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Investigation Of Whether Sedentary And Physically Active Conditions Lead To Altered Gabaergic Signaling In The Rvlm

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THE EFFECT OF SEDENTARY CONDITIONS ON GABAERGIC TRANSMISSION IN THE RVLM

by

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DEDICATION

I dedicate this work to my wonderful husband, Nicholas Michael Dombrowski. You always encouraged me to keep trying even when I wanted to quit. There is no way I could have reached my goal without your support. Thank you and I love you so much.

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LIST OF ABBREVIATIONS

Bicuculline	Bic
Cardiovascular disease	CVD
Caudal	C
Caudal ventrolateral medulla	CVLM
Cholera toxin B	CTB
Facial nucleus	FN
Gamma- aminobutyric acid	GABA
Glyceraldehyde 3-phosphate dehydrogenase	GAPDH
Heart Rate	HR
Ionotropic glutamate receptors	iGluRs
Kynurenic acid	Kyn
Mean arterial blood pressure	MAP
Millimolar	mM
Nanoliters	nl
Nucleus tractus solitarius	NTS
Paraventricular nucleus	PVN
Physically active	WR
Rostral	R
Rostral ventrolateral medulla	RVLM
Sedentary	SED
Sinoaortic denervation	SAD
Splanchnic sympathetic nerve activity	SSNA
Standard error of the mean	SEM
Sympathetic nerve activity	SNA
Sympathetic nervous system	SNS
Treadmill trained	ExTr

CHAPTER 1

INTRODUCTION

Significance

One major risk factor for developing cardiovascular disease (CVD) is a sedentary lifestyle (74). According to the World Health Organization (WHO), physical inactivity is the leading risk for death in 1 out 10 cases worldwide (WHO, http://www.who.int/mediacentre/factsheets/fs385/en/). Being sedentary or physically inactive has been shown to lead to chronic activation of the sympathetic nervous system (SNS) (75; 100). The SNS is important for controlling blood pressure (BP) and sympathetic nerve activity (SNA). An overactive SNS would lead to increased resting BP and SNA, which has been shown in sedentary and hypertensive animal models (60; 71; 87; 124). SNA and BP are regulated by brainstem nuclei in order to maintain homeostasis. It is possible that alterations in brainstem nuclei, such as the rostral ventrolateral medulla (RVLM), that are important for controlling sympathetic outflow, could lead to CVD (1; 45; 87; 93; 137). However, there may also be compensatory responses that develop in order to counteract these changes in order to maintain lower resting BP and SNA.

A sedentary lifestyle leads to chronic activation of the sympathetic nervous system

Physical inactivity is detrimental to our health and is a major risk factor for the development of CVD (132). Our laboratory has shown that physical inactivity leads to elevated resting splanchnic sympathetic nerve activity (SSNA) when compared to physically active conditions (87). Our laboratory has also shown that in response to glutamate microinjected into the RVLM, sedentary rats have greater increases in SSNA

(i.e. enhanced sympathoexcitation) when compared to physically active rats (87). Negrao *et al*. showed the response to sodium nitroprusside infusion led to attenuated increases in renal SNA exercise trained rats when compared to unexercised rats (100). These data demonstrate that exercise can help lower SNA in sedentary individuals. This enhancement of sympathoexcitation has been suggested to lead to overactivity of the SNS (27; 38; 111). The mechanisms that lead to alterations in the central nervous system in response to sedentary and physically active conditions are not completely understood. *This project is aimed to investigate the mechanisms that are playing a role in altering central control of the SNS in response to sedentary and physically active conditions.*

The role of the rostral ventrolateral medulla (RVLM) in the control of the SNS

The RVLM is important for the control of sympathetic outflow (38; 39). The activity of the RVLM is primarily regulated by multiple inputs that are both excitatory and inhibitory (12; 103). In the RVLM, there are several neurotransmitters and neuromodulators involved in signal transduction (108), but the primary excitatory and inhibitory neurotransmitters are glutamate (52; 91) and γ-aminobutyric acid (GABA) (80; 113), respectively. The RVLM is involved in tonic and reflex control of the SNS (113). When there is bilateral blockade of the RVLM, blood pressure decreases to levels similar to those observed after complete spinal cord transection (58; 141). This reliance of normal resting arterial blood pressure on spinal input from the RVLM demonstrates that the RVLM is important for maintaining resting blood pressure and sympathetic nerve activity. The RVLM contains populations of premotor neurons that are both tyrosine hydroxylase (TH) positive and negative and both contribute to the control of blood pressure (11; 70; 82; 112). Tyrosine hydroxylase is the rate limiting step in the

synthesis of catecholamines and is a marker for neurons in the RVLM (112) Premotor neurons in the RVLM project down to the intermediolateral cell column (IML) in the thoracic and lumbar regions of the spinal cord (37; 129). Not only does the RVLM control sympathetic outflow but it also receives information via the baroreceptor reflex about arterial blood pressure (15; 108; 113; 133). The arterial baroreceptor reflex tonically inhibits the RVLM via the caudal ventrolateral medulla (CVLM) (3; 39; 113). The CVLM is a key source of GABAergic input to the RVLM (3; 61; 113; 134). Changes at the level of the RVLM could lead to chronic activation of the SNS. Our laboratory, along with others, have shown that there is increased glutamate signaling in the RVLM in animal disease models which may lead to overactivity of the SNS (50; 87; 93; 122). However, the mechanisms responsible for changes at the level of the RVLM that lead to chronic activation of the sympathetic nervous system are not known. Figure 1 illustrates brainstem regions that are involved in control of sympathetic outflow and blood pressure. *The purpose of this project was to investigate how changes at the level of RVLM lead to altered SNA.*

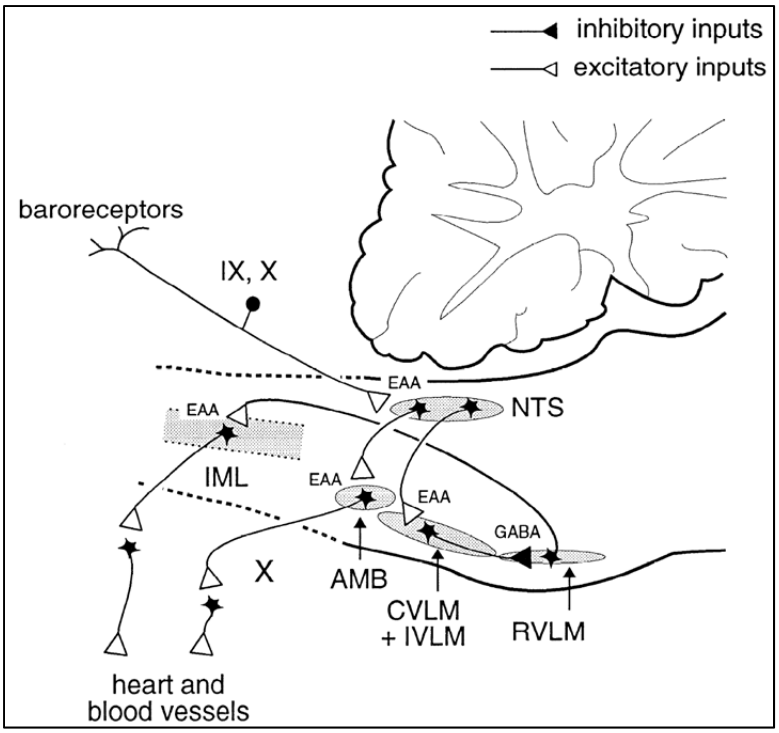

Figure 1. This diagram represents brainstem regions that are involved in reflex and tonic control of sympathetic outflow and blood pressure. (From Dampney, 1994.)

The role of GABA in the control of the SNS

GABA is the major inhibitory neurotransmitter in the RVLM (38). It plays an important role in control of sympathetic outflow (22). GABA is synthesized from glutamate via glutamic acid decarboxylase (GAD) 65 or GAD67. The actions of GABA are mediated via $GABA_A$ and $GABA_B$ receptors in the RVLM (16; 81; 90; 106; 114). $GABA_A$ receptors are fast acting ligand-gated chloride channel receptors while $GABA_B$ receptors are G-protein coupled receptors (68). The receptor that is most important for inhibitory signaling in the RVLM is the $GABA_A$ receptor (16; 24; 81; 113; 114). The $GABA_A$ receptors are heteropentametric channels and commonly consist of 2α, 2β and a γ subunit (68). Alteration in the expression of GABA receptors could lead to changes in inhibitory signaling within the RVLM. Hill *et al.* has shown that under different physiological states, different $GABA_A$ receptor subunit expressions are modified in the brain (41). The effect of sedentary conditions on $GABA_A$ receptor expression in the RVLM has not been reported. The α1 subunit of the $GABA_A$ receptor is in the RVLM and therefore may play a role in the control of the cardiovascular system (31; 105). *This project investigated whether $GABA_A$ receptor expression in the RVLM was altered by sedentary and physically active conditions.*

Glutamatergic and GABAergic neurotransmission in RVLM

As mentioned, the activity of neurons in the RVLM is controlled by numerous neurotransmitters; however the two most important neurotransmitters are the excitatory and inhibitory neurotransmitters glutamate and GABA respectively (24). Even though GABA tonically inhibits neurons in the RVLM, it is unknown what drives tonic excitation of RVLM neurons involved in blood pressure regulation.

One theory is that the neurons in the RVLM have the ability to generate

spontaneous action potentials. Guyenet's laboratory demonstrated that after intracerebroventricular administration of kynurenate, an ionotropic glutamate receptor blocker, RVLM neurons displayed a pacemaker-like activity via extracellular, single-unit recordings performed *in vivo* (127). From these studies and others performed in neonatal slice preparations, they concluded that spinally projecting RVLM neurons had an intrinsic ability to generate action potentials. In contrast, based on intracellular recordings performed *in vivo* by Lipski, synaptic input was shown to be responsible for the activity of RVLM neurons and was termed by Lipski and colleagues as the network theory (64). Thus, any intrinsic activity of RVLM neurons is likely modulated by extrinsic inputs such as glutamatergic projections from the paraventricular nucleus (PVN) and GABAergic projection from the CVLM and other regions. In order to explain the lack of response to blockade of glutamate receptors, Ito and Sved (52) proposed that glutamate simultaneously drives excitation and inhibition of the RVLM such that when excitatory amino acid (EAA) ionotropic receptors are blocked in the RVLM there is no change in SNA or blood pressure. Evidence supporting this model was provided by subsequent experiments in which the CVLM was inhibited prior to administration of EAA receptor blocker into RVLM. Under these conditions, EAA receptor blockade leads to a decrease in arterial pressure that is significantly lower than resting arterial pressure (52). These data would suggest that glutamate is driving both excitation and inhibition (52).

Keeping these findings in mind, there have been multiple studies that have demonstrated that there is enhanced sympathoexcitation in different CVD models and this enhancement could be due to a variety of mechanisms. First, enhanced sympathoexcitation could be due to increased post synaptic sensitivity to glutamate on

bulbospinal RVLM neurons. Evidence supporting this possibility is evident in physical inactivity and high salt diet models, where SNA responses to exogenous glutamate administration into the RVLM are enhanced (1; 76; 87; 92; 93). These data suggest that there is enhanced excitatory glutamate neurotransmission in the RVLM under certain physiological and pathophysiological conditions. Alternatively, a reduction in inhibitory input could also lead to sympathoexcitation. For example, it has been shown that there is a reduction in GABAergic input from CVLM to RVLM in animal models of hypertension and obesity (4; 46; 73). This information suggests that both enhanced glutamatergic or/and decreased GABAergic neurotransmission in the RVLM could lead to the enhanced sympathoexcitation that has been observed in the sedentary but not the physically active rats.

Our laboratory has shown that sedentary conditions lead to elevated baseline splanchnic SNA (87). SNA and blood pressure are modulated by the RVLM. Multiple laboratories have shown overactivity of the sympathetic nervous system is associated with CVD (55; 57; 59; 77; 87; 99). From the evidence mentioned above, it is clear that there is some mechanism allowing RVLM neurons to be more responsive to excitatory stimulation, thus leading to enhanced sympathoexcitation in CVD models. On the contrary, it has been shown that a high salt diet leads to both enhanced glutamate and GABA responsiveness (1). These data suggest that a high salt diet alone does not lead to chronic activation of the SNS but does lead to the development of a compensatory mechanism that can counterbalance the enhanced sensitivity to glutamate.

Similar results have been reported in our models of physical activity and inactivity. For example, we have demonstrated that not only is there enhanced glutamate sensitivity in the RVLM (87), but that following $GABA_A$ receptor blockade,

sedentary rats have greater increases in lumbar sympathetic nerve activity when compared to exercise trained rats (ExTr) (93). One possible explanation for this result is that there is enhanced GABAergic signaling occurring in response to sedentary conditions when compared to ExTr (93). However, it is also possible that enhance inhibitory inputs to the RVLM mask augmented excitation in sedentary animals. In support of this hypothesis, we have reported that upon disinhibition of the RVLM with bicuculline, a $GABA_A$ receptor antagonist, enhanced sympathoexcitation was observed in sedentary rats versus physically active rats (93). In another study, glutamate was microinjected into the RVLM and pressor responses were not initially different between sedentary and physically active rats (96). Following $GABA_A$ receptor blockade, however, pressor responses were enhanced in the sedentary rats only (96). These data suggests that there is increased GABAergic inhibition on glutamatergic neurons in the RVLM of sedentary animals that restrain excitation by exogenous activation of glutamate receptors. What is not known from this experiment is whether this GABAergic inhibition is due to increase $GABA_A$ receptor input or $GABA_A$ receptor mediated signal transduction on spinally projecting RVLM neurons. *The present study investigated how sedentary conditions may lead to alterations in GABAergic signaling in the RVLM.*

Summary

The health consequences of being sedentary are a major problem worldwide and one of the risk factors for CVD. Since sedentary conditions may lead to enhanced sympathoexcitation along with enhanced GABAergic signaling in RVLM, it is important to understand the mechanisms by which these changes are occurring. By understanding the mechanism, we can help improve therapeutic treatments including

individualizing exercise as a therapy to treat and prevent the development of CVD. *To determine whether sedentary conditions enhance GABAergic signaling in the RVLM via changes in GABA responsiveness, GABA input, or both, the following AIMs will be investigated:*

AIM 1. Determine *in vivo* whether sedentary conditions alter the responsiveness of the RVLM to GABA-mediated inhibition when compared to physically active conditions. We hypothesize that sedentary conditions enhance sympathoinhibitory responses to GABA inhibition of the RVLM.

AIM 2. Determine whether sedentary conditions alter $GABA_A$ receptor protein expression in the RVLM when compared to physically active conditions. We hypothesize that sedentary conditions enhance $GABA_A$ receptor protein expression in the RVLM.

AIM 3. Determine whether sedentary conditions lead to altered baroreceptor independent GABAergic input to RVLM when compared to physically active conditions. We hypothesize that sedentary conditions will lead to enhanced GABAergic input.

CHAPTER 2

THE EFFECT OF SEDENTARY AND PHYSICALLY ACTIVE CONDITIONS ON BLOOD PRESSURE AND SPLANCHNIC SYMPATHETIC NERVE RESPONSES TO GABAERGIC INHIBITION OF THE RVLM

Abstract

A sedentary lifestyle is a major risk factor for cardiovascular disease (CVD). CVD is often associated with enhanced activation of the sympathetic nervous system. The sympathetic nervous system is under tonic and phasic control by the rostral ventrolateral medulla (RVLM). The activity of neurons in the RVLM is modulated by the excitatory neurotransmitter, glutamate and the inhibitory neurotransmitter γ-amino-butyric acid (GABA). The release of GABA is tonically modulated by the arterial baroreceptor reflex. In a previous study, the $GABA_A$ receptor blocker, bicuculline, was bilaterally microinjected into the RVLM of sedentary and treadmill trained rats. Both groups responded with increases in mean arterial pressure (MAP) and lumbar sympathetic neve activity (LSNA). However, sedentary conditions enhanced the increase in LSNA when compare to physically active conditions. From these data we hypothesized two possibilities: 1.) There is more GABAergic inhibition in the RVLM of sedentary rats when compare to physically active rats, or 2.) There is similar GABAergic input suppressing the activity of glutamate sensitive neurons in the RVLM, and once GABAergic input is blocked, the remaining excitation is enhanced in sedentary rats when compared to physically active rats. Therefore, the purpose of this study was to investigate GABA sensitivity in the RVLM of sedentary and physically active rats. We hypothesized that sedentary conditions lead to increased responsiveness to GABA in RVLM when compared to physically active conditions. After chronic wheeling running or physical inactivity for 12-15 weeks, animals were instrumented. In Inactin

anesthetized, sedentary (SED) or physically active (WR) rats, mean arterial pressure (MAP), heart rate (HR) and splanchnic sympathetic nerve activity (SSNA) were recorded during unilateral microinjections of GABA (30 nl, 0.3-600 mM) into the left RVLM. Following GABA injections, the right RVLM was inhibited with muscimol (2 mM, 90 nl) and the GABA injections were repeated. There were no significant differences between SED or WR conditions for MAP, HR and SSNA responses to GABA before contralateral blockade of RVLM. However, after contralateral blockade of RVLM, SED rats had enhanced decreases in MAP to GABA compared to WR rats. In order to determine whether the arterial baroreceptor reflex was playing a role in buffering the GABA responses, we performed a sinoaortic denervation (SAD) on additional SED (n=11) and WR (n=11) rats. GABA microinjections under SAD conditions, prior to or after blockade of the contralateral RVLM caused decreases in MAP and SSNA that were not different between SED and WR rats. Our results suggest that the contralateral RVLM plays an important role in buffering responses to inhibition of the ipsilateral RVLM under sedentary but not physically active conditions. We conclude that a sedentary lifestyle not only leads to enhanced sympathoexcitation but also enhances sympathoinhibition in the RVLM. The enhanced sympathoinhibitory mechanisms may be a compensatory response to offset elevated sympathetic nervous system activity following sedentary conditions.

Introduction

A sedentary lifestyle is a major risk factor for developing cardiovascular disease (CVD) (10; 107; 132). CVD has been linked to elevated sympathetic nervous system (SNS) activity (71; 124). Sympathetic nerve activity (SNA) contributes significantly to resting blood pressure and is regulated in the brain by the rostral ventrolateral medulla

(RVLM) (38). Recently, overactivity of neurons in the RVLM has been proposed to lead to increased SNA and contribute to CVD (1; 46; 87; 93; 136). The mechanisms by which the activity of neurons in the RVLM is altered under conditions that contribute to CVD, like a sedentary lifestyle, are still not completely understood.

Although there are several neurotransmitters in the RVLM (108), most of the activity of the RVLM is controlled by the primary excitatory and inhibitory neurotransmitters glutamate (52; 91) and γ-aminobutyric acid (GABA) (46; 81), respectively. The primary source of tonic inhibition of the RVLM is the caudal ventrolateral medulla (CVLM) (81; 113; 114; 139). It has also been suggested that the RVLM receives inhibitory input from the contralateral RVLM (78) along with other brain regions (12; 19). The CVLM receives tonic excitatory stimulation from the NTS as part of the arterial baroreceptor reflex (113). Tonic activation of GABAergic neurons in the CVLM is due to both baroreceptor-dependent and baroreceptor-independent inputs and leads to tonic release of GABA in the RVLM, which maintains a lower level of resting activity of bulbospinal RVLM neurons (90; 114). The actions of GABA in the RVLM are mediated via $GABA_A$ and $GABA_B$ receptors (5; 16; 40); however, activation of $GABA_A$ receptors provides the predominant inhibition in the RVLM (23; 43; 44; 81; 89; 90; 96). In certain models of hypertension and obesity there is a reduction in GABAergic input from the CVLM (46; 125). It has also been shown that GABA neurotransmission, whether it is due to changes in receptor expression or GABA release, is altered under different physiological states (24; 41; 79; 93; 95). Interestingly, a study done to investigate the role of dietary salt on excitation and inhibition in the RVLM showed that increased salt intake leads to enhanced sensitivity to both glutamate and GABA in the RVLM (1). The data from this study suggests that increased dietary salt does not lead

to enhanced sympathoexcitation.

We have reported previously that $GABA_A$ receptor blockade in the RVLM of sedentary rats or exercise trained rats increases lumbar SNA, with responses being greater in the sedentary rats when compared to the physically active group (93). These data suggest that tonic GABAergic neurotransmission is important for restraining the activity of RVLM neurons in both sedentary and physically active rats, but once the influence of GABAergic input is removed, there is greater excitation remaining in the RVLM of sedentary compared to exercise trained rats (96). Another possibility is that in sedentary animals there is more GABA being released or an enhancement in GABA receptor number or function that restrains the activity of RVLM neurons to a greater extent than in physically active animals. These data demonstrate that tonic GABA input is important for suppressing the activity of the RVLM in order to maintain basal blood pressure and SNA, and that sedentary conditions may enhance GABAergic inhibition as well as glutamatergic excitation. However, the effect of sedentary conditions on GABA sensitivity in the RVLM is unknown. Therefore, the primary purpose of the current study was to determine whether sedentary conditions lead to enhanced GABA sensitivity when compared to active conditions. Since the RVLM is a bilateral structure and microinjections of short acting agents tend to be done unilaterally, it is possible that responses to GABAergic inhibition of one RVLM may be buffered by the baroreceptor reflex acting through the contralateral RVLM (72). Because of the potential buffering influence of the contralateral RVLM, we also looked at GABA sensitivity in the absence of baroreceptor input and the potential influence of the contralateral RVLM. We hypothesized that sedentary conditions would lead to enhanced GABA sensitivity in the RVLM when compared to active rats.

Methods

Animals

Male Sprague-Dawley rats (Harlan Sprague Dawley Inc., Indianapolis, IN; 75-100 g upon arrival) were used. Animals were housed under standard 12-hr dark/light cycle. Physically active rats were housed with a running wheel (Tecniplast, #2154F0105, 34cm in diameter, stainless steel) for 12-15 weeks and sedentary rats were housed without a running wheel for an equivalent period. This model has been validated by our laboratory and others in several previous studies (18; 87; 94; 101). The distances the animals ran were recorded by bicycle computers (Sigma Sport, Olney, IL) and documented daily. Experiments were performed in accordance with National Institutes of Health *Guide for Care and Use of Laboratory Animals* and with the approval of the Wayne State University Institutional Animal Care and Use Committee.

General surgical preparation for experiments

According to methods published previously (87; 93), rats were initially anesthetized with isoflurane (5% induction, 2% maintenance, (Henry Schein, Melville, NY), and femoral arterial and venous catheters were implanted to record arterial blood pressure and infuse drugs, respectively. A tracheostomy was performed for artificial ventilation, an electrode was implanted on the splanchnic nerve, and the brainstem was exposed for microinjections into the RVLM. Splanchnic sympathetic nerve activity (SSNA) was monitored with an audio monitor and oscilloscope. The animals were placed in a stereotaxic apparatus and the long acting anesthetic, Inactin (100mg/kg, i.v., Sigma Aldrich, St. Louis, MO), was infused. Supplements of Inactin (5-10 mg, i.v.) were given as needed to maintain lack of response to paw pinch and corneal stimulation. Isoflurane was then withdrawn. Body temperature and blood gases were monitored and

maintained at normal physiological levels (37°C; pCO_2 35-40 mmHg; pO_2>100 mmHg) by a heating pad and changes in ventilation, respectively.

Brainstem microinjections

All drugs used for microinjections were dissolved in artificial cerebrospinal fluid with the pH adjusted to 7.4. The head was placed into a position to allow the caudal tip of area postrema to be oriented 2.4mm posterior to the interaural line. In order to localize the RVLM, the following ranges of coordinates relative to calamus scriptorius were used: 0.9-1.1 mm rostral and 1.7-2.2 mm lateral to calamus scriptorius, and 3.2-3.6 mm ventral to the dorsal surface of the medulla. Similar to previous studies, the RVLM was identified bilaterally with 10 mM glutamate (30 nl injections) (2; 86; 96). A change in blood pressure of 10-15 mmHg indicated that the pipette was positioned in the region of the RVLM containing bulbospinal neurons involved in control of blood pressure. In addition, injection sites were confirmed postmortem by histological verification (see below). Once the RVLM was identified functionally, triple barrel pipettes containing different concentrations of GABA (0.3, 3, 30, 300 or 600 mM, equivalent to 0.009, 0.09, 0.9, 9 or 18 pmol) were placed into the left RVLM to perform GABA injections of varying concentrations at a fixed volume (30nl).

In order to test whether buffering of sympathetic nerve activity and blood pressure responses to GABA occurred via the contralateral RVLM, the long acting GABA agonist, muscimol (90 nl, 2 mM), was injected into the right RVLM. Following blockade of the contralateral RVLM, GABA injections were repeated on the remaining intact left side, in the same order as before. After completing the GABA microinjections, muscimol (90nl, 2 mM) was microinjected into the left RVLM to block neuronal activity in the remaining intact RVLM. In order to verify histologically that the microinjections were

placed in the RVLM, 2% Chicago Sky Blue 30 nl was microinjected bilaterally into both RVLMs after completion of all protocols.

At the end of the experiment, background noise was determined by injection of ganglionic blockers (hexamethonium 30 µg/kg, i.v. + atropine 0.1mg/kg, i.v.). Animals were then euthanized with Fatal Plus (0.2 ml, i.v., Vortech Pharmaceutical Dearborn, MI) and brains were removed and placed into vials that contained 4% phosphate buffered formalin solution. After post fixation for 5 to 7 days, brains were transferred to 20% sucrose for 24 to 48 hrs. and 30% sucrose for 24 to 48 hrs. The brains were then frozen and cut into 50 µm coronal sections on a cryostat (Thermo Scientific, Walldorf, Germany). The sections were mounted in an alternating fashion onto two sets of gel coated slides. One set of slides was left unstained and the other set stained with neutral red. Both sets were then cover slipped with Permount (Fischer Scientific, Pittsburg, PA) and allowed to dry. Using a bright-field microscope, dye spots were located, and the center of the dye spot was determined along with the spread of the dye. The use of a rat brain atlas helped to verify the anatomical location of the dye (104). The center of the dye injection was represented on a modified diagram from the rat atlas (104).

Sinoaortic denervation (SAD)

In another group of sedentary or physically active rats, SADs were performed similar to other laboratories (47; 83). Following a ventral incision in the neck, the carotid bifurcation was identified. Starting at the common carotid, surrounding nerves were carefully removed. Moving rostrally along the carotid artery, connective tissue and nerves were removed from the carotid bifurcation along with the internal and external carotid. The superior cervical ganglion was also removed and the superior laryngeal

nerve was transected bilaterally. Phenylephrine (1 μg/kg, i.v.) was given before and after SAD to test baroreflex function and verify the completeness of the SAD procedure, respectively (47; 83). A lack of a reflex decrease in heart rate (HR) and SSNA was used as evidence of an effective SAD (29; 117; 118).

Similar to studies in the baroreflex intact animals, the brainstem was exposed in order to locate the RVLM and perform GABA injections (0.3, 3, 30, 300 or 600 mM, equivalent to 0.009, 0.09, 0.9, 9 or 18 pmol) into the left RVLM. Following the first set of GABA injections, neuronal activity in the contralateral, right RVLM was blocked using muscimol (2 mM, 90 nl), and GABA injections were repeated in the remaining intact left RVLM.

Data analysis and statistics

All data from microinjection studies were stored using a computer based data acquisition system (PowerLab, ADInstruments, Colorado Springs, CO) and Chart software (version 6, ADInstruments). Changes in SSNA were expressed as a percentage of baseline and as absolute voltage (millivolts seconds (mV.s) after amplification). In order to determine whether there was a difference in resting SSNA, SSNA was expressed as absolute voltage and baseline levels were compared between groups.

Statistical analysis was performed using Sigma Stat 3.5 (Systat Software Inc, Chicago, IL.) for the two way ANOVA with repeated measures and the Student's t-tests. Statistical Package for the Social Sciences (SPSS, IBM Corporation, New York, New York) was used to perform a three-way ANOVA with repeated measures. Depending on the conditions that were compared (GABA dose; intact vs. contralateral RVLM inhibition; sedentary vs. active) we used a two or three way ANOVA with repeated

measures. An unpaired Student's t-test was used to test for statistical significance between sedentary and physically active rats for baseline MAP, absolute SSNA, HR and organ weights. For the responses to unilateral muscimol injections, an unpaired Student's t-test was also done in order to test whether there was a significant difference between groups for the peak change in MAP, HR, percent change in SSNA and absolute change in SSNA. In order to determine whether GABA dose responses were significantly different between sedentary and physically active rats a General linear model using SPSS was used to determine statistical significance (MAP and SSNA). Data are expressed as mean + SEM and a p value less than 0.05 was deemed significant.

Results

Histology

In rats (n=24), RVLM microinjection sites were verified histologically by 2% Chicago Sky Blue dye (30 nl) similar to our previous studies (1; 87; 96). Figure 2 represents the location of the microinjection sites in the left and right RVLM. Based on the histological analyses, on average microinjection sites were located rostral to the caudal pole of facial nucleus, 95 $\pm$ 48 μm on the right side and 85 $\pm$ 45 μm on the left side. The right and left microinjection sites were not statistically different (P=0.756). Furthermore, the microinjection sites were all centered lateral to the pyramidal tract, medial to the trigeminal tract, and ventral to nucleus ambiguus. Microinjection site locations were not different between sedentary (right 115 $\pm$ 30 μm and left 86 ± 31 μm) and physically active rats (right 75 ± 56 μm and left 73 ± 72 μm) (P=0.716).

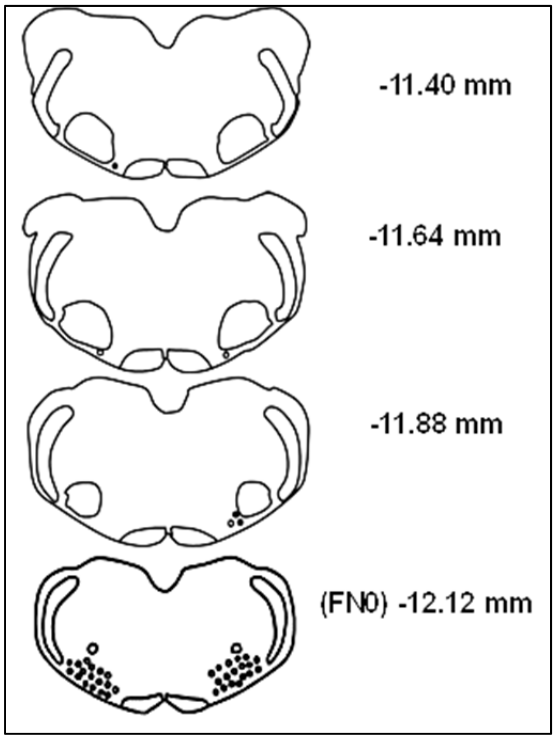

Figure 2. Histological verification of RVLM microinjection sites. Bilateral microinjection sites from sedentary and physically active rats plotted on a modified diagram from a standard rat atlas (Paxinos and Watson, 2007). FN, facial nucleus; NA, nucleus ambiguus; Py, pyramidal tract; SP5 spinal trigeminal tract. Open circles represent physically active rats and closed circles represent sedentary rats.

Baseline blood pressures, heart rates, absolute SSNA, and all organ weights, with the exception of the right adrenals in the intact group, were not significantly different between groups (Table 1 and Table 2). As expected, the sedentary rats had body weights that were significantly greater than the physically active rats. In addition, the weights of the right adrenal gland were significantly greater in physically active rats compared to the sedentary rats. The physically active rats ran 369 ± 46 km total over the entire 12-15 week period.

Table 1. Baseline cardiovascular parameters and body weights in sedentary versus physically active rats

Intact	Resting MAP (mm Hg)	Resting SSNA (mV.s)	Resting HR (bpm)	Body Weight (g)
Sedentary	114 ± 5	1.38 ±0.31	293 ± 9	438 ± 7
Physically active	110 ± 3	1.73 ± 0.33	284 ± 10	397 ± 10*
Pre SAD	Resting MAP (mm Hg)	Resting SSNA mV.s)	Resting HR (bpm)	Body Weight (g)
Sedentary	114± 5	1.36 ± 0.23	310± 9	439 ± 11
Physically active	110±6	0.9 ± 0.25	298± 10	401 ± 12*
Post SAD				
Sedentary	123 ± 5	1.97 ± 0.23	320 ± 9	
Physically active	113 ± 6	1.07 ± 0.25	288 ± 8	

MAP, mean arterial pressure; SSNA, averaged amplified splanchnic sympathetic nerve voltage; SAD, sinoaortic denervation. * $p<0.05$, effect of group.

Table 2. Organ weights from sedentary versus physically active rats

Intact	Left Heart (mg)	Right Heart (mg)	Right Adrenal (mg)	Left Adrenal (mg)	Soleus (mg)
Sedentary	780 ± 67	250 ± 20	23.7 ± 10	42.9 ± 20	140 ± 10
Physically active	810 ± 30	250 ± 20	38.5 ± 10*	27.4 ± 10	150 ± 10
PreSAD	Left Heart (mg)	Right Heart (mg)	Right Adrenal (mg)	Left Adrenal (mg)	Soleus (mg)
Sedentary	818 ± 30	280 ± 20	52.7 ± 18	34.9 ± 3	155 ± 5
Physically active	800 ± 30	316 ± 20	36.1 ± 2	38.6 ± 3	153 ± 5

SAD, sinoaortic denervation; mg, milligrams

The effect of sedentary conditions on GABA sensitivity in the RVLM when compared to physically active conditions

Unilateral microinjections of GABA in baroreceptor intact animals produced decreases in mean arterial pressure (MAP) and splanchnic sympathetic nerve activity (SSNA) in both groups of animals (Figure 3A). Changes in MAP and absolute SSNA and percent change SSNA decreased in a dose dependent manner in response to increasing doses of GABA ($p<0.05$, main effect of dose). Changes in heart rate in response to GABA microinjections were small (<11 bpm) and variable (data not shown). Despite the dose--dependent effects of GABA on MAP and SSNA, there were no significant differences in responses to increasing concentrations of GABA between sedentary and active groups for MAP or absolute or % change SSNA (Figure 3A) in the baroreceptor intact state.

Following GABA microinjections in intact animals, the contralateral RVLM was inhibited with muscimol. Muscimol into the right RVLM produced large decreases in MAP, SSNA and absolute SSNA in sedentary and physically active rats (Figure 4).

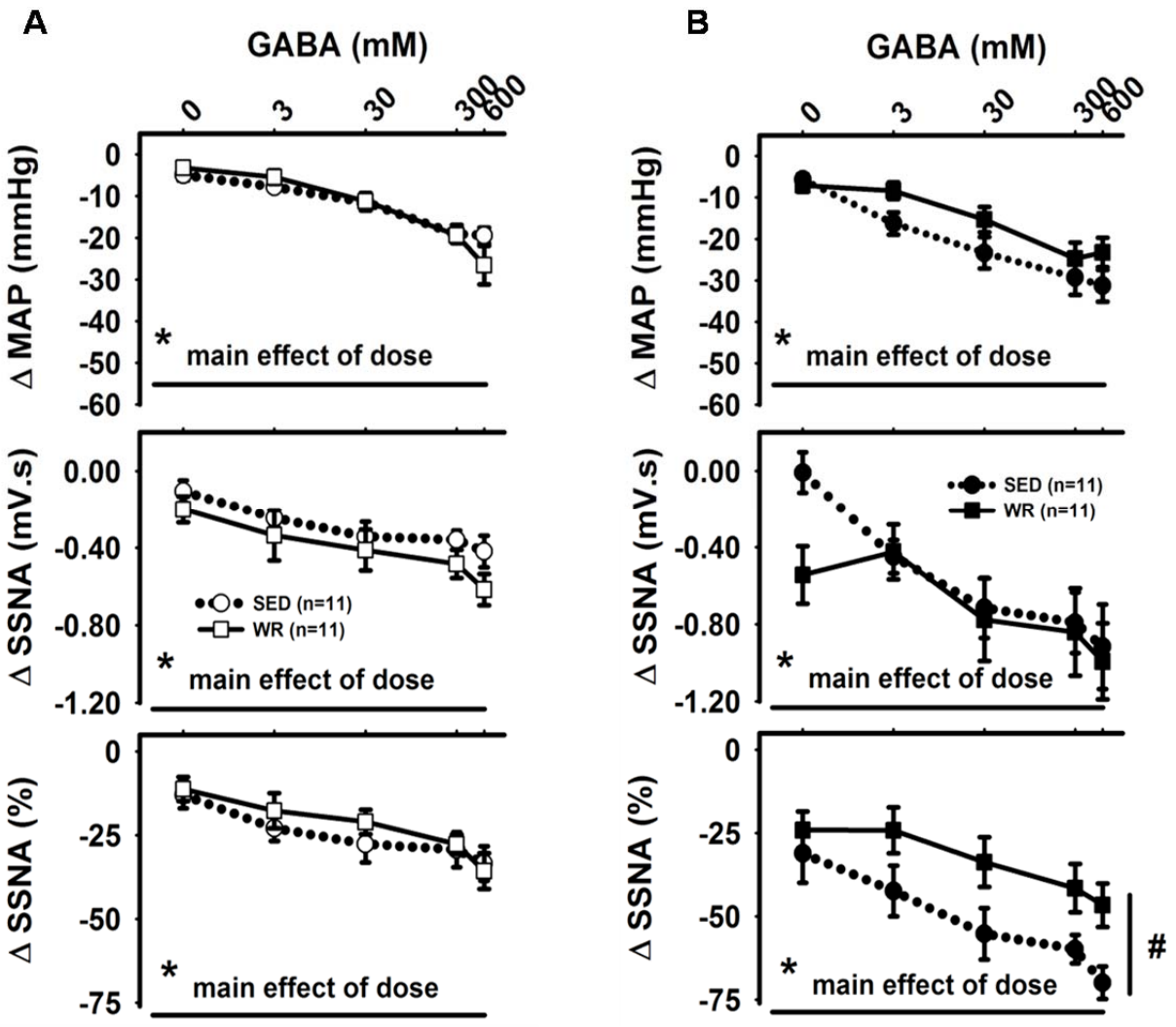

Figure 3. Peak mean arterial pressure (MAP), absolute splanchnic sympathetic nerve activity (SSNA), and percent change in SSNA responses to inhibition of the rostral ventrolateral medulla (RVLM) with γ-aminobutyric acid (GABA) in the absence (A) and presence (B) of unilateral RVLM blockade. Figure 3A represents GABA responses before inhibition of the contralateral RVLM. Figure 3B represents GABA (30 nl, at 0.3, 3, 30, 300, 600mM) responses after inhibition of the contralateral RVLM with the long acting $GABA_A$ receptor agonist muscimol (2mM, 90 nl). Sedentary and physically active rats had similar decreases in MAP and absolute SSNA and percent changes in SSNA before contralateral RVLM blockade. Following blockade of the contralateral RVLM, sedentary and physically active rats had similar decreased in MAP and absolute SSNA in response to acute inhibition of RVLM with GABA ($p>0.05$). However, the sedentary rats had significantly larger decreases in percent change in SSNA in response to GABA when compared to physically active rats ($p<0.05$). *, $p<0.05$ main effect of dose. #, $p<0.05$ group effect.

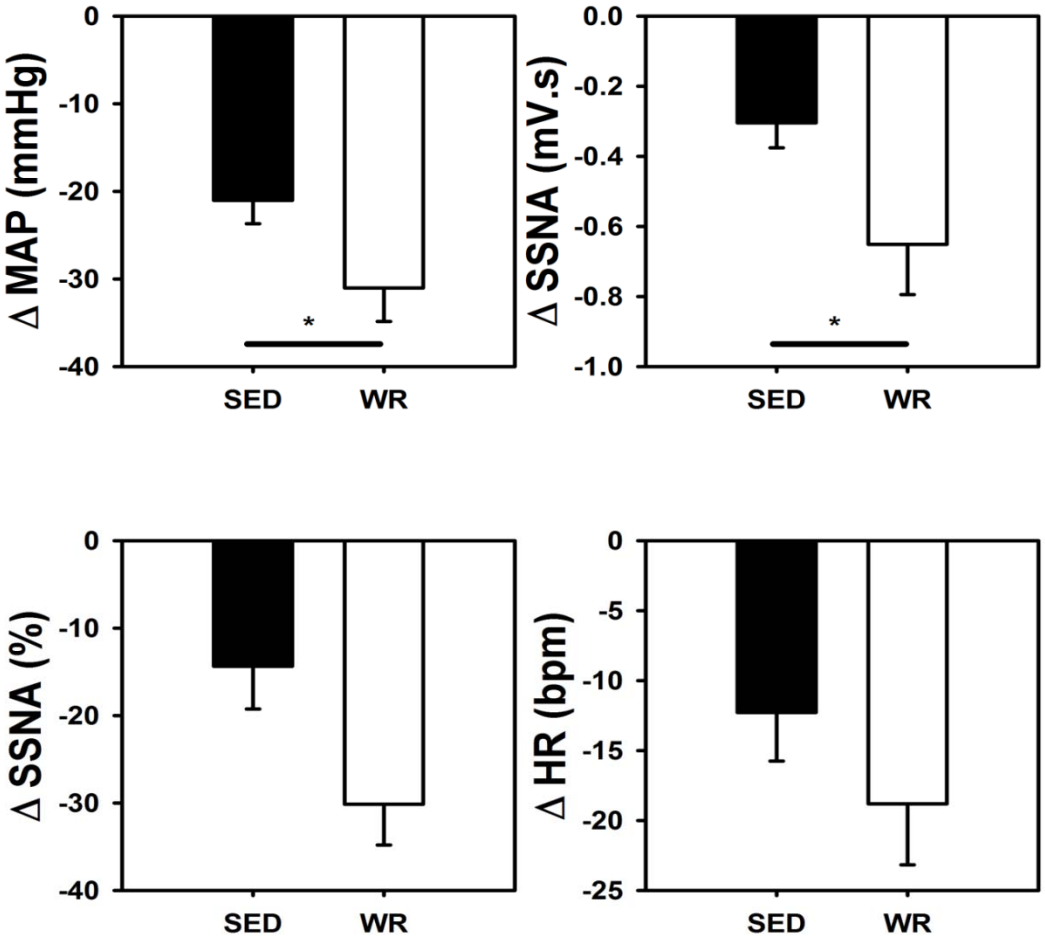

Figure 4. Peak change in MAP, absolute SSNA, percent SSNA and HR in response to contralateral RVLM blockade with muscimol (2 mM, 90 nl). Sedentary and physically active rats had similar decreases for percent change in SSNA and HR to muscimol. However, the physically active group had greater depressor responses and decreases in absolute changes in SSNA in response to muscimol. *p<0.05 when compared to sedentary conditions based on results from unpaired t-test.

However the decreases in MAP, and absolute SSNA were significantly attenuated in the sedentary rats when compared to physically active rats (MAP -21±3 and -31±4 mmHg, p=0.044; abs SSNA -0.3±0.07 and -0.65±0.14 p=0.043, respectively). GABA microinjections were repeated into the remaining intact RVLM and produced decreases in MAP and absolute and percent change SSNA that were dose dependent (Figure 3B; p<0.05, main effect of dose for all parameters).

In contrast to intact conditions, percent changes in SSNA to increasing concentrations of GABA in the remaining, intact RVLM were greater in sedentary rats when compared to active rats following contralateral RVLM blockade with muscimol (Figure 3B)($p<0.05$). There were no significant differences between sedentary and physically active rats for the peak decreases in MAP or absolute SSNA.

The effect of sedentary versus physically active conditions on GABA sensitivity in the absence of baroreceptor reflex function

We investigated responses to GABA administration in the RVLM in the absence of arterial baroreceptor input by performing SADs in additional groups of sedentary and physically active rats (Sed n=11, WR n=10). We verified the loss of baroreflex responsiveness after SAD by examining responses to phenylephrine (1µg/kg), similar to others laboratories (47; 83). Prior to SAD, pressor and baroreflex-mediated sympathoinhibitory response to phenylephrine were comparable between sedentary and physically active rats (Figure 5; open symbols). After SAD, phenylephrine injections lead to an increase blood pressure that was accompanied by no change in SSNA in both sedentary and physically active rats (Figure 5; closed symbols).

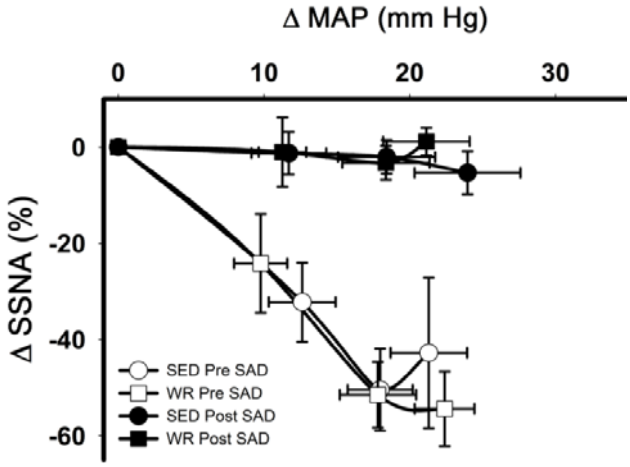

Figure 5. Verification of sinoaortic denervation using bolus i.v. injections of phenylephrine (1 µg/kg). The change in MAP and the change in SSNA in SEDs (n=6) and WRs (n=4) in response to 1µg/kg phenylephrine challenge was not significantly different. There was a trend for a difference in pre vs post SAD Δ SSNA (p=0.059).

The resting blood pressure and SSNA was not different between sedentary and physically active rats (Table 1). GABA microinjections into the RVLM produced decreases in MAP, percent change in SSNA, and absolute change SSNA (Figure 6A). Similar to the experiments in the animals that had intact baroreceptor function,

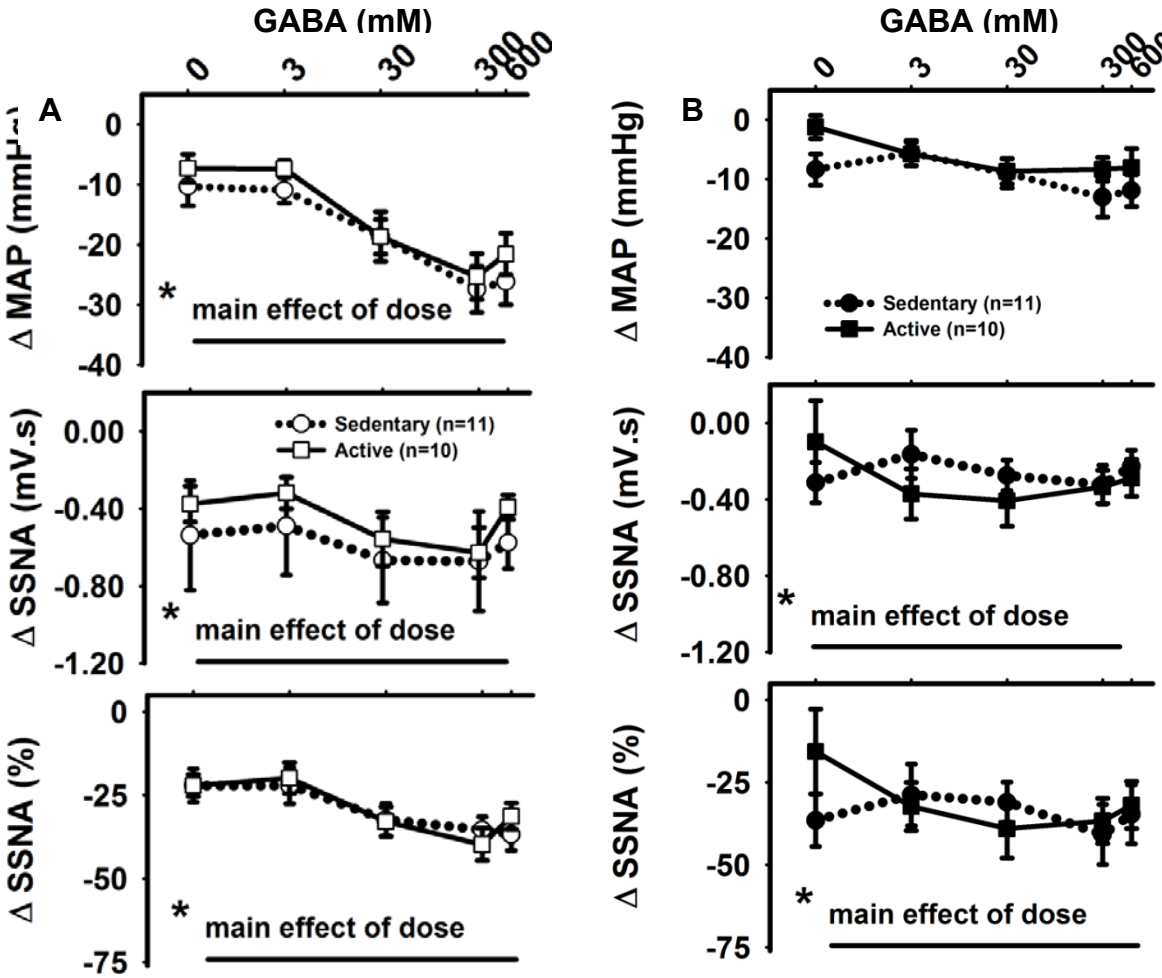

Figure 6. Peak changes in MAP, absolute SSNA, and percent SSNA in response to inhibition of RVLM with GABA in the absence (A) and presence (B) of unilateral RVLM blockade after sinoaortic denervation (SAD). Figure 6A represents GABA (30 nl, at 0.3, 3, 30, 300, 600mM) responses before inhibition of the contralateral RVLM with long acting $GABA_A$ receptor agonist Muscimol (2mM, 90 nl). SAD sedentary and physically active rats had similar decreases in MAP and absolute SSNA and percent change in SSNA before contralateral RVLM blockade ($p>0.05$). Figure 6B represents GABA (30 nl, at 0.3, 3, 30, 300, 600mM) responses after inhibition of the contralateral RVLM. Following blockade of RVLM, SAD sedentary and physically active rats had similar decreases in MAP, absolute SSNA and percent change in SSNA in response to acute inhibition of RVLM with GABA ($p>0.05$).*$p<0.05$ main effect of dose.

microinjections of increasing doses of GABA produced a dose dependent decrease in MAP and percent change and absolute change in SSNA ($p<0.05$; main effect of dose) (Figure 6A). The depressor and sympathoinhibitory responses to increasing concentrations of GABA were not different between sedentary and physically active rats (Figure 6A).

Subsequent microinjections of muscimol in the contralateral RVLM resulted in a decrease in MAP, HR, and SSNA in sedentary and physically active rats (Figure 7). The decreases in MAP, percent SSNA, and absolute SSNA were not different between the sedentary and the physically active rats (Figure 7). Following contralateral blockade

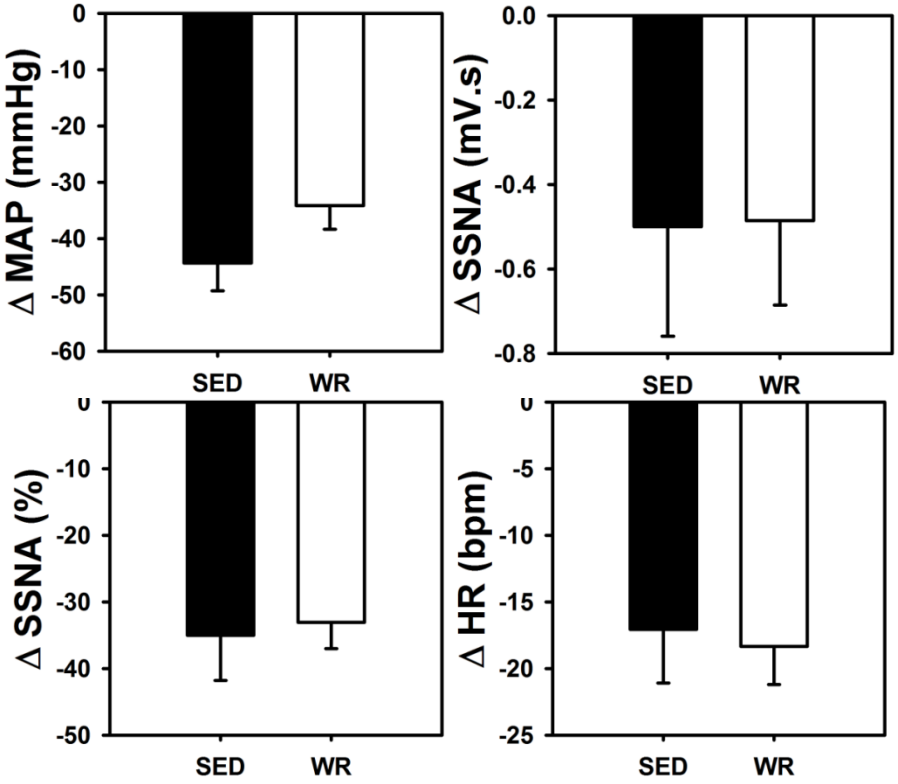

Figure 7. Peak change in MAP, absolute SSNA, percent SSNA and HR in response to contralateral RVLM blockade in sinoaortic denervated rats. Sedentary and physically active rats had similar decreases in MAP, percent change in SSNA, absolute change in SSNA and HR to muscimol (2 mM, 90 nl)($p>0.05$).

of RVLM, GABA microinjections into the remaining intact RVLM produced little change in MAP, but it did produce a dose dependent decrease for absolute SSNA and percent change in SSNA (Figure 6B). There was no difference between sedentary and physically active rats to GABA following unilateral RVLM blockade for MAP, absolute change in SSNA and percent change for SSNA.

Discussion

The purposes of this study were 1) to determine whether sedentary conditions lead to enhanced GABA sensitivity in the RVLM when compared to active conditions and 2) to determine if the arterial baroreflex is buffering responses to unilateral microinjections of GABA differently in sedentary versus physically active rats. The enhanced response to GABA after contralateral blockade of the RVLM could be explained by increased buffering via the baroreflex, the recently described inhibitory projection from the contralateral RVLM, or an interaction between both (81). In order to determine the mechanism by which unilateral RVLM inhibition leads to augmented GABA responses in sedentary rats, we performed additional experiments in SAD animals. Following SAD, the enhanced response to GABA observed after unilateral RVLM blockade in baroreflex intact animals was abolished. From these data we conclude that under sedentary conditions the baroreceptor reflex compensates decreases in sympathetic outflow in response to acute inhibition of the RVLM. Enhanced buffering may serve as a compensatory mechanism in order to counteract the developing sympathoexcitation.

The data from this study suggests that sedentary conditions lead to the development of a compensatory mechanism that involves the arterial baroreceptor reflex. DiCarlo and others demonstrated that exercise training leads to attenuated

baroreflex mediated sympathoexcitation when compared to sedentary rats (20; 26; 100). Similarly, Mischel and Mueller demonstrated that chronic, spontaneous wheel running in rats led to attenuated baroreceptor reflex mediated sympathoexcitation (87). Taking into consideration the data from these previous studies, we suggest that the enhanced response to GABA under sedentary conditions may be masked by baroreceptor reflex mediated buffering from the remaining RVLM in the intact state. This compensatory response could also involve the recently described commissural connections between the right and left RVLMs (78; 135). In the present study, we found that following unilateral RVLM blockade, the sedentary rats had enhanced sympathoinhibition in response to increasing doses of GABA when compared to the physically active group. In SAD animals, we observed no difference between sedentary and wheel running rats in response to GABA microinjections alone, whether the contralateral RVLM was inhibited or not. These findings suggest that in the sedentary group the baroreceptor reflex appears to compensate for the change in splanchnic sympathetic nerve activity whereas the physically active animals seem to lack this compensation. However, the role of the contralateral RVLM inhibitory projection cannot be completely ruled out (135). Although the physiological relevance of this connection still needs to be determined, McMullan and colleagues (78) showed that this connection is predominately inhibitory.

In order to test whether this commissural connection could be involved in the buffering of unilateral GABA microinjections, the contralateral RVLM was inhibited with the long acting GABA agonist muscimol. Similar to other studies performed in intact rabbits (42) and cats (28), unilateral RVLM inhibition in the present study produced only a modest fall in BP in intact animals. In the same study done by Dampney's group (42),

sinoaortic denervated rabbits had dramatic decreases in blood pressure and renal sympathetic nerve activity upon inhibition of one RVLM. These data suggest that the baroreceptor reflex is important to help maintain resting SNA and blood pressure via the remaining intact RVLM. We observed a similarly large fall in blood pressure and SSNA in both SAD groups when one RVLM was inhibited with muscimol. This finding, confirmed in rats in the present study, further demonstrates the importance of the baroreceptor reflex in maintaining blood pressure via the remaining intact RVLM.

Following inhibition of one RVLM in SAD animals, GABA sensitivity was not different between sedentary and wheel running rats. The simplest interpretation of the GABA dose response data would suggest that the commissural projection between the right and left RVLM may not play an obligatory role since inhibition of the right RVLM in barodenervated animals did not reveal a difference between groups. However, if the commissural pathway is dependent on baroreceptor input then our experimental design could have precluded us from observing an effect of altered commissural input in sedentary versus wheel running conditions. Unfortunately, we are unaware of a technique that would allow us to specifically test the influence of the commissural pathway on the regulation of sympathetic outflow and blood pressure responses to GABA in the absence of having an impact on arterial baroreflex pathways.

In the present study we found that intact sedentary and physically active rats have similar GABA responsiveness. Following the blockade of the contralateral RVLM sedentary rats display an increased responsiveness to GABA when compared to physically active rats. As mentioned above, this could be due to the removal of baroreceptor input that may be buffering GABA responses in the sedentary rat but not in the physically active rat. However, it may be possible that GABA receptor expression

and/or GABAergic inputs may be enhanced in the sedentary rat when compared to the physically active rat. In Chapter 3 and 4 of this dissertation, we further investigate how sedentary conditions could lead to changes in GABA input and receptor expression, respectively. A possible mechanism that could be causing enhanced GABA responses in the sedentary when compared to the physically active rats is increased GABA receptor expression. In contrast the results observed in our study may be independent of changes in $GABA_A$ receptor expression. In pregnant and non-pregnant rats, Foley *et al*. have shown that RVLM neurons had a greater level of protein and mRNA expression of $GABA_A$ α1 subunit when compared to the $GABA_A$ α2 protein and mRNA expression (30). However, there was no difference between pregnant and non-pregnant rats in their level of protein and mRNA expression of $GABA_A$ receptor subunits. If there is no difference between the sedentary and physically active rats in the number of $GABA_A$ receptors, there may be a difference in subunit composition (35). The enhanced response to GABA observed following unilateral RVLM in the sedentary could be due to enhanced GABAergic input or enhanced GABA receptor expression in RVLM. More studies are necessary in order to determine whether GABA receptor expression and/or GABAergic inputs are altered by sedentary conditions. Chapter 4 will investigate whether $GABA_A$ receptor expression is enhanced by sedentary conditions. Furthermore, we wanted to investigate whether $GABA_A$ receptor expression on spinally projecting neurons in the RVLM involved in the control of splanchnic sympathetic outflow was being enhanced by sedentary conditions.

Perspectives

Physical inactivity has been shown to increase the risk of developing cardiovascular disease (CVD) (121). Similar to a number of other studies, our data

suggests that there is a central nervous system component to the development of CVD (46; 46; 56; 56; 87; 87; 123). These alterations affect regions that directly or indirectly have control over sympathetic outflow (9; 13; 46; 56; 87). In our other studies, we tended to focus on the rostral ventrolateral medulla (RVLM) because it is the major source of control over the sympathetic nervous system (24). Past data from our lab has demonstrated enhanced sympathoexcitation in our sedentary model (87). Our data from the present study demonstrate that sedentary conditions enhance GABAergic modulation of the RVLM via an interaction with the arterial baroreceptor reflex. The enhanced inhibition may be a compensatory mechanism to offset enhanced sympathoexcitation that occurs via glutamatergic mechanisms as demonstrated in our previous studies (87; 96). However, this compensatory mechanism is not enough to completely correct the enhanced sympathoexcitation that is taking place since in previous studies have shown that enhanced sympathoexcitation still develops in response to sedentary conditions (87; 96). Therefore a net increase in excitation in RVLM can lead to higher sympathetic outflow and blood pressure, and ultimately increases the risk for developing cardiovascular disease. This net increase in excitation in the RVLM may be due to glutamate to some extent, and an increase in the responsiveness to direct glutamate excitation of the RVLM has been shown by our laboratory previously (87). However, there could be other sources of excitatory drive to RVLM under sedentary conditions that are enhanced. Chapter 3 investigates how much of the excitation that occurs following removal of all GABAergic input to the RVLM is due to glutamatergic versus non-glutamatergic input, and whether these different sources of excitatory input are altered by sedentary versus physically active conditions.

CHAPTER 3

IONOTROPIC GLUTAMATE RECEPTORS PARTIALLY MEDIATE THE PRESSOR AND SYMPATHOEXCITATION TO DISINHIBITION OF THE RVLM AFTER SINOAORTIC DENERVATION AND CONTRALATERAL RVLM INHIBITION IN SEDENTARY AND PHYSICALLY ACTIVE RATS

Abstract

Cardiovascular disease (CVD) is a major health problem globally. A major behavioral risk factor for developing CVD is a sedentary lifestyle. Our laboratory has shown that there is elevated splanchnic sympathetic nerve activity following sedentary versus physically active conditions. This elevated sympathetic outflow may be contributing to the development of CVD in individuals leading sedentary lifestyles. Sympathetic outflow is tonically controlled by the rostral ventrolateral medulla (RVLM). The RVLM is a bilateral region in the brainstem important for sympathetic control of the cardiovascular system. Although both right and left RVLMs are tonically active, their activity is restrained by ongoing γ-amino-butyric acid (GABA) mediated inhibition, in part due to pathways originating from the arterial baroreceptors. In the absence of baroreceptor input, blockade of activity in only one RVLM dramatically decreases mean arterial pressure (MAP) and renal sympathetic nerve activity (SNA), demonstrating the importance of the baroreflex in maintaining blood pressure via the remaining RVLM. Also under barodenervated conditions, a significant remaining GABAergic inhibition may be responsible for low AP and SNA observed rather than a loss of excitatory input. In addition, it is unknown if this input is altered following sedentary versus physically active conditions. The current study was aimed at testing the hypothesis that following removal of barosensitive and non-barosensitive GABAergic input to RVLM, there is sympathoexcitation that is enhanced under sedentary conditions when compared to

physically active conditions. Specifically, we tested the hypotheses that 1) ionotropic glutamate receptors contribute to increases in MAP and SSNA in response to $GABA_A$ receptor blockade, and 2) sedentary conditions enhance endogenous glutamatergic excitation. After chronic wheeling running or physical inactivity for 12-15 weeks, animals were instrumented to perform microinjections in Inactin-anesthetized, barodenervated sedentary (SED; n=7) and physically active (wheel running, WR; n=9) rats in which the right RVLM was already inhibited with muscimol (2 mM, 90nl), the long acting $GABA_A$ agonist. In response to muscimol both groups had dramatic decreases AP and SSNA. Responses to blockade of $GABA_A$ receptors with bicuculline (Bic 5 mM, 90nl) in the remaining intact, left RVLM were tested in the presence and absence of the ionotropic glutamate receptor blocker, kynurenic acid (Kyn 40 mM, 90nl). $GABA_A$ receptor blockade alone increased MAP and splanchnic SNA (SSNA) in SEDs and WRs equivalently. Pretreatment with Kyn attenuated increases in MAP and absolute change in SSNA to Bic in both SEDs and WRs. Sedentary conditions did not enhance the MAP and SSNA response to Bic in the presence of Kyn. Our data suggests that endogenous GABA is restraining the activity of RVLM neurons that are glutamate sensitive. Furthermore, other excitatory neurotransmitters seem to be playing a role in driving the activity of RVLM neuron in the absence of GABAergic input. We conclude that in the absence of barosensitive and non-barosensitive GABAergic input, the residual sympathoexcitation appears to be due to excitatory input which is partially dependent and partially independent of ionotropic glutamatergic receptors.

Introduction

A sedentary lifestyle is a major risk factor for developing cardiovascular disease (CVD). CVD has been associated with elevated sympathetic nerve activity (71; 142).

Alteration in sympathetic outflow could be occurring as a result of leading a sedentary lifestyle.

The rostral ventrolateral medulla (RVLM) is an important brainstem region involved in the control of sympathetic outflow (38). We have proposed that sedentary conditions lead to alterations in the RVLM that cause enhanced sympathetic nerve activity and ultimately may lead to the development of CVD (88). The RVLM receives multiple inputs that alter the activity of neurons in this region based on physiological needs (113). The arterial baroreceptor reflex is an example of a tonically active, inhibitory input to the RVLM that has been shown to be altered by physical inactivity (26; 100). Our laboratory and others have shown that sedentary conditions lead to enhanced sympathoexcitatory responses to a hypotensive challenge (i.e. unloading of the arterial baroreflex) (17; 26; 87; 100). However, the mechanism that leads to the development of this enhanced sympathoexcitation is still not completely understood.

The activity of neurons in the RVLM is regulated by the inhibitory neurotransmitter γ-amino butyric acid (GABA) and the excitatory neurotransmitter L-glutamate (53; 63; 81; 113). Other neurotransmitters have been shown to play a role in modulating the activity of RVLM neurons but GABA and glutamate are the primary neurotransmitters involved with regulating the activity of RVLM (2; 6; 15; 24). In a recent study from our laboratory, we confirmed previous reports that demonstrated that tonically active GABAergic input is important for suppressing the activity of the RVLM in order to maintain basal blood pressure and sympathetic nerve activity (23; 80; 89; 93; 96; 114; 131). Once this GABAergic input was removed, however, the blood pressure response to glutamate microinjection in the RVLM was enhanced in sedentary but not physically active rats (96). We concluded from these data that sedentary conditions a

tonic GABAergic inhibition of glutamate sensitive neurons in the RVLM of sedentary but not physically active rats (96). The mechanism responsible for the enhanced GABAergic drive to glutamate sensitive neurons in the RVLM of sedentary animals remains to be elucidated.

The CVLM provides GABAergic input to the RVLM that is both arterial baroreceptor-dependent and arterial baroreceptor-independent (21; 97). In disease models such as obesity and hypertension, it has been proposed that changes in GABA transmission in the RVLM may lead to enhanced sympathoexcitation (46; 120). A previous study by Morrison's laboratory has shown that in sinoaortic denervated rats, lesioning the CVLM still leads to increases in blood pressure and SSNA (21; 97). By definition, all arterial baroreceptor mediated GABA release has been eliminated by the sinoaortic denervation, the only GABA available (if there is any) to inhibit RVLM is GABA that is independent of the arterial baroreceptors (21) . These data demonstrate that GABAergic input that is *independent* of the baroreceptor reflex contributes to tonic inhibition of RVLM activity along with GABAergic input that arises as a result of the arterial baroreceptor reflex. To our knowledge no studies have been done to investigate tonic levels of both excitation and inhibition in the absence of baroreceptor-dependent GABAergic input and investigated these levels in models of physical activity and inactivity.

The purpose of this study was to address whether the pressor and sympathoexcitatory response to $GABA_A$ receptor blockade in the RVLM of barodenervated rats was due to activation of ionotropic glutamate receptors. We also wanted to investigate whether sedentary conditions enhance the contribution of ionotropic glutamate receptors to the pressor and sympathoexcitatory responses to

$GABA_A$ receptor blockade in the RVLM of barodenervated rats. Our hypotheses were: 1.) Ionotropic glutamate receptors (iGluRs) contribute to pressor and increased splanchnic sympathetic nerve activity (SSNA) responses to blockade of baroreceptor-independent GABAergic input to the RVLM. 2.) Sedentary conditions enhance ionotropic glutamatergic excitation of the RVLM independent of baroreceptor-dependent input.

Methods

Animals

Male Sprague-Dawley rats (Harlan Sprague Dawley Inc., Indianapolis, IN; 75-100 g upon arrival) were used for these studies. Animals were housed under standard 12-hr dark/light cycle. Physically active rats (n=9) were housed with a running wheel (Tecniplast, #2154F0105, 34cm in diameter, stainless steel) for 12-14 weeks and sedentary rats (n=7) were housed without a running wheel for an equivalent period. This model has been validated by our laboratory and others in several previous studies (7; 17; 87; 94; 101). The distances the animals ran were recorded by bicycle computers (Sigma Sport, Olney, IL) and documented daily. Experiments were performed in accordance with National Institutes of Health *Guide for Care and Use of Laboratory Animals* and with the approval of the Wayne State University Institutional Animal Care and Use Committee.

General Surgical preparation for experiments

According to methods published previously (87; 93), rats were initially anesthetized with isoflurane (5% induction, 2% maintenance) and femoral arterial and venous catheters were implanted to record arterial blood pressure and infuse drugs, respectively. A tracheostomy was performed for artificial ventilation; the brainstem was

exposed for microinjections into RVLM; and an electrode was implanted on the splanchnic nerve. Splanchnic sympathetic nerve activity (SSNA) was monitored with an audio monitor and oscilloscope. Body temperature and blood gases were monitored and maintained at normal physiological levels (37°C; pCO_2 <40 mmHg; pO_2>100 mmHg) by a heating pad and changes in ventilation, respectively.

Sinoaortic denervation (SAD)

Prior to the SAD, the long acting anesthetic Inactin (100mg/kg, i.v.) was infused. Supplements of Inactin (5-10 mg, i.v.) were given as needed to maintain lack of response to paw pinch and corneal stimulation. Isoflurane was then withdrawn. In sedentary or physically active rats, SADs were performed similar to other laboratories (47). Following a ventral incision in the neck, the carotid bifurcation was identified. Starting at the common carotid, surrounding nerves were carefully removed. Moving rostrally along the carotid artery, connective tissue and nerves were removed from the carotid bifurcation along the internal and external carotid. The superior cervical ganglion was also removed and the superior laryngeal nerve was transected bilaterally. Phenylephrine (1 ug/kg, i.v.) was given before and after SAD to test baroreflex function and verify SAD, respectively (47; 83). A lack of a reflex decrease in heart rate (HR) and splanchnic sympathetic nerve activity (SSNA) was used as evidence of an effective SAD (29; 118; 119).

Brainstem Microinjections

Similar to previous studies (87), the animals were placed in a stereotaxic apparatus and the RVLM was identified bilaterally with 10 mM glutamate (30 nl injections) using standard stereotaxic coordinates. The head was placed into the stereotax and the caudal tip of area postrema was positioned 2.4 mm posterior to the

interaural line. In order to find RVLM, the calamus scriptorius was used as a reference point and the following coordinates were used to locate the RVLM: 0.9-1.1 mm rostral and 1.7-2.2 mm lateral to calamus scriptorius, and 3.2-3.6 mm ventral to the dorsal surface of the medulla. In order to functionally verify the pipette was in the RVLM, a pipette containing 10 mM glutamate was positioned in the RVLM and a change in blood pressure of 10-15 mmHg indicated that the pipette was in the RVLM. In addition, injection sites were confirmed postmortem by histological verification (see below). Once the RVLM was identified, a single barrel pipette containing muscimol (90 nl, 2 mM), the long acting GABA agonist, was inserted into the right RVLM in order to block neuronal activity. The concentration and volume of muscimol used in this study was similar to others (33; 46; 49; 51; 114). Following blockade of the right RVLM with muscimol, bicuculline (Bic) (90 nl, 5 mM), the $GABA_A$ receptor antagonist was microinjected on the left side. The concentration and volume of Bic used in this study was similar to other studies (32; 52; 109). After a 45 minute recovery period, kynurenic acid (Kyn) (90 nl, 40 mM), the ionotropic glutamate receptor antagonist, was injected into the left RVLM. The concentration and volume of Kyn used was similar to others (58; 114). After 10 minutes Bic (90 nl, 5 mM) was microinjected into the left RVLM. After 45 minutes of recovery, muscimol (90 nl, 2 mM) was microinjected into the left RVLM in order to block neuronal activity. In order to verify that the microinjections were placed in RVLM, 2% Chicago Sky Blue 30 nl was microinjected bilaterally into RVLM after completion of all protocols. At the end of the experiment, background noise was determined by injection of ganglionic blockers (hexamethonium 30 µg/kg, i.v. + atropine 0.1 mg/kg, i.v.). Animals were then euthanized with Fatal Plus (0.2 ml, i.v., Vortech Pharmaceutical Dearborn, MI) and the brain was removed and placed into 4% formaldehyde. This tissue was

allowed to post fix for one week. After post fixation for five to seven days, brains were transferred to 20% sucrose for 24 to 48 hrs. and then 30% sucrose for 24 to 48 hrs. The brains were then frozen and cut into 50 μm coronal sections on a cryostat. The sections were mounted in an alternating fashion onto two sets of gel coated slides. One set of slides was left unstained and the other set was stained with neutral red (Sigma, St. Louis, MO). Both sets were then cover slipped with Permount (Fischer Scientific, Pittsburg, PA) and allowed to dry. Using a bright-field microscope, dye spots were located, and the center of the dye spot was determined along with the spread of the dye. The use of a rat brain atlas helped to verify the anatomical location of the dye (104). The dye was represented on a modified diagram from the rat atlas (104). All drugs used for microinjections were dissolved in artificial cerebrospinal fluid with the pH adjusted to 7.4.

Data analysis and statistics

All data from microinjection studies were stored using a computer based data acquisition system (PowerLab, ADInstruments, Colorado Springs, CO). Changes in SSNA were expressed as a percentage of baseline and as absolute voltage (mV.s after amplification). In order to determine whether there was a difference between sedentary and physically active rats for baseline levels of SSNA, SSNA was expressed as absolute voltage. Statistical analysis was performed using Sigma Stat 3.5 (SigmaStat 3.5, SPSS, Chicago, IL). We used two way ANOVA with repeated measures. When there was a significant interaction ($P< 0.05$), we used Holm-Sidak for the post hoc multiple comparisons. Student's t-test was used in order to determine if there were differences in organ weights between sedentary and physically active rats. Data are expressed as mean $\pm$ SEM and a p value less than 0.05 was deemed significant.

Results

Prior to the surgery, all animals were weighed in order to give the appropriate amount of anesthesia. Sedentary rats on average, weighed more than the physically active rats (438±12 vs. 390±9 grams, respectively; $P < 0.05$). Resting blood pressure, splanchnic sympathetic nerve activity and heart rate were similar between sedentary and physically active rats (Table 3). In order to verify that all microinjections were done in RVLM, 2% Chicago sky blue was microinjected at the end of all experiments. The average dye locations for both group for RVLM were -114 ± 174 μm on the right side and 85 ± 177 μm on the left side in reference to the caudal pole of facial motor nucleus in all the rats (n=7). There was no main effect between left and right sided injections

Table 3. Body weights and baseline cardiovascular measures in sedentary versus physically active rats.

Pre SAD Baselines		Sedentary	Physically active
	Body weight (g)	438±12	390±9 *
	MAP (mm Hg)	102±8	108±8
	SSNA (mV.s)	1.2±0.3	1.4±0.4
	HR (bpm)	325±8	321±14
SAD Pre Muscimol/ Post SAD Baselines	MAP (mm Hg)	116±6	113±8
	SSNA (mV.s)	2.0±0.8	0.9±0.1
	HR (bpm)	310±11	314±8
SAD Post Muscimol	MAP (mm Hg)	72±4‡	77±9‡
	SSNA (mV.s)	1.0±0.4‡	0.6±0.1‡
	HR (bpm)	289±9‡	293±10‡
Δ Muscimol responses	Δ MAP (mm Hg)	-43±7	-35±4
	ΔSSNA (%)	-44±8	-39±4
	ΔSSNA (mV.s)	-0.9±0.4	-0.3±0.2
	Δ HR (bpm)	-20±5	-21±5

(P=0.109, data not shown). There was also no main effect of the location of the dye spot between sedentary (right side -575 ± 100 μm and left side -150 ± 100 μm) and wheel running rats (right side 30 ± 174 μm and left side 180 ± 210 μm) (P= 0.203). Figure 8

represents the dye spots from 7 animals used in this study. All injection sites were located medial to the spinal trigeminal tract, lateral to the pyramidal tract and ventral to nucleus ambiguus, consistent with the location of the RVLM as mentioned in previous studies from our lab (87; 96). The physically active rats ran 283 ± 65 km total over the 12-14 week period.

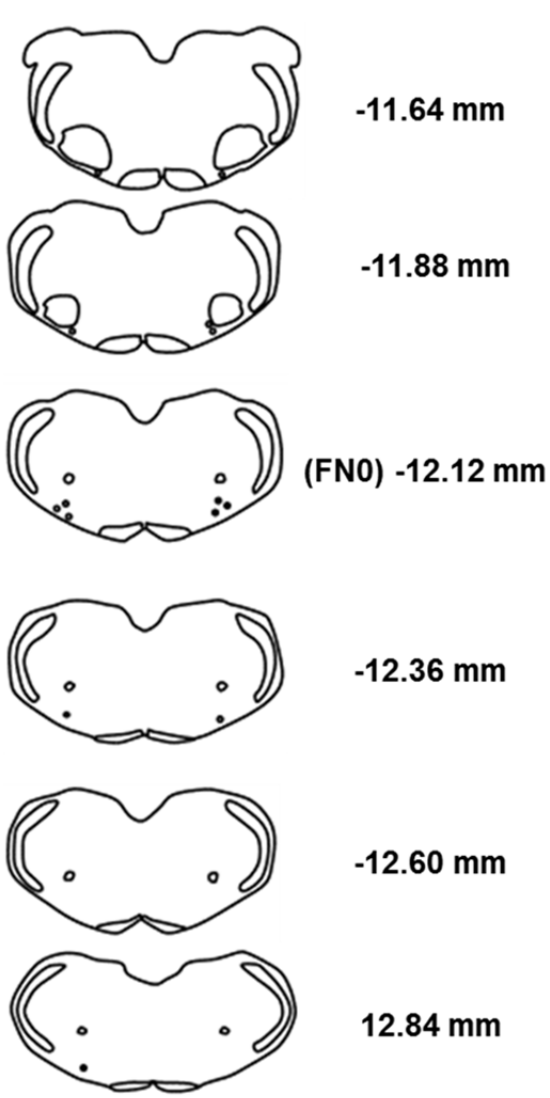

Figure 8. Histological verification of RVLM microinjection sites. Bilateral microinjection locations from sedentary and physically active rats plotted on a modified diagram from a standard rat atlas (Paxinos and Watson, 2007). FN, facial nucleus; NA, nucleus ambiguus; Py, pyramidal tract; SP5 spinal trigeminal tract. Open circles represent physical active rats and closed circles represent sedentary rats.

After completing the sinoaortic denervation, blood pressure (P=0.247), SSNA (P=0.704) and heart rate (P=0.158) did not significantly change from resting levels in both groups (Table 3). Blood pressure (P=0.939), splanchnic sympathetic nerve activity (P=0.355) and heart rate (P=0.885) following SAD were not different between sedentary physically active rats (Table 3). In order to verify that baroreceptor reflex function was present before and absent following the SAD, phenylephrine was given intravenously under both conditions. Prior to SAD, a bolus injection of phenylephrine (1 μg/kg)

increased blood pressure in both groups and caused a reflex decrease in splanchnic sympathetic nerve activity (Figure 9). Sedentary conditions did not affect the increase in blood pressure or the reflex decrease in splanchnic nerve activity (Abs or %) in response to the bolus injection of phenylephrine when compared to the physically active group. Following SAD, increases in blood pressure in response to phenylephrine were accompanied by little to no change in splanchnic sympathetic nerve activity in both groups. SAD SSNA responses were significantly blunted when compared to intact baroreceptor reflex function ($p<0.05$, Figure 9). However, being sedentary did not enhance blood pressure nor reflex changes in splanchnic sympathetic nerve activity to the same dose of PE.

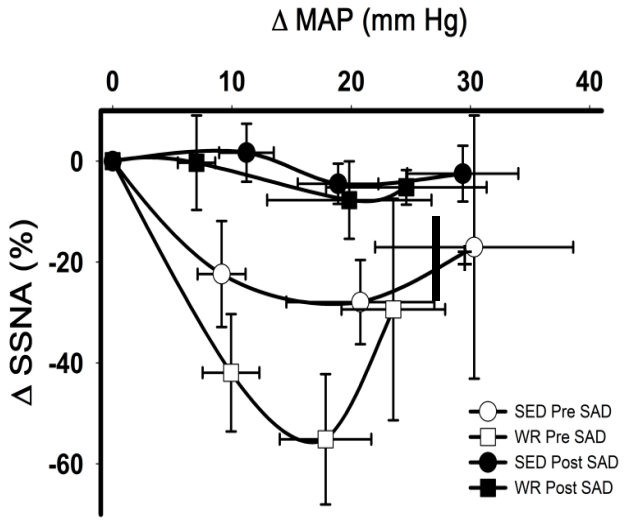

Figure 9. Verification of sinoaortic denervation. The change in mean arterial pressure (MAP) and the change in splanchnic sympathetic nerve activity (SSNA) in sedentary (SEDs, n=5) and physically active animals (chronic wheel running (WRs, n=5) in response to 1μg/kg phenylephrine challenge. There was a significant difference in the change in SSNA pre vs post SAD (‡, $p<0.05$).

For this study we wanted to functionally isolate one RVLM in order to understand the endogenous mechanisms that control the activity of the neurons in the region and minimize any influence of the contralateral RVLM when microinjecting unilaterally. Following SAD, we inhibited the right RVLM using the long acting $GABA_A$ receptor agonist, muscimol. Muscimol microinjections resulted in decreases in blood pressure,

splanchnic sympathetic nerve activity and heart rate in both groups that remained significantly lower than pre-muscimol levels (Table 3). Sedentary conditions did not affect the fall in blood pressure, splanchnic sympathetic nerve activity and heart rate in response to muscimol when compared to physically active conditions.

After inhibiting the right RVLM, the $GABA_A$ receptor antagonist, Bic was microinjected into the left RVLM. Figure 10A represents a 10 minute sample of a Bic microinjection from a physically active rat that includes blood pressure, rectified and

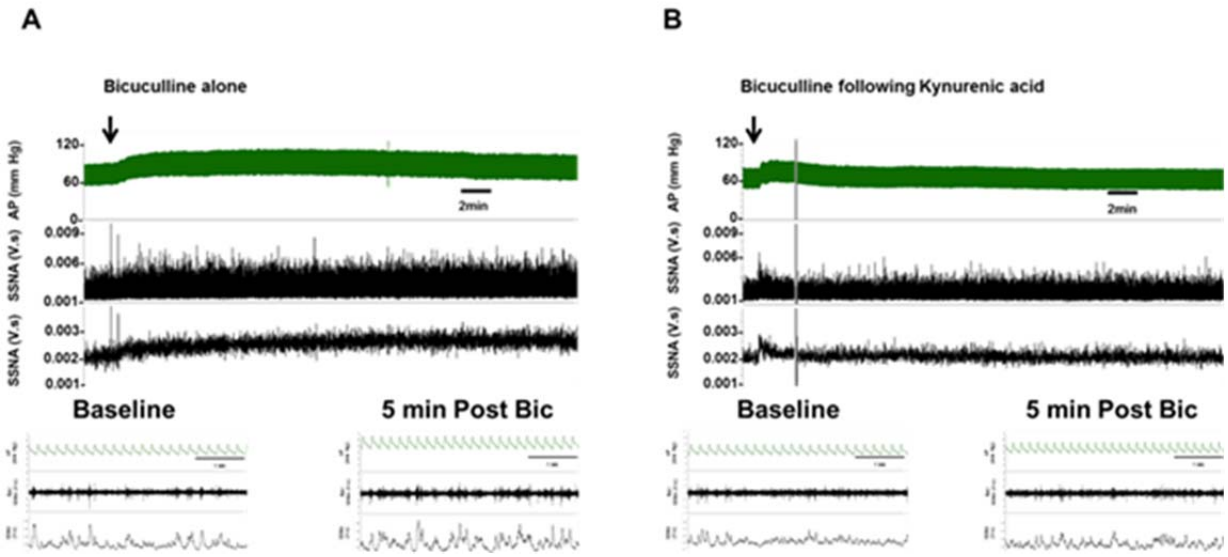

Figure 10. Individual examples of MAP, SSNA responses to unilateral blockade of $GABA_A$ receptors in the RVLM with bicuculline (2mM, 90 nl) before and after blockade of ionotropic glutamate receptors with kynurenic acid (40mM, 90 nl). Both sets of unilateral injections were performed following SAD and after the contralateral RVLM was inhibited with muscimol (2mM, 90 nl). (A) Response to bicuculline in a physically active rat. (B) Response to bicuculline following kynurenic acid in the same physically active rat shown in (A).

integrated SSNA and averaged SSNA. Bic increased blood pressure, percent and absolute change in SSNA in both groups (Figure 11A). Sedentary conditions did not significantly enhance the change in mean arterial pressure or the percent change in

SSNA (Figure 11). However, sedentary conditions did enhance the change in absolute SSNA when compared to physically active conditions (Figure 11). After the 45 minute

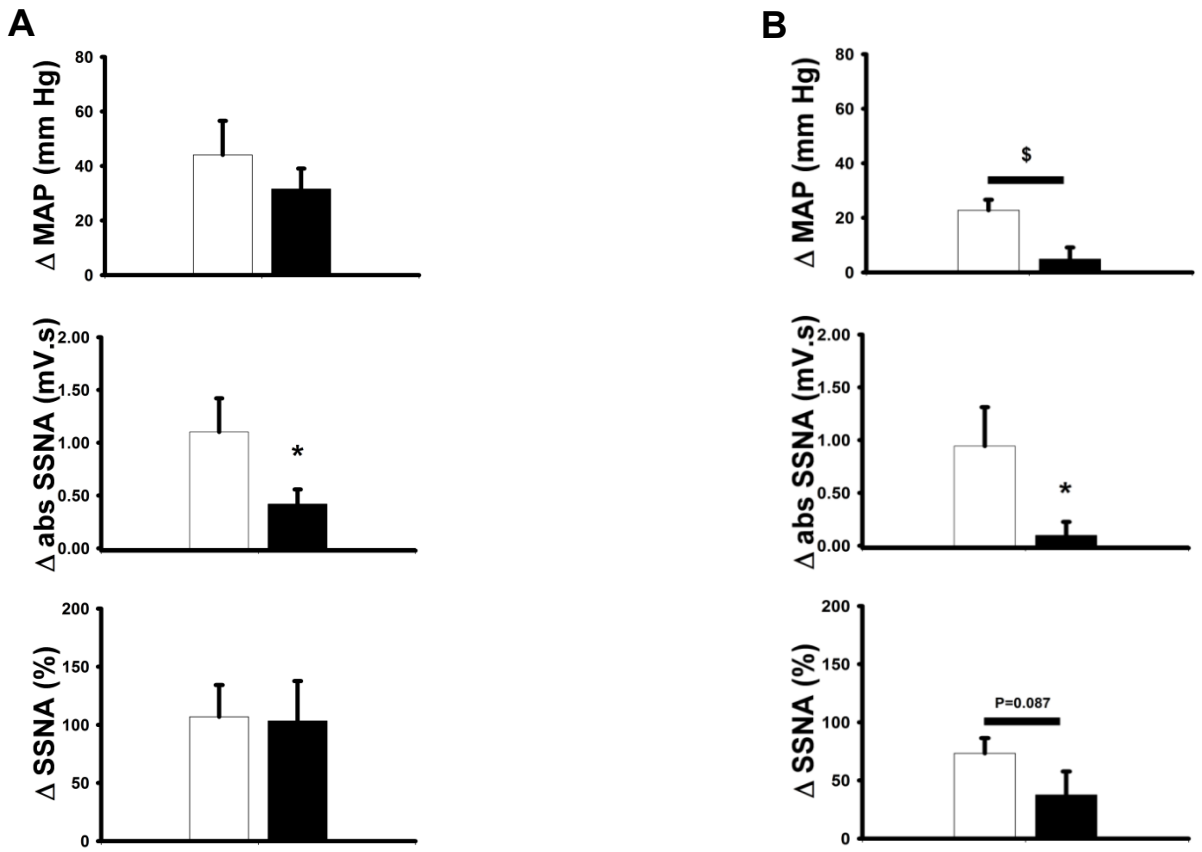

Figure 11. Peak change in MAP, absolute SSNA, and percent change in SSNA in response to disinhibition of rostral ventrolateral medulla (RVLM) with bicuculline (Bic) before and after blockade of ionotropic glutamate receptors with kynurenic acid (Kyn). Both sets of unilateral injections were performed following SAD and after the contralateral RVLM was inhibited with muscimol (2 mM, 90 nl). Figure 11A represents Bic (90 nl, 2 mM) response after inhibition contralateral RVLM alone. Figure 11B represents Bic (90 nl, 2 mM) response to Bic after blockade of ionotropic glutamate receptors in the RVLM with Kyn (40 mM, 90 nl). SAD sedentary and physically active rats had similar increases in MAP and percent change in SSNA to Bic alone in the RVLM. SAD sedentary rats had enhanced increases in absolute SSNA when compared to physically active rats (* $p<0.05$). Following disfacilitation of RVLM with Kyn, SAD sedentary and physically active rats had similar changes in MAP in response to Bic (Figure 9B). However, SAD sedentary rats had enhanced increases in absolute SSNA in response blockade $GABA_A$ receptors (* $p<0.05$). The sedentary rats tended to have larger increases for percent change in SSNA in response to Bic following disfacilitation of RVLM when compared to physically active rats. $, $p<0.05$ vs bicuculline alone.

recovery period, a single barrel pipette was used to microinject Kyn into the left RVLM and 5 to 10 minutes later Bic was microinjected again. Figure 10B represents a 10 minute sample of a Bic microinjection following a Kyn microinjection in the same physically active rat. Similar to Bic alone, Bic in the presence of Kyn increased MAP and SSNA in sedentary rats. However the peak change in MAP was significantly blunted when compared to Bic alone (Figure 11B vs. 11A). In the physically active rats, MAP did not increase in response to Bic after Kyn such that the MAP response to Bic following Kyn was significantly less than the pressor response to Bic alone (Figure 11B vs. 11A). The percent change in SSNA in response to Bic after Kyn pretreatment tended to be blunted when compared to Bic alone but the difference did not reach significance p=0.087 (Figure 11B vs. 11A). Following pretreatment with Kyn, sedentary conditions did enhance the change in absolute SSNA produced by Bic when compare to physically active conditions (Figure 11B).

Discussion

One of the purposes of this study was to address whether the pressor and sympathoexcitatory response to $GABA_A$ receptor blockade in the RVLM of barodenervated rats was due to activation of ionotropic glutamate receptors. We also investigated whether sedentary conditions enhance the contribution of ionotropic glutamate receptors to the pressor and sympathoexcitatory responses to $GABA_A$ receptor blockade in the RVLM of barodenervated rats. From this study we demonstrated three important findings: 1.) We extended findings from previous studies that demonstrated significant remaining inhibition of the RVLM in the absence of the baroreceptor reflex (42), and that this inhibition is driven by activation of $GABA_A$ receptors (114), 2.) In the absence of all GABAergic input, sedentary conditions

enhance excitatory drive to neurons that control SSNA and this excitatory drive is driven in part by ionotropic glutamate receptors, 3.) Finally, sedentary conditions appear to engage a non-ionotropic glutamatergic mediated excitation of RVLM neurons involved in splanchnic sympathetic outflow that is not present in physically active rats. Based on these findings we conclude that sedentary conditions lead to enhanced excitatory drive to RVLM neurons controlling SSNA and that this is being mediated by both ionotropic and non-ionotropic glutamatergic transmission.

One observation from our study was that in the absence of arterial baroreceptor input tonically released GABA continues to restrain the activity of RVLM neurons. The RVLM receives baroreceptor-dependent GABAergic input from the caudal ventrolateral medulla (CVLM) (21; 85; 116). Schreihofer demonstrated that in the absence of baroreceptor input (i.e. SAD), blockade of $GABA_A$ receptors in the RVLM still produces increases in blood pressure, demonstrating that there is non-barosensitive GABAergic input restraining the activity of RVLM neurons. Minson and colleagues (1997) used c-fos in order to demonstrate that there are glutamic acid decarboxylase 67 (GAD 67) neurons that were barosensitive and non-barosensitive in CVLM (85). Cravo and Morrison (1993) also demonstrated in SAD rats, that lesioning the CVLM, still produced an increase in blood pressure and SSNA (21). Interestingly, when CVLM neuronal activity is blocked, there is a persistent level of GABAergic input that presumably comes from other brain regions as speculated by Moffitt *et al*. (90). Anatomically it has been shown that there are multiple regions that provide GABAergic input to the RVLM; however, whether they are all active under physiological or pathophysiological conditions is still unknown (12). From this study we can conclude that there is inhibition independent of the baroreceptors that restrain the activity of RVLM neurons. This

inhibition is mediated through $GABA_A$ receptors in the RVLM.

We did observe that blockade of ionotropic glutamate receptors prior to blockade of $GABA_A$ receptors resulted in blunted changes in MAP and tended to blunt the percent change in SSNA when compared to $GABA_A$ receptor blockade alone. However, the change in absolute SSNA was not significantly different when compared to blockade of $GABA_A$ receptor blockade alone. We used absolute change because if there is a difference in baseline SSNA, the calculated percent change in SSNA may be similar when in fact the absolute change in SSNA could be different. For example, in the present study there was a significant increase in absolute SSNA for $GABA_A$ receptor blockade alone in sedentary rats and in the presence of ionotropic glutamate receptor blockade when compared to physically active rats. This difference between sedentary and physically active rats was not observed for the MAP and percent change in SSNA.

Several studies suggest that ionotropic glutamate receptors contribute to increases in excitatory drive to the RVLM (14; 50; 89), especially in physiological, pathophysiological or experimental (bicuculline) conditions of increased sympathetic outflow. Our laboratory has shown that sedentary conditions enhance sympathoexcitatory responses to glutamate microinjections in the RVLM when compared to physically active rats (87). These data show that sedentary rats may be more sensitive to excitatory drive. The question still remains as to what is responsible for this enhanced sensitivity to excitatory drive under sedentary conditions. Interestingly, when ionotropic glutamatergic receptors are blocked with Kyn alone in the RVLM of Sprague-Dawley rats, there is no change in blood pressure (36; 127; 128). These data suggested that ionotropic glutamate receptors do not play a role in driving the tonic activity of RVLM neurons, but may still modulate neuronal activity under

certain physiological states. Based on the findings from this study, in the absence of GABAergic and arterial baroreceptor input to the RVLM, tonic sympathoexcitatory drive is due at least in part to ionotropic glutamate receptors.

An additional finding revealed by our study is that the majority of the sympathoexcitation produced by removal of GABAergic transmission in the RVLM seems to be due to non-ionotropic glutamatergic input. This conclusion is based on the finding that much of the sympathoexcitation in response to bicuculline remained in sedentary rats even after blockade of ionotropic glutamate receptors with Kyn. The remaining excitation is most likely due to non-ionotropic glutamatergic input since we used a concentration and volume of Kyn similar to others that have shown that this concentration and volume will block ionotropic glutamate receptors (53; 93; 140). One possible source of non-ionotropic glutamatergic input could be angiotensin II. Sved and colleagues and others have shown that angiotensin II may be playing a tonic role in driving the activity of the RVLM in hypertension (8; 51; 130). Further experiments need to be done in order to determine whether angiotensin is playing a role in our model and driving the tonic activity of the RVLM.

In another study from our laboratory, we showed that the change in absolute SSNA was significantly enhanced in sedentary when compared to physically active rats in response to glutamate microinjections into the RVLM (87). We attributed the enhancement to the change in absolute SSNA that was not seen in the percent change in SSNA to differences in resting SSNA. We used absolute change because if there is a difference in baseline, the percent change in SSNA may be the same but the absolute change in SSNA could be different. In this same study, it was observed that sedentary conditions resulted in elevated resting arterial pressure and SSNA when compared to

physically active conditions. In this current study, we did not observe a statistically significant difference in resting SSNA; however, this may be due to a smaller sample size and differences in resting SSNA was not the primary focus of this study. In a previous study, we did see a difference in resting SSNA, but there were a larger number of animals in each group than compared to our current study (87). Table 3 indicates that after SAD the sedentary rats had on average twice as high SSNA when compared to physically active rats but this difference was not statistically significant ($p=0.355$).

Conclusion

In the absence of baroreceptor input to the RVLM, blockade of the remaining GABAergic tone produced sympathoexcitation that was observed in both sedentary and physically active rats that was partially mediated by glutamate. Sedentary conditions enhanced increases in splanchnic sympathetic nerve activity under these conditions, an observation not reported previously. Once the ionotropic glutamatergic input was removed there is still a residual pressor and sympathoexcitatory response to GABA receptor blockade, particularly prominent in sedentary rats. This remaining excitation was due to non-ionotropic glutamatergic transmission. Furthermore, sedentary conditions enhanced the non-ionotropic glutamatergic to neurons that control splanchnic sympathetic nerve activity. Since there is an increase in non-ionotropic glutamatergic transmission there could be a role for excitatory neurotransmitters such as angiotensin II.

Perspectives

Our study indicates a role for the central nervous system in the development of cardiovascular disease under sedentary conditions, a mechanism which has been shown in other CVD disease models (46; 56; 87). Different CVD models are associated

with alterations in glutamatergic and GABAergic transmission in the RVLM (1; 46; 87; 138; 140). The findings from this current study have demonstrated not only that glutamatergic input may be playing a role in enhanced excitation but that there may be non-glutamatergic mechanisms contributing to the overactivity of sympathetic nervous system under sedentary conditions. Other laboratories have implicated non-glutamatergic mechanisms, specifically angiotensin II, as a cause for elevated sympathetic nervous system activity in certain disease models such as hypertension, chronic heart failure and obesity (46; 56; 98). By identifying another potential pathway that may be contributing to the development of cardiovascular disease in sedentary individuals, it allows for the possibility of additional therapeutic interventions to help treat and prevent the development of cardiovascular disease, particularly in sedentary individuals.

CHAPTER 4

THE EFFECT OF SEDENTARY CONDITIONS ON GABAA RECEPTOR α1 SUBUNIT EXPRESSION IN THE RVLM

Abstract

The rostral ventrolateral medulla (RVLM) is important for sympathetic control of the cardiovascular system. RVLM neurons are known to be under strong tonic γ-amino-butyric acid (GABA)-mediated inhibition, due to activation of $GABA_A$ receptors. Previous *in vivo* studies suggests that sedentary conditions lead to enhanced GABA responsiveness in the RVLM when compared to physically active conditions (see Chapter 2). The purpose of the current study was to determine the potential mechanisms responsible for the enhanced sympathoinhibition following GABA microinjection into the RVLM of sedentary rats. We proposed that $GABA_A$ receptor α1 expression may be altered by sedentary compared physically active conditions. Specifically, we hypothesized: 1.) Sedentary conditions lead to increased expression of $GABA_A$ receptor α1 protein in the RVLM, and 2.) Sedentary conditions lead to enhanced $GABA_A$ receptor α1 expression on spinally projecting RVLM neurons that play a role in the control splanchnic sympathetic outflow. We examined expression of the $GABA_A$ receptor α1 subunit in the RVLM using western blot techniques in sedentary (SED) and physically active (Wheel runner, WR) male Sprague-Dawley rats. After fresh brainstem tissue was sectioned, we verified tissue punch location using cresyl violet staining. $GABA_A$ receptor α1 staining was normalized to GAPDH. There was no main effect of rostrocaudal expression of the $GABA_A$ receptor α1 subunit. Sedentary conditions did not enhance the protein expression of the $GABA_A$ receptor α1 subunit when compared to physically active conditions.

In order to investigate $GABA_A$ receptor α1 subunit expression on RVLM spinally projecting neurons, bilateral spinal cord injections of the retrograde tracer, cholera toxin B (CTB) were performed on SED and WR male Sprague-Dawley rats at spinal cord segments T9/T10. Double immunofluorescent labeling of RVLM neurons identified immunoreactivity for CTB and the $GABA_A$ receptor α1 subunit. Our data show that more than half all RVLM neurons that project to the T9-T10 region of the spinal cord also express the $GABA_A$ receptor α1 subunit. There was no main effect found between SED and WR rats in the number of spinally projecting neurons that co-express $GABA_A$ receptor α1, although our study may be somewhat under powered by the small number of animals examined (n=2-4). Similarly, there was no main effect of rostrocaudal position of bulbospinal, α1 subunit positive neurons with the same caveat that the study may be under powered. Nonetheless, for the first time, these anatomical observations support the involvement of RVLM $GABA_A$ receptors in inhibitory control of sympathetic outflow arising from the lower thoracic spinal cord. They do not, however, provide support for $GABA_A$ receptor α1a subunit involvement in enhanced sympathoinhibitory responses observed previously in SED versus WR animals.

Introduction

A sedentary lifestyle is a major risk factor for the development of cardiovascular disease (CVD). CVD has been linked to elevated sympathetic nervous system activity (71; 142). Elevated sympathetic outflow has been implicated in multiple pathophysiological diseases, such as hypertension, obesity, sleep apnea and chronic heart failure (45; 50; 122; 137). Our laboratory has shown previously that sedentary conditions lead to elevated mean arterial pressure and SSNA when compared to physically active conditions (87).

The rostral ventrolateral medulla (RVLM) is a brainstem region that is important for the tonic and reflex control of sympathetic nerve outflow and blood pressure (38). The RVLM contains two populations of neurons, the catecholaminergic (C1) and non-catecholaminergic (non-C1) neurons (69; 113). RVLM neurons project to the spinal cord and synapse on preganglionic neurons in the intermediolateral cell column (IML) (24; 102). These neurons then activate post ganglionic nerves that innervate the heart and vasculature, in order to modulate blood pressure (24; 102). Our laboratory is specifically interested in sympathetic control of the splanchnic circulation because in a previous study we showed that sedentary conditions lead to enhanced splanchnic sympathoexcitation (87). Based on these data, we are interested in whether sedentary conditions alter neurotransmission in the RVLM specifically to neurons that control splanchnic sympathetic outflow.

The activity of the RVLM is modulated by glutamate and γ-amino butyric acid (GABA). There are two major sources of GABAergic input to the RVLM, arterial baroreceptor-independent input and baroreceptor-dependent input (113). Baroreceptor-dependent GABAergic input to RVLM comes from the caudal ventrolateral medulla (CVLM) (113) via excitation from neurons in the nucleus tractus solitarius (NTS). The CVLM also provides baroreceptor-independent input to the RVLM (113). When GABA is released onto RVLM neurons it can bind to $GABA_A$ or $GABA_B$ receptors (22; 81; 113). The majority of GABAergic inhibition in the RVLM is mediated by $GABA_A$ receptors (22; 22; 24). $GABA_A$ receptors are ionotropic receptors that are ligand gated chloride channels (35). The structure of the $GABA_A$ receptor is made up of 5 subunits, typically two α, two β and a γ subunit (35). Different compositions of α, β, and γ subunits can change the $GABA_A$ receptor's affinity for GABA (ref?). It has been suggested that the

$GABA_A$ receptor α1 subunit predominates during adulthood and is present in the brain (34).

There multiple studies that have demonstrated the importance of GABA signaling in the RVLM. Huber *et al*. showed that obesity led to attenuated decreases in SSNA response as a result of activation of $GABA_A$ receptors in the RVLM with muscimol compared to lean rats (46). Kajekar *et al*. demonstrated that following acute exercise in hypertensive rats, resting RVLM neuronal activity was lower when compared to non-exercised hypertensive rats (54). Furthermore, they demonstrated that ionotophoresing bicuculline onto individually recorded RVLM neurons following an acute bout of exercise increased resting RVLM neuronal activity to a greater extent when compared to non-acutely exercised rats (54). The study from Kajekar and colleagues suggests that there may be greater GABAergic input to the RVLM as a result of a single bout of exercise. These studies provided functional data about how GABAergic transmission in the RVLM is modulated by different conditions. Foley *et al*. looked at $GABA_A$ receptor α1 subunit expression in the RVLM between pregnant and non-pregnant rats. They showed that neither condition altered the levels of $GABA_A$ receptor α1 present in the RVLM. A previous study from our laboratory used laser capture micro dissection coupled with polymerase chain reaction in order to investigate RNA levels of $GABA_A$ receptor α1 and $GABA_A$ receptor α2 (126). These data demonstrated that sedentary conditions did not alter $GABA_A$ receptor α1 gene expression in the RVLM (126). However as running distance increased so did the level of $GABA_A$ receptor α2 gene expression in the physically active group (126). These data demonstrated the presence of $GABA_A$ receptor α1 in the RVLM and the amount of exercise might play a role in regulated $GABA_A$ receptor gene expression in the RVLM. This study provided rationale for

investigating $GABA_A$ receptor protein expression in the RVLM and how sedentary and physically active conditions may be modulating the expression of $GABA_A$ receptors. Our current study will provide information that will help determine the effect physical inactivity on the expression of $GABA_A$ receptor α1 subunit in the RVLM of rats.

The purpose of the current study was to investigate whether physical inactivity alters $GABA_A$ receptor α1 subunit expression in the RVLM. Furthermore, since RVLM neurons project to the spinal cord and tonically control sympathetic outflow, we wanted to investigate $GABA_A$ receptor α1 subunit expression specifically on spinally projecting neurons. Our hypotheses were 1.) Sedentary conditions result in a greater amount of $GABA_A$ receptor protein expression in RVLM. 2.) Sedentary conditions increases the number of $GABA_A$ receptor positive neurons in the RVLM that project to T9-T10 when compared to physically active rats.

Methods

Animals

Male Sprague-Dawley rats (Harlan Sprague Dawley Inc., Indianapolis, IN; 75-100 g upon arrival) were used for these studies. Animals were housed under standard 12-hr dark/light cycle. Physically active rats were housed with a running wheel (Tecniplast, #2154F0105, 34cm in diameter, stainless steel) for 12-15 weeks and sedentary rats were housed without a running wheel for an equivalent period. This model has been validated by our laboratory and others in several previous studies (7; 17; 87; 94; 101). The distances the animals ran were recorded by bicycle computers (Sigma Sport, Olney, IL) and documented daily. Experiments were performed in accordance with National Institutes of Health *Guide for Care and Use of Laboratory Animals* and with the approval of the Wayne State University Institutional Animal Care and Use Committee.

Fresh Tissue Isolation, Gel electrophoresis and western blotting

Tissue Isolation

Animals were deeply anesthetized with diluted Fatal Plus (100 mg/kg, Pentobarbital, i.p.) and decapitated. Fresh brain, left adrenal, right adrenal, heart and soleus tissue from sedentary and physically active rats were collected and immediately put on a metal plate that was on dry ice until frozen and then stored in the -80 freezer. The entire brainstem was serially sectioned on a cryostat at 80 µm. The coronal sections were mounted onto a clean glass slide. Then using a 17 gauge stainless steel Hamilton needle bilateral punches were made from serial sections including the rostrocaudal extent of the RVLM. In order to determine which punches would be used for western blotting, crystal violet staining was performed on previously punched sections in order to visualize specific landmarks. A rat brain atlas was used as a reference guide (104). The tissue punches were placed into a modified Glasgow protein extraction buffer (150mM NaCl, 1.0mM EDTA, 50mM Tris, 1% NP40, 0.1% Triton X-100 and protease inhibitors). The buffer solution was sonicated 2-3 times for 2 seconds and then centrifuged for 5 minutes at 5000 rpm. The supernatant was collected and protein concentration of the samples was determined by a Bradford Assay (Sigma, St. Louis, MO).

SDS-PAGE

Once the protein concentrations were known, samples were prepared for electrophoresis. A 5 µg per 50 µl solution of protein solution was made by diluting samples with varying amounts of Laemmli sample buffer. The samples were loaded into different lanes on a 4-20 % Mini-Protean®TGX™ precast gel (Bio-Rad Laboratories, Hercules, CA cat. # 456-1084s) and the electrophoresis was performed.

Rostrocaudal samples from one sedentary and one physically active rat were loaded onto the gel in the following order in reference to the caudal pole of facial nucleus (FN0): molecular weight marker, C2 = 240-480 μm caudal to FN0, C1= 240-0 μm caudal to FN0, R1= 0-240 μm rostral to FN0, and R2 = 240-480 μm rostral to FN0 (see Results: Figure 12). Once the electrophoresis was completed the proteins from the gel were transferred to polyvinylindine difluoride (PVDF) membrane (Bio-Rad laboratories).

Western Blotting

The PDVF membrane was washed with Tris/Tween/saline buffer (TBST) and blocked with 5% non-fat dry milk in TBST. The primary antibody for $GABA_A$ receptor α1 subunit (1:250 lot no. AGA001AN1002; Alomone Labs) was diluted in TTBS that contained 4% chick egg ovalbumin. This solution was applied to the membrane and allowed to incubate overnight. The next day the primary antibody was removed and the PDVF membrane was washed (6x) with TTBS and before applying the secondary goat anti-rabbit conjugated horseradish peroxidase (1:2000, lot no. 2390378; Millipore) diluted in TBST. After the secondary was allowed to incubate for 1hr., the membrane was rinsed again (6x) with TBST and ECL (Fisher, Chicago, IL) was added to the membrane for one minute. The X-ray film (cat# F-9024-8x10, BioExpress, Kaysville, UT) was exposed to the membrane and then the film was developed.

After the bands for $GABA_A$ receptor α1 subunit were visualized. The PVDF membrane was rinsed 6x with TBST and then incubated with the primary for GAPDH (1:1000, cat# MAB374 Lot#NG1869834, Millipore, Billerica, MA). After a 24hr incubation period with the primary, the membrane was rinsed (6x) TBST and the Goat anti mouse IgG HRP conjugated secondary (1:5000, cat#12-349 Lot # NG1850774, Millipore, Billerica, MA) was added to the membrane for 1hr. The membrane was rinsed

(6x) with TBST and ECL was added to membrane for one minute and the film was exposed to the membrane and then developed. The $GABA_A$ receptor α1 subunit bands were normalized to GAPDH. The film was scanned and bands analyzed using Image J. X-Ray films were scanned using an image scanner II and Image J software 1.6 (NIH) was used to quantify band densities.

Retrograde labeling surgery

Prior to the start of surgery, animals were injected in the left hind quarter with ketoprofen (Ketofen™, Fort Dodge Animal Health, Fort Dodge, IA; 5 mg/kg S.C.). Animal were anesthetized with isoflurane (5% induction and 2% for maintenance, Henry Schein, Melville, NY). In order to label spinally projecting neurons in RVLM, each group of rats received an injection of cholera toxin B subunit (CTB, 1%, List Biological Laboratories, Inc., Campbell, CA), a retrograde tracer, into the T9-T10 region of the spinal cord as in previous studies (Quote Nick's and the NR1 paper). Briefly, rats were put in a stereotaxic head holder and the T9-T10 region of the thoracic spinal cord was exposed. CTB (3 X 30 nl) was microinjected bilaterally into the T9-T10 of the spinal cord. Injections were made with a glass micropipette in a grid pattern (3 rostral/caudal insertions at 0.8 depths) centered on the intermediolateral cell column and dorsolateral funiculus. After the injections were completed, the spinal cord was covered with a small piece of Gelfoam and the incision stapled closed using wound clips. The rats also received fluids (10 ml lactated ringers solution) subcutaneously immediately after surgery. The animals were given loose food pellets and DietGel (DietGel76A, CleatH_2O, Portland, MA). Animals were monitored after surgery and once they were awake and moving around, they were returned to the animal facility. The spontaneous wheel running rats (physically active) were put back in their cages with running wheels.

The sedentary rats remained in their cages without running wheel access. The first day following surgery, Ketoprofen (5 mg/kg S.C.) was given. After the first day of surgery Ketoprofen (5.0 mg/kg SC) was given only if the animal exhibited any signs discomfort. Rats were allowed to recovery for three days to allow for retrograde transport of CTB. If any animal displayed abnormal behavior or weight loss (>10 %), i

Perfusion, tissue fixation and sectioning

Following recovery, spinal cord injected rats were deeply anesthetized with diluted Fatal Plus (100 mg/kg, Pentobarbital i.p.), the chest was opened and heparin (1000 IU) was injected directly into the heart. Rats were perfused with oxygenated DMEM (Sigma D-8900 - 500ml) and 500ml of 4% formaldehyde in 0.1M phosphate buffer, pH 7.4 (10). The brains and spinal cords were removed and fixed in 4% formaldehyde in 0.1M phosphate buffer, pH 7.4 for 3 days at room temperature on a shaker. The tissue was rinsed and put into 20% sucrose for 48 hours and then into 30% sucrose for an additional 48 hours for cryoprotection. Brain tissue was cut in a 1:4 series into 30 µm sections on a cryostat and at -13°C.

Immunohistochemistry

Similar to other laboratories (66; 86), the sections were put into 0.1M phosphate buffer (PB), pH 7.4. The PB was removed from the sections and the tissue was rinsed with immunobuffer on a shaker for 3x10 minutes. The immunobuffer was removed and the tissue was incubated in 10% normal horse serum in immunobuffer for 30 minutes. Then the 10% horse serum was removed and a goat α-$GABA_A$ Rα1 primary antibody (1:100 Santa Cruz 7348) was added to detect α-$GABA_A$ Rα1 immunostaining. To reveal CTB immunostaining a rabbit primary anti-CTB antibody (1:200,000- 400,000; Biological Laboratories, Campbell, CA) was added to the tissue and incubated for 24

hrs. The tissue was then rinsed with 10 mM Tris, 0.9% NaCl, 0.05% thimerosal in 10 mM phosphate buffer, pH 7.4 (Tris-PBS). The secondary antibody for the goat α-$GABA_A$ Rα1 (1:500 biotinylated donkey α-goat, Jackson Laboratories, Sacramento, CA) was allowed to incubate with the tissue for 24 hrs along with the secondary antibody for rabbit anti-CTB (1:500 donkey α-rabbit conjugated to fluorescein isothiocyanate (FITC), Jackson Laboratories, Sacramento, CA). The following day the tissue was rinsed with Tris-PBS 3x10 minutes. In order to detect the $GABA_A$ receptor α1 subunit, the tissue was incubated in Cy3 conjugated to streptavidin (1:1000, Jackson Laboratories, Sacramento, CA) for four hours. The tissue was then rinsed again, mounted onto glass slides, and cover slipped with Vecta Shield (Vector Labs-Fischer, Burlingame, CA).

Verification of spinal cord injection site

We verified the T9-T10 injection site (48; 86) by examining the thoracic segment of the spinal cord which was removed from each rat after it was perfused. Specifically, the T7-L2 region was removed and was placed into 20 % sucrose for 48hrs and then put into 30% sucrose for 48 hrs. After the sucrose was allowed to infiltrate the spinal cord tissue, the T7-L2 segment was cut on a cryostat at 30 μm into a 1:4 series. Similar to the brainstem tissue above, CTB was detected using the same immunofluorescent staining protocol. The tissue was then mounted and cover slipped with Vecta Shield (Vector Labs-Fischer, Burlingame, CA).

Fluorescence microscope quantification/stereology

The criteria used to determine whether a neuron should be counted as being located in the RVLM were as follows: 1) it was located medial to the spinal trigeminal tract, 2) ventral to the nucleus ambiguus, and 3) lateral to the pyramidal tract. The caudal pole of facial nucleus (referred to as FN0) was used as a rostrocaudal

anatomical landmark. All sections were aligned prior to counting and labeled in reference to FN0 (caudal denoted -, rostral denoted +). The cells that were included in the counts had dendrites that were visible and they looked like a cell. The criteria used to determine whether a cell should be counted as a neuron included: 1) the nucleus had to be visible and void of fluorescence, and 2) the cell body of the neuron had to contain fluorescence. Micrographs of the sections were taken with a 30X objective on a Leica DM5000 microscope equipped with a fluorescence filter set (Leica Microsystems Inc., Buffalo Grove, IL). The micrographs were used to perform cell counts of CTB-immunoreactive cells and CTB positive cells that also expressed $GABA_A$ Rα1 immunoreactivity. Since the brain tissue was cut in a 1:4 series, each section in a given series was separated by 120 μm, which minimized the possibility of double counting the cell body of neurons in each section.

Statistical Analysis

SigmaStat v.3.5 (Systat Software, San Jose, CA) was used to test for differences between sedentary and physically active rats. Student's t-tests were done to detect any differences between body weights and organ weights between sedentary and physically active rats. For both protein expression and immunofluorescence-based cell counts, two-way ANOVAs with repeated measures were done in order to determine whether there was a main effect of rostrocaudal location and whether there was a main effect of sedentary versus physically active conditions. If a significant interaction was detected, a post hoc Holm-Sidak test was done in order to determine individual differences. Data are expressed as mean $\pm$ SEM and a p value less than 0.05 was deemed significant.

Results

There were no difference in body weights (P=0.084), detected between sedentary (n=7) and active rats (n=8). There were no differences detected between sedentary (n=4) and physically active (n=4) in organs weights from animals in the western blot study. As mentioned in the methods, organs were only collected from the western blot rats because the rats used in the immunofluorescent study were perfused and so the organ weights would not be comparable to the western blotting studies. The physically active rats (n=8) ran 253 ± 36 km total over the 12-15 week period.

Table 4. Body and organ weights of sedentary compared to physically active rats.

	Sedentary	Physically active
Body weight (g)	380 ± 19	373 ± 7
Left ventricle (mg)	774 ± 32	798 ± 42
Right ventricle (mg)	221 ± 56	155 ± 48
Left adrenal (mg)	41.1 ± 18	20.3 ± 7
Right adrenal (mg)	18.9 ± 2	18.2 ± 1
Right soleus (mg)	144 ± 6	134 ± 6

Protein expression

$GABA_A$ receptor α1 subunit was detected in the RVLM punches collected from sedentary and physically active rats. Since the micropunches were collected across the rostrocaudal area of RVLM, we investigated whether there was a difference in the amount of $GABA_A$ receptor α1 subunit protein distribution in RVLM. Figure 12 represents the rostrocaudal area from which tissue was obtained and the notation used to identify each different region. Each one of the tissue sections from which punches were obtained was 240 μm in thickness and represented a subregion of the ventrolateral medulla in the rostrocaudal plane. Figure 13A represents a western blot membrane that contains protein from one sedentary and one physically active rat. We

found that there were no differences at any rostral caudal level of the RVLM $GABA_A$ receptor α1 subunit/GAPDH ratio (Figure 13B).

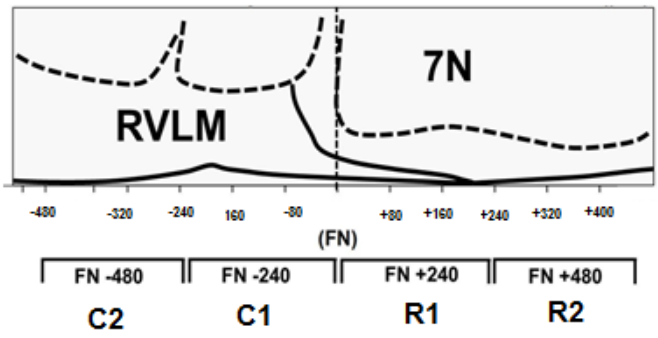

Figure 12. Rostrocaudal extent of the rostral ventrolateral medulla (RVLM) relative to the facial motor nucleus (7N). Diagram represents the area of the brainstem that was included in each of the four samples from each animal in the western blot study. Scale represents the number of microns rostral (positive numbers) and caudal (negative numbers) to the caudal pole of 7N, designated FN. C2 = punch taken from -240-(-)480 µm caudal to FN0; C1 = punch taken from 0-(-)240 µm caudal to FN0; R1 = punch taken from 0-+240 µm rostral to FN0; and R2 = punch taken from +240-(+)480 µm rostral to FN0.

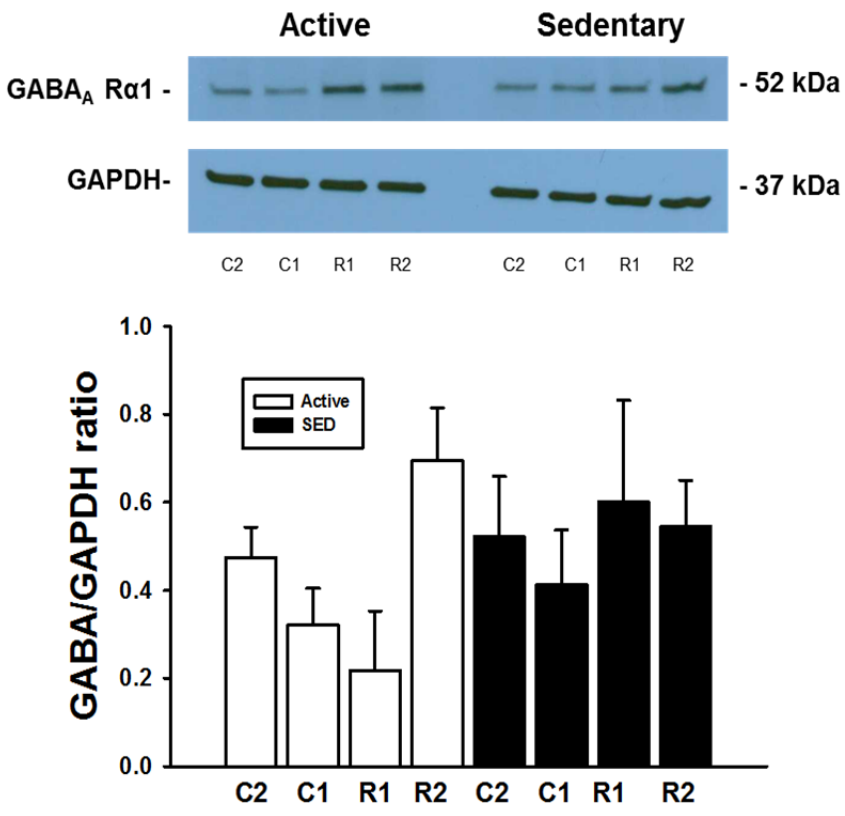

Figure 13. Western blotting of $GABA_A$ receptor α1 subunit in RVLM micropunches from sedentary and physically active rats. (A) Individual examples of $GABA_A$ receptor α1 subunit and GAPDH bands from one physically active (left) and one sedentary rat (right). (B) Quantitative analysis from groups of sedentary (n=4) and physically active (n=4) rats. No differences were found across the rostrocaudal area of the RVLM between the sedentary and physically active rats for the GABA/GAPDH ratio.

Quantification of immunoreactive bulbospinal neurons

Spinally projecting RVLM neurons were detected by the presence of green fluorescence (FITC). In this study we investigated only those neurons that were CTB positive because these neurons project to T9-T10 region of the spinal cord and control splanchnic sympathetic outflow. We also examined double-labelling for $GABA_A$ receptor

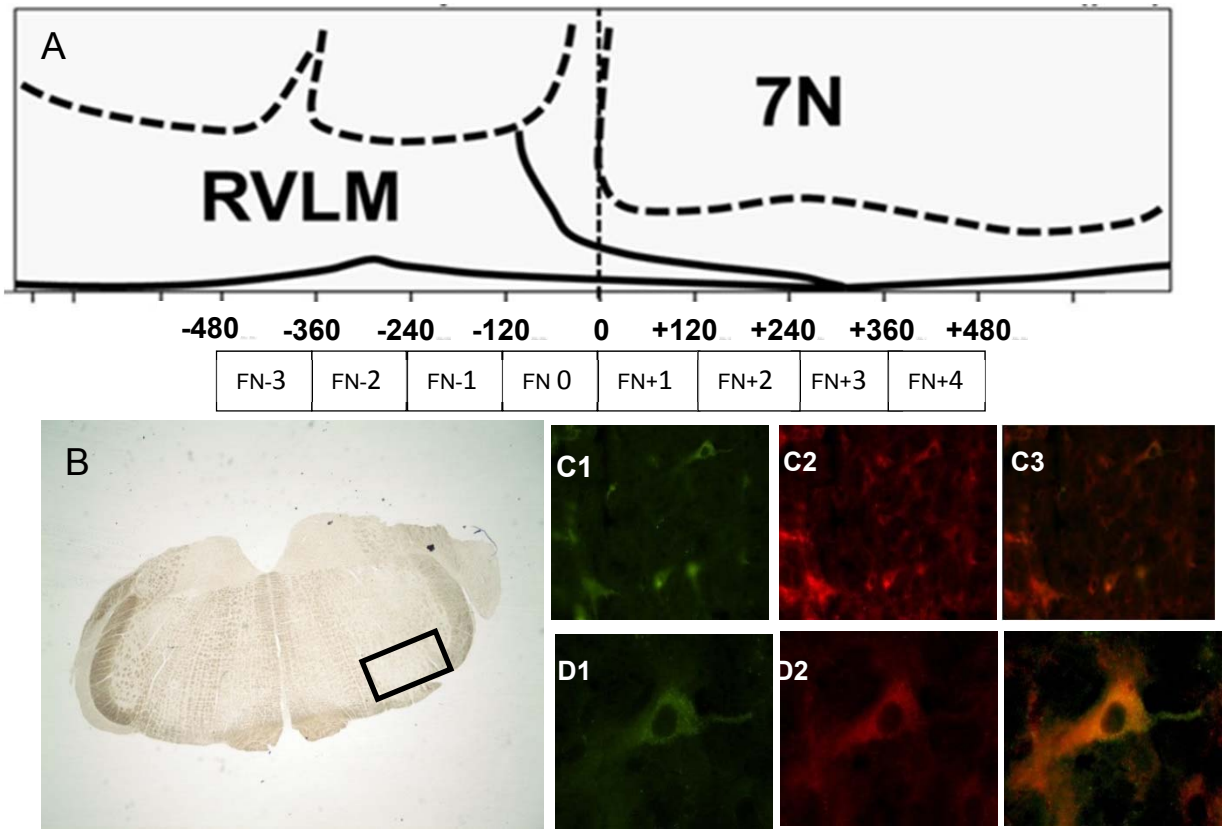

Figure 14. Location of immunofluorescent staining for $GABA_A$ alpha one subunit on spinally projecting RVLM neurons. (A) Diagrammatic representation of the rostrocaudal portion of the ventrolateral that was analyzed for this study. (B) Diagram is modified from a standard rat atlas (Paxinos and Watson, 2007) and denotes specific subregions of the RVLM examined in 120 µm increments relative to the caudal pole of the FN. (B) 2x bright field micrograph of a 30µm section from a sedentary rat. (C1) A micrograph of CTB positive neurons at 30 x magnification. (C2) A micrograph of $GABA_A$ α1 subunit positive neurons at 30 x magnification. (C3) A micrograph of spinally projecting neurons that are also CTB positive. (D1) A micrograph CTB positive neuron at 63 x magnification. (D2) A micrograph of $GABA_A$ α1 subunit positive neuron at 63 x magnification. (D3) A micrograph of a spinally projecting neuron that is also $GABA_A$ α1 subunit positive at 63 magnification.

α1 subunit positive (red, cy3). Figure 14A is a diagrammatic representation of the region of the ventrolateral medulla that was the focus of our study. We were primarily interested in sections located within 480 μm rostrocaudal to the caudal pole of facial nucleus (FN) (Figure 12A) because a previous study from our laboratory showed that there were CTB positive neurons located 500 μm rostral and 500 μm caudal to FN (86). Figure 14B represents a 2x brightfield micrograph from a sedentary rat that is 120 μm rostral to the caudal pole of the FN (designated FN +1). Figures 14C and 14D are magnified views of the highlighted area from Figure 14B taken under fluorescence illumination taken at 20x and 63x magnification, respectively. CTB positive neurons were present near the ventral surface just below nucleus ambiguus. They were clustered near the parapyramidal tract and the cluster became smaller as it approached the spinal trigeminal tract.

The cell counts for CTB positive along the rostrocaudal extent of RVLM were not significantly different at any level (P=0.192, Figure 15A). Furthermore, being sedentary did not increase the amount of CTB positive neurons at any rostrocaudal level when compared to physically active rats (P=0.085). The number of cells that were CTB positive and expressed GABAA α1 subunit were not different across the rostrocaudal extent of RVLM (P=0.185). Sedentary conditions did not alter the number of cells that were CTB positive and expressed GABAA α1 subunit when compared to the physically act rats (P=0.124).

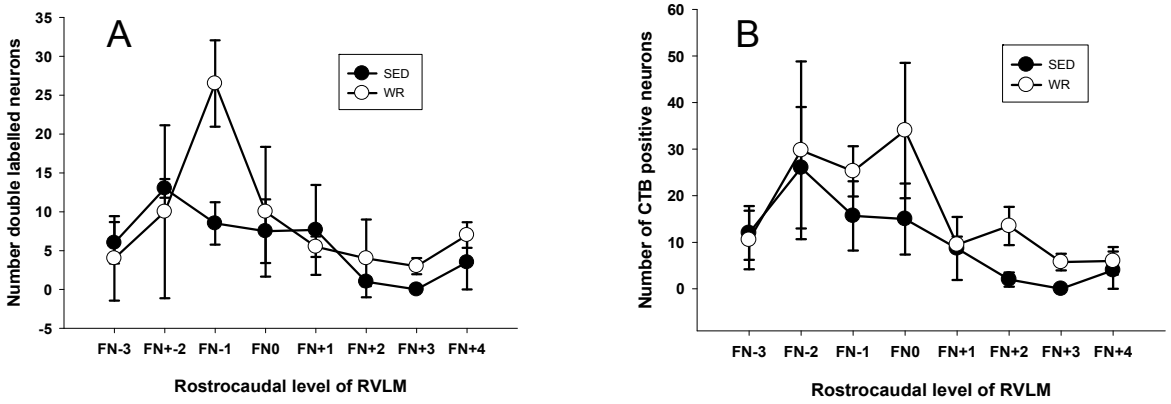

Figure 15. Immunofluorescent staining from a sedentary rat and the rostrocaudal area that was analyzed. Open circles represent physically active rats (WR) and closed circles represent sedentary rats (SED). (A) Represents the average data of the CTB neuronal counts over the rostrocaudal extent of the RVLM of sedentary and physically active rats. There were no differences in the number of CTB neurons over the rostrocaudal area of interest (main effect P=0.192). There were also no differences between sedentary and physically active rats in the number of CTB neurons present over the rostrocaudal area (main effect P=0.085). (B) Represents the average data from the immunofluorescence study. There were no differences found in the number of CTB neurons that also co-expressed $GABA_A$ receptor α1 subunit over the rostrocaudal area (main effect P=0.185). There was also no differences found in the number of CTB neurons that also co-expressed $GABA_A$ receptor α1 subunit between sedentary and physically active rats

Placement of spinal CTB injections

Figure 16 is a photomicrograph from one sedentary rat demonstrating the mediolateral and dorsoventral spread of CTB that was injected into the T9-T10 region of the spinal cord. The retrograde tracer spanned rostrocaudally between T8 and T12 (not shown).

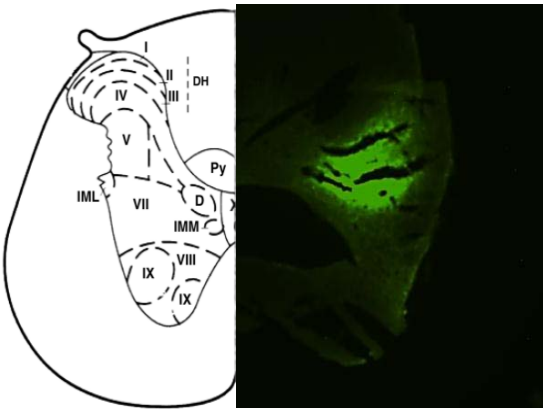

Figure 16. Verification of CTB injection into the T9/T10 region of the spinal cord. Diagram on the left is represents a left hemisection at the T12 level of the thoracic spinal cord taken from a rat atlas (Paxinos and Watson, 2007). The photomicrograph on the right represents CTB immunofluorescent staining in the same T12 region of the spinal cord taken from one sedentary rat]. The dye was present rostrocaudally in sections spanning from T8-T12 (not shown). The largest spread of the CTB injection appeared to be centered around the T9/T10 region.

Discussion

GABA is the major inhibitory neurotransmitter in the central nervous system. It plays an important role in restraining the tonic activity of RVLM neurons. Based on the data from our previous study (see Chapter 2), where we observed enhanced GABA responsiveness following inhibition of the right RVLM, the purpose of this study was to determine whether sedentary conditions increased $GABA_A$ receptor expression in the RVLM when compared to physically active conditions. The data from this study demonstrated that the $GABA_A$ receptor α1 subunit is present in the RVLM of male sedentary and physically active rats. This study also suggests that the $GABA_A$ receptor α1 subunit is expressed on spinally projecting neurons in the RVLM that are likely to play a role in the control splanchnic sympathetic outflow. From these studies we can conclude that $GABA_A$ receptors are present in the RVLM; however, sedentary and physically active conditions do not appear to alter $GABA_A$ receptor α1 subunit expression.

The activity of the RVLM is restrained by GABA, the major inhibitory neurotransmitter (24; 113). The tonic release of GABA in the RVLM is from projections from the caudal ventrolateral medulla, which is modulated tonically by the arterial baroreceptor reflex (113). The baroreceptor reflex helps to maintain resting blood pressure and sympathetic nerve activity (113). There are also other sources of GABAergic input to the RVLM that are independent of the baroreceptor reflex (12; 21; 84; 113). $GABA_A$ and $GABA_B$ receptors have been identified functionally in the RVLM (62; 81). Interestingly, when $GABA_A$ receptors are activated bilaterally in the RVLM, MAP drops to levels similar to ganglionic blockade (46; 114; 115). These data show that $GABA_A$ receptors are may be mediating the majority of inhibitory signaling in the

RVLM. Studies from Menezes and Fontes suggest that $GABA_A$ receptors in the RVLM may be functionally important for restraining the activity of RVLM neurons in conscious rats (81). They showed that microinjection of muscimol into the RVLM caused a significant decrease in MAP and HR. From these data they concluded that $GABA_A$ receptors are present in the RVLM and this receptor may play a role in inhibitory signaling in the RVLM. Multiple laboratories have shown similar functional data by using agonist to $GABA_A$ receptors and support the findings from the Menezes study (81; 114).

The studies above only show that the $GABA_A$ receptors are present in the RVLM, not that they are tonically active. Studies from other laboratories have demonstrated that $GABA_A$ receptors are tonically restraining the activity of RVLM neurons in order to help maintain resting blood pressure and sympathetic nerve activity (40; 90; 97). GABA is tonically released in the RVLM via projections from the CVLM which in turn are activated by the NTS and the arterial baroreceptor reflex (113). Schreihofer *et al*. showed that following sinoaortic denervation, $GABA_A$ receptor blockade in the RVLM still produces an increase in blood pressure (114). These data demonstrate that GABAergic inhibition of the RVLM can be independent of the arterial baroreceptor reflex. Since $GABA_A$ receptors play an important role in restraining the activity of RVLM neurons, any changes in $GABA_A$ receptor expression or function could alter inhibitory signaling.

There are multiple studies that have investigated the role of exercise in the modulation GABA signaling in CNS regions that are involved in the control of the cardiovascular system. Little *et al*. showed that chronic treadmill increased GAD gene expression in the caudal hypothalamus of spontaneously hypertensive rats (SHR) (65).

The study also showed blood pressure was lower in the exercise group when compared to the sedentary SHR (65). De Abreu *et al*. have also shown that blockade of $GABA_A$ receptors with bicuculline microinjections into the PVN caused greater increases in blood pressure in a swim trained group of rats when they were compared to a group of sedentary rats (25). These data suggest that swim training enhances GABAergic mediated inhibition in the PVN. This may be one possible mechanism that helps to lower sympathetic outflow and help prevent hypertension.

GABAergic input to the RVLM is partially mediated arterial baroreceptors (113). DiCarlo's laboratory performed a study in which they recorded renal sympathetic nerve activity and HR in conscious rabbits in response to increases or decreases in blood pressure before and after chronic treadmill training. This study showed that exercise trained rabbits have reduced baroreflex gain when compared to sedentary rabbits (26). In a study from our laboratory, sedentary conditions lead to greater increases in splanchnic sympathetic nerve activity in response to a hypotensive challenge (87). During a hypotensive challenge GABA release is decreasing from the CVLM. This decrease in GABA leads to an increase RVLM neuronal activity and an increase in sympathetic nerve activity. These data also support the idea that there may be enhanced GABAergic inhibition being mediated by the arterial baroreceptor reflex in response to sedentary conditions. This enhanced GABAergic inhibition may be a compensatory mechanism in order to offset the enhanced sympathoexcitation that has been observed following sedentary conditions (88). This current study demonstrated that physical activity did not appear to alter $GABA_A$ receptor α1 subunit in the RVLM when compared to sedentary conditions. From data obtained in this study, it is evident that the $GABA_A$ receptor α1 subunit is present on spinally projecting RVLM neurons that

likely play a role in the control of splanchnic sympathetic nerve activity since they were found on neurons back-labeled from T9/T10 region of the spinal cord. Sedentary conditions did not appear to alter α1 subunit expression in terms of the number of bulbospinal RVLM neurons containing the α1 subunit that also project to the lower thoracic portion of the spinal cord. Therefore it is possible that enhanced GABAergic transmission may be due to increased GABAergic inputs to RVLM.

$GABA_A$ receptors are ionotropic receptors that form a ligand gated Cl^- channel (68). When GABA binds to the $GABA_A$ receptor, the Cl- channel opens and Cl^- enters the cell causing the membrane potential to hyperpolarize and restrains neuronal activity (68). The $GABA_A$ receptor is a heteropentamer usually made up of two α subunits, two β subunits and one γ subunit (68). Though other subunits may substitute in this typical configuration, it has been shown that the α1 is the major subunit present during adulthood (67). Foley and colleagues showed that the $GABA_A$ receptor α1 subunit protein and mRNA were the most abundant α subunit in the RVLM but were unchanged by pregnancy versus non-pregnancy (30). Similar findings in the NTS, examining mRNA levels (110), Saha *et al* also showed that the $GABA_A$ receptor α1 protein was localized in cytoplasmic and sub-synaptic sites (110). The western blot data from the present study showed that α1 is present in the RVLM, and the immunofluorescence data showed that $GABA_A$ receptor α1 subunit is present on bulbospinal neurons in the RVLM that project to the T9-T10 region of the spinal cord, and does not seem to be altered by sedentary or physically active conditions.

There are some technical considerations to the current study that need to be addressed when interpreting the data. The western blot data represents the total amount of protein that is present in the RVLM. Unlike the immunofluorescence studies

there is no way of identifying whether GABA expression is altered on neurons that control a specific organ bed in this particular study.

We specifically investigated the α1 subunit of the $GABA_A$ receptor because we previously showed that it is present in the RVLM and is the main α subunit expressed during adulthood (31; 34; 126). However, the $GABA_A$ receptor complex consists of 5 subunits, including two α subunits, two β subunit s and one γ subunit. It is certainly possible that different subunits are altered by sedentary conditions. In addition, there are also different isoforms of each subunit (35; 68). For instance, it has been shown that the isoforms of the α subunit (α1-6) exist in the brain (35). Therefore further studies investigating $GABA_A$ receptor expression the RVLM may need to look at additional subunits and isoforms.

Based the data from this study alone, it appears that sedentary conditions do not enhance $GABA_A$ receptor expression in the RVLM. However, another possible mechanism that could be responsible for the enhanced GABAergic signaling in the RVLM is enhanced GABAergic input to the RVLM. More experiments need to be done in order to determine whether sedentary conditions lead to enhanced GABAergic inputs to RVLM when compared to physically active rats.

Summary/Perspectives

Based on studies from our laboratory and others evidence has been provided for a role of the CNS in the development of CVD (46; 56; 87). There are several risk factors that are associated with the development of CVD and laboratories have shown that these disease states cause changes in glutamatergic and GABAergic transmission in the RVLM (1; 46; 87; 138; 140). Previously, our laboratory has shown that sedentary conditions in rats lead to elevated resting blood pressure and splanchnic sympathetic

nerve activity (87). In this same study we demonstrated that sedentary conditions lead to enhanced sympathoexcitation in response to glutamate microinjections into the RVLM (87). However, no one had investigated how sedentary conditions modulate GABA signaling in the RVLM; specifically, whether or not $GABA_A$ receptor expression in the RVLM was altered by sedentary conditions. In Chapter 2 we identified a functional compensatory GABAergic mechanism following sedentary conditions, although it does not appear significant enough to counteract the enhanced excitation that is also occurring. It is possible that GABAergic input is being modulated by sedentary conditions and this could be investigated in future studies. By understanding the mechanisms that lead to enhanced GABA signaling we could manipulate and presumably potentiate this mechanism in order to help lower RVLM neuronal activity and ultimately sympathetic nerve activity. This would provide a new therapeutic option for preventing the development of CVD in sedentary individuals.

APPENDICES

Appendix A - Wayne State University IACUC Approval Letter

WAYNE STATE UNIVERSITY

RECEIVED
SEP 11 2012
By

INSTITUTIONAL ANIMAL
CARE AND USE COMMITTEE
87 E. Canfield, Second Floor
Detroit, MI 48201-2011
Telephone: (313) 577-1629
Fax Number: (313) 577-1941

ANIMAL WELFARE ASSURANCE # A 3310-01

PROTOCOL # A 12-11-11

Protocol Effective Period: March 1, 2012 – December 31, 2014

TO: Dr. Patrick Mueller
Physiology
School of Medicine
5263 Scott Hall

FROM: Lisa Anne Polin, Ph.D.
Chairperson
Institutional Animal Care and Use Committee

SUBJECT: Approval of Protocol # A 12-11-11
"Inactivity and Enhanced Sympathoexcitation: Role of Neuroplasticity in the RVLM"

DATE: March 1, 2012

Your animal research protocol has been reviewed by the Wayne State University Institutional Animal Care and Use Committee, and given final approval for the period effective **March 1, 2012** through **December 31, 2014**. The listed source of funding for the protocol is **Start-up Funds, NIH**. The species and number of animals approved for the duration of this protocol are listed below.

Species	Strain	Qty.	USDA Cat.
RATS	Sprague Dawley, Male, Adults	208	C
RATS	Sprague Dawley, Male, Adults	784	D

Be advised that this protocol must be reviewed by the IACUC on an annual basis to remain active. Any change in procedures, change in lab personnel, change in species, or additional numbers of animals requires prior approval by the IACUC. Any animal work on this research protocol beyond the expiration date will require the submission of a new IACUC protocol form and full committee review.

The Guide for the Care and Use of Laboratory Animals is the primary reference used for standards of animal care at Wayne State University. The University has submitted an appropriate assurance statement to the Office for Laboratory Animal Welfare (OLAW) of the National Institutes of Health. The animal care program at Wayne State University is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

Appendix B - Publication in Preparation

The Effect of Sedentary Conditions on GABAergic Transmission in the RVLM

MD Dombrowski, TA. Azar, PJ Mueller
Department of Physiology, Wayne State University, School of Medicine, Detroit, MI

A sedentary lifestyle is a major risk for cardiovascular disease. Cardiovascular disease has been associated with elevated basal sympathetic nerve activity. The rostral ventrolateral medulla is a bilateral brainstem region that is an important for the control of resting and reflex control of sympathetic nerve activity and blood pressure. The activity of these neurons in this region is tonically inhibited by the neurotransmitter γ-butyric amino acid (GABA). Interestingly, we have shown that following unilateral RVLM blockade, acute inhibition of the intact RVLM leads to decreases in splanchnic sympathetic nerve activity that are enhanced by sedentary compared to active conditions in rats. These data suggests that there is a compensatory mechanism that develops under sedentary conditions in order to offset the enhanced sympathetic nerve activity that is also present. In the current study we hypothesize that sedentary conditions will lead to increased GABAergic transmission when compared to active conditions. This dissertation had three specific aims in order to determine the mechanism(s) that were involved in the enhanced GABAergic transmission under sedentary conditions. **AIM 1** was designed to determine *in vivo* whether sedentary conditions alter the responsiveness of the RVLM to GABA-mediated inhibition when compared to physically active conditions. We hypothesized that sedentary conditions enhance sympathoinhibitory responses to GABA inhibition of the RVLM. In Inactin anesthetized, sedentary (SED) or physically active (WR) rats, mean arterial pressure (MAP), heart rate (HR) and splanchnic sympathetic nerve activity (SSNA) were

recorded during unilateral microinjections of GABA (30 nl, 0.3-600 mM) into the RVLM. Following GABA injections, the contralateral RVLM was inhibited with 90 nl of 2 mM muscimol and the GABA injections were repeated. There were no significant differences between SED or EX conditions for MAP, HR and SSNA responses to GABA before contralateral blockade of RVLM. However, after contralateral blockade of the RVLM, the SED rats had enhanced blood pressure responses compared to the WR rats. Based on our results, we conclude that sedentary conditions lead to enhanced inhibition of MAP only after blockade of the contralateral RVLM. These data suggest sedentary conditions lead to enhanced GABA sensitivity but only after removal of GABAergic input to the RVLM. The lack of group differences prior to removal of GABAergic input may be due arterial baroreceptor-mediated buffering via the contralateral RVLM. Once the contralateral RVLM is inhibited then differences are revealed. Since the arterial baroreceptor reflex provides tonic GABAergic input to RVLM, we tested whether this reflex plays a role in SAD sedentary and physically active rats and repeated the GABA dose response injection protocol performed in the intact animal study. We found no differences between sedentary and physically active before and after contralateral blockade of RVLM. These data suggests that the baroreceptor reflex may be playing a role in buffering decreases in sympathetic outflow under sedentary conditions. This may serve as a compensatory mechanism to offset the developing sympathoexcitation. **AIM 2** was designed to determine whether sedentary conditions lead to altered GABAergic input to RVLM when compared to physically active conditions. We hypothesized that sedentary conditions lead to enhanced GABAergic input. We tested the hypotheses that 1) ionotropic glutamate receptors (iGluRs) contribute to increases in MAP and SSNA in response to blockade of $GABA_A$ receptors and 2) SED conditions enhance

glutamatergic excitation. Prior to microinjections in Inactin-anesthetized SED (n=6) and WR (n=5) rats, SAD was performed and one RVLM was inhibited (muscimol 2 mM, 90nl). In Inactin-anesthetized SED (n=7) and WR (n=9) rats, SAD was performed and one RVLM was inhibited (muscimol 2 mM, 90nl). In responses to $GABA_A$ receptor blockade alone (bicuculline 5 mM, 90nl) alone in the intact RVLM there was a pressor and sympathoexcitatory response in SED and WR rats that were not significantly different between groups for MAP and percent change in SSNA. However, the absolute change in SSNA was significantly greater under SED when compared to WR conditions. Following $GABA_A$ receptor blockade, we microinjected the ionotropic glutamate receptor blocker kynurenic acid (40 mM, 90 nl) into the intact RVLM and then repeated the bicuculline microinjection. $GABA_A$ receptor blockade in the presence of iGluRs blockade produced pressor and sympathoexcitatory responses in SED and WR rats that were not significantly different. However, the change in absolute SSNA activity (mV.s) was significantly greater under SED when compared to WR conditions. These data suggests that baroreceptor-independent GABAergic input to the RVLM does play a role in suppressing sympathoexcitation under both SED and WR conditions. Once baro-independent GABAergic input to the RVLM is removed there is remaining sympathoexcitation that is mediated in part by glutamate receptors in the RVLM.. However, when ionotropic glutamate receptors are blocked prior to $GABA_A$ receptor blockade some excitation remains which is driven by non-ionotropic glutamatergic input under SED but not WR conditions. Finally **AIM 3** was designed to determine whether SED conditions alter $GABA_A$ receptor protein expression in the RVLM when compared to WR conditions. We hypothesize that SED conditions enhance $GABA_A$ receptor protein expression in the RVLM. In order to investigate $GABA_A$ receptor α1 subunit

expression on RVLM spinally projecting neurons, SED (n=3) and WR (n=4) male Sprague-Dawley rats were injected bilaterally with cholera toxin B (CTB) at spinal cord segment T9/T10. Double immunofluorescent labeling identified immunoreactivity for CTB (green, FITC) and the $GABA_A$ receptor α1 receptor subunit (red, Cy3) in RVLM. We found that there was no main effect of rostrocaudal CTB expression nor was there a difference found between sedentary and physically active rats. From this data we concluded that sedentary conditions do not alter the expression of $GABA_A$ receptor α1 subunit on spinally projecting neurons that control sympathetic outflow at the T9-T10 region of the spinal cord. We also looked at overall expression of $GABA_A$ receptor α1 subunit in the RVLM using western blot technique. SED and WR male Sprague-Dawley rats' fresh brainstem tissue was sectioned at 80 μm on cryostat. Each section was collected and mounted onto a clean glass slide. Micropunches were taken from each section bilaterally and then placed into a lysis solution. The sections were than stained with cresyl violet, coverslipped and looked at under a brightfield microscope in order to determine which section contained RVLM. Samples were pooled so that each contained a well-defined area of the RVLM that was 240 μm in rostrocaudal distance. The samples were prepared and loaded on a Mini-Protean® TGX precast gel and electrophoresis was performed. The protein was transferred to a PVDF membrane. The membrane was exposed to the $GABA_A$ receptor α1 subunit primary antibody overnight and then incubated with the secondary antibody the next day for 2 hours. The membrane was incubated in ECL and developed. The $GABA_A$ receptor α1 subunit was normalized to GAPDH. There was no main effect of rostrocaudal expression of $GABA_A$ receptor α1 subunit. Sedentary conditions did not enhance the protein expression of $GABA_A$ receptor α1 subunit when compared to physically active rats. These data

suggest that sedentary conditions do not enhance $GABA_A$ receptor expression. Overall, the data from this study show that sedentary conditions do enhance GABAergic transmission in the RVLM which be due to changes in GABA sensitivity or release.

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ABSTRACT

THE EFFECT OF SEDENTARY CONDITIONS ON GABAERGIC TRANSMISSION IN THE RVLM

by

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Major: Physiology

Degree: Doctor of Philosophy

Physical inactivity is detrimental to our health and is a major risk factor for the development of CVD (138). Our laboratory has shown that physical inactivity leads to elevated resting splanchnic sympathetic nerve activity when compared to physically active conditions(91). Our laboratory has also shown that in response to glutamate microinjected into RVLM sedentary rats have greater increases in SSNA (enhanced sympathoexcitation) when compared to physically active rats (91). Negrao et al showed that in response to angiotensin II and sodium nitroprusside infusion lead to sympathoexcitation but the exercise trained rats had greater increases in SSNA when compared to the unexercised rats (105). Roveda et al showed that muscle sympathetic nerve activity (MSNA) is elevated in heart failure patients when compared to healthy individuals (60). Following a single bout of exercise MSNA was lower in the heart failure patients (60). This enhanced sympathoexcitation has been suggested to lead to overactivity of the sympathetic nervous system (28; 39; 117). The mechanisms that lead to alterations in the central nervous system in response to sedentary and physically active conditions are not completely understood. This project is aimed to help provide

more information on the mechanisms that are playing a role in altering central control of the sympathetic nervous system in response to sedentary and physically active conditions.

AUTOBIOGRAPHICAL STATEMENT

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Education

2015 Ph.D. in Physiology, Wayne State University, School of Medicine

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Honors and Awards

2014 American Physiological Society minority Travel Fellows, San Diego, CA

2012 3rd place award for poster presentation at Graduate Exhibition, Wayne State University, Detroit MI

2010 Fellowship, Initiative for Maximizing Student Development, Wayne State University, Detroit MI

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Peer-reviewed Publications:

1. Mischel, N., Subramanian, M., **Dombrowski, M**., Llewellyn-Smith, I., Mueller, P., (In)activity-Related Neuroplasticity in Brainstem Control of Sympathetic Outflow: Unraveling Underlying Molecular, Cellular and Anatomical Mechanisms. Am J. Physio Heart Circ Physiol 309:H000-H000, 2015. Doi:0.1152/ajpheart00929.2014

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1. **Dombrowski, M**., Azar, T.A., I., Mueller, P., 2015. Ionotropic glutamate receptors partially mediate the pressor and sympathoexcitation to disinhibition of the RVLM after sinoaortic denervation and contralateral RVLM inhibition in sedentary and physically active rats. Abstract for poster presentation, Experimental Biology, Boston, MA.
2. **Dombrowski, M**., Azar, T.A., I., Mueller, P., 2014. Cardiovascular responses to disinhibition of rostral ventrolateral medulla (RVLM) following unilateral RVLM blockade in sedentary or physically active, sinoaortic denervated rats. Abstract for poster presentation, Experimental Biology, San Diego, CA.
3. **Dombrowski, M**., Llewellyn-Smith, I., Mueller, P., 2013. Immunofluorescence identifies the α1 subunit of the $GABA_A$ receptor on spinally projecting neurons in rostral ventrolateral medulla. Abstract for poster presentation, Experimental Biology, Boston, MA.
4. **Dombrowski, M**., Azar, T., Mueller, P., 2012. The effect of physical (in) activity on blood pressure and splanchnic sympathetic nerve responses to inhibition of the RVLM. Abstract for poster presentation, Experimental Biology, San Diego, CA.
5. **Dombrowski, M**., Azar, T., Mueller, P., 2012. The effect of physical (in) activity on blood pressure and splanchnic sympathetic nerve responses to inhibition of the RVLM. Abstract for poster presentation, Wayne State University and University of Michigan Physiology Symposium, Detroit, MI.
6. **Dombrowski, M**., Azar, T., Mueller, P., 2011. GABAergic Neurotransmission in the RVLM in response to sedentary conditions. Abstract for poster presentation, 15th annual Wayne State University Graduate Student Research Day, Detroit, MI.
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