UNIVERSITY OF OKLAHOMA

GRADUATE COLLEGE

DOES ACUTE MELATONIN SUPPLEMENTATION AFFECT CEREBROVASCULAR REACTIVITY IN HEALTHY YOUNG ADULTS?

A THESIS

SUBMITTED TO THE GRADUATE FACULTY

in partial fulfillment of the requirements for the

Degree of

MASTER OF SCIENCE

By

CHRIS MIXON

Norman, Oklahoma

2024

DOES ACUTE MELATONIN SUPPLEMENTATION AFFECT CEREBROVASCULAR REACTIVITY IN HEALTHY YOUNG ADULTS?

A THESIS APPROVED FOR THE

DEPARTMENT OF HEALTH AND EXERCISE SCIENCE

BY THE COMMITTEE CONSISTING OF

Dr. J. Mikhail Kellawan, Chair

Dr. Michael M. Markham

Dr. Hugo M. Pereira

Table of Contents

List of Tables

Lists of Figures

Abstract

Melatonin receptors have been found in the cerebrovasculature in animal models, and melatonin has been shown to improve systemic vascular control. While there is a correlation between low melatonin production and cardiovascular disease, the effect of acute melatonin supplementation on the cerebrovasculature, and more specifically, cerebrovascular reactivity (CVR), is unknown. **PURPOSE:** To determine if acute melatonin supplementation alters CVR via CO_2 rebreathing in healthy, young adults. **METHODS:** 14 healthy young adults (Age 23.7±4.39, 7 females tested during the first 5 days of their menstrual cycle) participated in a familiarization visit followed by 2 experimental visits separated by ³48hrs. Participants received a sublingual dose of either a placebo (PLA, mixture of mint extract and filtered water, to mimic taste of melatonin) or 5mg of melatonin (MEL, mint flavor) in a single-blind, randomized, crossover design. After ingestion, participants rested for 30 minutes prior to the experiment to allow for melatonin levels to reach peak concentration in the blood (Bartoli et al., 2013). After

which, mean arterial pressure (MAP, mmHg, finger photoplethysmography), middle cerebral artery velocity (MCAv, cm/s, Transcranial Doppler), and end-tidal CO_2 concentration ($PetCO_2$, mmHg, Gas Analyzer) were measured continuously for 5 minutes of supine rest while breathing atmospheric air followed by a CO_2 rebreathing challenge (CO_2=3%, O_2=40%, balanced N_2) until the participant displayed a $PetCO_2$ increase of ³10mmHg. Cerebrovascular conductance index was calculated as CVCi = MCAv/MAP (cm/s/mmHg). CVR was calculated in absolute $\Delta MCAv/\Delta PetCO_2$, $\Delta CVCi/\Delta PetCO_2$, and percent change from resting values. **RESULTS:** Data presented as change (Δ) from rest to CO_2 rebreathing, as mean ± SD. All values were not different between treatments during rest. No value was significantly different between treatments when comparing Δ from rest to CO_2 rebreathing (MCAv PLA Δ13.35±5.1 vs. MEL Δ11.62±6.03, p = 0.31, d = 0.28, MAP PLA Δ1.75±3.91 vs. MEL Δ-0.55±4.49, p = 0.20, d = 0.34, CVCi PLA Δ0.11±0.04 vs. MEL Δ0.11±0.05, p = 0.55, r = 0.16, CVR_{MCAv} PLA Δ2.75±1.30 vs. MEL Δ3.54±2.57, p = 0.92, r = 0.03, $\%CVR_{MCAv}$ PLA Δ18.41±7.07 vs. MEL Δ16.12±10.00, p = 0.36, r = 0.24, CVR_{CVCi} PLA Δ0.02±0.03 vs. MEL Δ0.02±0.02, d = 0.28, p = 0.31, $\%CVR_{CVCi}$ PLA Δ2.32±2.58 vs. MEL Δ1.52±1.83, d = 0.28, p = 0.31). **CONCLUSION:** Our results indicate that melatonin does not affect MCAv, CVCi, MAP, or CVR during rest or CO_2 rebreathing. Reactivity was variable individually and did not trend in any direction when comparing PLA treatment to MEL treatment. Thus, melatonin supplementation does not improve cerebrovascular function indicating melatonin supplementation may have differential responses between the cerebral and peripheral vasculature in humans.

Chapter I

Introduction

The brain is an extremely metabolically demanding organ with minimal intracellular substrate storage. Thus, the brain must rely on cerebral blood flow (CBF) for sustained neuronal metabolism (Tarumi et al., 2018). A constant supply of blood flow is required to maintain homeostatic conditions regardless of exogenous conditions, in order to avoid neurological damage, ischemic stroke, and possible death (Xing et al., 2017). In the United States, the lifetime risk of stroke has increase by >8.5% in the last 30 years, 87% of which are considered ischemic (Tsao et al., 2022). Since reliance on CBF is crucial for the maintenance of homeostatic conditions, proper cerebrovascular function is required to avoid disruption of neuronal homeostasis and to avoid neurological damage and death.

In daily life, humans experience numerous conditions that call for the redistribution of blood flow throughout the body. Simultaneously, because of the brain's high metabolic demand cerebral blood flow must remain constant not matter the metabolic demand of other tissues of the external environment. Cerebrovascular reactivity (CVR) represents the ability of cerebral vessels to dilate in response to increased tissue demand and is expressed as the magnitude of flow response (ΔCBF) per unit change in arterial CO_2 (ΔP_{aCO2}, Carr et al., 2021). To be clear, cerebral flow (CBF) if the total amount of blood traveling through a cerebral artery, while cerebral vascular reactivity (CVR) is the vessel's ability to respond to a stimulus and dilate or constrict. While the arterial concentration of CO_2, or $PaCO_2$, does not directly influence CVR, CO_2 is able to rapidly diffuse across the blood-brain barrier and induce changes in cerebrospinal fluid and interstitial fluid H^+ concentration, resulting in alterations to smooth muscle tone and thus significant cerebrovascular vasodilation, increasing blood flow (Willie et al., 2014; Hoiland et

al., 2019). Measurement of CVR, which will be described thoroughly in the methods and literature review sections, can be used as a metric to assess an individual's risk of experiencing a cerebrovascular event (Vernieri et al., 1999; Silvestrini et al., 2000; King et al., 2011; Hoiland et al., 2019). Dampened or dysfunctional CVR has been shown to have a link to an increase in ischemic event incidence and stroke (Hu et al., 1999; Chen et al., 2014; Hoiland et al., 2019).

Melatonin release is regulated via sympathetic innervation modulated by beta-adrenergic receptor stimulation through the pineal gland and is known to play a substantial role in regulating human circadian rhythm (Cajochen et al., 2003). Despite this, the melatonin receptor MT1 has been found in the aorta and left ventricle, while the melatonin receptor MT2 has been found in the brain vasculature, suggesting that the hormone may play a role in other unfounded mechanisms (Ekmekcioglu et al., 2003; Pandi-Perumal, 2007; Baker & Kimpinski, 2018). Both of these receptors have been associated with vasoconstriction and vasodilation (Pandi-Perumal, 2008; Baker & Kimpinski, 2018). Patients with cardiovascular disease have been shown to produce less nocturnal melatonin in comparison to healthy populations, meaning that there may be a link between low melatonin production and cardiovascular diseases (Brugger et al., 1995; Yaprak et al., 2003). While no mechanism of the lower release of melatonin in patients with cardiovascular disease has been identified, melatonin has been shown to both reduce free radical production and increase antioxidant reserves in humans and rats (Girouard et al., 2004; Koziróg et al., 2011; Reiter 2003) suggesting that lower melatonin release could at least in theory be related to the excess free radical production and damage consistently observed in patients with cardiovascular diseases (Panth et al., 2016; Moris et al., 2017; Peoples et al., 2019). In combination with reducing free radical production, melatonin has been shown to blunt α-adrenergic receptors in rats, reducing sympathetic outflow (Girouard et al., 2004). Furthermore,

potentially due to the blunting effect of α-adrenergic receptors, melatonin serves as a potent anti-hypertensive agent in humans (Holmes et al., 1976; Cunnane et al., 1980; Simko et al., 2007; Katsi et al., 2012; Baker & Kimpinski, 2018). Despite this, it has been demonstrated that proper cerebral autoregulatory function indicates that there should not be a noticeable change in CBF in healthy individuals when comparing a melatonin and a placebo treatment group (Mil et al., 2003). Therefore, as mentioned before, a stimulus that alters CBF directly can be used to measure a potential difference in change in CBF between taking melatonin and taking a placebo. In other words, a clear stimulus that alters CBF directly may highlight whether or not melatonin presents with any cerebrovascular vasoactive properties.

Because a major contributing factor that determines CVR is the effect of P_{CO2} on H^+ concentrations in the CSF and the interstitial fluid (ISF), the creation of hypercapnic conditions via CO_2 rebreathing serves as a valuable tool for observing differences in CVR between treatment groups. It has been demonstrated in rats that the pineal gland and melatonin play a role in the regulation of blood flow to the brain (Capsoni et al., 1995). Regardless, the direct effect of melatonin's cerebrovascular properties has not yet been studied in humans. Changes to CVR in humans during rebreathing under the effects of melatonin has also yet to be studied.

Purpose

The purpose of this study is to determine the role of acute melatonin supplementation on cerebrovascular reactivity and flow during rest and CO_2 rebreathing in healthy, young adults.

Research Questions

1. Does acute melatonin supplementation affect cerebrovascular flow during rest and CO_2 rebreathing?
2. Does acute melatonin supplementation affect cerebrovascular reactivity during rest and CO_2 rebreathing?
3. Does acute melatonin supplementation affect prefrontal cortex oxygenation during CO_2 rebreathing?

Research Hypotheses

1. Acute melatonin supplementation will increase cerebrovascular flow at rest and during CO_2 rebreathing in healthy, young adults.
2. Acute melatonin supplementation will increase cerebrovascular reactivity by means of increasing the cardiovascular conductance index (CVCi), decreasing mean arterial pressure (MAP), and increasing middle cerebral artery velocity (MCAv) during rest and CO_2 rebreathing in healthy, young adults.
3. Acute melatonin supplementation will increase prefrontal cortex oxygenation during rest and CO_2 rebreathing.

Significance of Study

Proper delivery of blood to the brain is essential for everyday function and to avoid neurological damage. While melatonin has been shown to attenuate sympathetic outflow in animal models, and melatonin receptors have been discovered in cerebrovasculature, the effect of melatonin on cardiovascular and cerebrovascular variables has not yet been studied during CO_2

rebreathing. Low melatonin production has been positively correlated with increasing cardiovascular disease, establishing a possible connection between the two. As a potent hypotensive agent as well as a sympathetic outflow attenuator in animal models, understanding the effects of melatonin on cerebrovascular reactivity has the potential to add valuable insight into possible therapeutic approaches towards populations with dampened cerebrovascular reactivity.

Delimitations

1. All subjects are considered healthy and do not have cardiovascular, metabolic, or pulmonary diseases, and have no history of autonomic dysfunction.
2. All subjects are normotensive and have a resting systolic blood pressure of < 130 mmHg and a resting diastolic blood pressure of < 85 mmHg.
3. All subjects are not taking any cardiometabolic medications that would interfere with the present study by interfering with cerebral or systemic blood flow.
4. All subjects are 18-35 years of age.
5. All subjects have a BMI of less than 30 kg/m^2.
6. All subjects are not pregnant.
7. All female subjects are tested between days 1 and 7 of the follicular phase of their menstrual cycle.
8. All subjects have not used nicotine products within the last 6 months.
9. All subjects do not regularly use any melatonin products.

Limitations

1. Middle cerebral artery velocity (MCA_v) has been measured and used to estimate flow and thus vasodilation rather than directly measuring middle cerebral artery vessel diameter.
2. Melatonin has been administered sublingually and not venously or intra-arterially.
3. CO_2 balloon volume was not measured directly during rebreathing.
4. Total brain oxygenation was not measured, only prefrontal cortex oxygenation has been measured.
5. Subject light exposure, which is a main determining factor for melatonin release, has not been controlled for outside of the experimental protocol.
6. Data may not adequately represent any population beyond young, healthy individuals.

Assumptions

1. All subjects have adhered to the pre-protocol requirements.
2. ΔMCA_v accurately represents Δcerebral blood flow.
3. ΔCVCi accurately represents Δcerebrovascular conductance.
4. Sublingually administered melatonin was absorbed into the blood stream as previously described (Bartoli et al., 2013).
5. 5mg of sublingually-administered melatonin reached peak concentration within the circulation ~30 minutes after administration as previously described (Bartoli, 2014).
6. Balloon volume did not limit CO_2 consumption during rebreathing protocol.
7. Subjects were properly blinded by placebo treatment.

Operational Definitions

1. **Cardiac Output (Q):** The amount of blood that leaves the heart over one minute, calculated by multiplying stroke volume and heart rate.
2. **Stroke Volume (SV):** The amount of blood pumped out of the left ventricle of the heart during one systolic contraction.
3. **Total Peripheral Resistance (TPR):** Total systemic resistance to blood flow in the circulatory system.
4. **Transcranial Doppler (TCD):** A rapid, noninvasive method used to measure cerebrovascular function and flow velocity. The Doppler effect allows for ultrasonic waves to reflect off moving erythrocytes to provide cerebrovascular information (Purkayastha & Sorond, 2012).
5. **Middle Cerebral Artery Velocity (MCA_v):** The speed at which blood travels within the middle cerebral artery.
6. **Cerebrovascular Conductance Index (CVCi):** An index that divides middle cerebral artery velocity by mean arterial pressure and represents conductance, or ease of flow, within an artery.
7. **Mean Arterial Pressure (MAP):** The average arterial pressure throughout one complete systolic and diastolic cardiac cycle (DeMers & Wachs, 2022).
8. **End-tidal CO_2 ($EtCO_2$):** The volume of CO_2 exhaled at the end of one breathing cycle.
9. **End-tidal O_2 (EtO_2):** The volume of O_2 exhaled at the end of one breathing cycle.
10. **Near-infrared Spectroscopy (NIRS):** A method that uses relative infrared light absorption wavelengths to determine oxygenated hemoglobin, deoxygenated hemoglobin, and total hemoglobin levels (Sanni & McCully, 2019).

11. **Total Saturation Index (TSI):** A ratio between oxygenated hemoglobin and total hemoglobin (Sanni & McCully, 2019)
12. **Oxygenated Hemoglobin (HbO_2):** Oxygen bound to hemoglobin complexes.
13. **Deoxygenated Hemoglobin (HHb):** Hemoglobin that does not have oxygen molecules bound to itself.
14. **Total Hemoglobin (tHb):** Total amount of hemoglobin in a sample of red blood cells, which can be used to measure oxygen transport capacity.
15. **Sympathetic Nervous System (SNS):** The part of the autonomic nervous system that prepares the body for periods of activity.
16. **CO_2 Rebreathing:** A method of inhaling large amounts of CO_2 to elicit a rise in $EtCO_2$ of at least 5 mmHg without a decrease in ventilation (Barrie & Beatty, 1995).
17. **Placebo (PLA):** A mixture of one microliter of McCormicks Pure Mint Extract and 29 mL of filtered water, administered via sublingual spray.
18. **Melatonin (MEL):** Consists of 5 mg of sublingual spray (10 total sprays, Onnit, Austin, Texas, United States).

Chapter II

Literature Review

Introduction

It has been demonstrated that melatonin is a hormone that is responsible for the regulation of sleep patterning and the Circadian Rhythm (Cajochen et al., 2003). Beyond this, recent evidence has shown that melatonin may have wider implications on systems like the cardiovascular system in both human and animal models (Cunnane et al., 1980; Simko et al., 2007; Katsi et al., 2012; Baker & Kimpinski, 2018). It has been suggested that α-adrenergic receptor inhibition may be related to melatonin production and thus exogenous melatonin supplementation, implying that melatonin produces anti-hypertensive effects in addition to anti-inflammatory and antioxidant effects (K-Laflamme et al., 1998; Baker & Kimpinski, 2018).

The brain is an organ that lacks substantial substrate storage and thus requires very controlled blood flow matching in order to meet metabolic demand (Tarumi et al., 2018). Because of the recent >8.5% increase in lifetime stroke risk in the United States, the proper establishment of potential therapeutic targets as well as exogenous aids with cardiovascular implications is of the upmost importance (Tsao et al., 2022). While no specific clinical significant difference has been determined detailing the risk between dampened CVR and stroke risk, studies have shown that there was a 9.8% increased annual risk of experiencing an ischemic brain event in those with lower CVR and a statistically increased risk of experience a first-time lacunar infarction (Molina et al., 1999; Silvestrini et al., 2000) Because measurement of CVR is commonly used to represent an individual's risk of an ischemic or traumatic brain event, and dampened CVR is typically associated with an increase in ischemic event incidence, using a CO_2

rebreathing challenge in order to evaluate CVR can be considered a potential predictive clinical tool (Silvestrini et al., 2000; Vernieri et al., 1999; King et al., 2011; Hu et al., 1999; Chen et al., 2014). The purpose of this literature review is to investigate the effects of melatonin supplementation on cerebrovascular blood flow and CVR during a CO_2 rebreathing challenge. Search terms were organized and utilized beginning in the order of the present review. Commonly used terms included CO_2 rebreathing, CO_2 and blood brain barrier, Middle Cerebral Artery Diameter, Melatonin, Melatonin Cerebrovasculature, Melatonin Receptors, Melatonin Receptors Cerebrovasculature, and other extensions or combinations of the above terms.

This chapter will discuss the production and secretion of melatonin from the pineal gland and extra-pineal locations, the functions and proposed functions of melatonin, the potential interaction of melatonin with cardiovascular control tissues, the pharmacokinetics and dosing of melatonin supplementation, cerebrovascular anatomy, sex differences in cerebrovascular blood flow and CVR, cerebrovascular responses to CO_2 rebreathing, the computation and interpretation of estimated flow changes during hypercapnic conditions, and the clinical implications of melatonin use on cardiovascular and cerebrovascular health.

Cerebrovascular Anatomy

Comprehensive understanding of the anatomy of the cerebral vasculature is important in forming a foundation of studying cerebrovascular health outcomes. Blood flows to the brain via four large cerebral arteries; the right and left vertebral arteries (VA) and the right and left internal carotid arteries (ICAs) (Traystman, 2017; Vavilala et al., 2002). The former is responsible for the distribution of blood to the posterior side of the brain while the latter is responsible for the

distribution of blood to the anterior part of the brain. The carotid arteries and the VAs then combine at the base of the brain's vasculature to form the Circle of Willis (Traystman, 2017). In particular, and in relevance to the present study, the anterior-supplying ICAs branch on both sides to the middle cerebral arteries (MCAs), which continue to branch into the anterior cerebral arteries (ACAs). In the posterior circulation, the right and left VAs integrate with one another, becoming the basilar artery (BA), which branch into the posterior cerebral arteries (PCAs). In redundant fashion, anterior and posterior communicating arteries integrate with the PCAs at the Circle of Willis, ensuring that there is an equal and adequate distribution of blood throughout the entire brain (Traystman, 2017). Through different branches blood then flows into arterioles, marking the point during which blood is no longer considered to be flowing in the large arteries of the brain. The distribution of nutrients and metabolites occurs at the level of capillary beds, which are fed by said arterioles.

Cerebral Vascular Anatomical Variability

Many of the common vascular pathways within the brain appear with differing degrees of variability, presenting an issue for the measurement and interpretation of cerebrovascular data. In particular and most relevant to the present study, the MCA is a vessel that is commonly bifurcated, trifurcated, or quadfurcated. Gunnal (2019) concluded in a cadaveric study with a sample size of 170 preserved brains that 64.70% had at least one bifurcated MCA, 12.35% had at least one trifurcated MCA, and 2.35% had at least one quadfurcated MCA. Other studies have concluded between 70 and 80 percent of individuals have at least one MCA bifurcation and between 9 and 30 percent of individuals have at least one MCA trifurcation (Idowu et al., 2002; Tanriover et al., 2003; Pai et al., 2005; Vuiller et al., 2008). Similar results have been found in different arteries associated with the Circle of Willis (Papantchey et al., 2013; Payne, 2016).

Abnormalities in cerebral vascular anatomy can make obtaining symmetrical data without some degree of variation difficult, as many of the variations in cerebral anatomy do not present with any obvious pathology (Alpers et al., 1959).

Cerebral Vascular Sex Differences

Alongside variations present in the cerebral vasculature of humans, there are measurable differences in both anatomical and blood flow-related variables when comparing males and females. It has been shown that females typically have higher global and regional cerebral blood flow compared to men, ranging depending on the study between an increase of ~6-11%, not normalized by volume of neural tissue, but as a change from a self-baseline (Rodriguez et al., 1988; Gur & Gur, 1990). Females also may present with more symmetrical flow in comparison to men (Rodriguez et al., 1988). End-tidal CO_2 concentrations were not different, indicating that there may be a hormonal difference that contributes to blood flow in females compared to men, which is an important factor when considering any sex difference analyses. Estrogen replacement therapy (ERT) has been shown to increase global cerebral blood flow in postmenopausal women, suggesting that the hormonal differences between males and females could contribute to cerebral blood flow responses both between males and females as well as within the same females at different stages of their menstrual cycles (Ohkura et al., 1994; Resnick et al., 1998; Nevo et al., 2007).

Cerebrovascular Blood Flow Control

Poiseuille's Law is fundamental to understanding cerebral hemodynamics, similar to any other part of the cardiovascular system. It states that the flow through a vessel is determined by

changes in blood velocity, viscosity, diameter, and the vessel length. The equation can be seen as:

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

ΔP = change in pressure, r = vessel radius, η = blood viscosity, l = vessel length

Under ideal conditions, the length of the vessel and viscosity of blood flowing through said vessel can typically be excluded when comparing groups. This leaves the effect of pressure and radius as two variables that can alter flow dramatically, with the most influential contribution being derived from changes in the radius of a blood vessel. Therefore, particular examination of vessel diameter alterations is crucial in terms of changes to cerebral blood flow.

Neurogenic Contributions to Flow

The degree of contribution of the sympathetic and parasympathetic nervous systems on vasodilatory and vasoconstrictive responses, particularly in the cerebrovasculature, is to this day debated within the scientific community. Most current research agrees that the parasympathetic nervous system either does not play a role or only plays a minor role in the vasodilatory and vasoconstrictive responses of the cerebral vasculature (Willie et al., 2014). It is known that the sympathetic nervous system does present adrenergic receptors within the vascular smooth muscle of the cerebral vasculature (Ainslie & Brassard, 2014; Brassard et al., 2017). Beyond this, adrenergic nerve endings have been discovered innervating the above-mentioned receptors (Ainslie & Brassard, 2014). It has not been concluded the extent to which sympathetic innervation contributes to cerebral blood flow control, but the influence of the SNA is evident, at least within

the larger arteries of the brain, where removal of sympathetic outflow results in higher total blood flow (Jeng et al., 1999; Claassen et al., 2021).

Cerebrovascular Responses to CO_2 Rebreathing – Metabolic Contributions to Flow

Arterial CO_2 concentration is inversely proportional to alveolar ventilation and directly proportional to the total volume of CO_2 production as described by:

$$P_aCO_2 = \frac{V_{CO2}}{V_A}$$

Where P_aCO_2 = arterial CO_2 concentration, V_{CO2} = whole-body CO_2 production, and V_A = alveolar ventilation (Willie et al., 2014)

So, arterial concentration of CO_2 can be derived through the measurement of CO_2 output and ventilation. Increases in arterial CO_2 concentration leads to a rapid passive diffusion of CO_2 across the blood-brain barrier, which changes hydrogen ion concentration via the bicarbonate buffering system in the intracellular and extracellular cerebrospinal and interstitial fluids of the brain, eventually leading to alterations in cyclic GMP production and causing vascular smooth muscle relaxation (Willie et al., 2014; Jensen et al., 1988). The subsequent change in the vascular tone of the cerebral vessels results in changes to CBF, and CO_2 concentrations have been established as the most potent regulator of cerebral tone itself (Hoiland et al., 2019). It has further been estimated that there is an ~4% increase in cerebral blood flow for every increase of 1 mmHg of CO_2 (Dagal & Lam, 2009). Therefore, controlled alterations in the concentration of inspired CO_2 will lead to possibly-predicted alterations in vasodilatory responses via the above-mentioned change in arterial tone, with arterial CO_2 concentration displaying a direct relationship with vascular smooth muscle relaxation and vasodilation (Hoiland et al., 2019).

CO_2 Rebreathing as a Tool to Assess Cerebrovascular Reactivity

Alteration of the concentration of inspired CO_2 will lead to possibly-predicted vasodilatory responses, and so CO_2 rebreathing can serve as a valuable tool to assess a vessel's ability to respond to a stimulus. To restate, if it is understood that a vessel will dilate in response to a particular stimulus, assessing the vessel's capability to dilate to that stimulus could be predictive of cerebrovascular health. Indeed, there is evidence to show that impaired CVR to stimuli is associated with predicted risk of ischemic or traumatic brain events (Vernieri et al., 1999; Silvestrini et al., 2000; King et al., 2011; Hoiland et al., 2019). Since it has been demonstrated that a dampened or weakened CVR is associated with an increase in ischemic event incidence, using CO_2 rebreathing as a tool to assess the capacity to which a vessel can react to stimulus proves extremely useful (Hu et al., 1999; Chen et al., 2014; Hoiland et al., 2019).

Production and Secretion of Melatonin

Melatonin Production from the Pineal Gland

Under low light conditions melatonin is secreted from the pineal gland. Through the retina, and in the presence of low light, a cascade beginning with activation of the suprachiasmatic nuclei (SCN) begins (Pandi-Perumal et al., 2008; Baker & Kimpinski, 2018). Activation of the SCN inhibits the paraventricular nucleus (PVN) which projects to the sympathetic intermediolateral nucleus in the spinal cord, projecting to the pineal gland via cervical ganglia (Pandi-Perumal et al., 2008; Baker & Kimpinski, 2018). Under light conditions, contrarily, the PVN remains inhibited and the cascade for melatonin release from the pineal gland ceases. When the cascade is allowed to continue, melatonin is released from the pineal

gland and can interact with targets in the brain that relate to the Circadian Rhythm but is also released into the peripheral and cerebrovascular bloodstreams as well as the cerebrospinal fluid where its proposed cardiovascular mechanisms may take form (Baker & Kimpinski, 2018). Physiologically, and because of the dependence of melatonin release on the presence or absence of light, the highest concentrations of melatonin are seen during the night and the lowest concentrations of melatonin are seen during the day in humans (Cajochen et al., 2003).

Melatonin Extra-Pineal Gland Production

Extra-pineal sources of melatonin include immune tissues, bone marrow cells, the gastrointestinal tract, and many more (Acuna-Castroviejo et al., 2014). Despite this, in most situations, extra-pineal melatonin release is local and does not leak into the peripheral circulatory system (Acuna-Castroviejo et al., 2014). One explanation for this is a molecule specifically designed to "buffer" melatonin to keep its highly lipophilic profile within the cell, but specific mechanisms have not yet been discovered (Acuna-Castroviejo et al., 2014). With recent investigation into the anti-inflammatory and antioxidant effects of melatonin, it would reason that extra-pineal and local melatonin release would be of high importance (Acuna-Castroviejo et al., 2014). Furthermore, locally produced and locally-maintained melatonin release would be able to exert its anti-inflammatory and antioxidant effects without leaking into the circulatory system.

Melatonin's Interaction with Cardiovascular Control Tissues

Melatonin Receptor Physiology

While it has been established that melatonin plays an important role in sleep and the regulation of the Circadian Rhythm, it also has been suggested that it plays an important role in various cardiovascular disease risk factor attenuation. Some of these areas include oxidative stress, diabetes, dyslipidemia, obesity, hypertension, arrythmias, and atherosclerosis (Imenashidi et al., 2020). Three types of melatonin receptors have been identified in humans, but only the G protein-coupled receptors MT1 and MT2 have thus far been demonstrated to exist in humans (Pandi-Perumal et al., 2008; Baker & Kimpinski, 2018). In humans, after the pineal melatonin release cascade through the SCN and PVN becomes active, melatonin binds to MT1 or MT2 receptors where its signaling is mediated via adenylate cyclase, guanylate cyclase, and calcium channels (Pandi-Perumal et al., 2008; Baker & Kimpinski, 2018). Typically, activation of MT1 receptors results in a vasoconstrictive response whereas activation of MT2 receptors results in a vasodilatory response (Pandi-Perumal et al., 2008). After intracellular signaling occurs, cyclic AMP production becomes inhibited and intracellular calcium concentrations increase (Baker & Kimpinski, 2018). The melatonin receptor MT3, otherwise known as the enzyme quinone reductase 2, acts as a mechanism that prevents oxidative stress by preventing electron transfer reactions of quinones, but have only been demonstrated thus far to be present in rats and hamster models (Pandi-Perumal et al., 2008).

Melatonin Receptors and Cerebrovascular Control

Previous work has found that there are melatonin receptors found outside of well-established locations like the hypothalamus and pineal gland. Besides the periphery, MT2

receptors have been discovered in the Circle of Willis in the brain, which suggests that there may be a mechanism of cardiovascular action derived from melatonin binding on the cerebral vasculature (Capsoni et al., 1994; Viswanathan et al., 1990). Separate from MT1 receptors, MT2 receptors also modulate guanylyl cyclase and resulting cGMP production, which can cause vasorelaxation and dilation by increasing conductance through potassium channels and decreasing vascular smooth muscle cell (VSMC) calcium sensitivity (Baker & Kimpinski, 2018; Hoiland et al., 2019). This implicates MT2 receptors as a potential location of melatonin's proposed cerebrovascular influence. Since MT2 receptors mediate the production of cGMP, and MT2 receptors have been found lining the large arteries of the cerebral vasculature, investigation of their activation and thus subsequent vasorelaxation is warranted.

The Effect of Melatonin on the Nervous System

It has been suggested that the mechanism through which melatonin exerts positive cardiovascular effects is via the attenuation of sympathetic α-adrenergic receptors and the stimulation of parasympathetic beta-receptors. Direct intravenous injection of melatonin resulted in increased concentrations of gamma aminobutyric acid (GABA), a central sympatho-inhibitory molecule, and decreased concentrations of glutamate, a central sympatho-excitatory molecule, which displayed a following decrease in MAP (Xia et al., 2008). This may have to do with the close connection between the SCN, which mediates melatonin release from the pineal gland, and the rostral ventrolateral medulla (RVLM), which extends into projections towards sympathetically-oriented cell bodies (Baker & Kimpinski, 2018). Furthermore, sympathetic activity was significantly decreased in response to simulated hypovolemic-induced stress after exogenous supplementation of melatonin (Ray, 2003). Numerous studies have also observed

decreases in MAP, pulsatility, and norepinephrine and epinephrine release following acute supplementation of exogenous melatonin (Arangino et al., 1999; Cagnacci et al., 1997).

The Effect of Melatonin as an Antioxidant and Anti-inflammatory Agent

When cells metabolize oxygen there is a potential for deleterious reactive oxygen species (ROS) if an imbalance between pro-oxidant and antioxidant enzymatic presence, potentially causing damage to proteins, DNA, and lipids (Touyz, 2004). Applied to hypertensive humans and rat models, ROS production is markedly enhanced with a subsequent reduction in antioxidant bioactivity (Touyz & Schiffrin., 2001). Melatonin has clearly demonstrated antioxidant effects to a large capacity by the direct clearance of ROS, through the stimulation of enzymes associated with antioxidant activity, and through the inhibition of enzymes associated with pro-oxidant activity (Reiter et al., 2016). Consideration of melatonin as a possible route of supplemental help towards clinical populations with hypertension or other cardiovascular disorders that are associated with increased levels of ROS and considering that melatonin has shown to improve antioxidant bioactivity in both human and rat models, has become more popular in recent years (Cunnane et al., 1980; Simko et al., 2007; Katsi et al., 2012; Baker & Kimpinski, 2018).

Following suit, as melatonin acts as an antioxidant hormone, its antioxidant activity produces anti-inflammatory responses that have been shown to benefit multiple chronic diseases which have an inflammatory underlying physiological cause or response (Nabavi et al., 2019). For instance, metabolic disease, hypertension, and obesity are typically associated with high levels of chronic and systemic inflammation (Prado et al., 2018). It has been demonstrated that melatonin supplementation is associated with a reduction in said inflammation in similar populations through multiple intracellular and extracellular processes, many of which are

associated with mitochondrial protective mechanisms as well as a reduction in the previously-mentioned ROS reduction rates (Prado et al., 2018).

Pharmacokinetics and Dosing of Melatonin

Exogenous supplementation of melatonin orally or intravenously ultimately ends up with excretion through the urinary system (Tordjman et al., 2017). As typically seen with exogenous supplementation of other substances, intravenous melatonin supplementation is effective more quickly and eliminated more quickly than oral supplementation, which can take up to 60 minutes to reach maximal plasma concentrations (Tordjman et al., 2017). Once concentrations are reached, the liver clears up to 90% of exogenous melatonin in a so-called "first-pass effect" within 30 to 60 minutes (Bartoli et al., 2013). Intake of a usual exogenous dose of melatonin can result in up to between 10 and 100 times normal physiological nocturnal peak concentrations with a return to basal values within 4 to 8 hours (Tordjman et al., 2017). Potential side effects of high dosages of melatonin include mood alterations, fatigue, a decrease in cognitive performance, and more rarely cardiovascular or endocrine system differences (Imenshahidi et al., 2020). Studies that displayed larger amounts of adverse effects, including endocrine or cardiovascular effects, were administering doses of between 20 and 100mg of melatonin, while typically only between 1 to 5mg of melatonin are used for research and daily purposes, including the present study (Imenshahidi et al., 2020).

In terms of the purposes of the present study, melatonin emulsion at the buccal mucosal level through a spray has been demonstrated to display no difference in absorption rate when compared to oral tablet intake (Bartoli et al., 2013). The larger surface area allows for more

absorption through the mucosa, avoiding some of the effects of the previously mentioned so-called “first-pass phase” (Bartoli et al., 2013). Furthermore, the total amount of melatonin that reaches the systemic circulatory system is statistically higher in melatonin consumed via a spray format than melatonin consumed via an oral tablet (Bartoli et al., 2013).

Computation of Estimated Flow during Hypercapnia

Limitation of Transcranial Doppler Ultrasonography in Calculating Flow

If it is to be assumed that MCA diameter remains constant then TCD, which measures blood velocity, serves as a potent estimator of blood flow responses (Bishop et al., 1986). Despite this, CO_2 challenges similar to the present study have displayed varying results as to whether or not MCA diameter changes during hypercapnic conditions (Serrador et al., 2000; Valdueza et al., 1997; Coverdale et al., 2014). More recently, and using a high-tesla MRI system, it was determined that there is a nonlinear relationship between 7.5 and 15 mmHg (or 1 and 2 kPa) $PetCO_2$ changes from baseline normocapnia and MCA vessel diameter (Verbree et al., 2014). This implies that the typical assumption of a static MCA diameter and thus proper estimation of blood flow through the measurement of blood velocity using TCD may underestimate true changes.

Computation of Normalized Estimated Cross-Sectional Area

The true outcome variable of the measurement of blood velocity using TCD is the derivation or assumption of blood flow alterations. As previously stated, and especially during hypercapnic conditions above 7.5 mmHg compared to normocapnia, TCD may underestimate blood flow response alterations. In using a 7-Tesla MRI system, it was concluded that there are

alterations in MCA vessel diameter under CO_2 challenges above 7.5 mmHg, so a third-order polynomial was derived and compared to MRI diameter measurements allowing for an estimation of vessel diameter change depending on CO_2 challenge magnitude (Verbree et al., 2014). The third-order polynomial is displayed as:

$$y = 0.93x^3 + 1.22x^2 + 1.99x + 99.64$$

Where y = MCA normalized area, x = $\Delta PetCO_2$ (Verbree et al., 2014)

Following Poiseuille's Law, this normalized area formula could serve as a key tool in more accurate estimation of blood flow alterations when measuring blood velocity changes using TCD under CO_2 challenges.

Novel Computation of Estimated Flow using Normalized Estimated Cross-Sectional Area

In following Poiseuille's Law once again, and under ideal circumstances in which vessel length and blood viscosity are not considered, blood flow can be calculated as:

$$Q = vA$$

Where v = blood velocity, A = vessel area

In applying the third order polynomial designed to derive normalized vessel area during CO_2 challenges it should then be possible, during CO_2 rebreathing within the present study, to estimate a more accurate change in blood flow based on the calculated vessel area by multiplying it by the blood velocity measured using TCD (Verbree et al., 2014). In doing so, we are estimating blood flow using an estimated normalized area, and so error becomes a relevant problem-source. Despite this, with the relative reported accuracy of estimating normalized vessel diameter using the above-stated polynomial ($R^2 = 0.51$), and with the knowledge that MCA

diameter does change when measured by a 7-Tesla MRI system during CO_2 challenges, it may be more apt to use estimations to derive blood flow than to assume that vessel diameter does not change, contradicting previous literature (Verbree et al., 2014).

Clinical Implications

The Effect of Melatonin on Cardiovascular Disease and Hypertension

While many of the mechanisms surrounding the cardiovascular effects are currently unknown on a physiological level, it is clear and has been demonstrated that acute exogenous melatonin supplementation improves blood pressure, parameters of oxidative stress, and serum lipid profile in clinical populations like metabolic syndrome (Koziróg et al., 2011). Multiple studies have also found that melatonin supplementation results in improved blood pressure profiles in patients diagnosed with hypertension (Sun et al., 2016). Furthermore, and while evidence linking the physiological cause to the clinical outcome is limited, there is a clear association between sleep deprivation or lack of sound sleeping and cardiovascular and metabolic diseases, implicating a possible role of melatonin in terms of cardiovascular health (Tobaldini et al., 2017). Melatonin's anti-inflammatory and antioxidant effects have also been associated with a protective mechanism against myocardial infarctions, hypertension, and ischemia-related injury (Chitimus et al., 2020). Therefore, while the physiological mechanisms that underly the protective effects of melatonin as well as the supplemental positive effects of exogenous melatonin supplementation, investigation regarding the mechanisms through which melatonin exerts its beneficial influences is worthwhile. Few studies have investigated the effect that melatonin has on cerebrovasculature, but even fewer studies have examined how melatonin

effects cerebrovascular reactivity under potent vasodilatory stimuli such as CO_2 rebreathing. Elucidation of how melatonin, with its potential α-adrenoreceptor inhibition and reduction in sympathetic outflow as a result, may affect how the brain vasculature responds to different stimuli may provide insight as to whether or not it is effective clinically in the prevention of ischemia and brain-related trauma events.

Conclusion

Melatonin has been established to play an important role in the regulation of sleep patterning and the Circadian Rhythm. Beyond this, it has been suggested and demonstrated that melatonin can exert cardiovascular effects. The presence of MT2 receptors, which have vasodilatory effects by acting on cGMP production, in large arteries of the brain vasculature suggests that some of the cardiovascular effects seen with exogenous melatonin supplementation may also be present in cerebrovasculature (Pandi-Perumal, 2007). As stated, it has been shown that there is an association between decreased melatonin release or absorption and cardiovascular disease. There is also an association between decreased CVR and ischemic or traumatic brain events, and it has been demonstrated that CO_2 rebreathing is an effective methodology for the assessment of CVR in humans (Hoiland et al., 2019). The combination of novel techniques that can be used to estimate flow via measurements in cerebral blood velocity and standardized MCA diameters as well as the knowledge that dampened melatonin release and CVR result in cardiovascular disease and disorder prompts a study that investigates a gap in knowledge regarding the effects of exogenous melatonin supplementation on CVR using effective CVR-assessment techniques.

Chapter III

Methodology

The ability for our cerebrovasculature to maintain proper flow is vital to supplying nutrients that are necessary for proper cognitive function as well as in avoiding neurological damage, stroke, and death. It has been established that CO_2 rebreathing is a useful technique in determining CVR (Silvestrini et al., 2000; Vernieri et al., 1999; King et al., 2011). The purpose of this study was to determine the role of acute melatonin supplementation on cerebrovascular reactivity and changes in flow during a CO_2 challenge. This chapter will elucidate the participants that have been included, ethical approval, the equipment and methods that have been implemented, threats to internal and external validity, and data collection and statistical analyses that has been completed.

Ethical Approval

This study has been approved by the Institutional Review Board of the University of Oklahoma Health Science Center (IRB #13084). All subjects were informed about the implications of participation before completion of the study and all guidelines have been abided by.

Participants

Prior to recruitment, a power analysis was performed using an a priori α value of 0.05. Data gathered from a pilot for the present study (n=10) was with CVR as the key dependent variable, displayed as the change in MCAv (cm/s) per unit change in CO_2 (mmHg). Pilot data was not normally distributed, so a Wilcoxon signed-rank test was specified in the program

G*Power as a two-tailed test comparing CVR during rebreathing between the placebo and Melatonin conditions (Faul et al., 2009). Because of the non-normal distribution of the data, a Spearman's rho correlation value was used alongside the mean and standard deviation of the placebo and Melatonin conditions in order to determine the effect size. To achieve a power of 0.85, 18 subjects were required to complete both experimental visits. Participants were between the ages of 18 to 35 years old. They were considered healthy with no conflicting comorbidities, neurological or cardiovascular disorders, and no absolute contraindications. All females were tested during the early follicular phase within the first 5 days of their menstrual cycles to avoid hormonal interferences in vasodilatory responses (Mannon et al., 2020). Sampling and recruitment was localized to the University of Oklahoma and surrounding areas via flyer-posting on the Norman campus, student base mass emails, and representative verbal recruitment during classes held in the Health and Exercise Science department at the University of Oklahoma. Participants were only enrolled in the study if they met the inclusion criteria outlined in this chapter.

Inclusion Criteria	Exclusion Criteria
• 18-35 years of age • No known metabolic, neurological, or cardiovascular disease • No autonomic dysfunction	• Use of any form of tobacco or nicotine • Regular use of melatonin as a supplement • Any history of autonomic dysfunction, cardiovascular disease, metabolic

• Females are premenopausal with regular cycles (26-30 days) • Systolic blood pressure of <130mmHg, diastolic blood pressure of <85mmHg	disease, or neurological disease (Harrell et al., 2014) • Females that are pregnant • Anyone with an allergic reaction to melatonin • Systolic blood pressure >130mmHg, diastolic blood pressure >85mmHg • Individuals engaging in sex hormone replacement therapies

Research Design

This study was a true experimental, randomized, placebo-controlled, crossover, single-blind (participant only) design. Threats to internal and external validity were be limited through the true experimental placebo-controlled design.

Experimental Protocol

Visit 1 – Informed Consent and Screening

Subjects were required to attend an initial familiarization and qualification session before participation in the protocol. Immediately upon attendance of the first visit, subjects were informed of the potential risks and benefits of participation, after which they signed an approved (IRB #13084) informed consent document. Health Insurance Portability and Accountability Act (HIPAA) authorization was then collected from the subject after informing the subject of the

implications of the use of their data on an approved authorization form. A Pittsburgh Sleep Quality Index (PSQI) form was completed by the subject in order to further understand sleeping habits, as melatonin is known to affect Circadian Rhythm signaling. The International Physical Activity Questionnaire (IPAQ) was then completed by the subject to determine their recent physical activity level. After completion of all forms and after all risks and benefits were discussed, if subjects agreed to continue, pregnancy tests were administered for female participants. To account for differences in cerebral blood flow and blood flow regulation across different menstrual cycle phases, it was ensured that each female participant experienced regular menstrual cycles with little variation (Peltonen et al., 2016). The participant then laid in a supine position while resting for 10 minutes, after which measurements of blood pressure were taken. After this, height, weight, waist and hip circumference, and finger circumference measurements were taken as 3 total measurements averaged with 1 minute breaks between each measurement. Gathered data was used to determine whether or not the participant met the inclusion criteria of the study. If the subject did, they were fitted for equipment, experienced CO_2 rebreathing sensation, and became familiarized with the protocol. Anatomical location, gate, and depth for the transcranial doppler ultrasonography probes were found to make experimental visit acquisition of the MCA quicker and easier during future experimental visits. Subjects were then scheduled for visit 2.

Visits 2 & 3

Upon arrival for visits 2 and 3 subjects indicated verbally that they had adhered to the pre-protocol requirements established earlier in this chapter. Participants were reminded of the protocol as they were shown during their first visit. They were instructed to use the restroom and don the Equivital ECG vest that they were fitted for during their first visit. Participants were then

instrumented with the equipment detailed below. During instrumentation, administration of either the melatonin treatment (5mg, Onnit, Austin, TX, mint flavor) or the placebo treatment (one µL of McCormicks Pure Mint extract and 29 mL of filtered water, to mimic flavor of melatonin treatment) was given sublingually in the form of 10 sprays under the tongue, randomized via a coin flip and not investigator-blinded. Whether the participant received the melatonin was randomized and counterbalanced with a placebo upon their next visit. Subjects were seated in the supine position and further instrumentation and verification of measurements occurred for 30 minutes (the amount of time melatonin has been described to reach peak blood concentration when consumed via a spray) (Bartoli et al., 2018). The laboratory was kept dark and temperature-controlled (22-24°C). After a measurement line-up, 5 minutes of baseline was followed by 2 CO2 rebreathing challenges, which occurred until the participant displayed a ≥ 10mmHg increase in End-tidal CO_2, was continuously measured.

Figure 1. Protocol Outline. Placebo (PLA); Melatonin (MEL). Rebreathing 1 and 2 was conducted until a physiological increase in end-tidal CO_2 of +10 mmHg was observed. Central cardiovascular variables described above, MCAv, CVCi, and TSI were collected during all stages.

Instrumentation

Transcranial Doppler Probes (Neurovision Transcranial Doppler Ultrasound, Multigon Industries, Elmsford, New York) are 2MHz probes that are ideally insonated at an ~90° angle to the middle cerebral artery to accurately measure flow velocity. The Equivital Life Monitor and Vest (EQ life monitor, Equivital Limited, Cambridge, United Kingdom) detects alterations in chest expansion and relaxation to output breathing frequency (BF) and uses a 2-lead ECG to

detect heart rate (HR). The Hans Rudolph V2 oro-nasal Mask (Hans Rudolph, Shawnee, KS) fits over the subject's nose and mouth and is attached to a custom 3-way valve allowing for breath-by-breath sampling and CO_2 rebreathing to be switched to seamlessly. The Finapres Finometer (Finapres® NOVA, Finapres Medical Systems B.V, Enschede, Netherlands) is a finger plethysmography unit that allows for the measurement of beat-to-beat mean arterial blood pressure (MAP), stroke volume (SV), total peripheral resistance (TPR), and cardiac output (Q) by means of small-balloon expansion within a cuff placed around the subject's middle finger.

Measurements

Middle Cerebral Artery Velocity (MCAv):

Alterations in middle cerebral artery velocity have been assessed using 2MHz transcranial robotic doppler probes. Just before or after administration of the treatment, subjects were seated while two technicians placed one probe on the temporal window of the skull of the participant. Each probe was affixed to an adjustable headset. Participants were asked to limit head and neck movement from the point of signal acquisition until the end of the experiment. Previous literature has established that TCD is an effective measurement of both blood velocity and blood flow in the middle cerebral artery (Bishop et al., 1986.).

Cardiac Output (Q), Stroke Volume (SV), Mean Arterial Pressure (MAP), and Total Peripheral Resistance (TPR):

These mentioned cardiovascular outcome variables were continuously measured using a finometer. Blood pressure tracking using this technique, which measure blood pressure waveforms and takes the difference between systolic and diastolic pressure to produce a mean

pressure, has been shown to be reliable and valid in other similar protocols in producing beat-by-beat MAP measurements (Reisner et al., 2007; Reisner et al., 2011).

Breathing Frequency (BF) and Heart Rate (HR):

HR and BF have been collected continuously using a 2-lead Equivital ECG vest capable of detecting alterations in chest expansion.

End-tidal CO_2 concentrations:

$EtCO_2$ has been derived with the use of breath-by-breath sampling at the mouth and Gemini O_2 and CO_2 gas analyzer (CWE Incorporated, Ardmore, Pennsylvania), which detects alterations in gas concentrations through changes in infrared absorption levels.

Threats to Validity

Threats to internal validity included participant sympathetic activation through the exposure to the new environment and procedure itself. While sympathetic activation could have occurred due to the instrumentation of multiple new pieces of equipment, as well as the audible volume of some stages of the protocol, this was controlled through the familiarization period that each participant was required to complete. Through exposure to the new stimuli, sympathetic response was assumed to be less variable from participant to participant independent of their given treatment on visits 2 or 3. Another possible threat to internal validity includes the possibility of technician error. Between 4 and 5 technicians are required to be present during each protocol, and a lack of practice on operating a certain piece of equipment is a threat to the integrity of data collection or instrumentation. To control for this, each technician was properly trained and instructed on every piece of equipment that they would need to run properly during each trial. Each piece of equipment has a very specific and detailed checklist associated with it,

and on each day of a protocol every technician signed an accountability form indicating that they were responsible for that piece of equipment on each specific day. Each technician was tested on their ability to run every piece of equipment, and every technician was signed off via an MOP by the laboratory principal investigator. Beyond this, a lack of intravenous melatonin concentration measurement could have threatened internal validity, as there was no verification that the sublingual melatonin supplementation caused changes to systemic or cerebrovascular melatonin concentrations. Despite this, previous literature has demonstrated that sublingual melatonin supplementation is safe and effective in altering intravenous melatonin concentrations (Bartoli et al., 2013). Melatonin receptor expression is also variable depending on a multitude of factors, including light exposure, duration of exposure to exogenous melatonin, and endogenous melatonin concentrations (Pandi-Perumal, 2007). Therefore, because the time of each visit was not regulated, it is possible that peak melatonin receptor concentrations were different between subjects as well as within subjects on different visits. Finally, it has been established that there is a clear reduction in cerebrovascular CO_2 reactivity and endothelium-dependent vascular reactivity early in the morning due to an unknown mechanism (Ainslie et al., 2007). Because of the fasting requirement for participation, many subjects chose to conduct their visits early in the morning, meaning that the influence of a reduction in cerebrovascular CO_2 reactivity may have had a direct substantial result on measured CVR. A threat to external validity includes a lack of applicability to populations other than young and healthy males and females between the ages of 18 and 35. Because young and healthy individuals between the ages of 18 and 35 are the target population, this is to be expected and possibly expanded upon in future research.

Data Acquisition and Analysis

All data was recorded and stored in LabChart (ADInstruments, CO, USA). Power Lab (ADInstruments, CO, USA) is a device that integrates inputs from multiple pieces of equipment associated with cardiovascular outcome variables and enables readability within the LabChart software at an approximate sampling rate of ~20kHz. Cardiovascular outcome variables were scripted and transferred into Microsoft Excel (Microsoft Corporation, Redmond, WA, USA). Calculation of the cardiovascular conductance index was completed through the use of the following equation:

$$CVCi = MCAv/MAP$$

Estimated MCA diameter change was calculated using a third-order polynomial from previous research using a 7-Tesla MRI system during a similar CO_2 rebreathing protocol that has displayed an R^2 value of up to 0.51 (Verbree et al., 2014). The polynomial itself can be shown as:

$$y = 0.93x^3 + 1.22x^2 + 1.99x + 99.64$$

Where y is representative of estimated vessel area change in a percentile and x is representative of $\Delta PetCO_2$ in kPa. The same normalized area was inserted into an equation capable of estimating flow in ideal conditions, not considering changes in pressure, changes in blood viscosity, or changes in vessel length, as displayed below:

$$Q = Av$$

Where Q displays middle cerebral artery blood flow in mL/min, A represents the cross-sectional area of the vessel in cm^2, and v represents the TCD-measured middle cerebral artery velocity in cm/s. Using established baseline data from the same 7-Tesla MRI analysis during CO_2 rebreathing, a multiplication of the percent change in area (calculated via the third-order

polynomial) and the baseline vessel area allows for us to estimate flow by multiplying our measured velocity values by the estimated area (Verbree et al., 2014). Our baseline vessel areas are derived from 7-Tesla MRI data and are a generalization based on sex (Verbree et al., 2014). Therefore, while each individual's vessel area will not be measured, using the generalized baseline areas allows for us to view changes in estimated flow. Not measuring each individual's vessel diameter is a limitation, so future methods should focus on taking individual measurements of MCA diameters and making comparisons with same-subject TCD data.

Statistical Analysis

Statistical analysis was performed during each rebreathing protocol against the last 30 seconds of the previous baseline's average. SPSS Version 27 (IBM, Chicago, IL, USA) and Microsoft Excel was used for statistical analysis. Shapiro-wilks tests of normality were conducted before carrying out paired samples t tests or Wilcoxon paired samples tests based on the normality of the data. Differences between estimated blood flow, MCAv, CVCi, TSI, and MAP were acquired and reported. Effect sizes were calculated using Cohen's d in the case of a paired samples t test or as an r value, shown in the following equation, in the case of non-normal data calling for a Wilcoxon analysis:

$$r = \frac{Z}{\sqrt{n_1} + \sqrt{n_2}}$$

Alpha was set a priori as 0.05.

Chapter IV

Results

Subject Demographics

A total of 22 subjects completed all three study visits, and 18 subjects (11 males, 7 females; 24.12 ± 4.06 years old) were included for the final analysis. Three subjects were not included due to poor MCAv signal acquisition during at least one of the experimental visits, and one additional subject's TSI values were excluded due to poor data quality. One subject opted to only complete one rebreathing trial during both experimental visits. All subjects matched the required population parameters; being between 18-35 years of age with no known neurological, cardiovascular, or metabolic diseases, having a systolic blood pressure of <130 mmHg, having a diastolic blood pressure of <85 mmHg, having not used nicotine products within the last 6 months, having not used prescription medications that could interfere with the present study, and having not used melatonin supplements on a regular basis. Female subjects were tested within the early follicular phase of their menstrual cycles.

Table 1. Subject Characteristics.

	Total (*n=18*)	**Males** (*n=11*)	**Females** (*n=7*)
Age (years)	24.12 ± 4.18	24.92 ± 4.06	22.71 ± 4.31
BMI (kg/m^2)	23.82 ± 3.44	25.16 ± 3.28	21.53 ± 2.49 *
SBP (mmHg)	114.16 ± 8.75	119.14 ± 6.88	105.61 ± 2.86 *
DBP (mmHg)	71.22 ± 3.68	71.49 ± 4.01	70.76 ± 3.26
Height (cm)	176.00 ± 9.32	181.33 ± 6.46	166.86 ± 5.46 *
Weight (kg)	73.84 ± 14.43	91.91 ± 9.95	60.00 ± 9.51 *

Waist Circ. (cm)	85.14 ± 10.18	88.21 ± 9.81	79.87 ± 9.13
Hip Circ. (cm)	99.76 ± 8.56	101.75 ± 9.22	96.36 ± 6.51
Chest Circ. (cm)	87.43 ± 11.75	94.13 ± 5.81	75.96 ± 10.43 *
HR (bpm)	57.30 ± 9.55	55.80 ± 7.91	59.86 ± 12.10
PSQI	6.64 ± 4.54	8.23 ± 5.23	4.14 ± 0.90 *
IPAQ	4496.31 ± 3528.31	3235.77 ± 2380.97	6477.14 ± 4281.69 *

*Values displayed as mean ± SD. * represents significant difference compared to males. BMI: Body Mass Index; SBP: Systolic Blood Pressure; DBP: Diastolic Blood Pressure; HR: Resting Average Heart Rate; PSQI: Pittsburgh Sleep Quality Index; IPAQ: International Physical Activity Questionnaire.*

Central Cardiovascular Variables

No statistically significant differences in any measured cardiovascular or pulmonary variables when measured between melatonin and placebo treatment conditions were found during the rebreathing trial (**Table 2,** $p > 0.05$). As expected, $P_{ET}CO_2$ significantly increased as an average from baseline to rebreathing (**Table 2**, PLA 42.59 ± 1.95 vs. 49.52 ± 2.22 mmHg, p = <0.001, d = -8.00; MEL 43.78 ± 3.12 vs. 50.49 ± 3.08 mmHg, p = <0.001, r = -0.88). Predictably, B_f also significantly increased during rebreathing when compared to baseline (**Table 2**, PLA 15.16 ± 3.22 vs. 56.40 ± 3.51 bpm, p = <0.001, d = -8.53; MEL 15.30 ± 3.72 vs. 57.51 ± 2.87 bpm, p = <0.001, d = -8.87). While MAP did visually increase during the transition from baseline to rebreathing (**Table 2, Figure 2**), no statistical differences were found. No other cardiovascular or pulmonary variable displayed a statistically significant difference when measuring from baseline to rebreathing (**Table 2**).

Table 2. Central Pulmonary and Cardiovascular Variables (*n = 18, 11 males, 7 females)*

	Baseline	***p Value, Effect Size***	**Rebreathing**	***p Value, Effect Size***
Q (L/min)				
PLA	6.24 ± 1.92	p = 0.469	6.22 ± 1.92	p = 0.616
MEL	6.06 ± 1.46	r = -0.166	5.99 ± 1.39	r = -0.118
SV (mL/beat)				
PLA	95.71 ± 23.15	p = 0.829	95.16 ± 23.16	p = 0.850
MEL	94.86 ± 19.75	d = 0.050	94.39 ± 19.48	d = 0.045
TPR				
PLA	1.09 ± 0.31	p = 0.469	1.11 ± 0.31	p = 0.913
MEL	1.11 ± 0.31	r = -0.166	1.11 ± 0.28	r = -0.026
HR (bpm)				
PLA	62.91 ± 7.96	p = 0.209	63.51 ± 7.41	p = 0.206
MEL	61.51 ± 9.21	d = 0.299	61.69 ± 8.20	d = 0.310
MAP (mmHg)				
PLA	107.88 ± 15.69	p = 0.968	109.43 ± 15.51	p = 0.647
MEL	107.19 ± 11.79	r = -0.092	107.43 ± 10.19	r = -0.108
$\mathbf{P_{ET}CO_2}$ (mmHg)				
PLA	42.59 ± 1.95 *	p = 0.212	49.52 ± 2.22 *	p = 0.472
MEL	43.78 ± 3.12 *	r = -0.286	50.49 ± 3.08 *	r = -0.170

B_f (bpm)				
PLA	15.16 ± 3.22 *	p = 0.828	56.40 ± 3.51 *	p = 0.298
MEL	15.30 ± 3.72 *	d = -0.050	57.51 ± 2.87 *	d = -0.253

*Values displayed as mean ± SD. * represents significant differences between baseline and rebreathing. PLA: Placebo Treatment Group; MEL: Melatonin Treatment Group; Q: Cardiac Output; SV: Stroke Volume; TPR: Total Peripheral Resistance; HR: Heart Rate; MAP: Mean Arterial Pressure; $P_{ET}CO_2$: End-tidal CO_2 measured during the last 30s of baseline and as an average over the entire rebreathing trial; B_f: Breathing Frequency.*

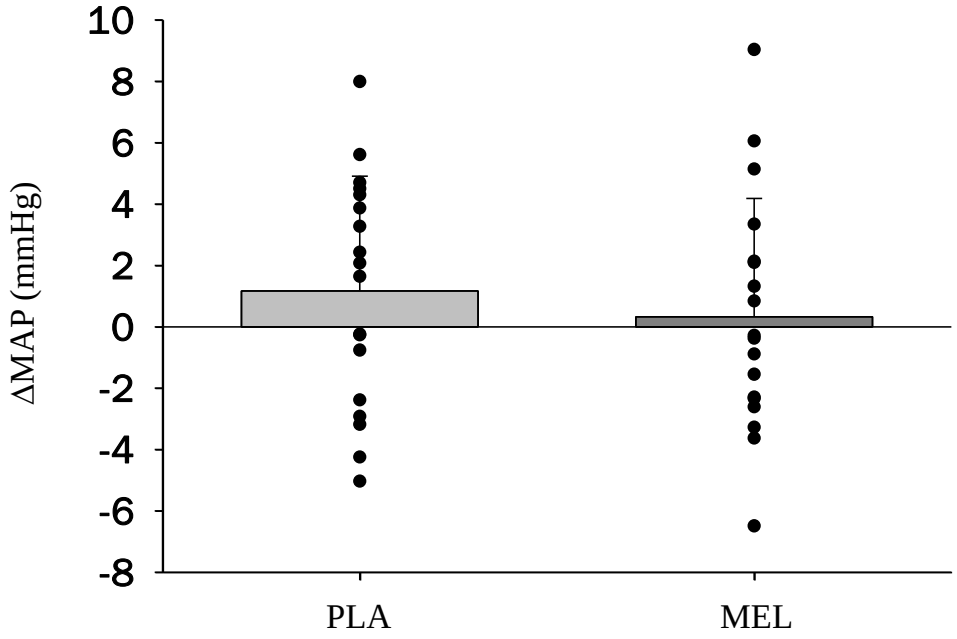

Figure 2. Data is displayed as mean ± SD. Shows alterations in mean arterial pressure from baseline to CO_2 rebreathing as a comparison between the placebo treatment and the melatonin treatment (n=18). ΔMAP was calculated as an average value, derived from subtracting an average value of the last 30s of the preceding baseline from each data point during rebreathing. Alpha was set a priori as $p < 0.05$. No significant difference was seen between treatment groups.

Cerebrovascular Variables

No cerebrovascular variable showed a statistically significant absolute difference between treatment groups during baseline or during the rebreathing trial (**Table 3, Table 4**). No measured variable showed any statistical differences between treatment groups when comparing the change seen during rebreathing calculated against the last 30s of baseline (**Table 3, Table 4**). MCAv increased significantly when transitioning from baseline to rebreathing (**Figure 3, Table 3**, PLA 70.90 ± 11.59 vs. 83.43 ± 13.39 cm/s, p = <0.001, d = -2.14; MEL 72.08 ± 15.77 vs. 83.95 ± 15.68 cm/s, p = <0.001, d = -2.20). CVCi also increased significantly when comparing the transition from baseline to rebreathing (**Figure 4, Table 3**, PLA 0.67 ± 0.14 vs. 0.78 ± 0.16 cm/s/mmHg, p = <0.001, d = -2.10; MEL 0.68 ± 0.16 vs. 0.79 ± 0.16 cm/s/mmHg, p = <0.001, d = -2.33). TSI increased significantly from baseline to rebreathing (**Figure 5, Table 3**, PLA 66.57 ± 6.99 vs. 68.12 ± 7.22 %, p = <0.001, r = -0.88; MEL 68.65 ± 5.95 vs. 69.62 ± 6.16 %, p = <0.001, r = -0.87). No particular visual or statistical trend was visible when comparing CVR calculated using MCAv or CVCi absolutely or as a percentage change from rebreathing onset between treatment groups (**Figure 6-9, Table 4**).

Table 3. Cerebral Vascular Variables (*n = 18, 11 males, 7 females; TSI n = 17, 11 males, 6 females*)

	Baseline	***p Value, Effect Size***	**Rebreathing**	***p Value, Effect Size***	**Δ BL-RB**	***p Value, Effect Size***
MCAv (cm/s)						
PLA	70.90 ± 11.59	p = 0.612	83.43 ± 13.39	p = 0.860	13.07 ± 5.74 *	p = 0.818
MEL	72.08 ± 15.77	d = -0.118	83.95 ± 15.68	d = -0.042	12.68 ± 5.65 *	d = 0.055

CVCi						
(cm/s/mmHg)						
PLA	0.67 ± 0.14	p = 0.759	0.78 ± 0.16	p = 0.755	0.11 ± 0.05 *	p = 0.926
MEL	0.68 ± 0.16	d = -0.072	0.79 ± 0.16	d = -0.075	0.12 ± 0.05 *	d = -0.022
TSI (%)						
PLA	66.57 ± 6.99	p = 0.528	68.12 ± 7.22	p = 0.906	1.39 ± 0.92 *	p = 0.106
MEL	68.65 ± 5.95	r = -0.149	69.62 ± 6.16	r = -0.029	0.99 ± 0.60 *	d = 0.415

*Values displayed as mean ± SD. * represents significant differences when comparing the transition from baseline and rebreathing. p Value represents a comparison between PLA and MEL treatments. MCAv: Middle Cerebral Artery Velocity; CVCi: Cerebrovascular Conductance Index. TSI: Total Saturation Index.*

Table 4. Rebreathing Cerebrovascular Reactivity (*n = 18, 11 males, 7 females*)

	Rebreathing	***p Value, Effect Size***	**RB % Change**	***p Value, Effect Size***
CVR_{MCAv}				
(cm/s/mmHg)				
PLA	1.83 ± 0.81	p = 0.949	5.37 ± 1.23	p = 0.148
MEL	1.84 ± 0.72	d = -0.015	6.01 ± 1.52	d = -0.357
CVR_{CVCi}				
$(\frac{\frac{cm}{s}}{mmHg}/mmHg)$				
PLA	0.02 ± 0.01	p = 0.500	4.98 ± 1.77	p = 0.315
MEL	0.02 ± 0.01	r = -0.159	5.51 ± 1.89	d = -0.244

Values displayed as mean ± SD. p Value represents a comparison between PLA and MEL conditions. CVR_{MCAv}: Cerebrovascular Reactivity calculated using MCAv; CVR_{CVCi}: Cerebrovascular Reactivity calculated using CVCi.

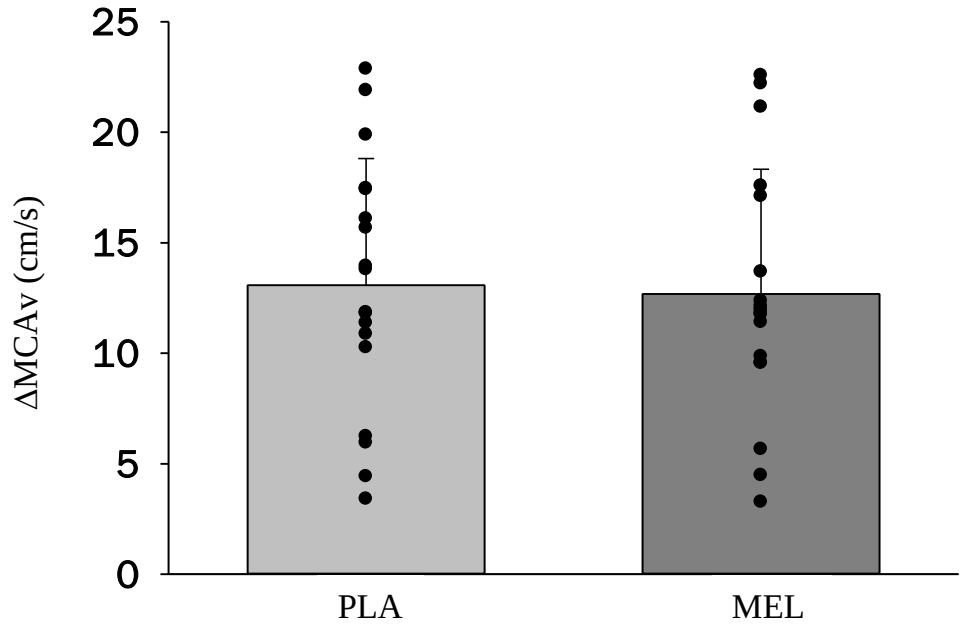

Figure 3. Data is displayed as mean ± SD. Shows alterations in middle cerebral artery velocity from baseline to CO_2 rebreathing as a comparison between the placebo treatment and the melatonin treatment (n=18). ΔMCAv was calculated as an average value, derived from subtracting an average value of the last 30s of the preceding baseline from each data point during rebreathing. Alpha was set a priori as $p < 0.05$. No significant difference was seen between treatment groups. MCAv significantly increased from baseline to rebreathing in both treatment groups.

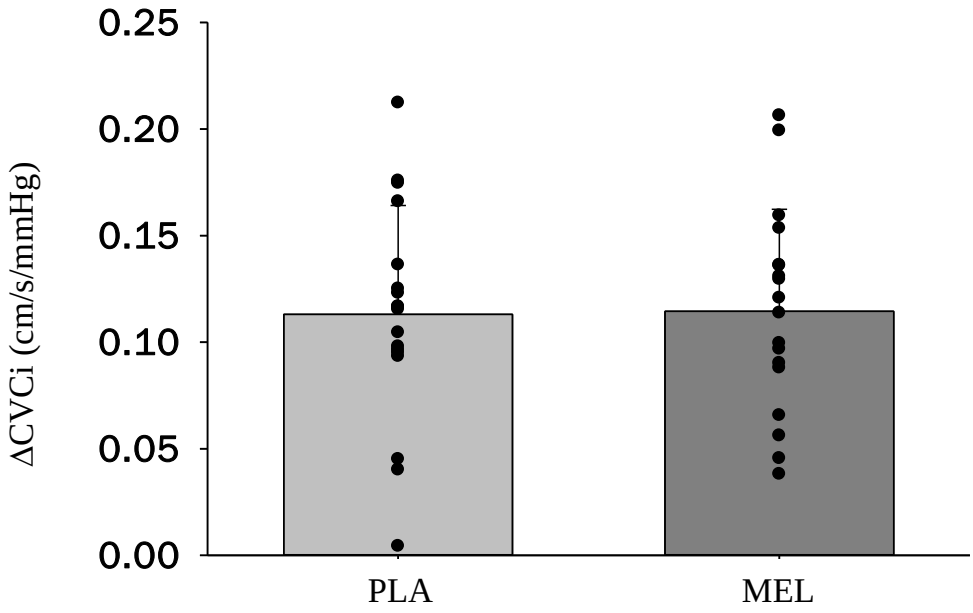

Figure 4. Data is displayed as mean ± SD. Shows alterations in the cerebrovascular conductance index from baseline to CO_2 rebreathing as a comparison between the placebo treatment and the melatonin treatment (n=18). ΔCVCi was calculated as an average value, derived from subtracting an average value of the last 30s of the preceding baseline from each data point during rebreathing. Alpha was set a priori as $p < 0.05$. No significant difference was seen between treatment groups. CVCi significantly increased from baseline to rebreathing in both treatment groups.

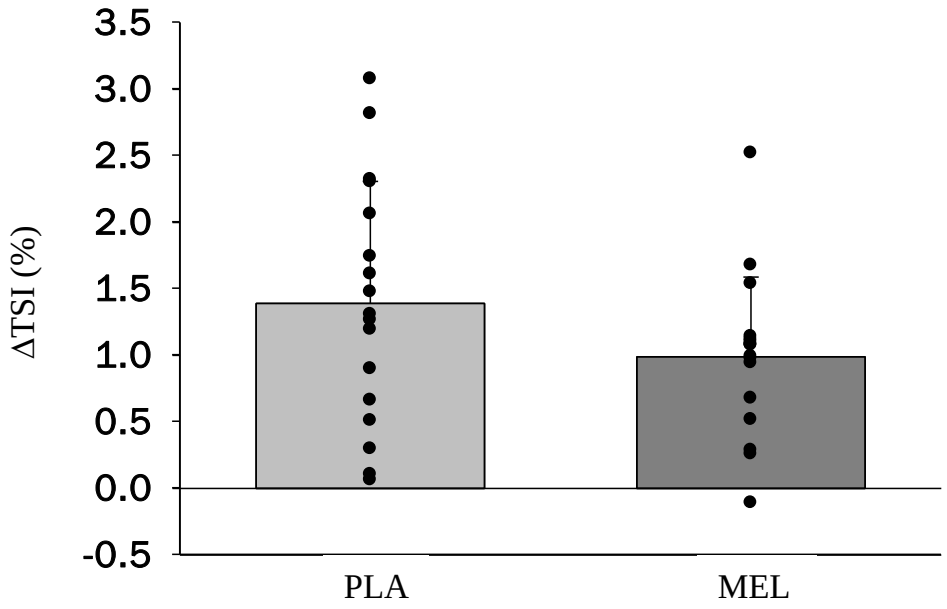

Figure 5. Data is displayed as mean ± SD. Shows alterations in the total saturation index from baseline to CO_2 rebreathing as a comparison between the placebo treatment and the melatonin treatment (n=17). ΔTotal Saturation Index (TSI) was calculated as an average value, derived from subtracting an average value of the last 30s of the preceding baseline from each data point during rebreathing. Alpha was set a priori as $p < 0.05$. No significant difference was seen between treatment groups. TSI significantly increased from baseline to rebreathing in both treatment groups.

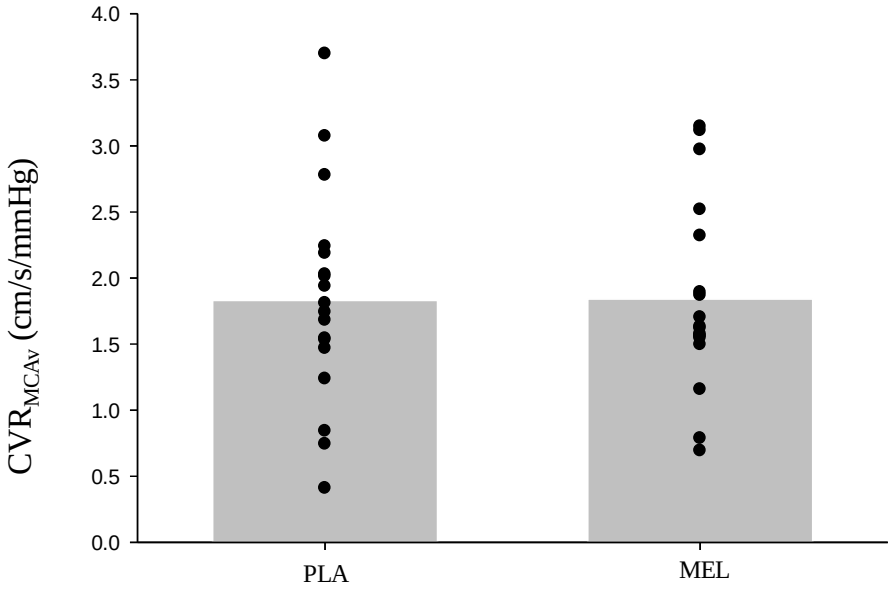

Figure 6. Data is displayed as mean ± SD. Shows alterations in the cerebrovascular reactivity calculated using MCAv from baseline to CO_2 rebreathing as a comparison between the placebo treatment and the melatonin treatment (n=18). CVR_{MCAv} was calculated as an average value, where ΔMCAv was divided by $\Delta PetCO_2$. Alpha was set a priori as $p < 0.05$. No significant difference was seen between treatment groups.

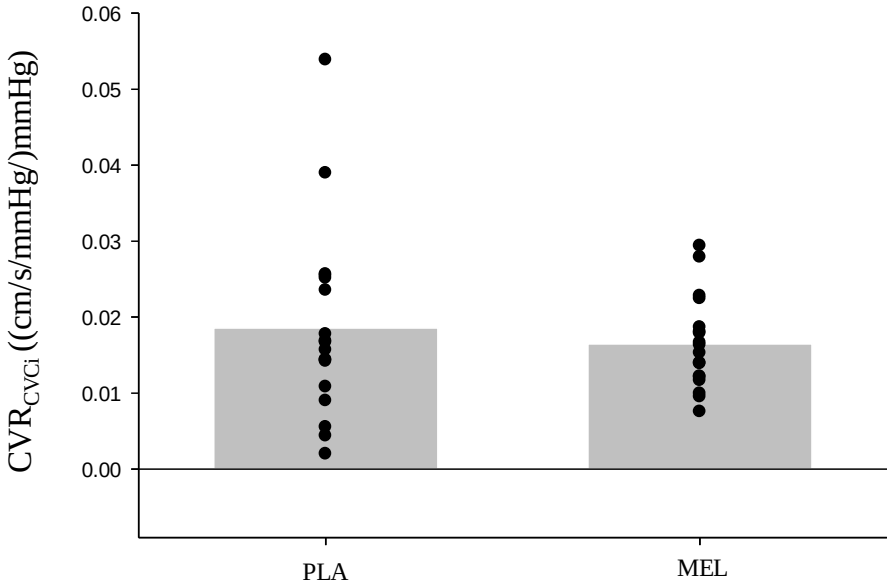

Figure 7. Data is displayed as mean ± SD. Shows alterations in the cerebrovascular reactivity calculated using CVCi from baseline to CO_2 rebreathing as a comparison between the placebo treatment and the melatonin treatment (n=18). CVR_{CVCi} was calculated as an average value, where ΔCVCi was divided by Δ$PetCO_2$. Alpha was set a priori as $p < 0.05$. No significant difference was seen between treatment groups.

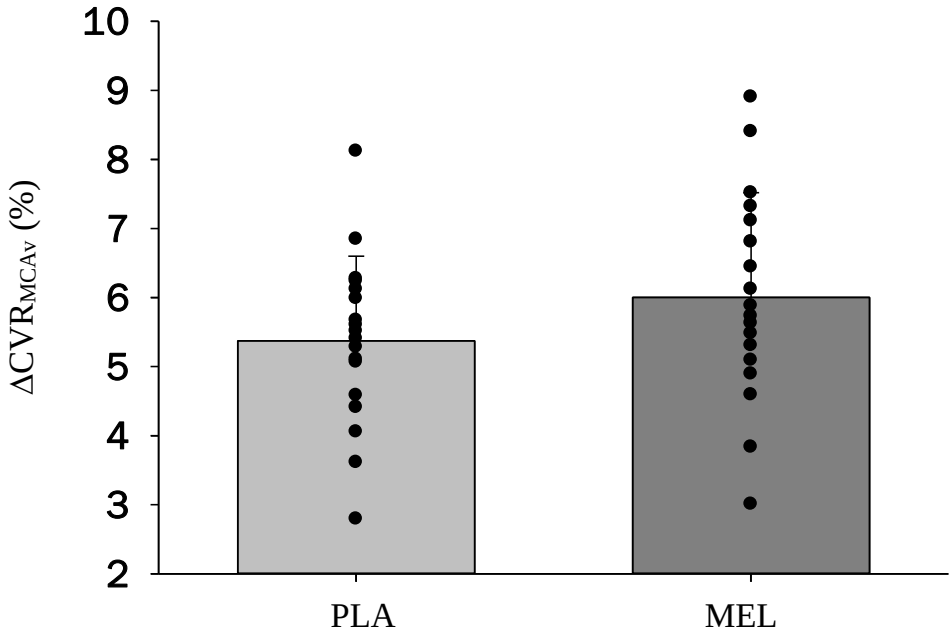

Figure 8. Data is displayed as mean ± SD. Shows alterations in the cerebrovascular reactivity calculated using MCAv from baseline to CO_2 rebreathing as a comparison between the placebo treatment and the melatonin treatment as a percent change (n=18). CVR_{MCAv} was calculated as the percent change in MCAv divided per unit change in end-tidal CO_2. Alpha was set a priori as $p < 0.05$. No significant difference was seen between treatment groups.

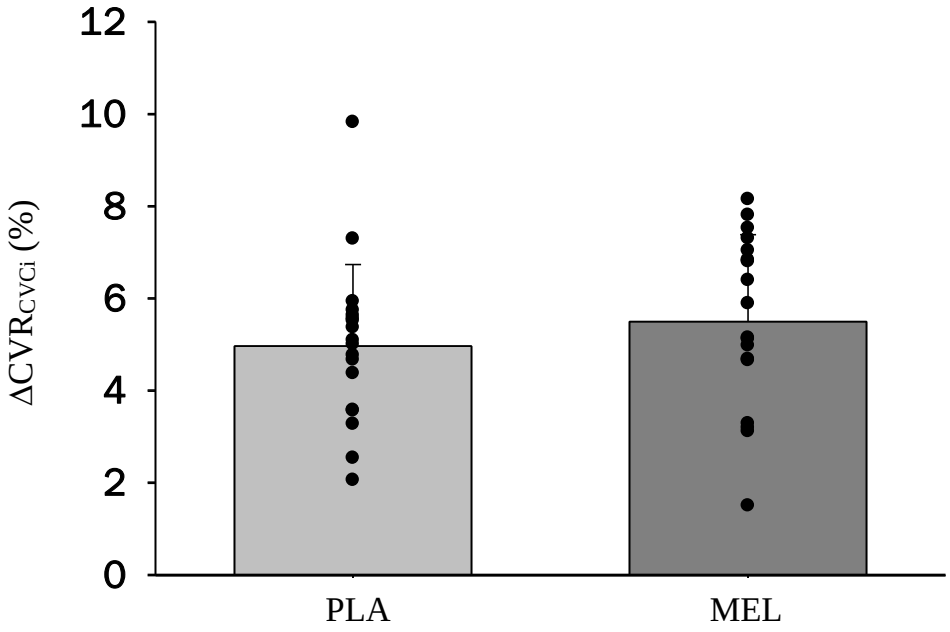

Figure 9. Data is displayed as mean ± SD. Shows alterations in the cerebrovascular reactivity calculated using CVCi from baseline to CO_2 rebreathing as a comparison between the placebo treatment and the melatonin treatment as a percent change (n=18). CVR_{CVCi} was calculated as the percent change in CVCi divided per unit change in end-tidal CO_2. Alpha was set a priori as $p < 0.05$. No significant difference was seen between treatment groups.

Time to Peak Analysis

The time to peak end-tidal CO_2 was calculated for both the first and second trials of rebreathing. No significant difference in the time to peak was seen comparing between treatment groups (**Table 5**). The time to peak was significantly shorter during the second trial of rebreathing for both the placebo group and the melatonin group (**Table 5**, PLA 100.10 ± 17.79 vs. 63.39 ± 24.78 seconds, p = <0.001, d = 1.40; MEL 95.68 ± 18.48 vs. 58.04 ± 21.22 seconds, p = <0.001, d = 2.06).

Table 5. Time to End-tidal CO_2 Peak during RB1 & RB2 (*RB1 n = 19, 12 males, 7 females, RB2 n = 18, 11 males, 7 females*)

	RB1	***p Value, Effect Size***	**RB2**	***p Value, Effect Size***	**p Value (RB1-RB2)**
TTP (seconds)					
PLA	100.10 ± 17.79	p = 0.241	63.39 ± 24.78	p = 0.493	<0.001
MEL	95.68 ± 18.48	d = 0.278	58.04 ± 21.22	d = 0.165	<0.001

Values displayed as mean ± SD. p Value represents a comparison between PLA and MEL conditions. TTP: Time to reach peak End-tidal CO_2 concentrations from the beginning of a rebreathing stage.

Experimental Estimated Flow Analysis

There was no statistically significant difference between treatment groups in terms of the estimated change of flow calculations, despite a visual reduced change in flow in the melatonin group when compared to the placebo group (**Figure 10, Table 6**, $p < 0.05$). When comparing the first trial of rebreathing to the second trial within each treatment group, no statistically significant difference was found (**Figure 10, Table 6**, $p < 0.05$).

Table 6. Estimated ΔCerebral Blood Flow (*n = 18, 11 males, 7 females*)

	Absolute Change	***p Value, Effect Size***	**CVR Percent Change**	***p Value, Effect Size***	**p Value (RB1-RB2)**

Estimated

Δ Flow (ml/min)

PLA	60.73 ± 26.64	p = 0.787	6.75 ± 1.19	p = 0.249	0.643
MEL	58.52 ± 27.26	d = 0.065	7.18 ± 1.24	d = -0.282	0.774

Values displayed as mean ± SD. p Value represents a comparison between PLA and MEL conditions. Estimated Δ Flow: Polynomial-based estimation of a change in cerebral blood flow using previous literature and measured flow velocity values (Verbree et al., 2014); CVR Percent Change: displays a calculation of CVR using estimated flow percentage changes per unit change in end-tidal CO_2. RB1-RB2 displays a comparison between the first trial of rebreathing to the second.

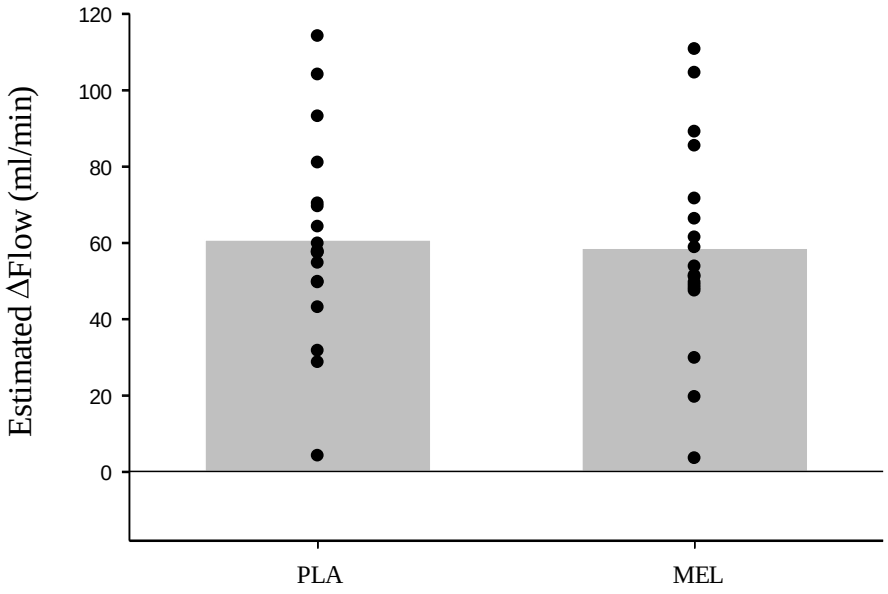

Figure 10. Data is displayed as mean ± SD. Shows alterations in the estimated change of cerebral flow in the middle cerebral artery from baseline to CO_2 rebreathing as a comparison between the placebo treatment and the melatonin treatment (n=18). ΔFlow was calculated as a multiplication of the estimated area derived from a previously

described 7-Tesla MRI origin study and our measured MCAv (Verbree et al., 2014). Alpha was set a priori as $p < 0.05$. No significant difference was seen between treatment groups.

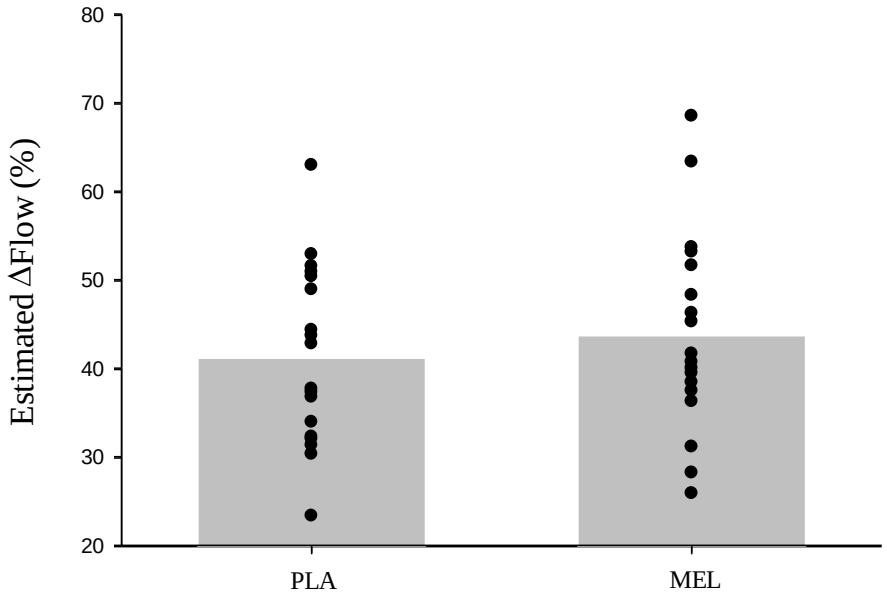

Figure 11. Data is displayed as mean ± SD. Shows alterations in the estimated change of cerebral flow in the middle cerebral artery from baseline to CO_2 rebreathing as a comparison between the placebo treatment and the melatonin treatment as a percent change (n=18). ΔFlow% was calculated using estimated baseline areas (Verbree et al., 2014). Alpha was set a priori as $p < 0.05$. No significant difference was seen between treatment groups.

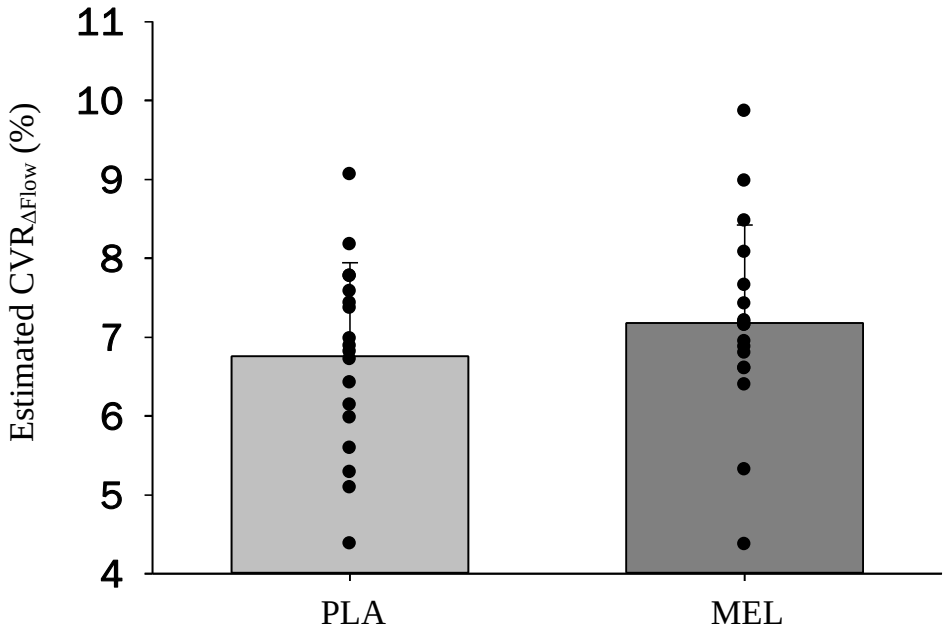

Figure 12. Data is displayed as mean ± SD. Shows alterations in the estimated change of cerebral flow in the middle cerebral artery from baseline to CO_2 rebreathing as a comparison between the placebo treatment and the melatonin treatment as a percent change per unit change of end-tidal CO_2 (n=18). $\Delta CVR_{Flow\%}$ was calculated using estimated baseline areas (Verbree et al., 2014). Alpha was set a priori as $p < 0.05$. No significant difference was seen between treatment groups.

IPAQ and PSQI Correlations

IPAQ and PSQI results were analyzed using a correlation test with our outcome CVR values both during individual treatment conditions (placebo or melatonin) and then as a combined group due to the lack of significant differences found between placebo and melatonin treatment conditions. The combined group incorporated both the placebo and melatonin conditions averaged with one another. When compared using individual conditions (placebo or

melatonin) or as a combined group, there was no statistically significant correlation (**Table 7**). Furthermore, there was no large effect size for any of the mentioned comparisons (**Table 7**).

Table 7. Correlations between IPAQ, PSQI and CVR outcomes (*n = 18, 11 males, 7 females*)

	CVR_{MCAv} Combined	**CVR_{MCAv} % Combined**	**CVR_{CVCi} Combined**	**CVR_{CVCi} % Combined**
PSQI				
p Value	0.531	0.857	0.669	0.587
r Coefficient	0.158	0.046	0.108	0.137
IPAQ				
p Value	0.249	0.853	0.268	0.284
r Coefficient	0.286	0.047	0.276	0.267

Values displayed as mean ± SD. p Value represents the significance of the Pearson or Spearman correlation. PSQI: Pittsburgh Sleep Quality Index; IPAQ: International Physical Activity Questionnaire; CVR_{MCAv} Combined: the combined CVR values between placebo and melatonin treatments using MCAv; CVR_{MCAv} % Combined: the combined CVR values as a percentage change and per unit change in CO_2 between placebo and melatonin treatments using MCAv; CVR_{CVCi} Combined: the combined CVR values between placebo and melatonin treatments using CVCi; CVR_{CVCi} % Combined: the combined CVR values as a percentage change and per unit change in CO_2 between placebo and melatonin treatments using CVCi.

Chapter V

Discussion

The purpose of the present study was to investigate the effects of melatonin supplementation on cerebrovascular hemodynamics during rest and CO_2 rebreathing in healthy, young adults. Contrary to our first hypothesis, melatonin supplementation did not alter MCA_v or estimated cerebrovascular flow during rest or the CO_2 rebreathing trial. Applied to our second hypothesis, melatonin supplementation did not alter cerebrovascular reactivity, did not significantly increase CVCi, and did not significantly decrease MAP. In opposition of our third hypothesis, acute melatonin supplementation did not increase prefrontal cortex oxygenation during either rest or rebreathing.

Melatonin and Cerebral Hemodynamics

The present study found that cerebral hemodynamic variables did not change during acute melatonin supplementation during a resting steady state. Few studies have investigated the effects of melatonin on the cerebrovasculature, but our findings come in contrast to previous literature, where acute melatonin supplementation reduced both circulating catecholamines and blood pressure within 90 minutes (Arangino et al., 1999). There was a visual decrease in blood pressure, with no statistical difference, but our inability to reproduce the blood pressure reduction seen in previous literature may be due to only allowing for 30 minutes to pass from the time of consumption to the beginning of the experimental protocol. Previous literature has primarily used either intravenous melatonin dosing or tablet dosing, which can take up to 60 minutes to reach maximal plasma concentrations (Tordjman et al., 2017). We based our dosing and the time between intake and experiment initiation on previous literature that showed both no

significant difference in absorption rate when compared to tablet dosing and a 30 minute time period with which melatonin may reach maximal plasma concentrations when consuming melatonin via a spray (Bartoli et al., 2013). As would be expected during a rebreathing trial, both MCAv and CVCi increased significantly from baseline to rebreathing. Despite this change, no significant difference between the placebo and melatonin treatment groups was found. Furthermore, TSI was also found to increase significantly from baseline to rebreathing, but there was no significant difference between the placebo and melatonin treatment groups.

This is the first time, to our knowledge, that cerebrovascular reactivity was measured under the effect of acute melatonin supplementation during rest and a potent vasodilatory stimulus such as CO_2 rebreathing. The melatonin receptor MT2 has been located in the large vessels of the brain and is known to influence the production of cyclic GMP, a molecule that plays an important role in the process of vasodilation (Petit et al., 1999; Yu et al., 2001). Despite melatonin's possible effect on cyclic GMP production, melatonin's ability to blunt sympathetic activity increases seen during lower-body negative pressure, and a lower total release of melatonin at night in patients with multiple cardiovascular diseases related to impaired cerebrovascular function, the promising possibility that melatonin could serve as a useful therapeutic option for those with impaired cerebral vascular reactivity did not display itself in the present study (Petit et al., 1999; Yu et al., 2001; Ray, 2003; Ovsenik et al., 2020). Our results indicated that our participants were within the healthy response range (~3-6%) during both the placebo and melatonin treatments in regards to the percent change in CVR during CO_2 rebreathing (Willie et al., 2014). Despite this, no discernable significant difference that would indicate that melatonin affected cerebrovascular reactivity when compared to the placebo group was found.

Melatonin Central Cardiovascular Effects and CO_2 Rebreathing

The physiological effects of CO_2 rebreathing on the cerebral vasculature are numerous. Supported by plentiful previous research, and because arterial CO_2 concentrations are directly proportional to the total volume of CO_2 production, the arterial concentration of CO_2 can be derived through the measurement of end-tidal CO_2 (Willie et al., 2014; Carr et al., 2021; Manferdelli et al., 2023). Increases in said arterial CO_2 concentration rapidly lead to passive diffusion of CO_2 across the blood-brain barrier. This ultimately alters hydrogen ion concentration via the bicarbonate buffering system in the intracellular and extracellular cerebrospinal and interstitial fluids, finally altering cyclic GMP production and causing a smooth muscle relaxation and vasodilation (Jensen et al., 1988; Willie et al., 2014). While the body displays a clear goal of returning to normal homeostasis following the start of concentrated CO_2 rebreathing by increasing Q, SV, HR, and MAP and clearing out the resulting excess hydrogen, the level to which end-tidal CO_2 was increased in our study was not enough to increase the mentioned central cardiovascular variables. One potential issue with the measurement of CBF during hypercapnia is hypercapnia's impairment of dynamic cerebral autoregulation, where hypercapnia-induced increases in MAP can "mask" a thorough interpretation of cerebrovascular reactivity (Birch et al., 1995; Zhang et al., 1998; Panerai, 2003; Ainslie et al., 2008; Ainslie & Duffin, 2009). The use of conductance calculated both by itself and as a measure of CVR, because of its incorporation of changes to MAP in the equation itself, also negate the possibility of changes in pressure altering the outcome of our results (**Figure 4, 7, 9**). Since MAP was not artificially increased during our rebreathing trials, our CVR measurements are more representative than if MAP had increased significantly, further involving autoregulatory responses. Furthermore, increased arterial CO_2 concentrations leads to chemoreceptor activation

and stimulation of the respiratory center in the medulla oblongata, which induces an increase in ventilation (Willie et al., 2014; Hoiland et al., 2019). Our data is consistent with this finding, as a statistically significant increase in end-tidal CO_2 concentrations led to a significant increase in breathing frequency.

We found that CO_2 rebreathing induced to a peak change in end-tidal CO_2 of 10 mmHg from the beginning of the trial did not significantly alter any central variables apart from breathing frequency. Furthermore, we found that there was no significant difference between the placebo and melatonin treatment groups in terms of any measured central cardiovascular or cardiopulmonary variable, indicating that 30 minutes post-melatonin spray consumption does not cause a significant difference in central or pulmonary cardiovascular variables during rest or CO_2 rebreathing.

Time to Peak Analysis

The time it took to reach a maximal end-tidal CO_2 concentration measuring from the beginning of each rebreathing stage was investigated in the present study to search for a temporal effect of melatonin on the cerebrovasculature. There was no difference during either the first or second trial of rebreathing between treatment groups. There was, however, a significant decrease in the time to peak for both treatment groups when transitioning from the first rebreathing stage to the second. This can more than likely be attributed to an increased chemoreceptor sensitivity seen in previous literature (Miyamura et al., 1976; Yamashiro et al., 2021). This mechanism is not fully understood but may have to do with central nervous system and carotid body chemoreceptor interactions, where activation of one set is critically dependent on the other,

leading to hypersensitivity if circulating concentrations of H^+ ions in the systemic circulatory system are not fully cleared (Blain et al., 2010).

Another, more probable explanation is that the time given between bouts of CO_2 rebreathing was not enough to reach a true baseline. While absolute values of end-tidal CO_2 did reach resting levels in between bouts of CO_2, we did not take blood samples to confirm circulating H+ ion concentrations. Future studies should investigate the mechanism behind the rapidity change between the first and second bouts of CO_2 rebreathing, as ensuring that baseline is achieved between bouts of CO_2 rebreathing is critical to replicating exactly similar responses between bouts.

Experimental Cerebral Flow Analysis

To our knowledge, this study is the first time that a 7-Tesla MRI-derived polynomial and standardized MCA diameters have been used to estimate changes in cerebral blood flow using only velocity measurements outside of the original description of the technique (Verbree et al., 2014). A previous study that induced hypercapnia through CO_2 rebreathing discovered a change in MCA flow using a 3-Tesla MRI of ~114 ± 68 ml/min, where our study found values of 60.73 ± 26.64 ml/min and 58.52 ± 27.26 in the PLA and MEL groups, respectively. This discrepancy could be due to subject sex distribution, as the mentioned study used a similar 19 subjects, 11 of which were female, where the present study used 18 subjects, 11 of which were male (Coverdale et al., 2014). MCA anatomy as well as responses are highly variable within each individual, but it has been shown that there may be a significant sex difference when measuring cerebral flow changes and comparing males and females (Alwatban et al., 2021). To mitigate this, we did control for menstrual phase, which is a major contributor to the differences in male and female

cerebral flow changes, but the sex-based discrepancy may still exist. Furthermore, our estimated percent change CVR values using the estimated changes in flow were higher when compared to our percent change CVR values measured using MCAv or CVCi alone. This would indicate that the consideration of a change in vessel diameter is vital when looking at vascular reactivity during conditions where the vessel diameter may be changing. TCD alone does not consider these vessel diameter changes, so attempting to derive flow during these conditions may be insufficient without using corrective factors such as the one described above. It would be wise to involve MRI flow measurements whenever possible when assessing changes in cerebral flow, particularly under conditions where the MCA is known to change such as CO_2 rebreathing to an increase of 10 or more mmHg end-tidal CO_2 (Coverdale et al., 2014; Verbree et al., 2014).

IPAQ and PSQI Correlations

There did not seem to be any statistical or visible relationship between IPAQ and PSQI scoring and CVR outcomes. This would indicate that sleeping habits, which has a large amount of variable between different individuals, did not show a relationship with their vascular reactivity. Physical activity levels also did not show any indication of acting as a predictive factor of differential CVR outcomes. To investigate further, a study would need to be more thorough with their monitoring and surveying of physical activity levels and sleep habits in subjects in order to solidify that no relationship exists with CVR. More specifically, a more controlled study that closely monitored physical activity levels and provided more comprehensive sleep quality measurements would be more appropriate in determining the relationship between these variables and cerebral vascular reactivity.

Implications of the Present Study

Our results indicate that acute melatonin supplementation does not play a role in MAP, MCAv, CVCi, TSI, cerebral reactivity as calculated using MCAv, or cerebral reactivity as calculated using CVCi during rest or CO_2 rebreathing (**Table 2, Table 3, Table 4**). Resting results are in accordance with previous literature, but to our knowledge, no other study has examined the effects of acute melatonin supplementation during repeated bouts of CO_2 rebreathing (Mil et al., 2003; Willie et al., 2014).

The time to peak analysis conducted to view the temporal aspect of melatonin's effect during rebreathing yielded no statistically significant results (**Table 5**). Repeated bouts of rebreathing became significantly faster when comparing the first and second rebreathing trials, but no difference in speed was shown when comparing the placebo and melatonin treatments, indicating that melatonin had no effect on the overall rapidity of reaching the goal +10 mmHg end-tidal CO_2.

Similar to our results for other cerebral vascular variables, there was no significant difference in our calculated change in cerebral flow between treatment groups (**Table 6**). While MCAv has been shown to be an accurate conduit of flow in conditions where the diameter of a vessel is not changing, it has been shown that an increase of +10 mmHg end-tidal CO_2 should cause an increase in the diameter of the middle cerebral artery (Serrador et al., 2000; Coverdale et al., 2014; Verbree et al., 2014). Using an area estimation polynomial derived from 7-Tesla MRI data serves as the closest available tool to assess any cerebral flow differences between the placebo and melatonin treatment groups in the event that our CO_2 rebreathing trial did in fact cause a substantial increase in diameter (Verbree et al., 2014). Our results showed lower total increases in absolute flow compared to a study that used a 3-Tesla MRI with a similar protocol

and with the same subject count, but an opposite distribution of male and female participants; a factor that would reason, as females tend to have higher global and regional cerebral flow when compared to men (Rodriguez et al., 1988; Gur & Gur, 1990; Coverdale et al., 2014). Furthermore, our calculated change in CVR using the estimated flow was reportedly higher than when using MCAv to calculate CVR, indicating that using corrective tools or estimation techniques may prove extremely interesting during conditions in which a vessel diameter may be changing. MRI is an extremely useful technique, but does not cover nearly as much temporally as the TCD (Aaslid et al., 1989; Anzola et al., 1995; Tiecks et al., 1995). So, a combination of confirmation of a vessel diameter change via MRI use and the temporal change in flow using the corrective factors described here would prove more thorough than either technique alone. Future studies should compare the same experimental analysis with results from the same subjects using an MRI confirmation, as to further assess the validity and accuracy of the described method, which would extend the usefulness of velocity-only TCD measurements towards conditions where vessel diameter is known to actively change (Verbree et al., 2014).

Limitations and Considerations

Multiple limitations may have interfered with the outcome of the present study. First and foremost, the accurate reporting of health status, medicine intake, menstrual phase, pre-study exclusion criteria, and physical activity levels was dependent on the honesty of the participants.

Secondly, we used transcranial doppler ultrasonography to estimate changes in flow, but we did not measure cerebral blood flow itself. Previous literature has demonstrated that the TCD serves as a potent tool for the estimation of changes to cerebral flow (Bishop et al., 1986; Serrador et al., 2000). Despite this, more recent literature has shown that MCA diameter may

change during hypercapnic conditions (Coverdale et al., 2014; Verbree et al., 2014). Because of our direct manipulation of end-tidal CO_2 concentrations, within the range where previous research tells us that MCA diameter may significantly change, it is possible that coming to conclusions regarding changes in cerebral flow based on our MCAv data would not be wise (Verbree et al., 2014). To mitigate this, we recorded the gate, depth, and anatomical location of each subject's MCA in order to as consistently as possible locate the MCA as an insonation angle of 90°. Furthermore, we conducted a further experimental analysis using standardized MCA diameter change values in combination with a polynomial based on end-tidal CO_2 changes in order to derive a potential solution to the TCD's only provides blood velocity problem (Verbree et al., 2014).

Third, we did not conduct any intravenous procedures to confirm that the level of circulating melatonin changed upon consumption of our mentioned oral spray. Despite this, it has been shown that the large buccal mucosal surface area that a melatonin spray contacts may avoid some of the negative effects of the so-called "first-pass phase" (Bartoli et al., 2013). It has also been stated in previous literature that the total amount of melatonin that reaches the general circulatory system is greater when melatonin is consumed via a spray in comparison to the consumption of a tablet (Bartoli et al., 2013).

Fourth, during the rebreathing stages, we did not directly measure our CO_2 balloon volume. While this could affect the concentration of CO_2 that each participant inhales in the case that there is a mask leakage and rebreathing does not occur properly, particularly towards the end of the stage if the balloon becomes depleted, we did not experience any situation during which the stage had to be stopped early due to a depletion of gas from the balloon.

Fifth, only prefrontal cortex oxygenation was measured, not global brain oxygenation. Despite this, there was a distinct lack of stimuli that would trigger a neurovascular-couple effect and thus an increased neural metabolic demand. Because of the location of the measured cerebral artery, the MCAv located in the prefrontal cortex, any alterations to cerebral oxygenation would more than likely permeate to other locations in the brain.

Sixth, the total amount of light exposure our participants received was not controlled for outside of the experimental protocol. Our protocol also did not consider participants traveling across other time zones, which may change their overall light exposure, as a factor. Because light exposure is the main determining factor of melatonin release, and in order to minimize any potential challenges that a lack of control of light exposure may pose, our study kept the protocol room dark, with only one small flood light to provide operator safety (Pandi-Perumal et al., 2008; Baker & Kimpinski, 2018).

Finally, previous literature has shown that there is a clear and established reduction in cerebrovascular CO_2 reactivity as well as endothelium-dependent vascular reactivity in the morning due to an undiscovered mechanism (Ainslie et al., 2007). Many of our participants chose to complete their experimental visits early in the morning due to our fasting requirement, so this may have directly affected our measured CVR results. In order to attempt to mitigate this and still be able to view changes within each single individual, we attempted to schedule each participant at a similar time of day to their previous visit.

Chapter VI

Conclusion

The purpose of the present study was to investigate the role of acute melatonin supplementation on cerebrovascular reactivity and flow during rest and CO_2 rebreathing in healthy, young adults.

Main Findings of the Current Study

1. We reject the first hypothesis that acute melatonin supplementation will increase cerebrovascular flow during rest and CO_2 rebreathing in healthy, young adults.
2. We reject the second hypothesis that acute melatonin supplementation will increase cerebrovascular reactivity by means of increasing CVCi, decreasing MAP, and increasing MCAv during rest and CO_2 rebreathing in healthy, young adults.
3. We reject the third hypothesis that acute melatonin supplementation will increase prefrontal cortex oxygenation during rest and CO_2 rebreathing in healthy, young adults.

Our results indicate that melatonin does not affect cerebrovascular flow, cerebrovascular reactivity, and does not beneficially affect prefrontal cortex oxygenation in healthy, young adults. Our results remain true whether measuring absolute values or changes between treatment groups in comparison to the previous baseline. Cerebral reactivity was variable individually and did not show any significant difference between treatment groups, despite being within the normal healthy response range for the percent change in CVR in response to CO_2 rebreathing (Willie et al., 2014). In conclusion, acute melatonin supplementation does not improve

cerebrovascular function, indicating that melatonin supplementation may display differential responses between the cerebral and peripheral vasculature in humans.

References

Aaslid R, Lindegaard KF, Sorteberg W, Nornes H. Cerebral autoregulation dynamics in humans. Stroke. 1989 Jan;20(1):45-52. doi: 10.1161/01.str.20.1.45. PMID: 2492126.

Acuña-Castroviejo, D., Escames, G., Venegas, C., Díaz-Casado, M. E., Lima-Cabello, E., López, L. C., Rosales-Corral, S., Tan, D. X., & Reiter, R. J. (2014). Extrapineal melatonin: sources, regulation, and potential functions. *Cellular and molecular life sciences : CMLS*, *71*(16), 2997–3025. https://doi.org/10.1007/s00018-014-1579-2

Ainslie, P. N., & Duffin, J. (2009). Integration of cerebrovascular CO2 reactivity and chemoreflex control of breathing: mechanisms of regulation, measurement, and interpretation. *American journal of physiology. Regulatory, integrative and comparative physiology*, *296*(5), R1473–R1495. https://doi.org/10.1152/ajpregu.91008.2008

Ainslie, P. N., & Brassard, P. (2014). Why is the neural control of cerebral autoregulation so controversial? *F1000Prime Reports*, *6*. https://doi.org/10.12703/p6-14

Ainslie, P. N., Murrell, C., Peebles, K., Swart, M., Skinner, M. A., Williams, M. J., & Taylor, R. D. (2007). Early morning impairment in cerebral autoregulation and cerebrovascular co2 reactivity in healthy humans: Relation to endothelial function. *Experimental Physiology*, *92*(4), 769–777. https://doi.org/10.1113/expphysiol.2006.036814

Alpers, B. J., Berry, R. G., & Paddison, R. M. (1959). Anatomical Studies of the Circle of Willis in Normal Brain. *A.M.A. Archives of Neurology & Psychiatry*, *81*(4), 409–418. https://doi.org/10.1001/archneurpsyc.1959.02340160007002

Anzola GP, Gasparotti R, Magoni M, Prandini F. Transcranial Doppler sonography and magnetic resonance angiography in the assessment of collateral hemispheric flow in patients with

carotid artery disease. Stroke. 1995 Feb;26(2):214-7. doi: 10.1161/01.str.26.2.214. PMID: 7831690.

Arangino, S., Cagnacci, A., Angiolucci, M., Vacca, A. M., Longu, G., Volpe, A., & Melis, G. B. (1999). Effects of melatonin on vascular reactivity, catecholamine levels, and blood pressure in healthy men. *The American journal of cardiology*, *83*(9), 1417–1419. https://doi.org/10.1016/s0002-9149(99)00112-5

Baker, J., & Kimpinski, K. (2018). Role of melatonin in blood pressure regulation: An adjunct anti-hypertensive agent. *Clinical and experimental pharmacology & physiology*, *45*(8), 755–766. https://doi.org/10.1111/1440-1681.12942

Barrie, J. R., Beatty, P. C. R. (1995). Rebreathing – Theoretical aspects of measurement. *Current Anesthesia & Critical Care, 6(3), 180-185.* https://doi.org/10.1016/S0953-7112(05)80209-1

Bartoli, A. N., De Gregori, S., Molinaro, M., Broglia, M., Tinelli, C., & Imberti, R. (2012). Bioavailability of a new oral spray melatonin emulsion compared with a standard oral formulation in healthy volunteers. *Journal of Bioequivalence and Bioavailability*, *4*(7), 96-99. https://doi.org/10.4172/jbb.1000120

Bishop, C. C., Powell, S., Rutt, D., & Browse, N. L. (1986). Transcranial Doppler measurement of middle cerebral artery blood flow velocity: A validation study. *Stroke*, *17*(5), 913–915. https://doi.org/10.1161/01.STR.17.5.913

Brassard, P., Tymko, M. M., & Ainslie, P. N. (2017). Sympathetic control of the brain circulation: Appreciating the complexities to better understand the controversy. *Autonomic Neuroscience, 207,* 37–47. https://doi.org/10.1016/j.autneu.2017.05.003

Brugger, P., Marktl, W., & Herold, M. (1995). Impaired nocturnal secretion of melatonin in coronary heart disease. *Lancet (London, England)*, *345*(8962), 1408. https://doi.org/10.1016/s0140-6736(95)92600-3

Cagnacci, A., Arangino, S., Angiolucci, M., Maschio, E., Longu, G., & Melis, G. B. (1997). Potentially beneficial cardiovascular effects of melatonin administration in women. *Journal of pineal research*, *22*(1), 16–19. https://doi.org/10.1111/j.1600-079x.1997.tb00297.x

Cajochen, C., Kräuchi, K., & Wirz-Justice, A. (2003). Role of melatonin in the regulation of human circadian rhythms and sleep. *Journal of neuroendocrinology*, *15*(4), 432–437. https://doi.org/10.1046/j.1365-2826.2003.00989.x

Capsoni, S., Viswanathan, M., De Oliveira, A. M., & Saavedra, J. M. (1994). Characterization of melatonin receptors and signal transduction system in rat arteries forming the circle of Willis. *Endocrinology*, *135*(1), 373–378. https://doi.org/10.1210/endo.135.1.8013371

Capsoni, S., Stankov, B. M., & Fraschini, F. (1995). Reduction of regional cerebral blood flow by melatonin in young rats. *Neuroreport*, *6*(9), 1346–1348. https://doi.org/10.1097/00001756-199506090-00029

Carr, J. M. J. R., Caldwell, H. G., & Ainslie, P. N. (2021). Cerebral blood flow, cerebrovascular reactivity and their influence on ventilatory sensitivity. *Experimental physiology*, *106*(7), 1425–1448. https://doi.org/10.1113/EP089446

Chen, J., Liu, J., Xu, W. H., Xu, R., Hou, B., Cui, L. Y., & Gao, S. (2014). Impaired dynamic cerebral autoregulation and cerebrovascular reactivity in middle cerebral artery stenosis. *PloS one*, *9*(2), e88232. https://doi.org/10.1371/journal.pone.0088232

Chitimus DM, Popescu MR, Voiculescu SE, et al. Melatonin's Impact on Antioxidative and

Anti-Inflammatory Reprogramming in Homeostasis and Disease. *Biomolecules*. 2020;10(9):1211. Published 2020 Aug 20. doi:10.3390/biom10091211

Claassen, J. A., Thijssen, D. H., Panerai, R. B., & Faraci, F. M. (2021). Regulation of cerebral blood flow in humans: Physiology and clinical implications of Autoregulation. *Physiological Reviews*, *101*(4), 1487–1559. https://doi.org/10.1152/physrev.00022.2020

Coverdale, N. S., Gati, J. S., Opalevych, O., Perrotta, A., & Shoemaker, J. K. (2014). Cerebral blood flow velocity underestimates cerebral blood flow during modest hypercapnia and hypocapnia. *Journal of applied physiology (Bethesda, Md. : 1985)*, *117*(10), 1090–1096. https://doi.org/10.1152/japplphysiol.00285.2014

Cunnane SC, Manku MS, Oka M, Horrobin DF. Enhanced vascular reactivity to various vasoconstrictor agents following pinealectomy in the rat: role of melatonin. Can J Physiol Pharmacol. 1980;58:287-293

Dagal, A., & Lam, A. M. (2009). Cerebral autoregulation and anesthesia. *Current Opinion in Anaesthesiology*, *22*(5), 547–552. https://doi.org/10.1097/ACO.0b013e32833020be

DeMers, D., & Wachs, D. (2022). Physiology, Mean Arterial Pressure. In *StatPearls*. StatPearls Publishing.

Ekmekcioglu, C., Thalhammer, T., Humpeler, S., Mehrabi, M. R., Glogar, H. D., Hölzenbein, T., Markovic, O., Leibetseder, V. J., Strauss-Blasche, G., & Marktl, W. (2003). The melatonin receptor subtype MT2 is present in the human cardiovascular system. *Journal of pineal research*, *35*(1), 40–44. https://doi.org/10.1034/j.1600-079x.2003.00051.x

Faul, F., Erdfelder, E., Buchner, A., & Lang, A.-G. (2009). Statistical power analyses using

G*Power 3.1: Tests for correlation and regression analyses. *Behavior Research Methods, 41,* 1149-1160

Georgiadis, D., Sievert, M., Cencetti, S., Uhlmann, F., Krivokuca, M., Zierz, S., & Werdan, K. (2000). Cerebrovascular reactivity is impaired in patients with cardiac failure. *European heart journal*, *21*(5), 407–413. https://doi.org/10.1053/euhj.1999.1742

Girouard, H., & de Champlain, J. (2004). Inhibitory effect of melatonin on alpha1-adrenergic-induced vasoconstriction in mesenteric beds of spontaneously hypertensive rats. *American journal of hypertension*, *17*(4), 339–346. https://doi.org/10.1016/j.amjhyper.2003.12.013

Gunnal, S., Farooqui, M., & Wabale, R. (2019). Study of middle cerebral artery in human Cadaveric Brain. *Annals of Indian Academy of Neurology*, *0*(0), 0. https://doi.org/10.4103/0972-2327.144289

Gur, R. E., & Gur, R. C. (1990). Gender differences in regional cerebral blood flow. *Schizophrenia Bulletin*, *16*(2), 247–254. https://doi.org/10.1093/schbul/16.2.247

Harrell JW, Schrage WG. Cyclooxygenase-derived vasoconstriction restrains hypoxia-mediated cerebral vasodilation in young adults with metabolic syndrome. Am J Physiol-heart C 306: H261–H269, 2014. doi: 10.1152/ajpheart.00709.2013.

Hoiland, R. L., Fisher, J. A., & Ainslie, P. N. (2019). Regulation of the cerebral circulation by arterial carbon dioxide. *Comprehensive Physiology*, 1101–1154. https://doi.org/10.1002/cphy.c180021

Holmes S, Sugden D. The effect of melatonin on pinealectomy induced hypertension in the rat. Br J Pharmacol. 1976;56:306P-361P

Hu, H. H., Kuo, T. B., Wong, W. J., Luk, Y. O., Chern, C. M., Hsu, L. C., & Sheng, W. Y. (1999). Transfer function analysis of cerebral hemodynamics in patients with carotid stenosis. *Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism*, *19*(4), 460–465. https://doi.org/10.1097/00004647-199904000-00012

Idowu, O. E., Shokunbi, M. T., Malomo, A. O., & Ogunbiyi, J. O. (2002). Size, course, distribution and anomalies of the middle cerebral artery in Adult Nigerians. *East African Medical Journal*, *79*(4). https://doi.org/10.4314/eamj.v79i4.8883

Imenshahidi, M., Karimi, G., & Hosseinzadeh, H. (2020). Effects of melatonin on cardiovascular risk factors and metabolic syndrome: a comprehensive review. *Naunyn-Schmiedeberg's archives of pharmacology*, *393*(4), 521–536. https://doi.org/10.1007/s00210-020-01822-4

Jeng, J.-S., Yip, P.-K., Huang, S.-J., & Kao, M.-C. (1999). Changes in hemodynamics of the carotid and middle cerebral arteries before and after endoscopic sympathectomy in patients with Palmar hyperhidrosis: Preliminary results. *Journal of Neurosurgery*, *90*(3), 463–467. https://doi.org/10.3171/jns.1999.90.3.0463

Jensen, K. E., Thomsen, C., & Henriksen, O. (1988). In vivo measurement of intracellular pH in human brain during different tensions of carbon dioxide in arterial blood. A31P-NMR study. Acta PhysiologicaScandinavica,134,295–298.

Katsi V, Karagiorgi I, Makris T, et al. The role of melatonin in hypertension. Cardiovasc

Endocrinol. 2012;1:13-18

King, A., Serena, J., Bornstein, N. M., Markus, H. S., & ACES Investigators (2011). Does impaired cerebrovascular reactivity predict stroke risk in asymptomatic carotid stenosis? A prospective substudy of the asymptomatic carotid emboli study. *Stroke*, *42*(6), 1550–1555. https://doi.org/10.1161/STROKEAHA.110.609057

Koziróg, M., Poliwczak, A. R., Duchnowicz, P., Koter-Michalak, M., Sikora, J., & Broncel, M. (2011). Melatonin treatment improves blood pressure, lipid profile, and parameters of oxidative stress in patients with metabolic syndrome. *Journal of pineal research*, *50*(3), 261–266. https://doi.org/10.1111/j.1600-079X.2010.00835.x

K-Laflamme A, Wu L, Foucart S, de Champlain J. Impaired basal sympathetic tone and alpha1-adrenergic responsiveness in association with the hypotensive effect of melatonin in spontaneously hypertensive rats. Am J Hypertens 1998;11:219-229.

Lynn, Aaron. (2011). The Influence of Osmoreceptors and Baroreceptors on Heat Loss Responses during a Whole-body Passive Heat Stress.

Manferdelli, G., Narang, B. J., Bourdillon, N., Debevec, T., & Millet, G. P. (2023). End-tidal carbon dioxide tension is a reliable surrogate of arterial carbon dioxide tension across different oxygen, carbon dioxide and barometric pressures. *ERJ open research*, *9*(2), 00507-2022. https://doi.org/10.1183/23120541.00507-2022

Mannon, E. C., Ray, S. C., Ryan, M. J., & Sullivan, J. C. (2020). Does sex matter?: An update on the implementation of sex as a biological variable in Research. *American Journal of Physiology-Renal Physiology*, *318*(2). https://doi.org/10.1152/ajprenal.00575.2019

Mil, A. H. M. van der H. et al. No Influence of Melatonin on Cerebral Blood Flow in Humans. J Clin Endocrinol Metabolism 88, 5989–5994 (2003).

Nabavi SM, Nabavi SF, Sureda A, et al. Anti-inflammatory effects of Melatonin: A mechanistic review. *Crit Rev Food Sci Nutr*. 2019;59(sup1):S4-S16. doi:10.1080/10408398.2018.1487927

Nevo, O., Soustiel, J. F., & Thaler, I. (2007). Cerebral blood flow is increased during controlled ovarian stimulation. *American Journal of Physiology-Heart and Circulatory Physiology*, *293*(6). https://doi.org/10.1152/ajpheart.00633.2007

Ohkura, T., Tejima, Y., Iwasaki, N., & Yaoi, Y. (1994). Poster presentation. *Menopause*, *1*(3), 165. https://doi.org/10.1097/00042192-199400130-00061

Pai, Sb., Varma, R., & Kulkarni, R. (2005). Microsurgical anatomy of the middle cerebral artery. *Neurology India*, *53*(2), 186. https://doi.org/10.4103/0028-3886.16406

Pandi-Perumal SR, Trakht I, Srinivasan V, et al. Physiological effects of melatonin: role of melatonin receptors and signal transduction pathways. Prog Neurobiol. 2008;85:335-353

Papantchev, V., Stoinova, V., Aleksandrov, A., Todorova-Papantcheva, D., Hristov, S., Petkov, D., Nachev, G., & Ovtscharoff, W. (2013). The role of Willis Circle Variations during unilateral selective cerebral perfusion: A study of 500 circles†. *European Journal of Cardio-Thoracic Surgery*, *44*(4), 743–753. https://doi.org/10.1093/ejcts/ezt103

Payne, S. (2016). *Cerebral Autoregulation*. Springer International Publishing. https://doi.org/10.1007/978-3-319-31784-7

Peltonen, G. L., Harrell, J. W., Aleckson, B. P., LaPlante, K. M., Crain, M. K., & Schrage, W. G. (2016). Cerebral blood flow regulation in women across menstrual phase: Differential contribution of cyclooxygenase to basal, hypoxic, and hypercapnic vascular tone. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, *311*(2). https://doi.org/10.1152/ajpregu.00106.2016

Petit, L., Lacroix, I., de Coppet, P., Strosberg, A. D., & Jockers, R. (1999). Differential signaling of human Mel1a and Mel1b melatonin receptors through the cyclic guanosine 3'-5'-monophosphate pathway. *Biochemical pharmacology*, *58*(4), 633–639. https://doi.org/10.1016/s0006-2952(99)00134-3

Prado NJ, Ferder L, Manucha W, Diez ER. Anti-Inflammatory Effects of Melatonin in Obesity and Hypertension. *Curr Hypertens Rep*. 2018;20(5):45. Published 2018 May 9. doi:10.1007/s11906-018-0842-6

Purkayastha, S., & Sorond, F. (2012). Transcranial Doppler ultrasound: technique and application. *Seminars in neurology*, *32*(4), 411–420. https://doi.org/10.1055/s-0032-1331812

Ray CA. Melatonin attenuates the sympathetic nerve responses to orthostatic stress in humans. *J Physiol*. 2003;551(Pt 3):1043-1048. doi:10.1113/jphysiol.2003.043182

Reisner, A. T., Xu, D., Ryan, K. L., Convertino, V. A., & Mukkamala, R. (2007). Comparison of cardiac output monitoring methods for detecting central hypovolemia due to lower body negative pressure. *Annual International Conference of the IEEE Engineering in Medicine and Biology Society. IEEE Engineering in Medicine and Biology Society. Annual International Conference*, *2007*, 955–958. https://doi.org/10.1109/IEMBS.2007.4352450

Reisner, A. T., Xu, D., Ryan, K. L., Convertino, V. A., Rickards, C. A., & Mukkamala, R. (2011). Monitoring non-invasive cardiac output and stroke volume during experimental human hypovolaemia and resuscitation. *British journal of anaesthesia*, *106*(1), 23–30. https://doi.org/10.1093/bja/aeq295

Reiter R. J. (2003). Melatonin: clinical relevance. *Best practice & research. Clinical endocrinology & metabolism*, *17*(2), 273–285. https://doi.org/10.1016/s1521690x(03)00016-2

Reiter RJ, Mayo JC, Tan DX, Sainz RM, Alatorre-Jimenez M, Qin L. Melatonin as an antioxidant: under promises but over delivers. *J Pineal Res*. 2016;61(3):253-278. doi:10.1111/jpi.12360

Resnick, S. M., Maki, P. M., Golski, S., Kraut, M. A., & Zonderman, A. B. (1998). Effects of estrogen replacement therapy on pet cerebral blood flow and neuropsychological performance. *Hormones and Behavior*, *34*(2), 171–182. https://doi.org/10.1006/hbeh.1998.1476

Rodriguez, G., Warkentin, S., Risberg, J., & Rosadini, G. (1988). Sex differences in regional cerebral blood flow. *Journal of Cerebral Blood Flow & Metabolism*, *8*(6), 783–789. https://doi.org/10.1038/jcbfm.1988.133

Sanni, A. A., & McCully, K. K. (2019). Interpretation of Near-Infrared Spectroscopy (NIRS) Signals in Skeletal Muscle. *Journal of functional morphology and kinesiology*, *4*(2), 28. https://doi.org/10.3390/jfmk4020028

Serrador, J. M., Picot, P. A., Rutt, B. K., Shoemaker, J. K., & Bondar, R. L. (2000). MRI

measures of middle cerebral artery diameter in conscious humans during simulated orthostasis. *Stroke*, *31*(7), 1672–1678. https://doi.org/10.1161/01.str.31.7.1672

Silvestrini, M., Vernieri, F., Pasqualetti, P., Matteis, M., Passarelli, F., Troisi, E., & Caltagirone, C. (2000). Impaired cerebral vasoreactivity and risk of stroke in patients with asymptomatic carotid artery stenosis. *JAMA*, *283*(16), 2122–2127. https://doi.org/10.1001/jama.283.16.2122

Simko F, Paulis L. Melatonin as a potential antihypertensive treatment. J Pineal Res. 2007;42:319-322

Sleight, E., Stringer, M. S., Marshall, I., Wardlaw, J. M., & Thrippleton, M. J. (2021). Cerebrovascular Reactivity Measurement Using Magnetic Resonance Imaging: A Systematic Review. *Frontiers in physiology*, *12*, 643468. https://doi.org/10.3389/fphys.2021.643468

Tanriover, N., Kawashima, M., Rhoton, A. L., Ulm, A. J., & Mericle, R. A. (2003). Microsurgical anatomy of the early branches of the middle cerebral artery: Morphometric analysis and classification with angiographic correlation. *Journal of Neurosurgery*, *98*(6), 1277–1290. https://doi.org/10.3171/jns.2003.98.6.1277

Tarumi, T., & Zhang, R. (2018). Cerebral blood flow in normal aging adults: cardiovascular determinants, clinical implications, and aerobic fitness. *Journal of neurochemistry*, *144*(5), 595–608. https://doi.org/10.1111/jnc.14234

Tiecks, F. P., Lam, A. M., Aaslid, R., & Newell, D. W. (1995). Comparison of static and dynamic cerebral autoregulation measurements. *Stroke*, *26*(6), 1014–1019. https://doi.org/10.1161/01.str.26.6.1014

Traystman, R. J. (2017). Cerebrovascular anatomy and hemodynamics. *Primer on Cerebrovascular Diseases,* 5–12. https://doi.org/10.1016/b978-0-12-803058-5.00001-1

Tsao, C. W., Aday, A. W., Almarzooq, Z. I., Alonso, A., Beaton, A. Z., Bittencourt, M. S., Boehme, A. K., Buxton, A. E., Carson, A. P., Commodore-Mensah, Y., Elkind, M. S. V., Evenson, K. R., Eze-Nliam, C., Ferguson, J. F., Generoso, G., Ho, J. E., Kalani, R., Khan, S. S., Kissela, B. M., Knutson, K. L., … Martin, S. S. (2022). Heart Disease and Stroke Statistics-2022 Update: A Report From the American Heart Association. *Circulation*, *145*(8), e153–e639. https://doi.org/10.1161/CIR.0000000000010523.

Tobaldini E, Costantino G, Solbiati M, et al. Sleep, sleep deprivation, autonomic nervous system and cardiovascular diseases. *Neurosci Biobehav Rev*. 2017;74(Pt B):321-329. doi:10.1016/j.neubiorev.2016.07.004

Tordjman, S., Chokron, S., Delorme, R., Charrier, A., Bellissant, E., Jaafari, N., & Fougerou, C. (2017). Melatonin: Pharmacology, Functions and Therapeutic Benefits. *Current neuropharmacology*, *15*(3), 434–443. https://doi.org/10.2174/1570159X14666161228122115

Touyz RM. Reactive oxygen species, vascular oxidative stress, and redox signaling in hypertension: what is the clinical significance?. *Hypertension*. 2004;44(3):248-252. doi:10.1161/01.HYP.0000138070.47616.9d

Touyz RM, Schiffrin EL. Increased generation of superoxide by angiotensin II in smooth muscle cells from resistance arteries of hypertensive patients: role of phospholipase D-dependent NAD(P)H oxidase-sensitive pathways. *J Hypertens*. 2001;19(7):1245-1254. doi:10.1097/00004872-200107000-00009

Valdueza, J. M., Balzer, J. O., Villringer, A., Vogl, T. J., Kutter, R., & Einhäupl, K. M. (1997). Changes in blood flow velocity and diameter of the middle cerebral artery during hyperventilation: assessment with MR and transcranial Doppler sonography. *AJNR. American journal of neuroradiology*, *18*(10), 1929–1934.

Vavilala, M. S., Lee, L. A., & Lam, A. M. (2002). Cerebral blood flow and vascular physiology. *Anesthesiology Clinics of North America*, *20*(2), 247–264. https://doi.org/10.1016/S0889-8537(01)00012-8

Verbree, J., Bronzwaer, A. S., Ghariq, E., Versluis, M. J., Daemen, M. J., van Buchem, M. A., Dahan, A., van Lieshout, J. J., & van Osch, M. J. (2014). Assessment of middle cerebral artery diameter during hypocapnia and hypercapnia in humans using ultra-high-field MRI. *Journal of applied physiology (Bethesda, Md. : 1985)*, *117*(10), 1084–1089. https://doi.org/10.1152/japplphysiol.00651.2014

Vernieri, F., Pasqualetti, P., Passarelli, F., Rossini, P. M., & Silvestrini, M. (1999). Outcome of carotid artery occlusion is predicted by cerebrovascular reactivity. *Stroke*, *30*(3), 593–598. https://doi.org/10.1161/01.str.30.3.593

Virani, S. S., Alonso, A., Benjamin, E. J., Bittencourt, M. S., Callaway, C. W., Carson, A. P., Chamberlain, A. M., Chang, A. R., Cheng, S., Delling, F. N., Djousse, L., Elkind, M. S. V., Ferguson, J. F., Fornage, M., Khan, S. S., Kissela, B. M., Knutson, K. L., Kwan, T. W., Lackland, D. T., Lewis, T. T., … American Heart Association Council on Epidemiology and Prevention Statistics Committee and Stroke Statistics Subcommittee (2020). Heart Disease and Stroke Statistics-2020 Update: A Report From the American Heart Association. *Circulation*, *141*(9), e139–e596. https://doi.org/10.1161/CIR.0000000000000757

Viswanathan, M., Laitinen, J. T., & Saavedra, J. M. (1990). Expression of melatonin receptors in arteries involved in thermoregulation. *Proceedings of the National Academy of Sciences of the United States of America*, *87*(16), 6200–6203. https://doi.org/10.1073/pnas.87.16.6200

Vuillier, F., Medeiros, E., Moulin, T., Cattin, F., Bonneville, J.-F., & Tatu, L. (2008). Main anatomical features of the M1 segment of the middle cerebral artery: A 3D time-of-flight magnetic resonance angiography at 3 T study. *Surgical and Radiologic Anatomy*, *30*(6), 509–514. https://doi.org/10.1007/s00276-008-0360-3

Willie, C. K., Tzeng, Y. C., Fisher, J. A., & Ainslie, P. N. (2014). Integrative regulation of human brain blood flow. *The Journal of physiology*, *592*(5), 841–859. https://doi.org/10.1113/jphysiol.2013.268953

Xia, C. M., Shao, C. H., Xin, L., Wang, Y. R., Ding, C. N., Wang, J., Shen, L. L., Li, L., Cao, Y. X., & Zhu, D. N. (2008). Effects of melatonin on blood pressure in stress-induced hypertension in rats. *Clinical and experimental pharmacology & physiology*, *35*(10), 1258–1264. https://doi.org/10.1111/j.1440-1681.2008.05000.x

Xing, C. Y., Tarumi, T., Meijers, R. L., Turner, M., Repshas, J., Xiong, L., Ding, K., Vongpatanasin, W., Yuan, L. J., & Zhang, R. (2017). Arterial Pressure, Heart Rate, and Cerebral Hemodynamics Across the Adult Life Span. *Hypertension (Dallas, Tex. : 1979)*, *69*(4), 712–720. https://doi.org/10.1161/HYPERTENSIONAHA.116.08986

Yaprak, M., Altun, A., Vardar, A., Aktoz, M., Ciftci, S., & Ozbay, G. (2003). Decreased nocturnal synthesis of melatonin in patients with coronary artery disease. *International journal of cardiology*, *89*(1), 103–107. https://doi.org/10.1016/s0167-5273(02)00461-8

Yu, M., Sun, C. W., Maier, K. G., Harder, D. R., & Roman, R. J. (2002). Mechanism of cGMP

contribution to the vasodilator response to NO in rat middle cerebral arteries. *American journal of physiology. Heart and circulatory physiology*, *282*(5), H1724–H1731. https://doi.org/10.1152/ajpheart.00699.2001

Supplementary Materials

Interestingly, and while the absolute values still showed no significance, ΔTSI as measured from baseline to the second trial of rebreathing showed that the placebo group increased more than the melatonin group, in direct opposition of our third hypothesis. Because this is the first time that TSI has been measured during acute melatonin supplementation and during a CO_2 rebreathing trial, the inherent large amount of individual anatomical variation and cerebral response variation, the first trial of rebreathing showing no significant difference, and no absolute value differences being found during either trial, it is most likely that individual variation responses contributed to our significant result (Idowu et al., 2002; Tanriover et al., 2003; Pai et al., 2005; Vuiller et al., 2008; Papantchey et al., 2013; Payne, 2016). It is entirely possible that there is another unknown mechanism at play through which melatonin may be affecting the cerebral vasculature and cerebral oxygenation after repeated bouts of CO_2 rebreathing, and this may warrant further investigation.

Melatonin seemingly inhibited a change in oxygenation during the second trial of rebreathing in comparison to the placebo treatment (**Table 3**). This is likely a result of individual anatomical and cerebral vascular response variation, as absolute values displayed no significant difference during either rebreathing trial when comparing treatments (**Table 3**). Despite this, an unknown mechanism that is contrary to our third hypothesis may be at play. Future research should focus on the investigation of melatonin's interaction with MT2 receptors in the cerebral vasculature and how that interaction, and subsequent possible cyclic GMP production alterations, may have an effect either minutely or in cases similar to CO_2 rebreathing; where there is a potent vasodilatory stimulus present.

Table 2. RB1 Central Pulmonary and Cardiovascular Variables (*n = 19, 12 males, 7 females)*

	Baseline	**Rebreathing 1**
Q (L/min)		
PLA	6.07 ± 1.97	6.13 ± 2.01
MEL	5.98 ± 1.51	5.98 ± 1.40
SV (mL/beat)		
PLA	94.14 ± 23.35	93.99 ± 23.51
MEL	93.80 ± 19.86	93.29 ± 18.68
TPR		
PLA	1.10 ± 0.32	0.11 ± 0.31
MEL	1.13 ± 0.34	1.12 ± 0.29
HR (bpm)		
PLA	61.74 ± 8.23	63.46 ± 7.49
MEL	61.13 ± 8.95	62.21 ± 9.47
MAP (mmHg)		
PLA	105.76 ± 15.19	107.38 ± 14.42
MEL	106.90 ± 11.56	107.23 ± 10.83
$\mathbf{P_{ET}CO_2}$ (mmHg)		
PLA	43.03 ± 1.93	49.19 ± 2.10 *
MEL	44.09 ± 3.49	50.19 ± 3.12 *
$\mathbf{B_f}$ (bpm)		
PLA	14.90 ± 3.15	57.87 ± 4.68 *
MEL	15.35 ± 3.49	58.31 ± 3.28 *

*Values displayed as mean ± SD. * represents significant differences between baseline and rebreathing. PLA: Placebo Treatment Group; MEL: Melatonin Treatment Group; RB1: The First Trial of Rebreathing until Achieving a Physiological Change of +10 mmHg; Q: Cardiac Output; SV: Stroke Volume; TPR: Total Peripheral Resistance; HR: Heart Rate; MAP: Mean Arterial Pressure; $P_{ET}CO_2$: End-tidal CO_2; B_f: Breathing Frequency.*

Table 3. RB2 Central Pulmonary and Cardiovascular Variables (*n = 18, 11 males, 7 females)*

	Baseline	**Rebreathing 2**
Q (L/min)		
PLA	6.40 ± 1.90	6.32 ± 1.80
MEL	6.14 ± 1.43	6.11 ± 1.43
SV (mL/beat)		
PLA	97.28 ± 23.19	95.87 ± 22.56
MEL	95.91 ± 19.82	95.30 ± 20.00
TPR		
PLA	1.08 ± 0.31	1.10 ± 0.31
MEL	1.09 ± 0.28	1.09 ± 0.28
HR (bpm)		
PLA	64.07 ± 8.12	64.09 ± 7.67
MEL	61.89 ± 9.65	62.39 ± 8.53
MAP (mmHg)		
PLA	109.99 ± 16.36	111.13 ± 16.53
MEL	107.48 ± 12.42	108.19 ± 10.36

$P_{ET}CO_2$ (mmHg)		
PLA	42.15 ± 2.40	49.81 ± 2.73 *
MEL	43.47 ± 3.03	50.76 ± 3.05 *
B_f (bpm)		
PLA	15.43 ± 4.30	55.09 ± 5.40 *
MEL	15.26 ± 3.78	56.73 ± 5.67 *

*Values displayed as mean ± SD. * represents significant differences between baseline and rebreathing. RB2: The Second Trial of Rebreathing until Achieving a Physiological Change of +10 mmHg; Q: Cardiac Output; SV: Stroke Volume; TPR: Total Peripheral Resistance; HR: Heart Rate; MAP: Mean Arterial Pressure; $P_{ET}CO_2$: End-tidal CO_2.*

Table 4. RB1 Cerebral Vascular Variables (*n = 19, 12 males, 7 females; TSI n = 18, 12 males, 6 females*)

	Baseline	**Rebreathing 1**	**Δ BL-RB**
MCAv (cm/s)			
PLA	71.33 ± 12.09	83.76 ± 12.97	13.34 ± 5.74 *
MEL	72.22 ± 16.44	84.50 ± 15.55	13.41 ± 6.44 *
P-Value	0.702	0.803	0.962
CVCi (cm/s/mmHg)			
PLA	0.69 ± 0.14	0.79 ± 0.15	0.12 ± 0.05 *
MEL	0.69 ± 0.17	0.80 ± 0.17	0.12 ± 0.06 *
P-Value	0.947	0.931	0.653

TSI (%)			
PLA	66.69 ± 6.72	67.93 ± 7.07	1.24 ± 1.17 *
MEL	68.40 ± 5.88	69.71 ± 5.80	1.30 ± 0.74 *
P-Value	0.616	0.616	0.846

*Values displayed as mean ± SD. * represents significant differences between baseline and rebreathing. P-Value represents a comparison between PLA and MEL treatments. MCAv: Middle Cerebral Artery Velocity; CVCi: Cerebrovascular Conductance Index.*

Table 5. RB2 Cerebral Vascular Variables (*n = 18, 11 males, 7 females; TSI n = 17, 11 males, 6 females*)

	Baseline	**Rebreathing 2**	**Δ BL-RB**
MCAv (cm/s)			
PLA	70.47 ± 11.44	83.49 ± 14.32	13.21 ± 7.83 *
MEL	71.94 ± 15.58	83.63 ± 15.84	12.09 ± 7.64 *
P-Value	0.552	0.964	0.672
CVCi (cm/s/mmHg)			
PLA	0.66 ± 0.14	0.77 ± 0.17	0.12 ± 0.07 *
MEL	0.68 ± 0.15	0.78 ± 0.15	0.11 ± 0.07 *
P-Value	0.510	0.931	0.653
TSI (%)			
PLA	66.45 ± 7.30	68.13 ± 7.24	1.52 ± 0.88 *

MEL	68.89 ± 6.08	69.51 ± 6.37	0.65 ± 0.68 *
P-Value	0.446	0.943	0.003 †

*Values displayed as mean ± SD. * represents significant differences between baseline and rebreathing. † represents significant differences between treatment groups. P-Value represents a comparison between PLA and MEL treatments. MCAv: Middle Cerebral Artery Velocity; CVCi: Cerebrovascular Conductance Index.*

Table 6. Rebreathing Cerebrovascular Reactivity (*RB1 n = 19, 12 males, 7 females, RB2 n = 18, 11 males, 7 females*)

	RB1	**RB1 % Change**	**RB2**	**RB2 % Change**
CVR_{MCAv} (cm/s/mmHg)				
PLA	1.78 ± 0.95	0.19 ± 11.49	1.45 ± 0.97	-0.70 ± 3.65
MEL	1.92 ± 0.98	2.83 ± 8.67	1.30 ± 0.93	-1.15 ± 6.31
P-Value	0.561	0.355	0.651	0.616
CVR_{CVCi}				
PLA	0.02 ± 0.02	0.75 ± 4.60	0.01 ± 0.01	1.73 ± 7.66
MEL	0.02 ± 0.01	-0.99 ± 2.80	0.01 ± 0.01	0.06 ± 2.42
P-Value	0.460	0.198	0.867	0.845

Values displayed as mean ± SD. P-Value represents a comparison between PLA and MEL conditions. CVR_{MCAv}: Cerebrovascular Reactivity calculated using MCAv; CVR_{CVCi}: Cerebrovascular Reactivity calculated using CVCi.

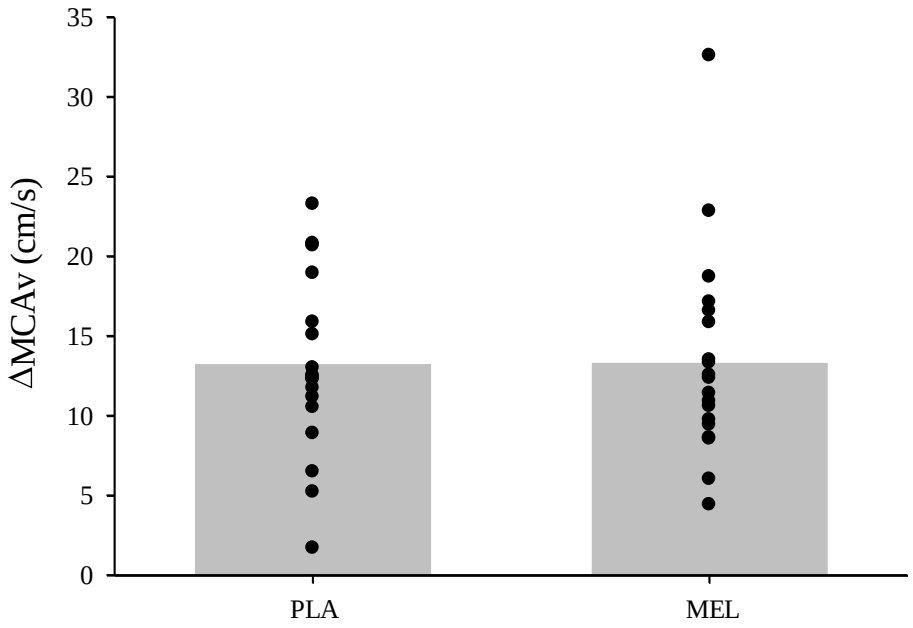

Figure 2. Data is displayed as mean ± SD. Shows alterations in middle cerebral artery velocity from baseline to the *first* trial of CO_2 rebreathing as a comparison between the placebo treatment and the melatonin treatment (n=19). ΔMCAv was calculated as an average value, derived from subtracting an average value of the last 30s of the preceding baseline from each data point during rebreathing. Alpha was set a priori as $P < 0.05$. No significant difference was seen between treatment groups. MCAv significantly increased from baseline to rebreathing in both treatment groups.

RB1 dMCAv

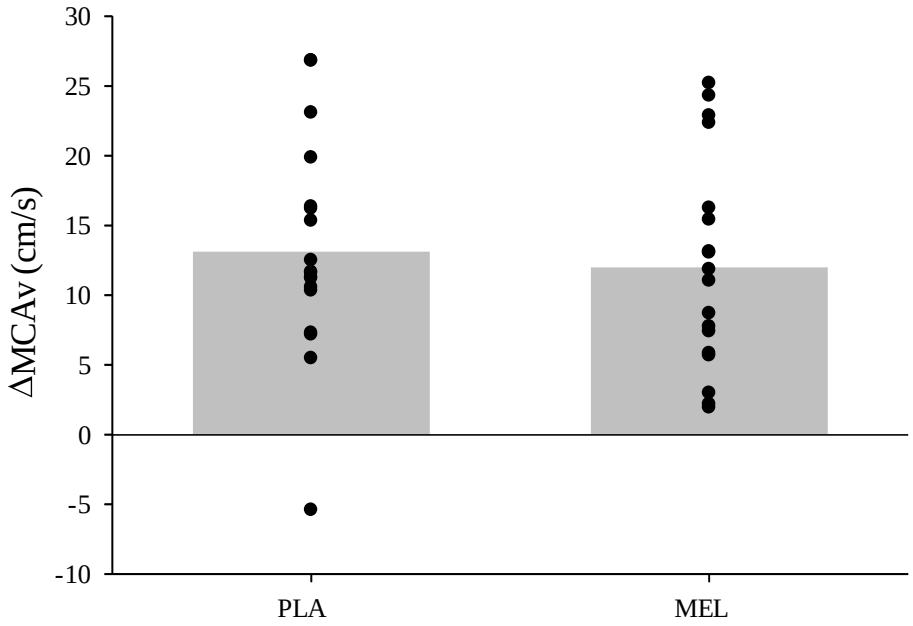

Figure 3. Data is displayed as mean ± SD. Shows alterations in middle cerebral artery velocity from baseline to the *second* trial of CO_2 rebreathing as a comparison between the placebo treatment and the melatonin treatment (n=18). ΔMCAv was calculated as an average value, derived from subtracting an average value of the last 30s of the preceding baseline from each data point during rebreathing. Alpha was set a priori as $P < 0.05$. No significant difference was seen between treatment groups. MCAv significantly increased from baseline to rebreathing in both treatment groups.

RB2 dMCAv

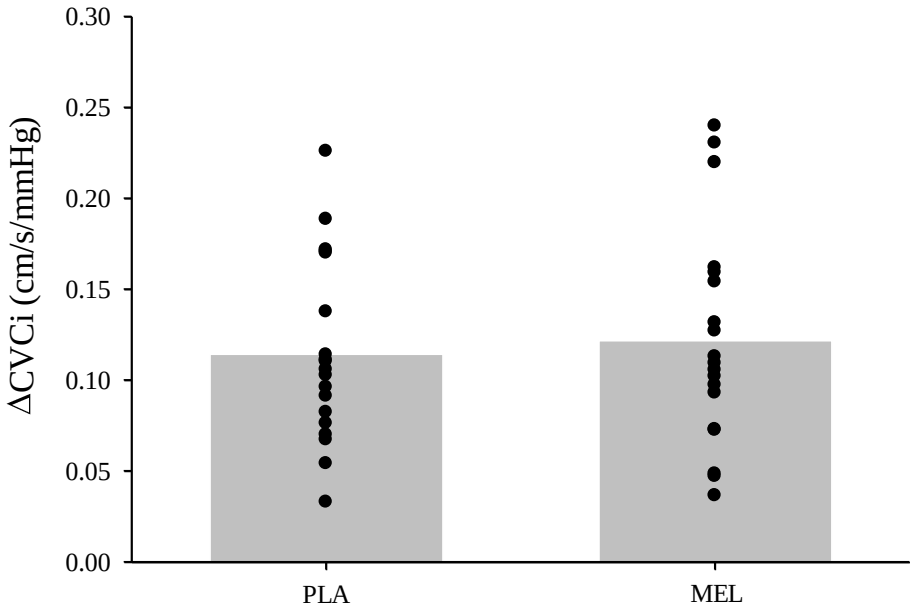

Figure 4. Data is displayed as mean ± SD. Shows alterations in the cerebrovascular conductance index from baseline to the *first* trial of CO_2 rebreathing as a comparison between the placebo treatment and the melatonin treatment (n=19). ΔCVCi was calculated as an average value, derived from subtracting an average value of the last 30s of the preceding baseline from each data point during rebreathing. Alpha was set a priori as $P < 0.05$. No significant difference was seen between treatment groups. CVCi significantly increased from baseline to rebreathing in both treatment groups.

RB1 dCVCi

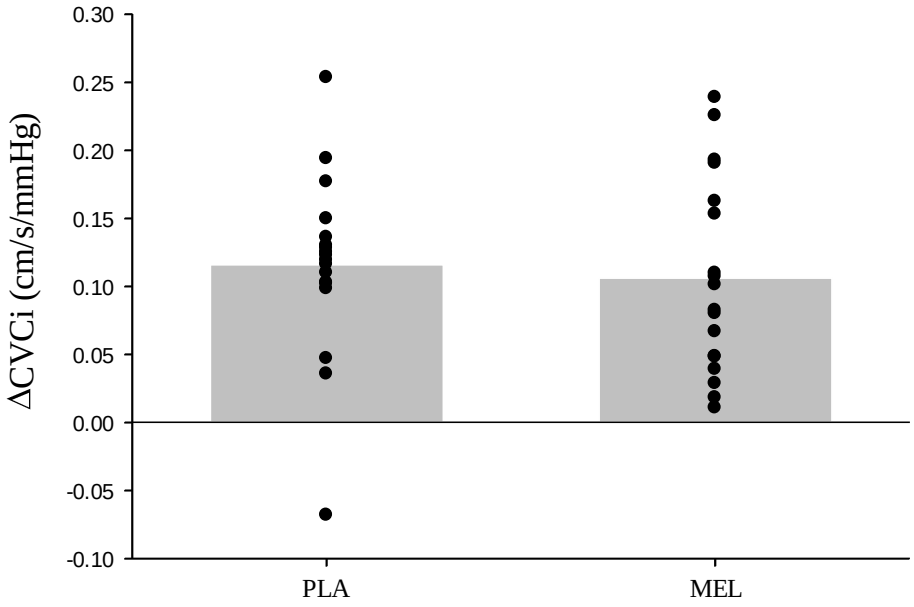

Figure 5. Data is displayed as mean ± SD. Shows alterations in the cerebrovascular conductance index from baseline to the *second* trial of CO_2 rebreathing as a comparison between the placebo treatment and the melatonin treatment (n=18). ΔCVCi was calculated as an average value, derived from subtracting an average value of the last 30s of the preceding baseline from each data point during rebreathing. Alpha was set a priori as $P < 0.05$. No significant difference was seen between treatment groups. CVCi significantly increased from baseline to rebreathing in both treatment groups.

RB2 CVCi

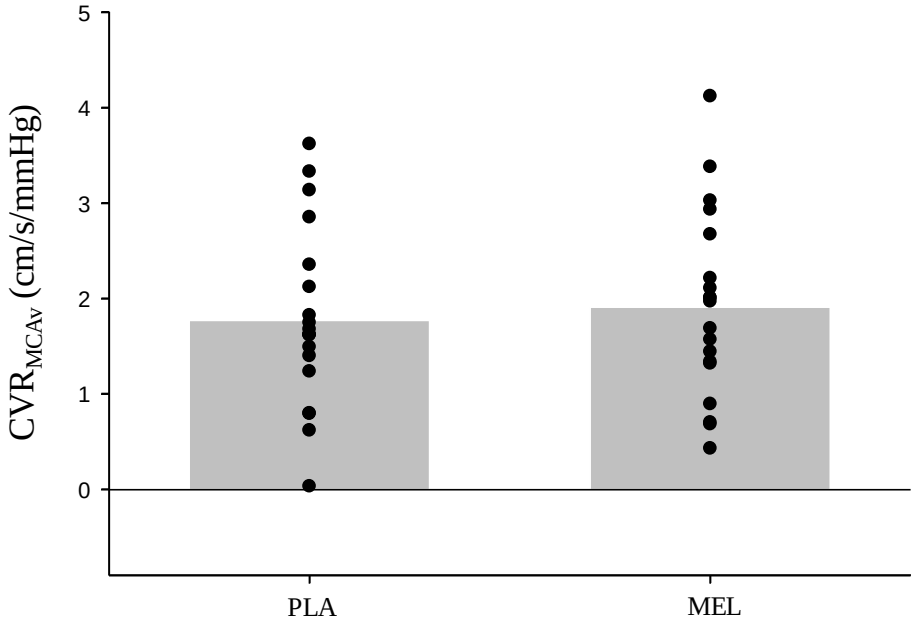

Figure 6. Data is displayed as mean ± SD. Shows alterations in the cerebrovascular reactivity calculated using MCAv from baseline to the *first* trial of CO_2 rebreathing as a comparison between the placebo treatment and the melatonin treatment (n=19). CVR_{MCAv} was calculated as an average value, where ΔMCAv was divided by $\Delta PetCO_2$. Alpha was set a priori as $P < 0.05$. No significant difference was seen between treatment groups.

RB1 CVRMCAv

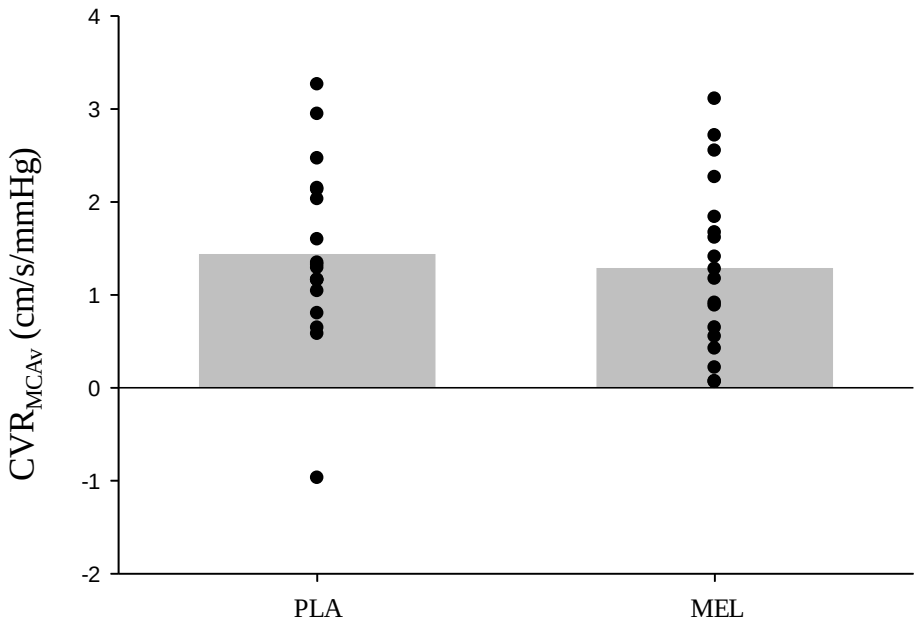

Figure 7. Data is displayed as mean ± SD. Shows alterations in the cerebrovascular reactivity calculated using MCAv from baseline to the *second* trial of CO_2 rebreathing as a comparison between the placebo treatment and the melatonin treatment (n=18). CVR_{MCAv} was calculated as an average value, where ΔMCAv was divided by $\Delta PetCO_2$. Alpha was set a priori as $P < 0.05$. No significant difference was seen between treatment groups.

RB2 CVRMCAv

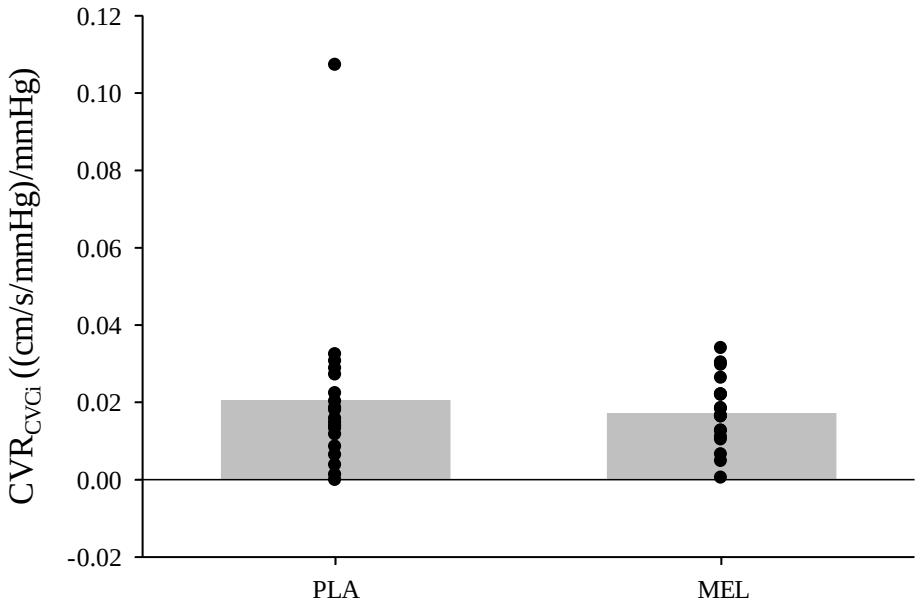

Figure 8. Data is displayed as mean ± SD. Shows alterations in the cerebrovascular reactivity calculated using CVCi from baseline to the *first* trial of CO_2 rebreathing as a comparison between the placebo treatment and the melatonin treatment (n=19). CVR_{CVCi} was calculated as an average value, where ΔCVCi was divided by Δ$PetCO_2$. Alpha was set a priori as $P < 0.05$. No significant difference was seen between treatment groups.

RB1 CVRCVCi

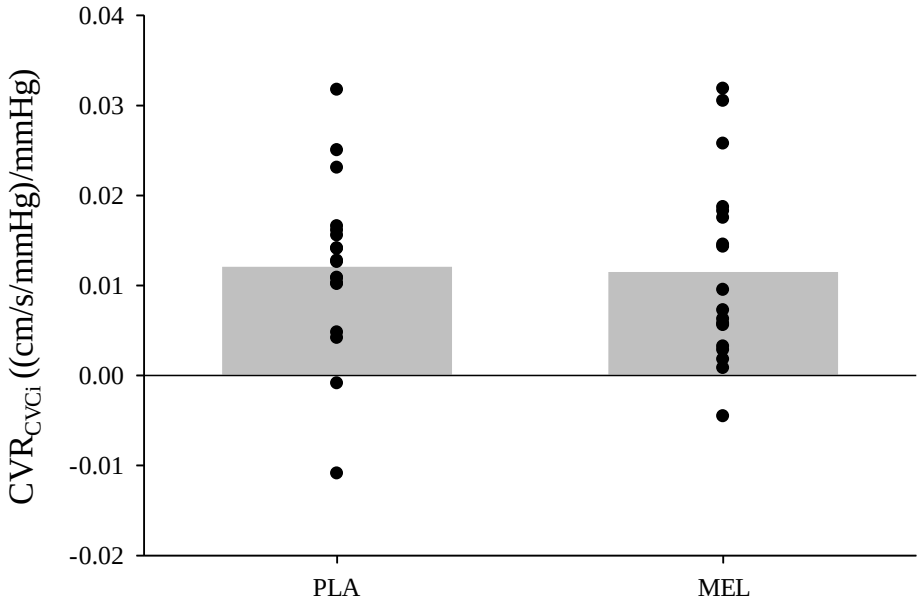

Figure 9. Data is displayed as mean ± SD. Shows alterations in the cerebrovascular reactivity calculated using CVCi from baseline to the *second* trial of CO_2 rebreathing as a comparison between the placebo treatment and the melatonin treatment (n=18). CVR_{CVCi} was calculated as an average value, where ΔCVCi was divided by $\Delta PetCO_2$. Alpha was set a priori as $P < 0.05$. No significant difference was seen between treatment groups.

RB2 CVRCVCi

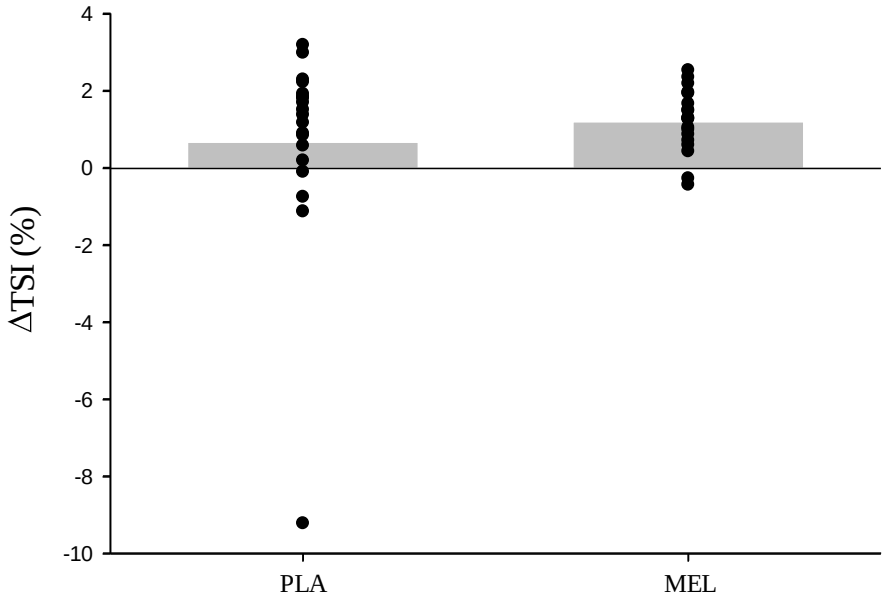

Figure 10. Data is displayed as mean ± SD. Shows alterations in the total saturation index from baseline to the *first* trial of CO_2 rebreathing as a comparison between the placebo treatment and the melatonin treatment (n=18). ΔTSI was calculated as an average value, derived from subtracting an average value of the last 30s of the preceding baseline from each data point during rebreathing. Alpha was set a priori as $P < 0.05$. No significant difference was seen between treatment groups. TSI significantly increased from baseline to rebreathing in both treatment groups.

RB1 dTSI

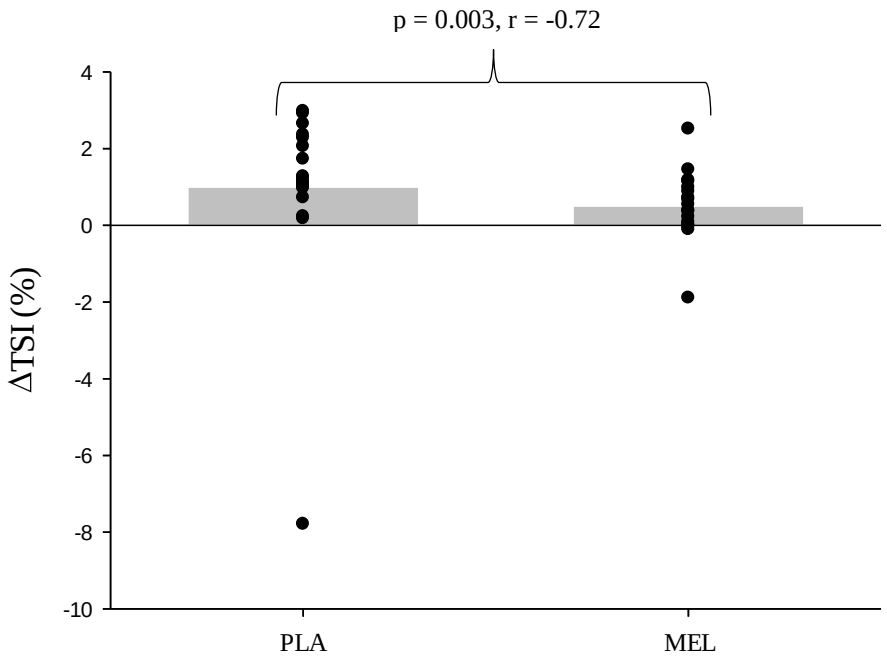

Figure 11. Data is displayed as mean ± SD. Shows alterations in the total saturation index from baseline to the *second* trial of CO_2 rebreathing as a comparison between the placebo treatment and the melatonin treatment (n=17). ΔTSI was calculated as an average value, derived from subtracting an average value of the last 30s of the preceding baseline from each data point during rebreathing. Alpha was set a priori as $P < 0.05$. The change in TSI was significantly lower during the melatonin treatment when compared with placebo. TSI significantly increased from baseline to rebreathing in both treatment groups.

RB2 dTSI

Table 7. Time to End-tidal CO_2 Peak during RB1 & RB2 (*RB1 n = 19, 12 males, 7 females, RB2 n = 18, 11 males, 7 females*)

	RB1	**RB2**	**P-Value (RB1-RB2)**
TTN (seconds)			
PLA	100.10 ± 17.79	63.39 ± 24.78	<0.001
MEL	95.68 ± 18.48	58.04 ± 21.22	<0.001

P-Value	0.241	0.493

Values displayed as mean ± SD. P-Value represents a comparison between PLA and MEL conditions. TTN: Time to reach peak End-tidal CO_2 concentrations from the beginning of a rebreathing stage.

Table 8. Estimated ΔCerebral Blood Flow (*RB1 n = 19, 12 males, 7 females, RB2 n = 18, 11 males, 7 females*)

	RB1	**RB2**	**P-Value (RB1-RB2)**
Estimated			
ΔFlow			
(ml/min)			
PLA	61.28 ± 22.69	62.47 ± 38.38	0.643
MEL	59.70 ± 30.28	57.01 ± 38.62	0.774
P-Value	0.837	0.660	

Values displayed as mean ± SD. P-Value represents a comparison between PLA and MEL conditions. Estimated Δ Flow: Polynomial-based estimation of a change in cerebral blood flow using previous literature and measured flow velocity values (Verbree et al., 2014).

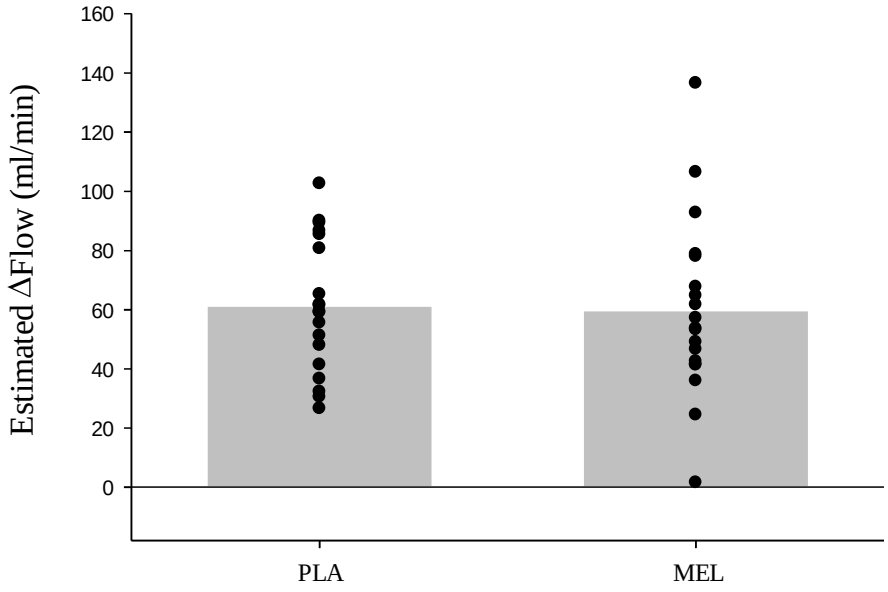

Figure 12. Data is displayed as mean ± SD. Shows alterations in the estimated change of cerebral flow in the middle cerebral artery from baseline to the *first* trial of CO_2 rebreathing as a comparison between the placebo treatment and the melatonin treatment (n=19). ΔFlow was calculated as a multiplication of the estimated area derived from a previously described 7-Tesla MRI origin study and our measured MCAv (Verbree et al., 2014). Alpha was set a priori as $P < 0.05$. No significant difference was seen between treatment groups.

RB1 Experimental dFlow

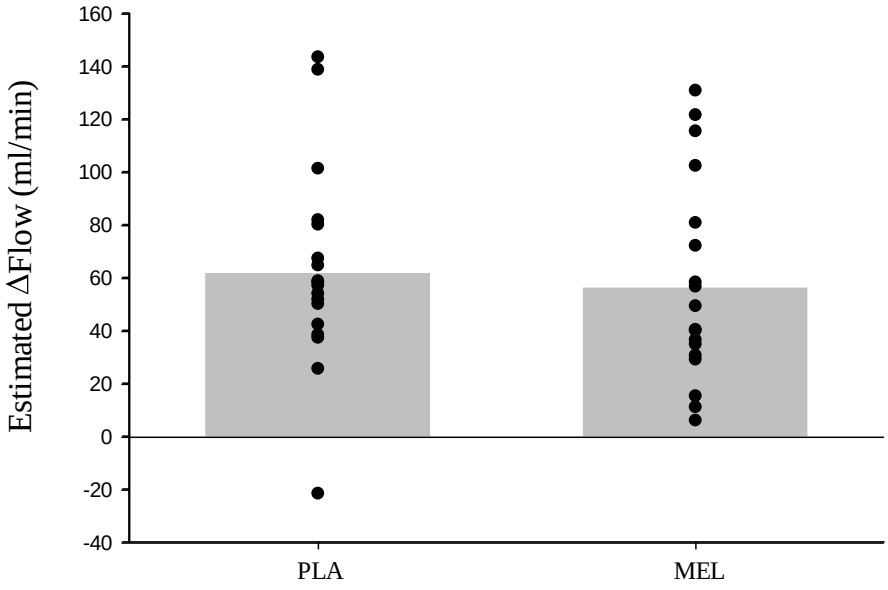

Figure 13. Data is displayed as mean ± SD. Shows alterations in the estimated change of cerebral flow in the middle cerebral artery from baseline to the *second* trial of CO_2 rebreathing as a comparison between the placebo treatment and the melatonin treatment (n=18). ΔFlow was calculated as a multiplication of the estimated area derived from a previously described 7-Tesla MRI origin study and our measured MCAv (Verbree et al., 2014). Alpha was set a priori as $P < 0.05$. No significant difference was seen between treatment groups.

RB2 Experimental dFlow

Females

#	CSA-cm2
1	0.09
2	0.079
3	0.075
4	0.075
5	0.07
6	0.07
7	0.068
8	0.068
9	0.063
10	0.059
11	0.051
Mean CSA - cm2	**0.069818181818**
Diameter - cm	**0.29815309831095**

Males

#	CSA-cm2
1	0.101
2	0.091
3	0.085
4	0.08
5	0.08
6	0.079
7	0.07
8	0.068
Mean CSA - cm2	**0.08175**
Diameter - cm	**0.322625685248555**

(Verbree et al., 2014).

Appendices

	CVRmcav		CVRcvci		CVRmcav % Change		CVRcvci % Change	
Sub ID	PLA	MEL	PLA	MEL	PLA	MEL	PLA	MEL
101	1.938147	2.97143	0.016831	0.027922	5.283856	5.736286	3.548953	5.130007
103	2.013498	1.495732	0.053859	0.016315	6.278105	6.122026	5.624133	6.794093
104	3.697442	3.117431	0.038955	0.029393	6.244395	8.905603	4.754339	8.142611
105	0.743539	1.559823	0.005519	0.015288	3.615427	3.836367	2.046392	1.490882
106	1.541805	1.576673	0.017742	0.009516	5.068628	6.810477	4.654495	7.513661
108	0.40969	0.69339	0.004385	0.009932	4.411765	3.009441	5.506316	3.179717
109	1.743451	0.786887	0.01082	0.007557	5.517179	7.115531	3.563161	7.030899
110	1.236588	1.54611	0.008981	0.018053	6.123255	5.883802	5.923321	7.798022
111	1.680318	1.701931	0.014192	0.016641	5.10653	7.519315	4.989496	7.293617
112	2.028121	2.321028	0.016675	0.012175	5.99009	6.446082	5.551219	5.102803
114	1.5329	1.563097	0.014371	0.013887	6.849271	4.894147	7.282563	3.102691
201	0.841253	2.51883	0.001998	0.022803	2.795046	5.632598	5.078637	6.385686
203	1.811244	1.892913	0.014349	0.011675	4.056702	5.094825	2.526375	3.270974
204	3.073619	1.870562	0.025641	0.018653	5.674894	4.592817	5.355904	4.642722
207	2.240667	1.631308	0.023542	0.013952	8.122982	5.481248	9.814711	6.823901
210	2.778886	3.146496	0.025467	0.022447	4.583099	8.405124	3.261881	5.882195
211	2.187804	1.157192	0.025156	0.012087	5.608017	5.305486	5.732783	4.963476
213	1.468113	1.620505	0.015669	0.017859	5.407859	7.319244	4.363699	4.66912
Mean	1.831505	1.842852	0.018564	0.016453	5.374283	6.006134	4.976577	5.51206

	CVRcvci		% Change Flow		CVRflow	
Sub ID	PLA	MEL	PLA	MEL	PLA	MEL
101	69.62266	104.6731	44.29873	51.60813	6.720171	7.159121
103	104.2149	47.58591	42.75851	37.44083	8.178576	8.081361
104	114.2434	110.8541	62.92766	68.48477	7.584918	9.869988
105	31.82765	49.01307	32.27117	31.12266	5.09414	5.323168
106	54.86186	48.27162	32.01448	39.98484	6.817193	6.603176
108	49.92419	3.678802	31.30839	25.86139	6.143809	4.375136
109	81.14475	29.90739	50.88012	36.24013	6.889755	6.801019
110	28.76978	58.8862	37.6713	41.62366	7.77659	7.663768
111	57.64846	66.39585	51.53798	63.3122	6.424522	8.479158
112	59.98462	89.24315	52.86508	53.1509	7.372071	7.156506
114	57.93989	61.55227	43.66395	45.24417	7.778833	6.398711
201	4.304855	71.72355	23.31628	48.25631	4.382171	7.213953
203	49.72134	49.72134	33.91505	39.43396	5.594301	6.877246
204	93.26601	53.93151	48.88821	28.17783	6.983962	6.608708
207	70.47753	51.16065	50.36078	40.67727	9.066567	7.196194
210	64.34464	85.53305	37.29289	53.66503	5.98165	8.984266
211	57.53003	19.73178	36.73777	38.40893	7.436963	6.946115
213	43.24365	51.51519	30.30185	46.23845	5.289487	7.426254
Mean	60.72613	58.52103	41.27834	43.82953	6.750871	7.175769

COLLEGE OF ARTS AND SCIENCES
DEPARTMENT OF HEALTH AND EXERCISE SCIENCE
The UNIVERSITY of OKLAHOMA

The Effects of Acute Melatonin Supplementation on Cardiovascular Responses to Sympathetic Activation

Are you interested in helping us determine if

Taking Melatonin improves cardiovascular function at rest and during exercise?

What is Melatonin? Melatonin is natural hormone that helps regulate sleep. Recent studies have also found that taking melatonin which is available as an over-the-counter supplement may have positive benefits for the cardiovascular system. This experiment is looking to understand if Melatonin improves cardiovascular responses and how it may do so.

Time commitment: 3 visits (~9 hours total)

What will be measured: Brain and Muscle Blood flow and Oxygenation, Blood Pressure, Heart Rate, Breathing Rate. ***All measures are noninvasive***.

Eligibility: Healthy women* and men between the ages of 18-35 years who do not regularly take melatonin, are not allergic to melatonin, do not use nicotine/tobacco products.

**Women need to have a regular menstrual cycle*

- Brain Oxygenation
- Muscle Oxygenation
- Handgrip Exercise

Compensation: A gift card valued at $50 for Amazon.com will be given for completing all study requirements.

If interested, please contact:

MS student: Sarah Skillett; sarah.skillett@ou.edu

Principal Investigator: Dr. Mikhail Kellawan; kellawan@ou.edu; (405) 325-9028

OR Scan the QR CODE!

The University of Oklahoma is an equal opportunity institution (IRB# 13084)

IRB NUMBER: 13084
IRB APPROVAL DATE: 01/26/2023

Consent Form to Participate in a Research Study

University of Oklahoma Health Sciences Center (OUHSC)
University of Oklahoma- Norman Campus

Study Title: The Effects of Acute Melatonin Supplementation on Cardiovascular Responses to Sympathetic Activation

Sponsor: Department of Health and Exercise Science

Principal Investigator: J. Mikhail Kellawan
Phone Number: 405-325-9028

KEY INFORMATION ABOUT THE RESEARCH STUDY

You are being asked to participate in a research study. Research studies are voluntary and include only people who choose to take part. This consent form begins with a 'Key Information' section to provide important information to help you decide whether or not to participate in this study. More detailed information is provided after the key information. Please take your time, discuss this with family and friends, and ask the investigator and study team any questions you may have.

WHY HAVE I BEEN ASKED TO PARTICIPATE IN THIS STUDY?

You are being asked to participate in this research study because you are a healthy individual that does not use melatonin and is not allergic to melatonin.

WHY IS THIS STUDY BEING DONE AND HOW LONG WILL IT LAST?

The purpose of this study is to find out what effects (good and bad) on the cardiovascular system that melatonin has on you and other healthy people. We think that you will be in the study for as little as 1 week or as long as three months dependent on when your schedule allows you to complete the three study visits.

WHAT WILL I BE ASKED TO DO IN THIS STUDY?

If you decide to participate in this study, you will be asked to complete three study visits. The first visit is to ensure you qualify for study and to measure your cardiovascular responses to: a) placing your lower body into a device that causes suction during rest and during handgrip exercise b) rebreathing a gas mixture that has higher levels of oxygen and carbon dioxide than the outside air. Putting your lower body in this device and causing suction while measuring cardiovascular responses is a common non-invasive test called "Lower Body Negative Pressure or LBNP". The breathing of the high oxygen, high carbon dioxide mix while measuring cardiovascular responses is a common non-invasive test called vascular reactivity test or "hypercapnia" test. You will also complete a Dual-energy X-ray absorptiometry (DXA) scan to determine the amount of fat, muscle and bone is in your body. The second and third visits are to test if a dose of melatonin, which is an "over-the-counter" supplement used to help with sleep, changes cardiovascular responses to LBNP during rest and during handgrip exercise and to hypercapnia. In visits two and three you will be randomly chosen to receive either melatonin treatment or a placebo treatment. Both treatments are a liquid spray under your tongue. You will not know which treatment you are receiving on any visit. However, whatever treatment you receive on visit two, you will receive the opposite treatment on visit three.

WHY MIGHT I WANT TO PARTICIPATE IN THIS STUDY?
If you agree to take part in this study, there is no direct medical benefit to you. We hope that the information learned from this study will benefit people with diseases that affect the cardiovascular system in the future.

WHY MIGHT I NOT WANT TO PARTICIPATE IN THIS STUDY
You may not want to be exposed to the radiation of a DXA scan. DXA uses less than one-tenth of the dose of radiation than that used in a standard chest X-ray, which is considered extremely low and is generally safe for most people. This amount of radiation is similar to the amount of background radiation received by an average person over a typical day. However, any amount of radiation may harm an unborn baby. Excessive radiation exposure may increase cancer risk, but the amount of radiation used in a DXA scan remains relatively low, and the benefits often outweigh potential cancer risks. You may not want to participate because some of the procedures/measurement may cause you some discomfort. However, the researchers will do everything possible to minimize your discomfort. Further, there is a rare possibility that you will have an adverse response to melatonin). The researchers do not know all the side effects that could happen. For a complete description of known risks, refer to the Detailed Information section of the consent form.

WHAT OTHER OPTIONS ARE THERE?
You may choose to receive no therapy at this time and receive only care to help you feel more comfortable. You may choose not to participate in this study. Please talk to your regular doctor about these and other options.

HOW WILL PARTICIPATING IN THE STUDY AFFECT ME FINANCIALLY?
There is no additional cost to you if you participate in this study.
If you chose to participate and complete all three study visits you will receive a gift card for $50 for Amazon.com.

DETAILED INFORMATION ABOUT THE RESEARCH STUDY

The following pages of the consent form will provide you with more information about this study. Please take your time in reviewing this information and ask the investigator and study team any questions you may have.

HOW MANY PEOPLE WILL TAKE PART IN THE STUDY?
About *150* people will take part in this study.

WHAT IS THE STATUS OF THE SUPPLEMENT USED IN THIS STUDY?
Melatonin is an "Over-the-counter" supplement that is typically used as a sleep aid. It falls under the US Food and Drug Administration's Dietary health and Education Act as a **dietary supplement**. Therefore, melatonin is not an investigational drug and not approved or regulated by the US Food and Drug Administration

WHAT IS INVOLVED IN THE STUDY?
If you agree to be in this research, you will be asked to complete three study visits. The first visit is to ensure you qualify for study and to measure your cardiovascular responses to lower body negative pressure (LBNP) during rest and during handgrip exercise and during hypercapnia. Putting the lower part of your body in chamber and adding suction while measuring cardiovascular responses is a common non-invasive test to challenge your cardiovascular systems to regulate blood pressure. Breathing the hypercapnia gas responses is a common non-invasive test that causes your blood vessels to open wider. The second and third visits are to test if a dose of melatonin, which is an "over-

the-counter" supplement used to help with sleep, changes cardiovascular responses to LBNP during rest and during handgrip exercise and to hypercapnia. In visits two and three you will be randomly chosen to receive either melatonin treatment or a placebo treatment. Both treatments are a liquid spray under your tongue. You will not know which treatment you are receiving on any visit. However, whatever treatment you receive on visit two, you will receive the opposite treatment on visit three. Female participants will take a pregnancy test prior to any experimental visit.

Visit 1 (Screening): ~2 hours. The screening visit will include informed consent, measurements of height, weight, waist and hip circumference, and questionaries about your health history, sleeping, and physical activity. . Women will also have to complete a pregnancy test. These are done to ensure you are eligible for the study and that it is safe for you to participate. If you qualify, you will then undergo a complete a time control experiment. Once you have been deemed eligible to participate, you will complete a Dual-energy X-ray absorptiometry (DXA) scan to determine the amount of fat, muscle and bone is in your body. After the DXA scan, your forearm grip strength in your non-dominate arm will be measured. Additionally, the proper positioning of the handgrip dynamometer will be determined for forearm exercise. The proper position of a transcranial doppler ultrasound device will also be determined.

Visit 2 & 3 (Study day): ~3 hours. On these study days you will receive a dose of melatonin or placebo via a spray under your tongue. You will not be told on which visit you will receive melatonin and which day you will receive the placebo. Next, we will setup and attach multiple pieces of equipment which measure several cardiovascular variables. All pieces of equipment used are described in the list below under "Instrumentation". You will rest for 30 minutes while all pieces of equipment are setup to start the experiment. After the rest period, a lower body negative pressure (LBNP) machine will be activated for 2 minutes. LBNP will cause a redistribution of your blood from your upper body to the lower body as well as an increase in heart beats per minute. You will then rest for 10 more minutes after which, you will undergo 2 trials of hypercapnic rebreathing separated by 5minutes of rest. After hypercapnia, you will have another 10min of rest. Then you will start handgrip exercise at 20% of your maximal grip strength. You will squeeze a handgrip exercise device for 1 second and relax for 2 seconds in time with a metronome. You continue this exercise for 7 minutes. 5 minutes into the exercise an investigator will start the LBNP for 2 minutes. You will continue to exercise during the LBNP exposure for the remaining 2 minutes. After exercise is completed, you will rest for 10 minutes. Then a blood pressure cuff will inflate around the arm that you were exercising for 5 minutes. After 5 minutes, the cuff will deflate, and recovery measurements will be collected - after which, the visits end.

Instrumentation: These are the devices what we will place on you. All of these devices are non-invasive.

- Forearm and Brain Oxygenation: These are measured via Near-Infrared Spectroscopy (NIRS). We will place probes on your forehead and forearm secured with a tensor bandage
- Heart Rate (HR): will be measured with a heart rate monitor that is you strap on your torso directly on your skin
- Blood Pressure: Is measured non-invasively via Finger photoplethysmography. In which a larger blood pressure cuff is placed on both of your upper arm and small blood pressure cuff on your middle finger which is connected to monitor that is strapped on your wrist
- Blood Oxygenation: Is measured via infrared light with a clip on your ear
- End-Tidal Carbon Dioxide ($EtCO_2$): End-Tidal Carbon Dioxide ($EtCO_2$) will be measured non-invasively using a mask and recorded using a metabolic cart.
- Cerebral Blood Velocity (MCAv): A non-invasive Transcranial Doppler Ultrasound with bilateral 2MHz robotic probes will be used for MCA_v on the left and right portions of the brain

- Forearm Blood Flow: Non-invasive Echo and Doppler Ultrasound with a linear array probe in B-mode will be used to measure brachial artery diameter and blood velocity.
- Maximal Voluntary Contraction (MVC): using a Handgrip dynamometer. Using your non-dominate hand you will squeeze the dynamometer as hard as you can 3 sperate times.
- Handgrip Exercise: Using your non-dominant hand at 20% MVC, 1:2s contraction-relaxation cycle, for 7 minutes
- Lower Body Negative Pressure (LBNP): Your lower body up to the top of your hips will be inserted into a plexiglass chamber attached to a vacuum. When turned on the device will "suck" at a pressure of -20 mmHg for 2 minutes.
- Hypercapnia (HC): While lying down you will breathe through a mouthpiece connected to a three-way sliding valve. A meteorological ballon filled with hyperoxic (%40 O_2), hypercapnic (3% CO_2) mix of gas will be filled to a volume 1 liter above estimated vital capacity based on sex, height, and age. The valve will be switched such that you will rebreathe the mixture until you $EtCO_2$ levels are 10mmHg above baseline levels (~2min)
- Pregnancy Test: Female participants will take a commercially available pregnancy test prior to participation in any experimental visit.
- Body Composition: Total body composition will be determined by use of a Dual-energy X-ray absorptiometry (DXA) scan
- Placebo: 1 µL McComicks Pure Mint Extract mixed with 29 ml filter water, which you will spray under your tongue
- Melatonin: 5mg commercially available melatonin spray (Onnit, Mint Flavor), which you will spray under your tongue

CAN I WITHDRAW FROM THE STUDY?

You can stop participating in this study at any time. However, if you decide to stop participating in the study, we encourage you to talk to the researcher and your regular doctor first. To withdraw from the study, simply inform one of the researchers of your decision.

There may be circumstances under which your participation may be terminated by the investigator without your consent.

- He/She feels that it is in your medical best interest.
- New information becomes available.
- You fail to follow study requirements.

WHAT ARE THE RISKS OF THE STUDY?

In addition to the risks described in the Key Information section, you may also be at risk for these side effects. You should discuss these with the researcher and/or your regular doctor. Other drugs may be given to make side effects less serious and uncomfortable. Many side effects go away shortly after the melatonin/fasting/exercise/LBNP are stopped. The treatment or procedure may involve risks that are currently unforeseeable.

Risks and side effects related to melatonin and the procedures we are studying include:

This experiment is non-invasive. Therefore, we do not anticipate any risk or benefit to you personally. However, nothing is without risk, there is a rare chance that you may have an adverse event. Some risks associated with the study may include:

a. Melatonin supplement: Melatonin is an "Over-the-counter" supplement that is typically used as a sleep aid. Adverse responses to melatonin are rare but include headache, short-term feelings of depression, daytime sleepiness, dizziness, stomach cramps, nausea, and irritability. *We recommend that you do not drive or use machinery for four to five hours after taking melatonin.*

b. 8-hour fast: Side effects of an 8-hour fast include feeling hungry or weak. To reduce this risk, we will encourage you to eat a substantial meal prior to the fast, drink plenty of water while fasting. If you feel sign/symptoms such as tiredness, headache, stomachache, unsteadiness, anxiety, etc. that interfere with daily living, subjects should discontinue the fast. To avoid these sensations, we recommend fasting after a night-time meal and visiting the lab in the early morning.

c. Anthropometric Measurements: There are no risks to measuring anthropometrics height, weight, heart rate, etc. Measuring blood pressure with a forearm cuff and finger cuff may cause a temporary increase in pressure at the area of skin below the cuff, however, this discomfort is temporary and subsides when the cuff pressure is reduced.

d. Abstaining from exercise, caffeine, NSAIDs: This poses minimal risk to you. Abstaining from caffeine may result in irritability and headache. Acute abstinence from exercise has no risk. There is no risk form abstaining from NSAIDs considering regular use of NSAIDS for a clinical condition would exclude you from participation.

e. NIRS: A NIRS will be used to measure oxygenation of your brain and muscle. It is a non-invasive device that continuously monitors regional tissue oxygenation safely. It is secured on your forehead and forearm with a tensor bandage. This may feel tight on your forehead and arm.

f. Blood pressure monitoring: Photoplethysmography uses a non-invasive, automatic blood pressure cuff around the upper arm and finger. Around the finger a near-infrared light is used to measure changes in pressure on a beat-by-beat basis. When both the cuffs are inflated it may feel uncomfortable while inflated but this is temporary. These blood pressure measures are considered very safe.

g. Total Liable Signal determination: Uses a non-invasive, automatic blood pressure cuff around the upper arm. When the cuff is inflated it may feel uncomfortable while inflated but this is temporary. It will be inflated for 5 minutes then released. While inflated, there may be some feeling some numbness and tingling in your hands when the cuff is inflated. Once released those sensations will subside quickly. These procedures are considered very safe

h. Radiation Exposure: If you participate in the research, you will receive a DXA scan, a type of x ray procedure. The DXA scan will be performed for research purposes only and is not required for your medical care. The amount of additional radiation to which you will be exposed is approximately the amount that you receive in one day from natural, background sources of radiation. The risk of radiation exposure is cumulative over your lifetime.

i. Lower Body Negative Pressure: Requires placing the lower half of your body (up to the top of your hips) in a chamber that when turned on creates suction at -20 mmHg for 2-5 minutes. The risk of LBNP is you may feel uncomfortable or light-headed when the machine is running.

j. Breach of Confidentiality: Personal information such as name, gender, date of birth, and medications will be stored in a locked file cabinet in the HCRL laboratory. Study records will be coded with a number and only study personnel will have access to the link connecting your name to the collected data. After the study is complete, we will remove all identifying information so that study data is coded during analysis and publication. Your information will be coded to remove any personal identifiers during data analysis or research publications.

We do not anticipate that there will be any direct benefits to you for participating.

For more information about risks and side effects, ask the researcher.

TO WHAT EXTENT WILL MY INFORMATION BE KEPT CONFIDENTIAL?

Efforts will be made to keep your personal information confidential. You will not be identifiable by name or description in any reports or publications about this study. We cannot guarantee absolute confidentiality. Your personal information may be disclosed if required by law. You will be asked to sign a separate authorization form for use or sharing of your protected health information.

There are organizations outside the OUHSC that may inspect and/or copy your research records for quality assurance and data analysis. These organizations may include the US Food & Drug Administration and other regulatory agencies. The OUHSC Human Research Participant Program office, the OUHSC Institutional Review Board, OUHSC Office of Compliance, and other University administrative offices may also inspect and/or copy your research records for these purposes.

Posting Study on ClinicalTrials.gov:

A description of this clinical trial will be available on http://www.ClinicalTrials.gov, as required by U.S. Law. However, this website will not include information that can identify you. At most, the website will include a summary of the study and results. You can search this website at any time.

Identifiable Private Information:
Your information may be used for future studies without your additional consent. We will remove direct identifiers from your information and assign a code. The key to this code will be kept separately and only the researcher for this study will have access to the code. If your information is shared with another investigator for research purposes, they will not have access to the key code and will not be able to re-identify you.

WHAT ARE THE COSTS?
There are no direct costs to participating in the study.

WHAT IF I AM INJURED OR BECOME ILL WHILE PARTICIPATING IN THIS STUDY?
In the case of injury or illness results from this study, emergency medical treatment is available.

Emergency medical treatment should be sought at the nearest medical center and the study P.I J. Mikhail Kellawan should be contacted immediately (405-325-9028, kellawan@ou.edu).

You or your insurance may be charged for this treatment

No other funds have been set aside by the University of Oklahoma Health Sciences Center, University of Oklahoma – Norman Campus to compensate you in the event of injury, illness, or for other damages related to your event of injury or illness.

WHAT ARE MY RIGHTS AS A PARTICIPANT?
Taking part in this study is voluntary. You may choose not to participate. Refusal to participate will involve no penalty or loss of benefits to which you are otherwise entitled.

If you agree to participate and then decide against it, you can withdraw for any reason and leave the study at any time. However, please be sure to discuss leaving the study with the principal investigator or your regular doctor. You may discontinue your participation at any time without penalty or loss of benefits to which you are otherwise entitled.

You have the right to access the medical information that has been collected about you as a part of this research study. However, you may not have access to this medical information until the entire research study has completely finished. You consent to this temporary restriction.

DO I HAVE ANY OTHER RIGHTS OVER MY DATA?
Depending on where the sponsor for your study is located and other factors, you may have additional rights over your personal data collected in this study. For example, the European Union General Data Protection Regulation (GDPR) and some state privacy laws might apply. If the GDPR applies, generally you may have the following rights:

1. The right to request the information collected to be corrected.

2. The right to withdraw your consent for the use of your personal information at any time.
3. The right, in some circumstances, to receive your personal information in a structured, commonly used and machine-readable format and the right to provide your information to a third party.
4. The right to strict confidentiality of your personal data when it is used/shared.
5. The right to limit the use/sharing of your personal information in certain circumstances.
6. The right under some circumstances to request the erasure of your personal data.
7. The right to file a complaint with a privacy protection regulator if you believe any of the rights above have been violated.

You can receive more information regarding these rights in the Privacy Notice for Research Participants, located on the OUHSC Office of Human Research Participant Protection (HRPP) website at *https://compliance.ouhsc.edu/HRPP/Participant/Privacy-Notice*.

If you have any questions and requests, please contact the HRPP Office at 405-271-2045.

WHOM DO I CALL IF I HAVE QUESTIONS, SUGGESTIONS, OR CONCERNS?
If you have questions, concerns, or complaints about the study or have a research-related injury, contact *J. Mikhail Kellawan* at *405-325-9028*.

If you cannot reach the Investigator or wish to speak to someone other than the investigator and for questions about your rights as a research participant, contact the OUHSC Director, Office of Human Research Participant Protection, at 405-271-2045.

SIGNATURE:
By signing this form, you are agreeing to participate in this research study under the conditions described. You have not given up any of your legal rights or released any individual or entity from liability for negligence. You have been given an opportunity to ask questions. You will be given a copy of this consent document.

I agree to participate in this study:

PARTICIPANT SIGNATURE (age ≥18)	**Printed Name**	**Date**
SIGNATURE OF PERSON OBTAINING CONSENT	**Printed Name**	**Date**

AUTHORIZATION TO USE or SHARE
HEALTH INFORMATION[1] THAT IDENTIFIES YOU FOR RESEARCH
An Informed Consent Document for Research Participation may also be required.
Form 2 must be used for research involving psychotherapy notes.

Title of Research Project: **[The Effects of Acute Melatonin Supplementation on Cardiovascular Responses to Sympathetic Activation**

Leader of Research Team: **J. Mikhail Kellawan**

Address: **1401 Asp AVE. Norman, OK, USA, Rm 112, 73019**

Phone Number: **405-325-9028**

If you decide to sign this document, University of Oklahoma Health Sciences Center (OUHSC) researchers may use or share information that identifies you (protected health information) for their research. Protected health information will be called PHI in this document.

PHI To Be Used or Shared. Federal law requires that researchers get your permission (authorization) to use or share your PHI. If you give permission, the researchers may use or share with the people identified in this Authorization any PHI related to this research from your medical records and from any test results. Information used or shared may include all information relating to any tests, procedures, surveys, or interviews as outlined in the consent form; medical records and charts; name, address, telephone number, date of birth, race, government-issued identification numbers, and [Heart rate, Forearm & Brain Oxygenation, Blood Pressure, Blood Oxygentation, End-Todal Carbon Dioxide, Brain and Forarm Blood Flow, Pregnacy test, and Body Composition during rest, exercise, and lower body negative pressure testing there is also survey data on your sleeping and physical activity habits.

Purposes for Using or Sharing PHI. If you give permission, the researchers may use your PHI to To determine if acute melatonin supplementation alters central and peripheral responses to lower body negative pressure at rest and during rhythmic exercise and to hypercapnia

Other Use and Sharing of PHI. If you give permission, the researchers may also use your PHI to develop new procedures or commercial products. They may share your PHI with other researchers, the research sponsor and its agents, the OUHSC Institutional Review Board, auditors and inspectors who check the research, and government agencies such as the Food and Drug Administration (FDA) and the Department of Health and Human Services (HHS), and when required by law. The researchers may also share your PHI with no one else

[1] **Protected Health Information includes all identifiable information relating to any aspect of an individual's health whether past, present or future, created or maintained by a Covered Entity.**

Confidentiality. Although the researchers may report their findings in scientific journals or meetings, they will not identify you in their reports. The researchers will try to keep your information confidential, but confidentiality is not guaranteed. The law does not require everyone receiving the information covered by this document to keep it confidential, so they could release it to others, and federal law may no longer protect it.

YOU UNDERSTAND THAT YOUR PROTECTED HEALTH INFORMATION MAY INCLUDE INFORMATION REGARDING A COMMUNICABLE OR NONCOMMUNICABLE DISEASE.

Voluntary Choice. The choice to give OUHSC researchers permission to use or share your PHI for their research is voluntary. It is completely up to you. No one can force you to give permission. However, you must give permission for OUHSC researchers to use or share your PHI if you want to participate in the research and, if you cancel your authorization, you can no longer participate in this study.

Refusing to give permission will not affect your ability to get routine treatment or health care unrelated to this study from OUHSC.

Canceling Permission. If you give the OUHSC researchers permission to use or share your PHI, you have a right to cancel your permission whenever you want. However, canceling your permission will not apply to information that the researchers have already used, relied on, or shared or to information necessary to maintain the reliability or integrity of this research.

End of Permission. Unless you cancel it, permission for OUHSC researchers to use or share your PHI for their research will never end.]

Contacting OUHSC: You may find out if your PHI has been shared, get a copy of your PHI, or cancel your permission at any time by writing to:

Privacy Official University of Oklahoma Health Sciences Center PO Box 26901 Oklahoma City, OK 73190	or	Privacy Board University of Oklahoma Health Sciences Center PO Box 26901 Oklahoma City, OK 73190

If you have questions, call: (405) 271-2511 or (405) 271-2045.

Access to Information. You have the right to access the medical information that has been collected about you as a part of this research study. However, you may not have access to this medical information until the entire research study is completely finished. You consent to this temporary restriction.

Giving Permission. By signing this form, you give OUHSC and OUHSC's researchers led by the Research Team Leader permission to share your PHI for the research project listed at the top of this form.

Patient/Participant Name (Print): _____

_____ _____
Signature of Patient-Participant Date
or Parent if Participant is a minor

Or

_____ _____
Signature of Legal Representative** Date

**If signed by a Legal Representative of the Patient-Participant, provide a description of the relationship to the Patient-Participant and the authority to act as Legal Representative:

_____
OUHSC may ask you to produce evidence of your relationship.

A signed copy of this form must be given to the Patient-Participant or the Legal Representative at the time this signed form is provided to the researcher or his representative.

PI: J. Mikhail Kellawan

Short Title: MEL

Subject ID: _____ Sex: M / W Date: / /

Medical History (General)

Body System	Have you ever had any conditions affecting these body systems?	*Only complete if 'Yes' for Diagnosed Condition*			
		Diagnosis/Condition/Surgery	Onset Date	Is it a current problem?	Are you currently taking a prescribed medication?*
Cardiovascular					
☐ Heart Attack	☐ Yes ☐ No			☐ Yes ☐ No	☐ Yes ☐ No
☐ Stroke	☐ Yes ☐ No			☐ Yes ☐ No	☐ Yes ☐ No
☐ Hypertension	☐ Yes ☐ No			☐ Yes ☐ No	☐ Yes ☐ No
☐ Coronary Artery Disease	☐ Yes ☐ No			☐ Yes ☐ No	☐ Yes ☐ No
☐ Other Cardiovascular	☐ Yes ☐ No			☐ Yes ☐ No	☐ Yes ☐ No
☐ Your 1st Degree Relatives (e.g. mother, brother, daughter)	☐ Yes ☐ No	List family members, their diagnosis, and approximately when they were diagnosed:			
Lungs					
☐ Asthma	☐ Yes ☐ No			☐ Yes ☐ No	☐ Yes ☐ No
☐ Exercise-Induced Bronchospasm	☐ Yes ☐ No			☐ Yes ☐ No	☐ Yes ☐ No
☐ Obstructive Lung Disease	☐ Yes ☐ No			☐ Yes ☐ No	☐ Yes ☐ No
☐ Other	☐ Yes ☐ No			☐ Yes ☐ No	☐ Yes ☐ No
Musculoskeletal					
☐ Knee	☐ Yes ☐ No			☐ Yes ☐ No	☐ Yes ☐ No
☐ Hips	☐ Yes ☐ No			☐ Yes ☐ No	☐ Yes ☐ No
☐ Back					
☐ Other					
Head/Eyes/Ears/ Nose/Throat/Neck	☐ Yes ☐ No			☐ Yes ☐ No	☐ Yes ☐ No
Endocrine/Metabolic					
Diabetes	☐ Yes ☐ No			☐ Yes ☐ No	☐ Yes ☐ No

High blood sugar	☐ Yes ☐ No			☐ Yes ☐ No	☐ Yes ☐ No
Liver	☐ Yes ☐ No			☐ Yes ☐ No	☐ Yes ☐ No
Thyroid	☐ Yes ☐ No			☐ Yes ☐ No	☐ Yes ☐ No
Kidney	☐ Yes ☐ No			☐ Yes ☐ No	☐ Yes ☐ No
Pituitary Gland	☐ Yes ☐ No			☐ Yes ☐ No	☐ Yes ☐ No
Neurological					
Nerve or Neurologic disorders	☐ Yes ☐ No			☐ Yes ☐ No	☐ Yes ☐ No
Autonomic Neurological disorders	☐ Yes ☐ No			☐ Yes ☐ No	☐ Yes ☐ No
Other	☐ Yes ☐ No			☐ Yes ☐ No	☐ Yes ☐ No
Immunological					
Autoimmune disease	☐ Yes ☐ No			☐ Yes ☐ No	☐ Yes ☐ No
Other	☐ Yes ☐ No			☐ Yes ☐ No	☐ Yes ☐ No
Psychological					
Clinical depression	☐ Yes ☐ No			☐ Yes ☐ No	☐ Yes ☐ No
Other	☐ Yes ☐ No			☐ Yes ☐ No	☐ Yes ☐ No

Allergies	☐ Yes ☐ No			☐ Yes ☐ No	☐ Yes ☐ No
Tobacco Use					
Ever Smoked or used tobacco products (smoke, smokeless, vapor	☐ Yes ☐ No	For how long? (years): ___		*If no, when did you quit? ___/___/___	

ADDITIONAL NOTES: _____

_____

_____

Additional Questions (all subjects)

Have you recently experienced any of the following?	Yes	No	When?
Pain in the neck, jaw, or arms?			
Dizziness or fainting?			
Swelling in the ankles?			
Rapid heart rate while at rest?			
Leg pain or cramping while walking, relieved with rest?			
Has a doctor ever told you that you have a heart murmur?			
Unusual fatigue with usual activities?			

Additional COVID-19 Questions (all subjects)

Have you recently experienced any of the following in the 14 days?	Yes	No	When?
Fever or Chills?			
Cough?			
Shortness of Breath or Difficulty Breathing?			
Unusual fatigue with usual activities?			
Muscle of body aches?			
Headache?			
Sore Throat?			
Congestion or runny nose?			
Nausea or vomiting?			
Diarrhea?			
If you have answered YES to any of the above, **have you been Tested for COVID-19?**			

Have you experienced any of the following?	Yes	No	When?
Reason to believe you have had been infected with COVID-19?			
Received a positive test or confirmed diagnosis of COVID-19?			
Been hospitalized for COVID-19?			
Been Infected with COVID-19 and experienced symptoms?			
Been Infected with COVID-19 and experienced **NO** symptoms (Asymptomatic)?			
If you have had a confirmed case of COVID-19, did you experience any of the following symptoms:			
Fever or Chills?			
Cough?			
Shortness of Breath or Difficulty Breathing?			
Unusual fatigue with usual activities?			
Muscle of body aches?			
Sore Throat?			
Congestion or runny nose?			
Nausea or vomiting?			
Diarrhea?			
Have you recovered from your COVID-19 infection? (if applicable)			
Has a medical professional told you that you have you recovered from your COVID-19 infection? (if applicable)			

Please list all Medications or Supplements You Take

Medications/Supplements
Are you taking **ANY** Prescribed medications? (list below):
Are you taking hormone replacement (e.g., estrogen) therapy?
Do you take over the counter medications &/or supplements (aspirin, vitamins, etc.)?

Do you have any reason you believe you should not participate in this research study? ☐ Yes ☐ No

Explain:

Are you currently enrolled in any other research studies or have you participated in any other research studies in the past 30 days? ☐ Yes ☐ No

If yes, when was your last study visit (MM/DD/YYYY)?

If yes, what is the date of your next visit (MM/DD/YYYY)?

Female Subjects Only ☐ N/A - subject is male

Subject currently pregnant? ☐ Yes ☐ No
Subject plans to become pregnant? ☐ Yes ☐ No

Currently using birth control? ☐ Yes ☐ No

If yes, method of birth control *[Select All That Apply]:*

☐[1] Oral Contraceptives	☐[5] NuvaRing	☐[10] Post-menopausal for ≥ 1 year
☐[2] Hormonal Injections	☐[6] Intrauterine device	☐[11] Tubal ligation, bilateral oophorectomy, or hysterectomy
☐[3] Hormonal Implants (i.e. Implanon)	☐[7] Hormonal Intrauterine device	☐[12] Abstinence
☐[4] Contraceptive Patches	☐[8] Non-hormonal Barrier method	☐[13] Other (specify in Reproductive field)
	☐[9] Spermicide	
Start Date of Birth Control (MM/DD/YY):	Brand Name: _____	

What is the date do you expect you next period? _____

Do you have a regular menstrual cycle (last 3 cycles consecutive)? ☐ Yes ☐ No

Past Menstrual History

Start Date of LAST menstrual cycle (MM/DD/YY):_____

End Date of LAST menstrual cycle (MM/DD/YY):_____

Have you ever consulted a doctor about menstrual problems (specifically, about irregular or missing periods)?

_____

Have you ever consulted a doctor about any problems relating to your hormonal system? If so, please explain.

_____

For HCRL Staff Only

Form Verified by:_____ **Date:**_____

INTERNATIONAL PHYSICAL ACTIVITY QUESTIONNAIRE (October 2002)

LONG LAST 7 DAYS SELF-ADMINISTERED FORMAT

FOR USE WITH YOUNG AND MIDDLE-AGED ADULTS (15-69 years)

The International Physical Activity Questionnaires (IPAQ) comprises a set of 4 questionnaires. Long (5 activity domains asked independently) and short (4 generic items) versions for use by either telephone or self-administered methods are available. The purpose of the questionnaires is to provide common instruments that can be used to obtain internationally comparable data on health–related physical activity.

Background on IPAQ

The development of an international measure for physical activity commenced in Geneva in 1998 and was followed by extensive reliability and validity testing undertaken across 12 countries (14 sites) during 2000. The final results suggest that these measures have acceptable measurement properties for use in many settings and in different languages, and are suitable for national population-based prevalence studies of participation in physical activity.

Using IPAQ

Use of the IPAQ instruments for monitoring and research purposes is encouraged. It is recommended that no changes be made to the order or wording of the questions as this will affect the psychometric properties of the instruments.

Translation from English and Cultural Adaptation

Translation from English is encouraged to facilitate worldwide use of IPAQ. Information on the availability of IPAQ in different languages can be obtained at www.ipaq.ki.se. If a new translation is undertaken we highly recommend using the prescribed back translation methods available on the IPAQ website. If possible please consider making your translated version of IPAQ available to others by contributing it to the IPAQ website. Further details on translation and cultural adaptation can be downloaded from the website.

Further Developments of IPAQ

International collaboration on IPAQ is on-going and an ***International Physical Activity Prevalence Study*** is in progress. For further information see the IPAQ website.

More Information

More detailed information on the IPAQ process and the research methods used in the development of IPAQ instruments is available at www.ipaq.ki.se and Booth, M.L. (2000). *Assessment of Physical Activity: An International Perspective*. Research Quarterly for Exercise and Sport, 71 (2): s114-20. Other scientific publications and presentations on the use of IPAQ are summarized on the website.

INTERNATIONAL PHYSICAL ACTIVITY QUESTIONNAIRE

We are interested in finding out about the kinds of physical activities that people do as part of their everyday lives. The questions will ask you about the time you spent being physically active in the **last 7 days**. Please answer each question even if you do not consider yourself to be an active person. Please think about the activities you do at work, as part of your house and yard work, to get from place to place, and in your spare time for recreation, exercise or sport.

Think about all the **vigorous** and **moderate** activities that you did in the **last 7 days**. **Vigorous** physical activities refer to activities that take hard physical effort and make you breathe much harder than normal. **Moderate** activities refer to activities that take moderate physical effort and make you breathe somewhat harder than normal.

PART 1: JOB-RELATED PHYSICAL ACTIVITY

The first section is about your work. This includes paid jobs, farming, volunteer work, course work, and any other unpaid work that you did outside your home. Do not include unpaid work you might do around your home, like housework, yard work, general maintenance, and caring for your family. These are asked in Part 3.

1. Do you currently have a job or do any unpaid work outside your home?

   ☐ Yes

   ☐ No → ***Skip to PART 2: TRANSPORTATION***

The next questions are about all the physical activity you did in the **last 7 days** as part of your paid or unpaid work. This does not include traveling to and from work.

2. During the **last 7 days**, on how many days did you do **vigorous** physical activities like heavy lifting, digging, heavy construction, or climbing up stairs **as part of your work**? Think about only those physical activities that you did for at least 10 minutes at a time.

   _____ **days per week**

   ☐ No vigorous job-related physical activity → ***Skip to question 4***

3. How much time did you usually spend on one of those days doing **vigorous** physical activities as part of your work?

   _____ **hours per day**
   _____ **minutes per day**

4. Again, think about only those physical activities that you did for at least 10 minutes at a time. During the **last 7 days**, on how many days did you do **moderate** physical activities like carrying light loads **as part of your work**? Please do not include walking.

   _____ **days per week**

   ☐ No moderate job-related physical activity → ***Skip to question 6***

5. How much time did you usually spend on one of those days doing **moderate** physical activities as part of your work?

_____ **hours per day**
_____ **minutes per day**

6. During the **last 7 days**, on how many days did you **walk** for at least 10 minutes at a time **as part of your work**? Please do not count any walking you did to travel to or from work.

_____ **days per week**

☐ No job-related walking → ***Skip to PART 2: TRANSPORTATION***

7. How much time did you usually spend on one of those days **walking** as part of your work?

_____ **hours per day**
_____ **minutes per day**

PART 2: TRANSPORTATION PHYSICAL ACTIVITY

These questions are about how you traveled from place to place, including to places like work, stores, movies, and so on.

8. During the **last 7 days**, on how many days did you **travel in a motor vehicle** like a train, bus, car, or tram?

_____ **days per week**

☐ No traveling in a motor vehicle → ***Skip to question 10***

9. How much time did you usually spend on one of those days **traveling** in a train, bus, car, tram, or other kind of motor vehicle?

_____ **hours per day**
_____ **minutes per day**

Now think only about the **bicycling** and **walking** you might have done to travel to and from work, to do errands, or to go from place to place.

10. During the **last 7 days**, on how many days did you **bicycle** for at least 10 minutes at a time to go **from place to place**?

_____ **days per week**

☐ No bicycling from place to place → ***Skip to question 12***

11. How much time did you usually spend on one of those days to **bicycle** from place to place?

_____ **hours per day**
_____ **minutes per day**

12. During the **last 7 days**, on how many days did you **walk** for at least 10 minutes at a time to go **from place to place**?

_____ **days per week**

☐ No walking from place to place → ***Skip to PART 3: HOUSEWORK, HOUSE MAINTENANCE, AND CARING FOR FAMILY***

13. How much time did you usually spend on one of those days **walking** from place to place?

_____ **hours per day**
_____ **minutes per day**

PART 3: HOUSEWORK, HOUSE MAINTENANCE, AND CARING FOR FAMILY

This section is about some of the physical activities you might have done in the **last 7 days** in and around your home, like housework, gardening, yard work, general maintenance work, and caring for your family.

14. Think about only those physical activities that you did for at least 10 minutes at a time. During the **last 7 days**, on how many days did you do **vigorous** physical activities like heavy lifting, chopping wood, shoveling snow, or digging **in the garden or yard**?

_____ **days per week**

☐ No vigorous activity in garden or yard → ***Skip to question 16***

15. How much time did you usually spend on one of those days doing **vigorous** physical activities in the garden or yard?

_____ **hours per day**
_____ **minutes per day**

16. Again, think about only those physical activities that you did for at least 10 minutes at a time. During the **last 7 days**, on how many days did you do **moderate** activities like carrying light loads, sweeping, washing windows, and raking **in the garden or yard**?

_____ **days per week**

☐ No moderate activity in garden or yard → ***Skip to question 18***

17. How much time did you usually spend on one of those days doing **moderate** physical activities in the garden or yard?

_____ **hours per day**
_____ **minutes per day**

18. Once again, think about only those physical activities that you did for at least 10 minutes at a time. During the **last 7 days**, on how many days did you do **moderate** activities like carrying light loads, washing windows, scrubbing floors and sweeping **inside your home**?

_____ **days per week**

☐ No moderate activity inside home → ***Skip to PART 4: RECREATION, SPORT AND LEISURE-TIME PHYSICAL ACTIVITY***

19. How much time did you usually spend on one of those days doing **moderate** physical activities inside your home?

_____ **hours per day**
_____ **minutes per day**

PART 4: RECREATION, SPORT, AND LEISURE-TIME PHYSICAL ACTIVITY

This section is about all the physical activities that you did in the **last 7 days** solely for recreation, sport, exercise or leisure. Please do not include any activities you have already mentioned.

20. Not counting any walking you have already mentioned, during the **last 7 days**, on how many days did you **walk** for at least 10 minutes at a time **in your leisure time**?

_____ **days per week**

☐ No walking in leisure time → ***Skip to question 22***

21. How much time did you usually spend on one of those days **walking** in your leisure time?

_____ **hours per day**
_____ **minutes per day**

22. Think about only those physical activities that you did for at least 10 minutes at a time. During the **last 7 days**, on how many days did you do **vigorous** physical activities like aerobics, running, fast bicycling, or fast swimming **in your leisure time**?

_____ **days per week**

☐ No vigorous activity in leisure time → ***Skip to question 24***

23. How much time did you usually spend on one of those days doing **vigorous** physical activities in your leisure time?

_____ **hours per day**
_____ **minutes per day**

24. Again, think about only those physical activities that you did for at least 10 minutes at a time. During the **last 7 days**, on how many days did you do **moderate** physical activities like bicycling at a regular pace, swimming at a regular pace, and doubles tennis **in your leisure time?**

_____ **days per week**

☐ No moderate activity in leisure time → ***Skip to PART 5: TIME SPENT SITTING***

25. How much time did you usually spend on one of those days doing **moderate** physical activities in your leisure time?
_____ **hours per day**
_____ **minutes per day**

PART 5: TIME SPENT SITTING

The last questions are about the time you spend sitting while at work, at home, while doing course work and during leisure time. This may include time spent sitting at a desk, visiting friends, reading or sitting or lying down to watch television. Do not include any time spent sitting in a motor vehicle that you have already told me about.

26. During the **last 7 days**, how much time did you usually spend **sitting** on a **weekday?**

_____ **hours per day**
_____ **minutes per day**

27. During the **last 7 days**, how much time did you usually spend **sitting** on a **weekend day?**

_____ **hours per day**
_____ **minutes per day**

This is the end of the questionnaire, thank you for participating.

Name:_____ Date:_____

Pittsburgh Sleep Quality Index (PSQI)

Instructions: The following questions relate to your usual sleep habits during the past month only. Your answers should indicate the most accurate reply for the majority of days and nights in the past month. **Please answer all questions.**

1. During the past month, what time have you usually gone to bed at night? _____
2. During the past month, how long (in minutes) has it usually taken you to fall asleep each night? _____
3. During the past month, what time have you usually gotten up in the morning? _____
4. During the past month, how many hours of actual sleep did you get at night? (This may be different than the number of hours you spent in bed.) _____

5. During the past month, how often have you had trouble sleeping because you…	Not during the past month	Less than once a week	Once or twice a week	Three or more times a week
a. Cannot get to sleep within 30 minutes				
b. Wake up in the middle of the night or early morning				
c. Have to get up to use the bathroom				
d. Cannot breathe comfortably				
e. Cough or snore loudly				
f. Feel too cold				
g. Feel too hot				
h. Have bad dreams				
i. Have pain				
j. Other reason(s), please describe:				
6. During the past month, how often have you taken medicine to help you sleep (prescribed or "over the counter")?				
7. During the past month, how often have you had trouble staying awake while driving, eating meals, or engaging in social activity?				
	No problem at all	Only a very slight problem	Somewhat of a problem	A very big problem
8. During the past month, how much of a problem has it been for you to keep up enough enthusiasm to get things done?				
	Very good	Fairly good	Fairly bad	Very bad
9. During the past month, how would you rate your sleep quality overall?				