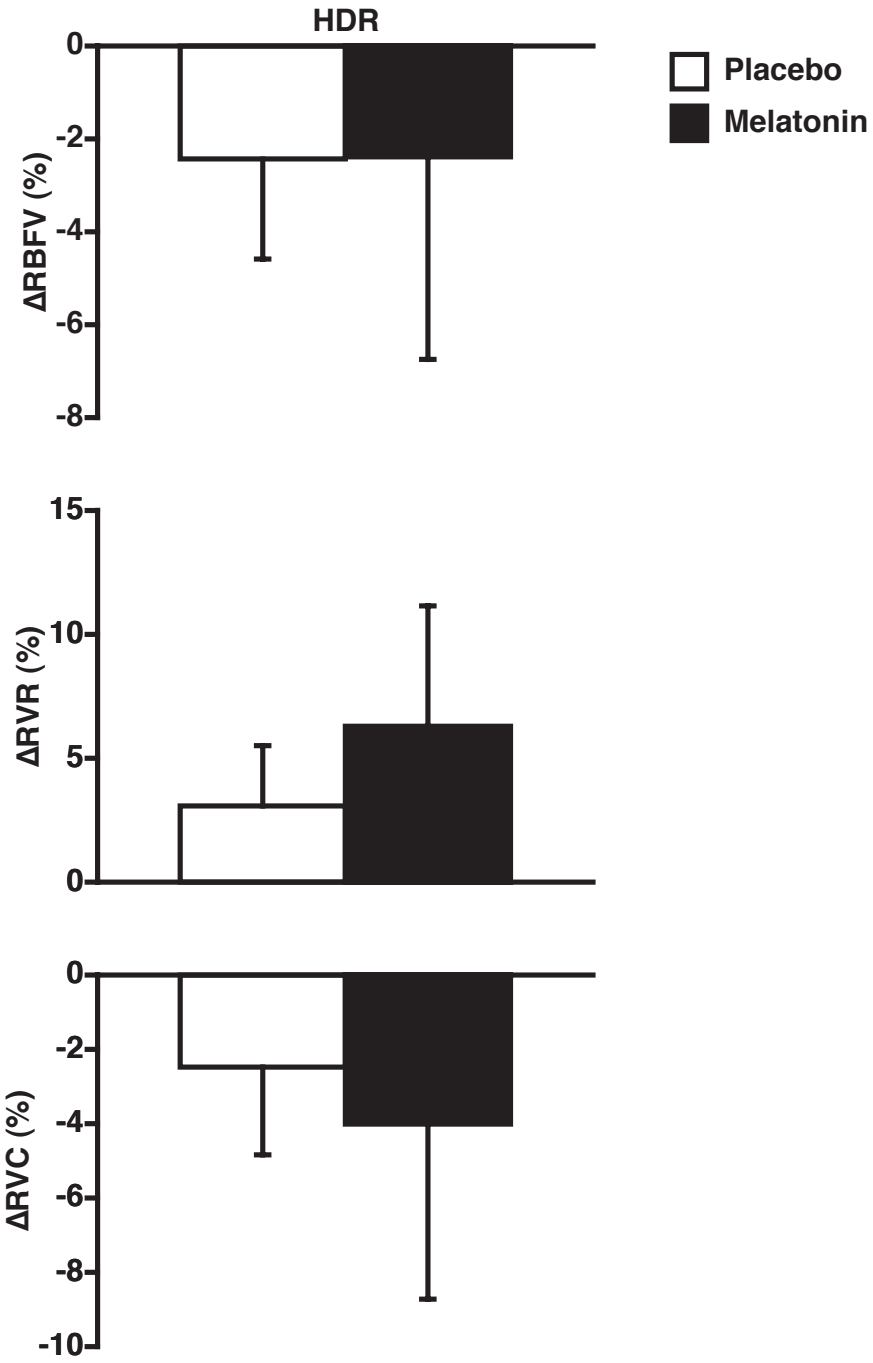
_____	_____	_____	_____
Signature of Participant	Date	Time	Printed Name

Person Explaining the Research: Your signature below means that you have explained the research to the participant/participant representative and have answered any questions he/she has about the research.

_____	_____	_____	_____
Signature of person who explained this research	Date	Time	Printed Name

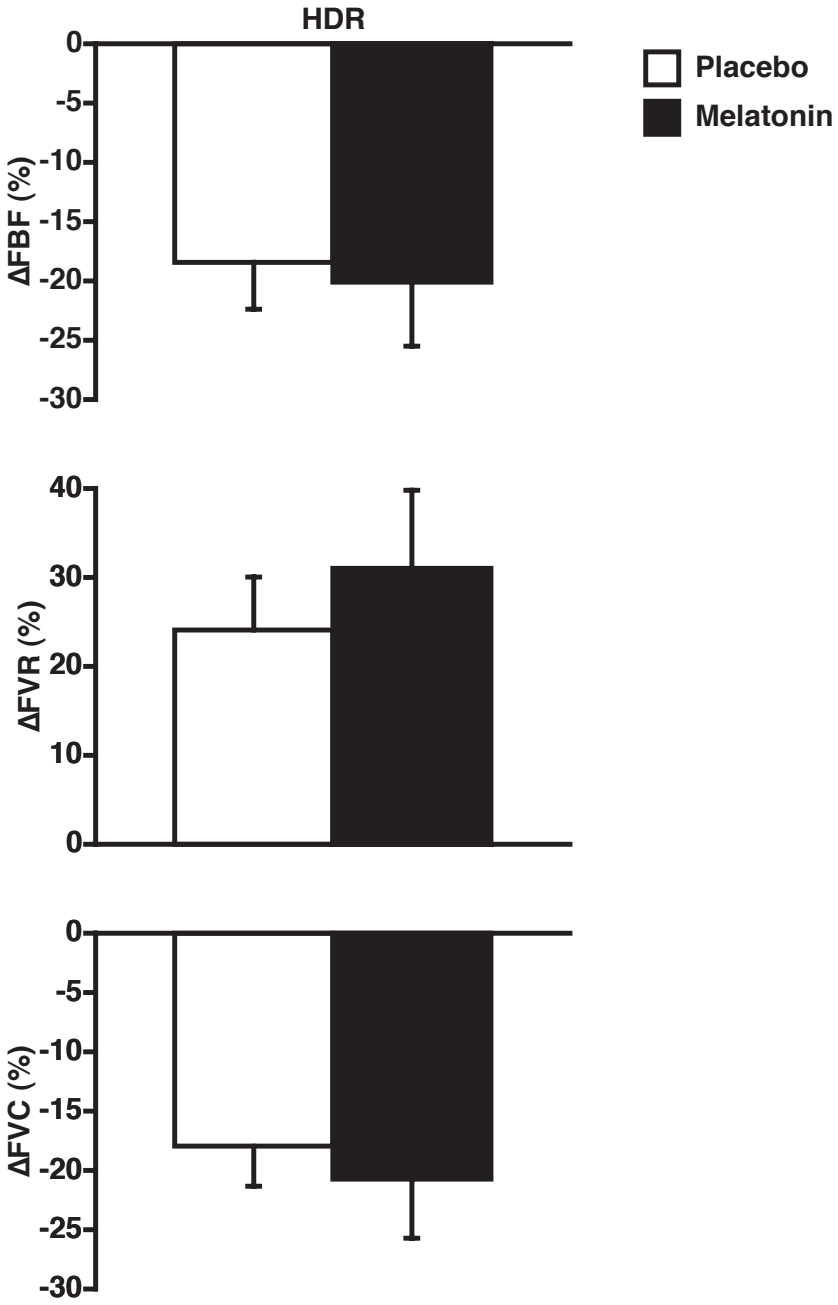
Appendix D

Effect of Melatonin on Renal Blood Flow During Head-down Rotation



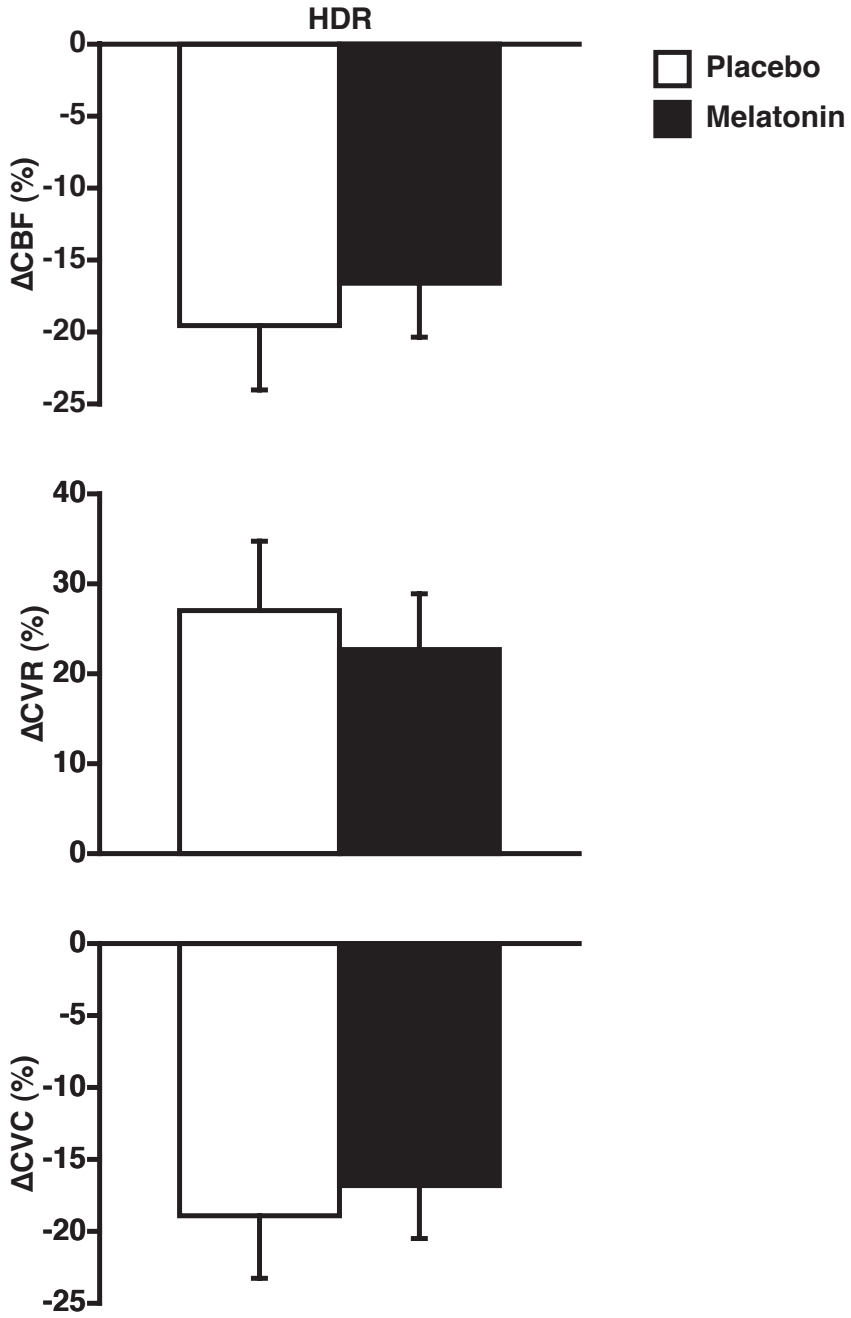
Appendix E

Effect of Melatonin on Forearm Blood Flow During Head-down Rotation



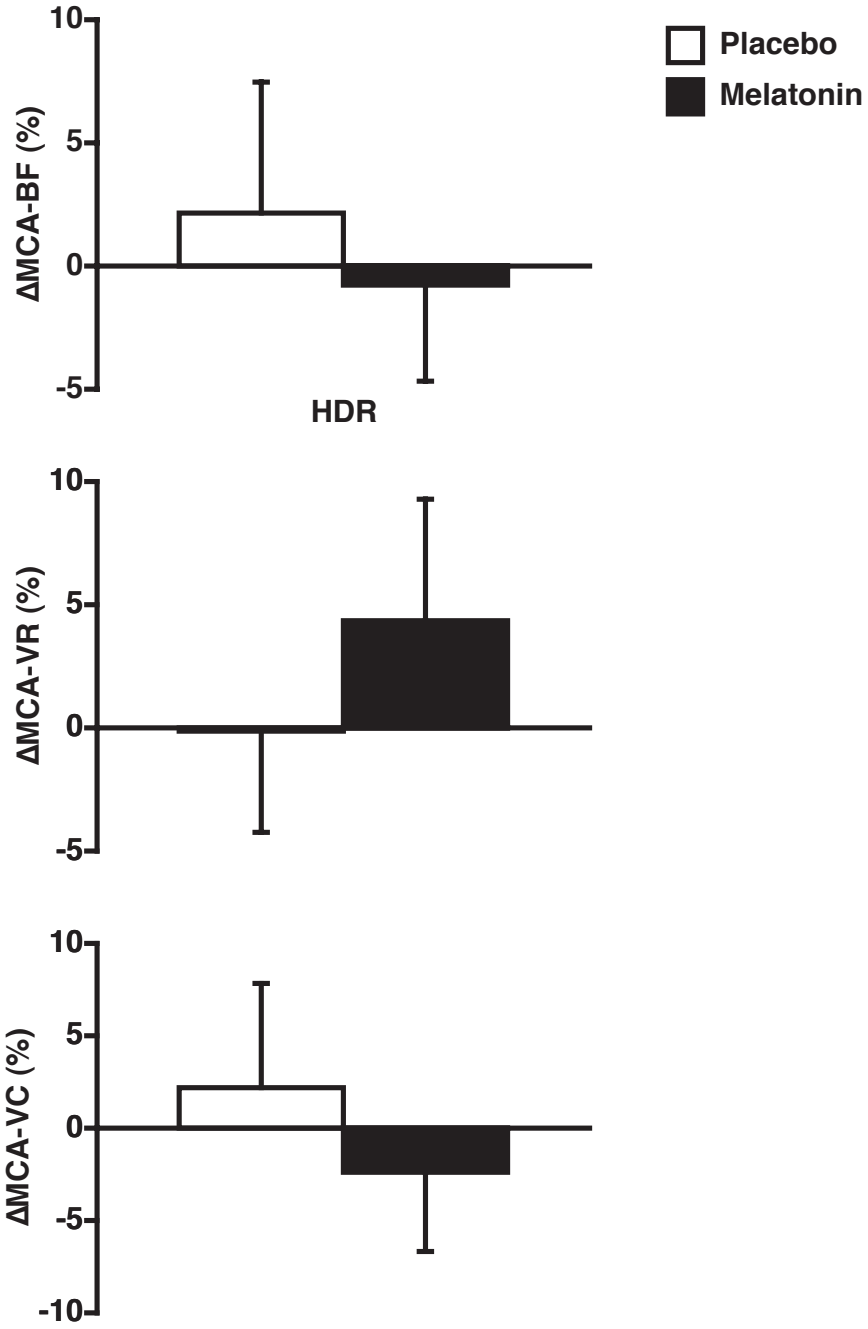
Appendix F

Effect of Melatonin on Calf Blood Flow During Head-down Rotation



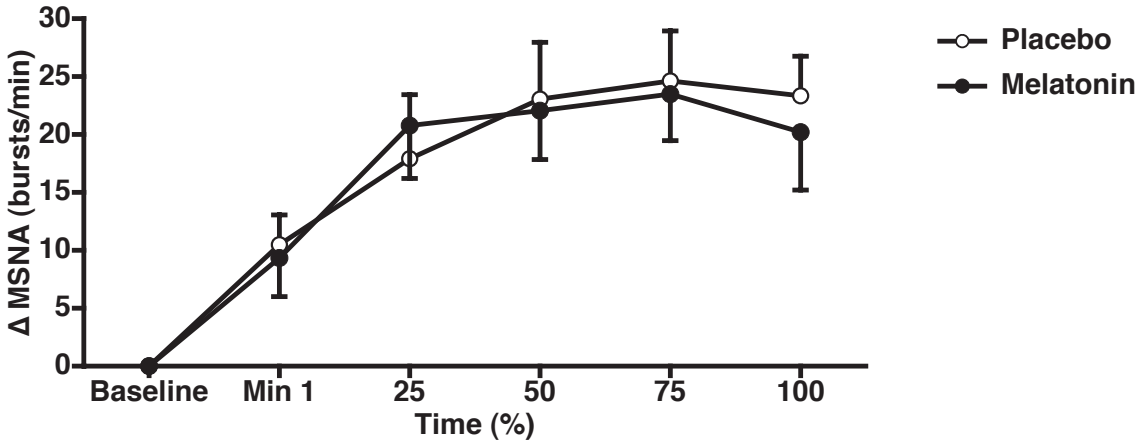
Appendix G

Effect of Melatonin on Cerebral Blood Flow During Head-down Rotation



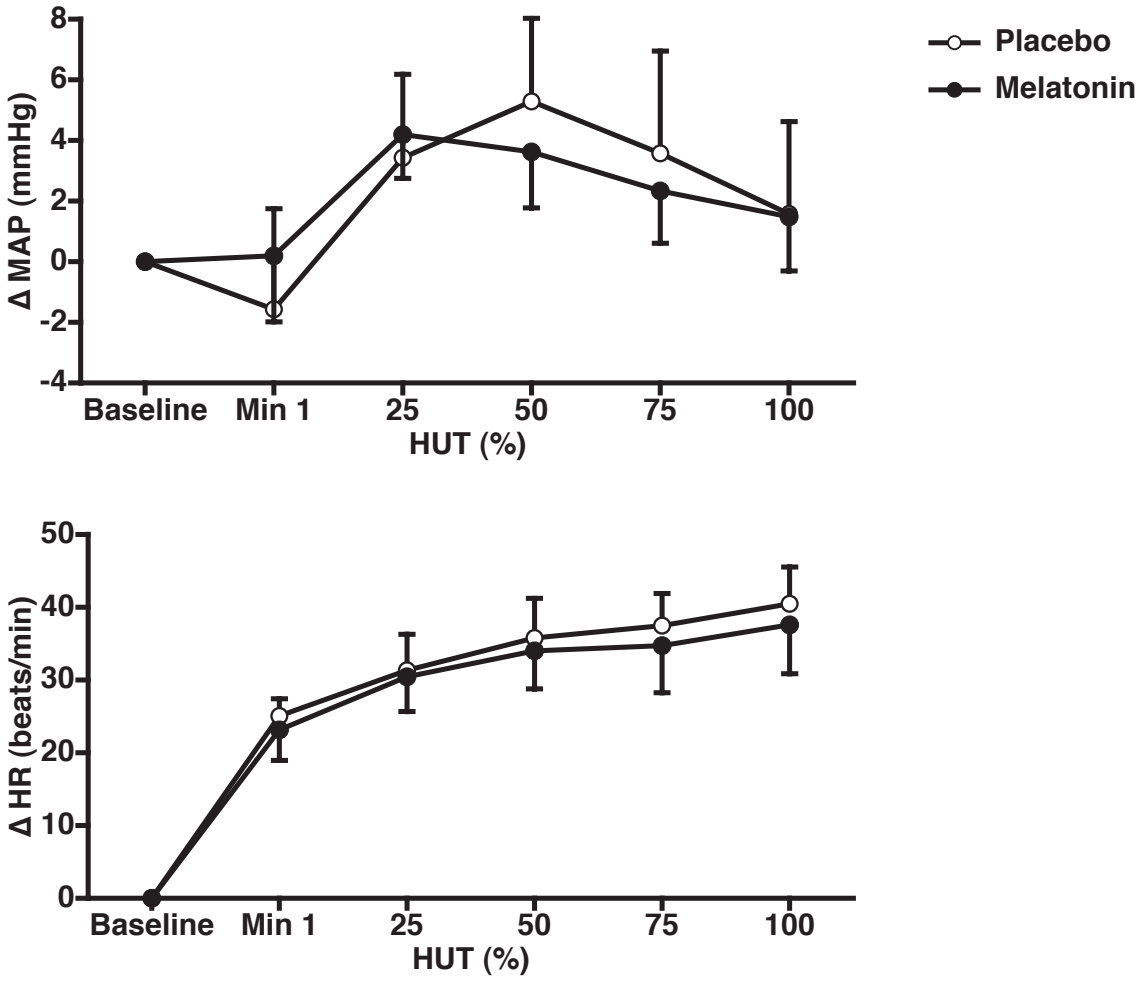
Appendix H

Effect of Melatonin on MSNA During Head-up Tilt



Appendix I

Effect of Melatonin on Mean Arterial Blood Pressure and Heart Rate During Head-up Tilt



VITA

Jonathan S. Cook

Education

Pennsylvania State University College of Medicine	Ph.D.	Physiology	2010
University of Texas, Austin	M.A.	Kinesiology	2006
University of Michigan, Ann Arbor	B.S.	Movement Science	2005

Fellowships

NASA Space Grant Fellowship	PA Space Grant Consortium	2009-2010

Awards

Howard Morgan Travel Award, Dept. of Cellular and Molecular Physiology	2010
Caroline tum Suden Professional Opportunity Award, APS	2010
Patrick G. Quinn Award for Outstanding Ph.D. Candidate	2009-2010
Rogel Scholarship, University of Michigan, Ann Arbor	2002-2005

Professional Activities

Senator – University Planning Committee, Faculty Senate	2009-2010
Co-Chair, Career Day 2009	2009-2010
Secretary, Graduate Student Assembly	2009-2010
Academic Integrity Representative, Graduate Student Assembly	2008-2010

Publications

Cook, J.S. and C.A. Ray. Modulation of muscle sympathetic nerve activity to muscle heating during dynamic exercise. *American Journal of Physiology-Regulatory, Comparative and Integrative Physiology* 296: R1439-1444, 2009.

Cook, J.S. and C.A. Ray. Melatonin attenuates the vestibulosympathetic but not vestibulocollic reflexes in humans: selective impairment of the utricles. Submitted to *Journal of Applied Physiology*

Cook, J.S., C.L. Sauder, and C.A. Ray. Melatonin differentially affects vascular blood flow in humans. Submitted to *AJP – Heart and Circulatory Physiology*

Cook, J.S., S.A. Chin-Sang, and C.A. Ray. Circadian rhythm of muscle sympathetic nerve activity: implications for melatonin. In preparation