8-2021

The Impacts of Aging, Sedentarism, And Exercise on Neurotrophic Factor Expression and Innervation in the Heart and the Effects of Treatment With α-CGRP On Heart Function

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THE IMPACTS OF AGING, SEDENTARISM, AND EXERCISE ON NEUROTROPHIC FACTOR EXPRESSION AND INNERVATION IN THE HEART AND THE EFFECTS OF TREATMENT WITH α-CGRP ON HEART FUNCTION

by

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A dissertation submitted to the Graduate College
in partial fulfillment of the requirements
for the degree of Doctor of Philosophy
Biological Sciences
Western Michigan University
August 2021

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Neurotrophic factors (NFs) are important molecules responsible for development, differentiation, regeneration, and maintenance of new and mature neurons. Neurotrophic factors act as neurocytokines and may assist with the regulation of axonal and dendritic arrangements and synaptic plasticity between neurons themselves or with other non-neural target tissues. In this study, we analyze the levels of two NFs: glial cell line-derived neurotrophic factor (GDNF) and nerve growth factor (NGF). Cardiomyocytes produce these neurotrophic factors which assist with the innervation pattern of the heart. The heart is innervated by the two branches of the autonomic nervous system; namely the sympathetic nervous system and the parasympathetic nervous system, as well as the sensory nervous system. Changes in neurotrophic factor protein content in the heart may cause modifications in structural plasticity of all branches of the nervous system, which may contribute to, or prevent development of, cardiac diseases. Aging, sedentarism, and exercise are factors known to contribute to changes in neurotrophic factor levels in a diversity of tissues throughout the body, including the heart. The goal of this research was to investigate the impact of aging, sedentarism, and exercise on NFs levels and to examine structural plasticity of all branches of the nervous system that innervate the heart throughout the entire lifetime of rats.

Calcitonin gene-related peptide (CGRP) plays an important role in physiology as a potent vasodilator, which may help prevent cardiac and pulmonary hypertension, ischemia, migraine, and ultimately, improve blood flow distribution, and wound healing. It has been suggested that CGRP may play a role in cardiovascular regulation; however, the effects of exogenous CGRP on cardiac physiology has not been adequately investigated. An additional goal of this research was to investigate the effects of exogenous αCGRP on heart function.

Sprague-Dawley rats were used for the aging/exercise studies in this thesis. NGF and GDNF levels in the heart, innervation pattern of the heart, blood pressure (BP) and heart rate (HR) were examined throughout the entire animals' lifetime. For the CGRP studies, adult bullfrogs (*Lithobates catesbeianus*) were used. Following CGRP treatment alone or in combination with autonomic antagonists, the force of contraction (FOC) and HR were examined in the heart.

Resting HR and BP were significantly increased in young Sprague Dawley rats compared to older rats. Specifically, eighteen months old and 24 months old sedentary animals had significantly higher HR and BP when compared to 6mo-sed and 12mo-sed. In addition, the mean arterial blood pressures in older sedentary Sprague Dawley rats were greater than 100mmHg, which is characterized as hypertension. At 18mo-sed and 24mo-sed, NGF levels were significantly lower when compared to all younger ages. Voluntary exercise significantly increased NGF and GDNF levels in all heart chambers when compared to the age-matching sedentary groups. From 4wk-sed to 14wk-sed, GDNF protein levels significantly increased in all heart chambers. From 6mo-sed and older groups, GDNF protein levels progressively and significantly decreased in all heart chambers. Our data demonstrates that, throughout the animal's lifespan, aging combined with sedentary behavior can lead to an increase in

sympathetic nerve density and a decrease in parasympathetic and sensory nerve densities in the heart. Our results suggest that exercise may significantly increase parasympathetic nerve density in the heart and reduce resting BP and HR.

The data collected by this thesis suggests that neurotrophic factor content in the heart peaks in young animals and declines with aging. GDNF content declines with aging earlier and more drastically than NGF content. These results support the hypothesis that NGF primarily supports sympathetic nervous system, which does not seem to change much with age, while GDNF supports the parasympathetic system, which does decline with age. NGF supports the sensory nervous system. Therefore, the changes in NGF content that were observed in these studies may be linked to the changes in sensory nerve density. Density of parasympathetic and sensory innervation decline with aging, while sympathetic innervation does not decline as much with aging, which may be the cause for an increase in BP and HR. Therefore, both BP and HR increase with age, as balance between sympathetic and parasympathetic innervation is impaired. With exercise, GDNF content increases, parasympathetic innervation increases, and BP and HR decrease. The effects of exercise on neurotrophic factor expression may be a possible mechanism by which exercise exerts positive effects on cardiac innervation, promoting the prevention and treatment of cardiovascular diseases.

All bullfrogs had lower FOC and lower HR within 5-10 minutes after CGRP treatment when compared to the untreated controls. Our results demonstrate that combined treatments using atropine, a non-selective muscarinic acetylcholine receptor antagonist, and CGRP, promoted negative inotropic and chronotropic effects in the heart. Evidence obtained from immunocytochemical studies suggest that CGRP is available in nerve fibers in the wall of the

frog heart. CGRP could be released by these nerve fibers, possibly through varicosities, to cause the effects observed in these studies.

This data suggests that exogenous CGRP treatment significantly reduces heart rate and force of contraction in the heart of frogs, even when the parasympathetic nervous system was blocked, and can influence cardiac physiology. CGRP and the sensory nervous system may actively play additional and important roles in the heart and other organs and systems.

ACKNOWLEDGMENTS

I first would like to acknowledge Dr. John Spitsbergen, a wonderful mentor throughout my career at Western Michigan University. His friendship, disposition, and passion has guided and supported me through the graduate program for growth, opportunities, and success. I will always be grateful for his guidance, his friendship, and the friendship of his incredible family.

I would also like to acknowledge the members of my committee, Dr. Cindy Linn, Dr. Pamela Hoppe, and Dr. James Springstead. Their help and suggestions during my research were fundamental to enhance the quality of my project and augment my knowledge in science. Our conversations have introduced me to many disciplines within the scientific field.

I would like to acknowledge my family for all their support. Despite being across the globe, I appreciate their words of love, support, and encouragement throughout my educational career in the United States of America. While I was in Brazil, their abundant love and support were the foundation that allowed me to achieve prosperity in a different country.

Finally, I would like to acknowledge my wife, Dr. Anel Arias. I witnessed Anel's journey through her own doctorate program in counseling psychology at Western Michigan University. Her perseverance and fortitude were inspirational to me. Anel's unconditional support, motivation, companionship, and love have supported me throughout my entire program, and I am eternally grateful for it.

Once again, thank you very much to my family, Dr. Spitsbergen and family, and committee members for being in my life, for helping me grow, and for sharing memories.

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CHAPTER I

INTRODUCTION

Neurotrophic factors (NFs) are a type of biological molecule responsible for regulation of development, differentiation, regeneration, and maintenance of new and mature neurons. Neurotrophic factors act as neurocytokines, assisting with the regulation of axonal and dendritic arrangements and synaptic plasticity between neurons themselves or with other non-neural target tissues. Examples of neurotrophic factors explored in this thesis are glial cell line-derived neurotrophic factor (GDNF) and nerve growth factor (NGF).

Early Studies With GDNF

GDNF belongs to the GDNF family ligands (GFLs), which is composed of neurotrophic factors including artemin, persephin, neurturin and GDNF (Lin et al, 1993; Heuckeroth et al, 1996; Baloh et al, 1998; Milbrandt et al, 1998; Rosenblad et al, 2001). GDNF research led by Lin et al. (1993 & 1994) isolated GDNF from B49 rat glial cells in culture. These studies reported that GDNF increased dopamine uptake by dopaminergic neurons and that GDNF may augment dopaminergic neural differentiation and survival rates (Lin et al., 1993 & 1994). Shortly after the discovery of GDNF, researchers found GDNF mRNA and protein in a variety of cells and tissues throughout the body. Suter-Crazzolara and Unsicker (1994) found GDNF mRNA in the heart, blood, kidney, liver, lung, spleen, bone, and sciatic nerve. Additionally, this research team found a shorter length of GDNF mRNA in these tissues, which suggests that GDNF mRNA may undergo alternative splicing (Suter-Crazzolara and Unsicker 1994). Springer et al (1994) identified two variants of GDNF mRNA in all regions of the central nervous system (CNS) in rats, specifically in the hippocampus, cortex, striatum, and spinal cord of humans. This research group also reported GDNF mRNA in the dorsal root ganglia of 24-hour postnatal rats (Springer

et al, 1994). Trupp et al. (1995) found GDNF mRNA for both GDNF isoforms in developing gonads, stomach, whisker pad, skin, skeletal muscle, adrenal glands, lungs, spinal cord, dorsal root ganglion (DRG) and superior cervical ganglion in rats (Trupp et al., 1995). In adult humans, the highest levels of GDNF mRNA were found in muscles and in the spinal cord respectively (Yamamoto & Yamamoto 1996). Springer et al (1995) identified GDNF protein in two isoforms (GDNFα and GDNFβ) in rat skeletal muscle and Schwann cells. Results of this study also suggests that GDNF may operate as a target-derived trophic factor, in which GDNF is released by the tissues in the peripheral target tissues of the nervous system and promote neural plasticity (Springer et al., 1995).

GDNF Expression and Processing

The precursor form of GDNF protein contains 211 amino acids and is called pro-GDNF in mammalian cells. Pro-GDNF is transported to the endoplasmic reticulum before being secreted. As pro-GDNF is secreted, it folds by forming di-sulfide bonds, dimerizes and goes through proteolytic processing via N-linked glycosylation by furin, PACE4, and protein convertases PC5A, PC5B, and PC7, to become the mature GDNF peptide with 134 amino acids (Lin et al. 1993; Lin et al. 1994; Piccinini et al. 2013; Lonka-Nevalaita et al. 2010; reviewed by Cintrón-Colón et al. 2020). GDNF genes can undergo alternative splicing to generate a long mRNA version called pre-(α)pro-GDNF and a short mRNA version pre-(β)pro-GDNF. Both mRNA versions can be cleaved and generate mature versions of GDNF protein (Suter-Crazzorola and Unsicker 1994; Matsushita et al. 1997; Grimm et al. 1998; Penttinen et al. 2018). The shorter version of GDNF mRNA has been found in humans and rodents. It has a 78 pair deletion in the exon I of the GDNF mRNA transcript, which will lead to a 26 amino acid deletion in the pro-region of the protein (Cristina et al. 1995; Schaar et al. 1994; Wang et al. 2008).

GDNF Trafficking

Target cells release trophic factors, which will contribute to the growth and maintenance of neural populations that innervate them. The trophic factors that are released by target cells arrive at the axon terminals and are carried away via retrograde transport to the cell bodies of the neurons, promoting survival. Retrograde signaling occurs following internalization of the neurotrophic factor-receptor complex at the axon terminal. Following internalization, compartmentalization of neurotrophic factor-receptor complex occurs in signaling endosomes, which are carried to the cell body via transport proteins (Wu et al. 2009; Zahavi et al. 2015; Zahavi et al. 2017).

The processes regulating long-distance trafficking and internalization of GDNF are not well understood. However, one of the best characterized GDNF receptors, a receptor known as the RET receptor, has been shown to couple with AP2 and clathrin in the plasma membrane, which could enable clathrin-mediated endocytosis (Beattie et al. 2000; Crupi et al. 2015; Howe et al. 2001). Retrograde and anterograde transport of GDNF has been reported in adult organisms (Henderson et al. 1994; Nguyen et al. 1998; Leitner et al. 1999; Haase et al. 2002; Rind and von Bartheld 2002; Russell et al. 2000; Zahavi et al. 2015). To further understand the retrograde transport of GDNF between muscles and neurons, Zahavi et al. (2015) created a modern *in vitro* microfluidic platform containing motor neuron (MN) cell bodies in one chamber and muscle cells in another connected chamber. MNs grew from the cell bodies, extended through the microgrooves, and connected to the muscle cells forming functional neuromuscular junctions (NMJs). Then, researchers applied GDNF into the MN cell bodies, which promoted survival via the AKT signaling pathway, a well-known pathway that promotes cell growth and survival. GDNF was also applied in the muscle compartment side, which led to axonal growth at the axon

tips and enhanced innervation of muscle cells. The team of researchers was also able to visualize retrograde transport of GDNF from muscle cell to neuron (Zahavi et al. 2015; reviewed by Franke et al. 2003).

GDNF Receptors

The members of the GFLs are homodimers (Lin et al., 1994) and utilize the receptors RET and glycosylphosphatidylinositol-linked GDNF receptor (GFRα), which may be GFRα1, GFRα2, GFRα3, or GFRα4 (Jing et al., 1996; Sariola and Saarma 2003). By binding to the RET-GFRα complex, GDNF activates its receptor and triggers a variety of intracellular second message pathways such as Erk, Akt, phosphoinositositide-3-kinase (PI-3K) and mitogen-activated protein kinase (MAPK) which are known pathways that aid cell proliferation and survival (Airaksinen and Saarma 2002; Sariola and Saarma 2003; Kim and Kim 2018). GFRα has also been shown to interact with another membrane protein, known as the neural cell adhesion molecule (NCAM), which forms another receptor for GDNF as demonstrated by Paratcha et al. (2003). This GFRα-NCAM signaling stimulates axonal growth and Schwann cell migration in cultured hippocampal and cortical neurons. GFRα-NCAM activation leads to downstream activation of protein tyrosine kinases/FYNs, or focal adhesion kinases (FAKs), known to promote cytoskeleton reorganization (Paratcha et al., 2003; Beggs et al., 1997). NCAM has been shown to participate in regulation of synaptic plasticity, neurite outgrowth and cell migration (Schachner 1997; Crossin and Krushel 2000; Rønn et al. 2000). Work from Pozas and Ibáñez (2005) suggests that GDNF promotes migration and differentiation of embryonic cortical GABAergic neurons, cells in which RET and NCAM receptors are absent (Pozas and Ibáñez, 2005). Bespalov et al. (2011) suggest that most members of the GFL can bind to a transmembrane heparan sulfate proteoglycan known as syndecan-3. The interaction between

GFL-syndecan-3 promotes cell spreading and neurite outgrow by activation of Src family kinase pathways (Bespalov et al., 2011). Src family pathway is known to promote neurite outgrowth, cytoskeletal reorganization, development, and segregation of focal contact/adhesion formation (Ignelzi et al., 1994; Beggs et al., 1997; Kinnunen et al., 1998; Rauvala et al., 2000; Volberg et al., 2001; Hienola et al., 2006; Bespalov et al., 2011).

GDNF in Development

Evidence that GDNF is an important neurotrophic factor for the development and maintenance of the central and peripheral nervous systems is overwhelming in the literature. GDNF receptors are highly expressed during the embryonic phase in mice. Using in-situ hybridization, researchers found GDNF receptors in the developing central and peripheral nervous systems; respiratory, digestive, endocrine, and urogenital systems; as well as on skin, bone and muscles. GFL members were found in many target tissues such as trigeminal regions, urogenital system, kidneys, mesenchyme/epithelial induction, submandibular gland, and limbs during embryonic development. In adult mice, the expression of GDNF receptors and GFL members were decreased. These findings suggests that GFL members and their receptors are essential for the development of the central and peripheral nervous systems (Golden et al, 1999). Results of other studies have shown that at the time of apoptosis, during development, trophic factor levels are low (Oppenheim 1989; Yuen et al., 1996).

GDNF is also essential to normal morphogenesis of the ureteric bud in developing kidneys and in Sertoli cells in the testis (Schuchardt et al. 1994; Cacalano et al. 1998; Enomoto et al. 1998; reviewed by Hofmann, 2008). Other members of the GFL are also known to promote development and neural plasticity. Neurturin, artemin, persephin, and GDNF promoted survival and differentiation of tyrosine hydroxylase (TH) immunoreactive neurons in cultured E14 rat

ventral mesencephalon cells (Zihlmann et al, 2004). The GDNF receptor RET is present in peripheral and central nervous system during development in rats (Sanicola et al. 1997).

GDNF in the Periphery

GDNF protein has been found to be a potent neurotrophic factor by promoting survival of motor neurons (MN) in the peripheral nervous system and it is predicted to be an excellent candidate to treat MN diseases (Henderson et al. 1994). GDNF was found to prevent naturally occurring programmed cell death of motor neurons (MN) in an avian model. GDNF treatment increased the survival of cultured motor neurons and prevented cell death and atrophy of spinal motor neurons after peripheral axotomy (Wang et al, 1995). It is suggested that GDNF may be involved in reinnervation of skeletal muscle. By analyzing muscle biopsies from Duchenne muscular dystrophy patients, researchers found elevated levels of GDNF in denervated muscle. GDNF levels were also elevated in rapidly-progressive neurogenic atrophy in amyotrophic lateral sclerosis patients when compared to patients with chronic atrophy. Denervation caused by MN lesion is suggested to trigger GDNF expression, as an attempt of the muscle to recover its innervation (Lie & Weis, 1998). Muscle from mice overexpressing GDNF displayed hyperinnervation of neuromuscular junctions, suggesting that GDNF is a powerful target-derived neurotrophic factor (Nguyen et al., 1998; Springer et al., 1995).

GDNF in the Heart

The heart is innervated by sympathetic, parasympathetic, and sensory nervous systems and they respond to most GFL members (Hiltunen et al., 2000). Researchers found RET and GFRα2 transcripts in neurons present in the heart muscle, and GFRα1 and GFRα3 mRNAs in non-neural cells in the heart ganglia. Researchers were also able to observe GFRα2 immunoreactivity in cardiac ganglion neurons and their respective nerve fibers. mRNA from

GFL receptors was found in the endocardium, valves, atria, and pulmonary trunk. Results from these studies suggests that GFRα2/RET are required for normal cholinergic innervation of the heart. GFRα2 knockout mice had 40% reduction of cholinergic innervation in the ventricles and 60% reduction of the ventricular conduction system (Hiltunen et al. 2000). Cardiomyocytes may promote the growth, development, and maintenance of all branches of the nervous system by secreting GDNF and other members of the GFL. GDNF mRNA was found in atrial and ventricular myocytes in rats. Additional findings suggest that GDNF protein is synthesized by cardiomyocytes in the heart of normal and sympathectomized animals. In normal rats, GDNF levels were found to be higher in 37-day-old animals than in 60-day-old animals. GDNF protein levels were significantly higher 7 days after sympathectomy and dropped to control level 30 days after this procedure, suggesting that GDNF may participate in nerve maintenance and regeneration in the heart of rats (Martinelli et al., 2002). The literature supports that GDNF is produced by cardiomyocites and may regulate nerve growth, development, and maintenance in the heart. However, it is still unknown how aging, exercise, and sedentary lifestyle may affect GDNF levels in the heart.

GDNF and Exercise

Exercise has been suggested to promote recovery of central and peripheral nerve injuries and delay the progression of neurodegenerative diseases directed by neurotrophic factor signaling (reviewed by Cobianchi et al. 2017). Work from Wehrwein et al. (2002) found that a 4-week walking exercise plan resulted in increased GDNF production by soleus, gastrocnemius, and pectoralis major muscles of rats. Two weeks of hindlimb unloading provoked a decrease in GDNF production in the hindlimb muscles, but an increase in GDNF production by the pectoralis major. These results suggest that GDNF production by skeletal muscles in rats is

activity-dependent, indicating that exercise may promote remodeling and recovery of NMJs in injury and disease (Wehrwein et al. 2002). Work from McCullough et al. (2013) found that different modalities of exercise, such as involuntary and voluntary running, and swimming caused significant increase in GDNF protein levels in the spinal cord. Low intensity running also led to an increase in GDNF protein levels in the spinal cord of old animals. The results suggest that when GDNF protein levels increase, MN cell body size and vesicle-like structures containing GDNF also increase in the spinal cord of exercised animals (McCullough et al. 2013).

Studies from Gyorkos et al. (2014) suggest that swimming and running increase GDNF protein content in soleus muscle, which contains predominantly Type I slow twitch fibers. The swimming group showed a trend to increase in GDNF protein content in the extensor digitorum longus muscle which contains Type II fast-twitch fibers. NMJ morphology was also examined and demonstrated that the total end plate area increased in the swimming exercise regimen group (Gyorkos et al. 2014). Taken together, these studies strongly suggested that exercise increases GDNF production by target tissues of the nervous system such as muscles, promoting growth, regeneration, maintenance of the nervous system and synaptic potentiation at the NMJ.

Research from Alves et al. (2019) investigated the impacts of exercise in neurotrophic factor protein content in the heart of healthy and chagasic animals. Trained controls, trained chagasic mice, and sedentary chagasic mice had significantly higher GDNF protein content in their hearts than in sedentary controls. These results suggest that exercise increases GDNF levels in the heart. Chronic infections, such as Chagas disease caused by *Trypanosoma cruzi*, may also increase GDNF content, suggesting additional roles of this neurotrophic factor to maintain cardiac function near to its normal parameters. NGF mRNA and protein levels were significantly higher in the heart twenty days after inoculation with trypomastigotes, suggesting that NGF may

participate in regenerative events after an acute myocarditis (Alves et al. 2019; Martinelli et al. 2006). Ultimatelly, exercise may exert positive effects in neurotrophic factor levels and may potentially help mitigate the effects of cardiovascular diseases. More research is necessary to determine the effects of exercise on NGF and GDNF protein levels in the heart throughout different ages.

GDNF in Aging

Neurotrophic factor levels have been shown to decline with aging in the central and peripheral nervous systems. Neurotrophic factors such as GDNF, NGF and brain-derived neurotrophic factor (BDNF) are known to decline with aging in humans and in several animal models, and these low levels of neurotrophic factor may be related to cognitive impairment, Alzheimer's disease (AD), Parkinson's disease (PD) and age-related frailty (reviewed by Budni et al. 2015).

Studies from Yurek & Fletcher-Turner (2001) using long-living hybrid rats (F344BNF1), suggest that BDNF and GDNF levels are significantly increased in young animals as well as in young animals affected by unilateral 6-hydroxydopamine (6-OHDA) lesion of the nigrostriatal pathway, when compared to old normal and old-affected animals. At two weeks post-lesion, GDNF and BDNF levels were higher in the young-affected group when compared to the young healthy group, suggesting a compensatory mechanism of the nigrostriatal system in young animals. There was no compensatory increase in neurotrophic factor levels in the old-affected group when compared to the old healthy group, indicating that aging may limit neurotrophic factor production in the brain (Yurek & Fletcher-Turner, 2001). Boger et al. (2006) used a wild-type (WT) and a heterozygous GDNF+/− mice to evaluate the impacts of chronic reduction of GDNF in motor function and the nigrostriatal dopamine system during the aging process. Their

results suggest that both animal models had age-related depletion in exercise, however, it happened 4 months earlier in the GDNF+/− mice (12 months-old). Using an accelerating rotarod apparatus and comparing the performances of young (8 months-old) and aged (20 months-old) WT and GDNF+/− mice, Boger et al. (2006) found that aged WT mice performed as well as their young counterparts, and aged GDNF+/− mice underperformed compared to all other groups. Using tyrosine hydroxylase (TH) immunostaining, researchers found that age-related decrease in substantia nigra was accelerated in the GDNF+/− mice (Boger et al. 2006).

GDNF treatment is suggested to prevent and treat age-related sequels. After selecting old Fisher 344 rats with poor performance in the Morris water maze, Pertusa et al. (2008) injected lentiviral vectors encoding human GDNF that are specifically transduced by astrocytes in the hippocampus. Their results suggest that GDNF released by astrocytes improved neuron activity by increasing the production of neurotransmitters such as serotonin, dopamine, and acetylcholine. Significant cognitive enhancements such as spatial learning and memory were observed two weeks after gene transduction (Pertusa et al. 2008). Work from Xie et al. (2018) investigated the effects of exogenous GDNF via intrathecal administration. Three groups of Sprague-Dawley rats were used that included adult-controls, aged-controls, and aged-injected animals. Immunostaining was used to detect activation of microglia/monocyte as a marker for neurodegeneration. Recordings of compound muscle action potentials was used to test muscular function, while Western blots was performed to quantify GDNF, GFRα1, neuregulin-1, and other markers. Their results suggested that aged groups had significantly lower levels of GDNF, GFRα1, and neuregulin-1 comparing to the adult group. Exogenous GDNF attenuated neuromuscular dysfunction in aged-injected animals, however, it did not prevent activation of spinal microglia/monocyte, but increased levels of GFRα1 and neuregulin-1 (Xie et al. 2018).

Research from Adly et al. (2016) suggests that GDNF and GFRα1 levels diminish with aging in the epidermis of humans. The levels of these proteins are the highest in children (5–18 years) and gradually decrease in the other groups: adults (19–60 years) and elderly (61–81 years) respectively. GDNF and GFRα1 are highly expressed in the stratum basale. However, their levels gradually decrease towards the top layers of the epidermis and are not detected in the stratum corneum. In elderly individuals, GDNF and GFRα1 expression is restricted in the stratum basale and is found in much lower levels compared to the other age-groups (Adly et al. 2016).

The literature is compelling on the powerful roles that GDNF possesses. This potent neurotrophic factor can aid neuroprotection, development, and regeneration of new and existing neurons in the central and peripheral nervous systems. GDNF levels are depleted by aging but increased with exercise, and it may also support synaptic potentiation and structural plasticity. Finally, delivering GDNF exogenously may be a potential treatment for neurodegenerative diseases, age-related peripheral neuromuscular dysfunction, and age-related cognitive depletions.

Early Studies With NGF

NGF belongs to the neurotrophin family of neurotrophic factors, which is composed of NGF, BDNF, neurotrophin-3 (NT-3), and neurotrophin-4 (NT-4). These trophic factors are also important for the development, growth, maintenance, and regeneration of neurons in the central and peripheral nervous systems. NGF research began in the lab of Levi-Montalcini, who first observed that a soluble growth factor was released by cancerous sarcoma tissue to promote outgrowth of sensory and sympathetic fibers (Levi-Montalcini and Hamburger, 1951, Cohen et al., 1954, Levi-Montalcini and Cohen, 1960).

NGF Expression and Processing

NGF mRNA and protein are expressed by various cell types in an animals body, such as hepatocytes, smooth muscle cells, heart cells, epithelial cells, fibroblasts and myofibroblasts; cells from the immune system including mast cells, eosinophils, lymphocytes, granulocytes, and antigen presenting cells; and accessory cells of the nervous system such as astrocytes, Muller cells, and glial cells (Furukawa et al. 1984; Spitsbergen et al. 1995; Clemow et al. 1998; Micera et al. 2003; Lambiase et al 2004). NGF and BDNF mRNAs are both expressed at high levels in the heart (Yamamoto and Yamamoto 1996). Similar to GDNF, NGF and other members of the neurotrophin family are produced and secreted by neurons themselves (autocrine), nearby cells (paracrine), or target cells, then bind to their receptors in central and peripheral neurons where they are retrogradely transported to the nerve cell bodies to promote survival and maintenance of these neurons (Thoenen 1995; Bothwell 1995; Yuen et al., 1996; Sobue et al., 1998; Ginty 2002; reviewed by Skaper 2012).

All members of the neurotrophin family are translated as precursor molecules (pro-neurotrophins) and then intracellularly cleaved into their mature forms by furin and proconvertases. The precursor molecules' pro-regions assist with proper folding and secretion pathway (Suter et al. 1991; Seidah et al. 1996; Rattenholl et al. 2001; Lee 2001). After secretion, pro-NGF is further processed by extracellular plasmin, matrix metalloproteinase type 7 and 9 (Bruno & Cuello, 2006).

NGF Receptors

NGF binds to two different classes of receptor; the high affinity tropomyosin-related kinase (TrkA) receptor and the common low affinity $p75^{NTR}$. The literature strongly supports that activation of p75 receptor may trigger deleterious effects in neurons. During development or an

inflammatory condition, the interaction between NGF and its receptor $p75^{NTR}$ is suggested to promote cell death in several cell types, such as oligodendrocytes, and neurons in the CNS, in sciatic MNs, and in neointimal smooth muscle cells (Casaccia-Bonnefil et al., 1996, Roux et al., 1999, Terrado et al., 2000, Beattie et al., 2002, Kraemer, 2002). Wiese et al. (1999) performed facial nerve axotomy in wild-type mice and p75-null mice treated with NGF. The results suggest that NGF treatment increased MN loss in wild-type mice, but not in p75-null mice, supporting the hypothesis that cell apoptosis promoted by NGF is dependent on the p75 receptor (Wiese et al. 1999). Adult mice lacking p75 recovered their whisker movement from facial nerve crush earlier than mice that express p75. Additionally, re-expression of p75 in aged animal augmented MN predisposition to undergo cell apoptosis (Ferri et al. 1998).

Immunohistochemical investigations have found immunoreactivity of NGF receptors in active multiple sclerosis (MS) lesions in humans. The receptor p75 was immunoreactive in astrocytes, microglia, and macrophages in those lesions. These results suggest that NGF may play a role in regulating the autoimmune response in the glia and immune cells (Valdo et al., 2002). p75 immunoreactivity is increased in MN of an ALS mouse model, while TrkA and TkB are absent (Seeburger et al., 1993; Copray et al., 2003). Additional studies using an ALS mouse model, suggest that p75-null female mice had delayed manifestations of the disease (Küst et al. 2003). Work from Turner et al. (2003) used antisense peptide nucleic acid (PNA) constructs to knockdown the p75 receptor's gene. Their research suggest that this treatment reduced apoptotic signaling by NGF in Schwann cell cultures. Mice treated with PNA had significant delay in locomotor impairment and mortality compared to the control, and PNA treatments increased the survival of spinal cord neurons (Turner et al., 2003). Many other studies have shown the role that pro-NGF, NGF, and the receptor p75 in neurodegeneration in central and peripheral nervous

system via activation of apoptotic cascade (Lee, 2001; Beattie et al., 2002; Peng et al., 2004; Harrington et al., 2004; Nykjaer et al., 2004; Pedraza et al., 2005; Teng et al., 2005; Volosin et al., 2006; Domeniconi et al., 2007).

Expression of p75 is increased in spinal motor neurons after rats are born, but decreases as rats age. P75 expression is not detected two weeks into the post-natal phase (Yan & Johnson, 1988). This may suggest that NGF and p75 may have a role on natural programmed cell death of motor neurons during development. However, p75 may be re-expressed later in life. Work from Xie et al. (2003) found that p75 is re-expressed in the spinal motor neurons of aged and axotomized rats, in which 60% of spinal MN were immunopositive to p75 (Xie et al., 2003). Work from Hasenöhr et al. (1997) suggests that rats with increased levels of p75 in the basal forebrain performed better in the water maze test than those animals with lower levels of this receptor. Their results suggest that this increase of p75 may be a compensatory or adaptive mechanism of the nervous system to maintain its normal roles in aged animals (Hasenöhr et al. 1997).

Altogether, the literature strongly suggests that pro-NGF and NGF are involved with apoptotic signaling pathways via activation of their receptor $p75^{NTR}$, which lead to nerve degeneration during aging, disease, and natural programmed cell death of central and peripheral nerves.

On the other hand, the literature suggests that the signaling effects caused by NGF biding to TrkA receptors have completely opposite effects than NGF-$p75^{NTR}$ signaling. TrkA signaling has been suggested to promote neurite outgrow, survival, and maintenance of sympathetic and sensory neurons. TrkA knockout mice had severe sympathetic and sensory deficiencies and die a few weeks after birth (Martin-Zanca et al., 1986). Similar observations were seeing with NGF

knockout mice (Smeyne et al., 1994, Huang and Reichardt, 2001). Smeyne et al. (1994) suggests that TrkA knockout mice had considerable cholinergic basal forebrain and cortical projection deficits, and serious sympathetic and sensory neuropathies (Smeyne et al., 1994). *In-vitro* research by Yoon et al. (1998) using cultured oligodendrocytes suggests that activation of TrkA receptor promoted cell survival and inactivated $p75^{NTR}$ by suppression of c-jun kinase activity (Yoon et al. 1998). C-jun kinase activity is triggered by $p75^{NTR}$ and it is suggested to promote cell apoptosis (Yoon et al. 1998; Parkinson et al. 2004).

The receptor $p75^{NTR}$ is not always related to apoptotic signaling. Research suggests that the presence of $p75^{NTR}$ can enhance NGF-TrkA binding, further collaborating to growth and survival of neurons. However, these effects are still dependent on TrkA signaling cascade. Neurotrophin signaling via Trk receptors' cascade triggers several intracellular responses such as receptor cross talk with G-protein coupled receptors (GPCR), $p75^{NTR}$, vanilloid receptor (VR1), and c-Ret. These signaling are known to promote regulation of ion channels, retrograde signaling in a phosphorylated state, local axonal and dendritic growth, survival and proliferation of neurons, synaptic functions, assembly of cytoskeleton, and activation of protein kinase C (Ehlers et al., 1995; Cunningham & Greene, 1998 Qian et al., 1998; Wooten et al., 1999; Foehr et al., 2000; Kaplan & Miller, 2000; Latina et al., 2017; reviewed by Huang & Reichardt, 2003).

Deficit in NGF/TrkA signaling pathway has been suggested to play a role in presynaptic dysfunction and Alzheimer's Disease through activation of proapoptotic activity via beta-mediated phosphorylation and formation of amyloid precursor protein (Tarr et al., 2002; Matrone et al. 2008, 2009, 2011). p75-mediated CDK5 and Src intracellular signaling have been suggested to suppress TrkA phosphorylation as both are known to be involved in amyloid formation (Williamson et al., 2002; Cruz & Tsai, 2004; Reynolds et al., 2008). Partial silencing

of p75 RNA (p75i) prevented neuron death and TrkA phosphorylation. Amyloidogenesis is significantly reduced in p75-silenced and NGF-deprived neurons. Additionally, p75 and TrkA co-silencing is suggested to promote neuron survival when NGF is absent (Matrone et al., 2009).

NGF in the Periphery

NGF is fundamental for the development and survival of sympathetic and sensory neurons in the peripheral nervous system (Davies, 1994; Ibáñez, 1995). NGF-depleted adult rats submitted to inferior alveolar nerve crush had decreased nerve regeneration and low satellite cell response compared to control animals (Anderson et al., 1998). Spitsbergen et al. (1995) investigated the levels of NGF in vascular smooth muscle cells (VSMCs) from normotensive Wistar-Kyoto (WKY) rats and spontaneously hypertensive rats (SHR). ELISA was used to quantify NGF in VSMCs conditioning medium and in serum-free medium (SFM). Their results suggest that NGF protein secretion was higher in VSMCs maintained in SFM from SHR than in WKY. After treating these VSMCs with molecules analogous to sympathetic neurotransmitters, NGF levels increased in SHR but not in WKY. Treatment with isoproterenol, a beta-1 and beta-2 adrenergic receptor agonist, promoted a decrease in NGF secretion by the normotensive WKY VSMCs, but no changes in the SHR. NGF secretion promoted by the sympathetic neurotransmitter receptors in VSMCs of SHR and WKY are different, and they may play a role in NGF level and sympathetic innervation in this peripheral tissue (Spitsbergen et al., 1995). Clemow et al. (1998) suggests that NGF mRNA is elevated in a hypertensive strain of WKY rats (WKHT) compared to a hyperactive strain of WKY rats (WKHA). NGF protein content was raised in aorta, tail, and mesenteric arteries of WKHT compared to the WKHA. NGF protein secretion was also elevated on VSMCs of WKHT. Disturbances in alpha-adrenergic, purinergic, and peptidergic receptors caused elevated secretion of NGF in VSMCs of WKHT and had no

effect in WKHA cultures. Altogether, these investigations indicate that faulty beta-adrenergic, alpha-adrenergic, purinergic, and peptidergic regulation may influence NGF production in animal models of hypertension. This may contribute to an overexpression of vascular NGF which will lead to sympathetic hyperinnervation of the vasculature and the development of hypertension (Head, 1989; Spitsbergen et al., 1995; Clemow et al., 1998).

Exogenous injections of NGF and peripheral nerve injuries increase TrkA and NGF protein expression in Schwann cells, and increase TrkA expression in sympathetic neurons, DRG, sciatic nerve, and in other peripheral sensory neurons. In a scenario of a nerve injury, NGF binds to TrkA (NGF-TrkA) in the proximal and distal segments of the nerve cell and triggers regenerative signaling in NGF-responsive neurons. NGF-TrkA are tyrosine phosphorylated and retrogradely transported to the cell body, changing gene expression and promoting neural regeneration (Schwab & Thoenen, 1985; Taniuchi et al., 1986; Ehlers et al., 1995).

NGF and Exercise

Undoubtedly, alteration in NGF protein levels will impact the development, survival, structural plasticity, and synaptic potentiation of peripheral sympathetic and sensory neurons with their target tissues. Besides neuropathies and nerve injuries, exercise has also been suggested to change NGF levels. Six weeks of exercise endurance training increased levels of NGF and BDNF mRNAs, and NGF and BDNF proteins in the cerebellum of healthy and diabetic rats (Taheri et al., 2020). Research from Rebimbas-Cohen (2005), utilizing ELISA and immunohistochemical techniques, suggests that NGF decreases with aging in the mesenteric vessels of sedentary rats, and innervation pattern shift from a balanced sympathetic/sensory innervation in young rats to predominantly sympathetic innervation in older animals. Immunohistochemical analysis revealed that NGF colocalizes with Calcitonin Gene-Related

Peptide (CGRP), a marker for sensory innervation, in mesenteric vessels of young rats. In old rats, NGF only colocalized with TH fibers.

Typically, blood pressure (BP) increases in old rats. However, voluntary exercise regimen was able to lower blood pressure, restore NGF protein content, and reverse the imbalance of the sympathetic/sensory innervation in the mesenteric vessels of old animals. These results suggest that exercise increases NGF protein content in mesenteric vessels and may participate in restoring the balance of sympathetic/sensory innervation (Rebimbas-Cohen, 2005). Exercise contributing to sympathetic attenuation have also been reported to control BP in SHR (Krieger et al., 1999). Other types of exercise regimen have shown to have positive impacts in BP. Heavy resistance strength training normalizes BP in old men and women (Martel et al., 1999). Cycling has been shown to increase BDNF and NGF levels in patients with multiple sclerosis. Patients that underwent this exercise regimen had increased neural plasticity and improved cognitive function (Petajan & White, 1999; Gold et al., 2003). Exercise has also been found to promote neurogenesis in hippocampus and recover septohippocampal cholinergic arrangement and function with concomitant increase in NGF levels in rats (Chae et al., 2013; Hall et al., 2018). Altogether, exercise can increase NGF levels producing several protective and regenerative responses in the central and peripheral nervous systems. Therefore, exercise may have protective roles in the nervous system via changes in NGF expression by the target tissue.

NGF in Aging

As previously discussed in this chapter, research from Rebimbas-Cohen (2005) suggests that NGF decreases with aging in the mesenteric vessels of sedentary rats and exercise may counterbalance the effects of aging by increasing NGF levels in old rats (Rebimbas-Cohen, 2005). Aging is known to be one of the main risk factors for the onset of cognitive impairments,

dementia, AD, and occurs with concomitant changes in neurotrophic factor levels such as NGF, BDNF and GDNF (reviewed in Budni et al., 2015). It is suggested that NGF plays contrasting roles in the animal's body in different ages. At young ages, NGF promotes the development and survival of nociceptive sensory neurons and sympathetic neurons. At older ages, NGF may promote inflammation, apoptosis, and hyperalgesia (Oppenheim, 1989; Lewin & Mendell, 1993; Della Seta et al., 1994; Levi-Montalcini et al., 1997; Yuen et al., 1996; Pedraza et al., 2005). This alteration in pro-NGF and NGF roles in the aging brain may be caused by oxidative molecules such as peroxynitrite, which has been shown to alter pro-NGF and NGF signaling and decrease the activity of other antioxidant enzymes in the brain (Bruno & Cuello, 2012; Yamakura et al., 1998; Aoyama et al., 2000; Bayır et al., 2006; Filipović et al., 2007; Demicheli et al., 2007). Aged rats with impairments regarding spatial learning in a water maze and in recognition memory in a spontaneous novel object had increased levels of pro-NGF and $p75^{NTR}$ receptors and decreased levels of mature NGF in the prefrontal cortex and hippocampus (Terry et al., 2011). Utilizing a novel way to deliver NGF intranasally, researchers were able to study the effects of exogenous NGF in age-related hypogonadism in aging male mice. Their results suggest that NGF therapy given twice a day for 12 weeks improved the animals' sexual function, sperm quality, and rehabilitated fertility (Luo et al., 2018).

NGF in the Heart

Research suggests that NGF mRNA levels in the heart increases from day 17 in the embryonic phase to maximum levels 10-14 days postnatally. After that, NGF mRNA levels decrease twofold and remains at a constant level as measured in adult rats. The increase in NGF mRNA levels coincides with important development events in the heart; specifically, sympathetic nerve terminal differentiation. Sympathectomy in neonatal rats did not alter NGF

mRNA levels, suggesting that innervating sympathetic neurons in the heart have no effect on NGF mRNA levels. These levels may be maintained by either or both sensory innervation and/or cardiomyocytes themselves (Clegg et al., 1989). Circulating and cardiac levels of NGF increase dramatically for hours after a myocardial infarction (MI), and it is suggested that this leads to sympathetic nerve sprouting and cardiac arrhythmias. However, an increase in NGF levels may also help with the healing process. Months after a MI, cardiac levels of NGF level drops below normal, which may collaborate to faulty innervation in heart failure (HF) (reviewed by Govoni et al., 2011). Chronic HF leads to decreased levels of NGF in sympathetic and sensory neurons. Sensory afferent nerves are involved in sympathetic responses to exercise via a reflex mechanism. Research suggests that low NGF levels may be responsible for the development of muscle reflex-mediated abnormal sympathetic overactivity remarkably present in chronic HF and hypertension (Xing et al., 2014; Julius & Nesbitt, 1996).

Little is known about the roles of the sensory innervation in the heart. One of the major sensory neurotransmitters is CGRP, and research suggests that CGRP treatment may increase NGF mRNA levels in the brain, and may protect the heart by maintaining mitochondrial membrane potential, diminishing oxidative stress, and blocking apoptotic signaling (Hashikawa-Hobara et al., 2015; Guo et al., 2018). CGRP is also known to increase BDNF levels in the endothelium, and promote angiogenesis, neurogenesis and survival of stem cells and reduces neuroinflammation in the brain (reviewed by Borkum, 2019).

Calcitonin Gene-related Peptide (CGRP)

CGRP is a 37 amino-acid peptide that serves as a neurotransmitter in enteric, somatic motor, and sensory neurons. CGRP is processed into two distinct isoforms (α & β), which arise from different genes (reviewed on Russel et al., 2014). βCGRP is found in the enteric and central

nervous systems as well as in the pituitary and thyroid glands (Petermann et al., 1987). βCGRP is produced by splicing of the CALCII gene and plays a similar role to αCGRP (Alevizaki et al., 1986; Brain & Grant, 2004; Muddhrry et al., 1988; Steenbergh et al., 1986). αCGRP is produced by alternative splicing of the CALCI gene and stored in vesicles in sensory and somatic motor nerve terminals. αCGRP can be found in peripheral and central neural tissues throughout the body and may regulate cell and tissue function (Alevizaki et al., 1986; Steenbergh et al., 1986; Russel et al., 2014).

CGRP Receptor

CGRP receptor is a combination of two receptors known as the calcitonin-like receptor (CLR) and the receptor activity modifying protein 1, 2, or 3 (RAMP1, RAMP2, RAMP3) (Hay et al., 2008; Fluhmann et al., 1995; Russell et al., 2014). The receptors are translated into the endoplasmic reticulum (ER) and are combined to form a binding site for CGRP. CLR:RAMP complexes are transported from the ER into the plasma membrane where CGRP can bind. CLR or RAMP are unable to recognize CGRP by themselves and must be combined (CLR:RAMP) to recognize the CGRP molecule (McLatchie et al., 1998; Spielman & Parameswaran, 2012). CGRP has an affinity with the CLR:RAMP3 complex, also known as adrenomedullin 2 (AM2) receptor (Choksi et al., 2002; Muff et al., 1998; Russell et al., 2014). Receptor component protein (RCP) is an intracellular peripheral membrane protein that binds to the second intracellular loop of the CLR, making it a third component of the CGRP receptor. Inhibiting RCP causes a shutdown of the CGRP receptor-intracellular signaling (Evans et al., 2000; Luebke et al., 1996). CGRP may also cause the release of calcium by the endoplasmic reticulum, through the production of inositol trisphosphate (reviewed on Russel et al., 2014). Binding of CGRP to its receptor can also increase intracellular levels of diacylglycerol, which may activate protein

kinase C (Pin & Bahr, 2008). In addition, a well-known pathway triggered by CGRP binding to its receptor is the activation of adenylate cyclase, which increases intracellular cyclic AMP (cAMP), leading to activation of protein kinase A (PKA). PKA downstream pathways will activate extracellular signal-related kinases and transcription factors (Drake et al., 1999, 2000). Phosphorylated PKA may also open potassium channels and promote smooth muscle relaxation in arteries (Nelson et al., 1990).

CGRP is a Potent Vasodilator

CGRP is a potent vasodilator and may help to prevent cardiac and pulmonary hypertension, ischemia, migraine, and ultimately, improving blood flow distribution, and wound healing (Jonhagen, 2006; Hasbak et al., 2001, 2003; Schlier et al., 2009; Russel et al., 2014; Toda et al., 2008; Vause & Durham, 2010). CGRP also promotes vasodilation of *in-vitro* parenchymal microvessels from hippocampal slices of rat brains (Fergus et al., 1995). CGRP immunoreactive fibers are found in coronary arteries in the heart of rats, and the action of CGRP regulates blood flow in these vessels (Goto et al., 1991). A study using anesthetized rats and conscious dogs, treated them with intravenous human CGRP and found that CGRP caused dose-dependent peripheral vasodilation. More findings from this same study suggested that treatment with angiotensin II increases mRNA levels of CLR and RAMP1 in wild-type animals, but not in the αCGRP knockout (αCGRPKO) animals. Their results also suggested that αCGRP mRNA was elevated in the aorta, mesenteric vessels, and dorsal root ganglia of WT animals treated with angiotensin II compared to the WT control animals (Smillie et al., 2014). Therefore, CGRP is suggested to have potente relaxative effects in muscles. However, little is known about the effects of CGRP in the heart.

CGRP and Exercise

Research has shown that exercise may increase CGRP production and secretion by the sensory nerve fibers present in the skeleton and cardiac muscle (Sun & Pan, 2014; Jonhagen, 2006; Onuoha et al., 1998; Russel et al., 2013). As previously discussed in the chapter, exercise increases CGRP-positive sensory nerve fibers density in arteries and veins of exercised rats (Rebimbas-Cohen, 2005).

CGRP in Cardiovascular Regulation

The two branches from the autonomic nervous system (ANS), the sympathetic (SNS) and parasympathetic (PNS) nerve systems work antagonistically to regulate all the heart's function (Bush et al., 2016; Hiltunen et al., 2000). CGRP is suggested to play a role in cardiovascular regulation by inhibiting sympathetic nervous system activity in mice (Kurihara et al., 2003). Pre-treatment with a CGRP receptor antagonist, CGRP 8-37, prevented the vasodilatory actions of CGRP. The same study suggested that CGRP has a greater effect on increasing blood flow in rats' hearts than producing vasodilatory effects in the brain. Nevertheless, by systemically blocking β-adrenergic receptors, the increase of regional blood flow caused by CGRP in these two organs was identical. Their findings suggest that CGRP increases blood flows in coronary, renal, and carotid arteries of dogs (Shen et al., 2001). Intravenous injection of CGRP in humans caused an increase in heart rate, decreased blood pressure, and produced skin redness, which indicates vasodilation (Lundberg et al., 1989). More studies have shown that the inhibitory actions of CGRP may be regulated by vagal parasympathetic outflow (Li et al., 1998).

The changes in neurotrophic factor protein content and the changes in structural plasticity of all branches of the nervous system in the heart caused by aging, exercise, and sedentary lifestyles are still unknown. Moreover, it is unclear what are the effects of direct exogenous

CGRP treatment on heart function. We aimed to examine age-related changes in the innervation pattern of the heart; and in GDNF and NGF protein content in the heart of rats, and to examine the effects of exercise in the innervation pattern of the heart; and in GDNF and NGF protein content in the heart. We hypothesized that 1: Neurotrophic factor expression in heart tissues will decline with age in sedentary animals; 2: The pattern and density of cardiac innervation will be altered with age in sedentary animals; 3: Blood pressure and heart rate will increase with age in sedentary animals; 4: Levels of expression of neurotrophic factors in cardiac tissues will be increased following exercise; 5: Exercise will restore a more normal pattern and density of innervation in cardiac tissues; 6: Blood pressure and heart rate will be lowered in exercised animals. The results from this thesis research provides a novel understanding of the roles of the sensory nervous system in the heart and further understanding on the impacts of aging, sedentarism, and exercise on NF levels and structural plasticity of all branches of the nervous system in the heart throughout the entire lifetime of normotensive animals.

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CHAPTER II

THE IMPACTS OF AGING, SEDENTARISM, AND EXERCISE ON NEUROTROPHIC FACTOR EXPRESSION AND INNERVATION IN THE HEART

Abstract

Neurotrophic factors (NFs) are molecules which promote the development, growth, survival, and repair of neural tissues. Two neurotrophic factors are examined in this study: nerve growth factor (NGF) and glial cell line-derived neurotrophic factor (GDNF). Aging, sedentarism, and exercise are factors known to contribute to changes in neurotrophic factor expression in many tissues throughout the body, including the heart. The heart is innervated by the two branches of the autonomic nervous system, the sympathetic nervous system (SNS) and the parasympathetic nervous system (PNS), as well as by the sensory nervous system. Changes in neurotrophic factor protein content in the heart may cause modifications in structural and function of all branches of the nervous system, which may contribute to, or prevent, development of cardiac disease.

The goal of this research was to investigate the impacts of aging, sedentarism, and exercise on NF levels and nervous system structure in the heart, throughout the entire life span of rats. Our results suggest that neurotrophic factor content in the heart peaks in young animals and declines with age. GDNF content declined with aging earlier and more dramatically than NGF content. It may be that NGF primarily supports sympathetic nervous system distribution, which does not seem to change much with age, while GDNF supports the parasympathetic system distribution of fibers, which does decline with age. NGF also supports the sensory nervous system. Therefore, the changes in NGF content that were observed in these studies may be linked to the changes in sensory nerve density. The density of parasympathetic and sensory innervation decline with age, while sympathetic innervation does not, which may be the cause for an increase

in BP and HR. Therefore, it is hypothesized that BP and HR both increase with age as the balance between sympathetic and parasympathetic innervation is impaired. With exercise, GDNF content increases, parasympathetic innervation increases, and BP and HR decrease. The effects of exercise in neurotrophic factor expression may be a possible mechanism by which exercise exerts positive effects on cardiac innervation, promoting the prevention and treatment of cardiovascular diseases.

Introduction

Nerve Growth Factor

NGF belongs to the neurotrophin family of neurotrophic factors, which includes NGF, brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4 (NT-4). These NFs are important for the development, growth, maintenance, and regeneration of neurons in the central and peripheral nervous systems. NGF research began with Dr. Levi-Montalcini, who observed a soluble molecule released by cancerous sarcoma tissue that promoted outgrowth of sensory and sympathetic fibers from nerve cells allocated nearby (Levi-Montalcini and Hamburger, 1951, Cohen et al., 1954, Levi-Montalcini and Cohen, 1960). After that, many other researchers have reported that various cell types, such as cardiomyocytes, other types of muscle cells, hepatocytes, epithelial cells, fibroblasts, myofibroblasts, cells from the immune system, and cells from the nervous system are known to express NGF mRNA and protein (Furukawa et al. 1984; Spitsbergen et al. 1995; Clemow et al. 1998; Micera et al. 2003; Lambiase et al 2004).

NGF is known to promote the development and survival of sympathetic and sensory neurons in the peripheral nervous system, including the heart (Davies 1994; Ibáñez 1995). NGF levels vary with age and may assist with development. Research from Clegg et al. (1989) found that NGF mRNA levels in the heart of rats increase from day 17 in the embryonic phase to

maximum levels at 10-14 days postnatally. After 14 days, levels decrease twofold and remain at the reduced level in adults. The increase in NGF mRNA levels up to 14 days postnatally coincides with sympathetic nerve terminal differentiation. Sympathectomy in neonatal rats does not alter NGF mRNA levels, suggesting that innervating sympathetic neurons in the heart do not regulate NGF mRNA expression. Instead, NGF mRNA expression is suggested to be regulated by sensory neurons innervating the heart or by cardiomyocytes themselves (Clegg et al. 1989). When cardiovascular diseases are present, NGF expression changes. NGF levels is in cardiac tissues and in the circulation increase dramatically hours after myocardial infarction (MI), which may lead to sympathetic nerve sprouting and possibly the development of cardiac arrhythmias. Increased NGF expression following MI may also help with the healing process. Months after a MI, cardiac levels of NGF drop below normal levels, which may contribute to faulty innervation seen with heart failure (HF) (reviewed by Govoni et al. 2011). Chronic HF leads to decreased levels of NGF in sympathetic and sensory neurons. As sensory afferent nerves are involved in sympathetic responses to exercise via a reflex mechanism, research suggests that low NGF levels may be responsible for the development of muscle reflex-mediated abnormal sympathetic overactivity remarkably present in chronic HF and hypertension (Xing et al. 2014; Julius & Nesbitt 1996). Nevertheless, little is known about how aging, exercise, and sedentarism impact NGF levels in the heart, and how these changes in NGF levels may impact the innervation pattern of the heart.

Glial Cell Line-derived Neurotrophic Factor

GDNF belongs to the GDNF family ligands (GFLs), which includes artemin, persephin, and neurturin (Lin et al, 1993; Heuckeroth et al, 1996; Baloh et al, 1998; Milbrandt et al, 1998; Rosenblad et al, 2001). GDNF research was first studied by Lin et al. (1993 & 1994), who

isolated GDNF from B49 rat glial cells in culture. These studies reported that GDNF increases dopamine uptake by dopaminergic neurons and that GDNF may augment their differentiation and survival rates (Lin et al., 1993 & 1994). Suter-Crazzolara and Unsicker (1994) found GDNF mRNA in the heart, blood, kidney, liver, lung, spleen, bone, and sciatic nerve. Additionally, this study found a shorter length of GDNF mRNA in these tissues, which suggests that GDNF mRNA may undergo alternative splicing (Suter-Crazzolara and Unsicker 1994; Buj-Bello et al. 1995). In adult humans, the highest levels of GDNF mRNA have been found in skeletal and cardiac muscle and in the spinal cord (Yamamoto & Yamamoto 1996).

Cardiomyocytes may promote the growth, development, and maintenance of all branches of the nervous system by secreting GDNF and other members of the GFL. GDNF mRNA is expressed in atrial and ventricular myocytes in rats and GDNF protein is synthesized by cardiomyocytes in the heart of normal and sympathectomized animals. In control rats, GDNF levels were found to be higher in 37-day-old animals than in 60-day-old animals. GDNF protein levels were significantly higher 7 days after sympathectomy and dropped to control level 30 days after this procedure, suggesting that GDNF may promote reinnervation in the heart of rats (Martinelli et al., 2002). Hiltunen et al. (2000) found GDNF receptors mRNA in neurons present in the heart of rats, and GDNF mRNAs in non-neural cells in the heart ganglia. GFL receptor mRNA was also found in the endocardium, valves, atria, and pulmonary trunk. GDNF receptor knockout mice had a 40% reduction of cholinergic innervation in the ventricles and 60% reduction of the ventricular conduction system, suggesting a role for GDNF in regulating cholinergic innervation.

Neurotrophic Factors and Innervation of the Heart

The heart is innervated by sympathetic, parasympathetic, and sensory nervous systems and they respond to most GFL members (Hiltunen et al., 2000) and to the neurotrophin family (Levi-Montalcini and Hamburger, 1951). Deficiencies in the innervation of the heart have been related to the onset of many cardiovascular (change throughout) diseases such as heart failure, myocardial infarction (MI), arrythmias, and hypertension (Barron and Lesh, 1996; Nolan et al., 1998; La Rovere et al., 1998; Floras, 2003; Shen and Zipes, 2014; reviewed by Hanna et al., 2017). Neuromodulatory therapies, such as bilateral cardiac sympathetic decentralization and vagus nerve stimulation, have been used to treat some cardiac diseases (reviewed by Hanna et al. 2017). A MI has been proposed to increase NGF levels causing sympathetic nerve sprouting. In addition, exogenous infusion of NGF in dogs with a chronic MI and AV block, increased sympathetic nerve sprouting and caused ventricular tachycardia, ventricular fibrillation, and sudden cardiac death (Cao et al., 2000; Zhou et al. 2004). Decreased NGF expression in congestive heart failure leads to alterations in sympathetic neuronal function and neuroanatomy of the heart (Kaye et al. 2000). NGF has been implicated in playing a pivotal role in the development and maintenance of sensory innervation in the heart (Trupp et al. 1995; Kuruvilla et al. 2004). Research from Alves et al. (2019) showed the impacts of exercise in neurotrophic factor protein content in the heart of healthy and chagasic animals. Trained controls, trained chagasic mice, and sedentary chagasic mice had significantly higher GDNF protein content in their hearts than in sedentary controls. These results suggest that exercise increases GDNF levels in the heart; and that chronic infections, such as Chagas disease caused by *Trypanosoma cruzi*, may also increase GDNF content, suggesting additional roles of this neurotrophic factor to maintain cardiac function near to its normal parameters. Moreover, NGF mRNA and protein

levels were significantly higher in the heart of rats 20 days after inoculation with trypomastigotes, suggesting that NGF may participate in regenerative events after an acute myocarditis (Alves et al. 2019; Martinelli et al. 2006).

Aging and Exercise

It is suggested that NGF plays contrasting roles in an animal's body at different ages. At young ages, NGF promotes the development and survival of nociceptive sensory neurons and sympathetic neurons. At older ages, NGF may promote inflammation, apoptosis, and hyperalgesia (Oppenheim, 1989; Lewin & Mendell, 1993; Della Seta et al., 1994; Levi-Montalcini et al., 1997; Yuen et al., 1998; Pedraza et al., 2005). Research from Rebimbas-Cohen (2005) suggests that NGF decreases with aging in the mesenteric vessels of sedentary rats; and exercise may counterbalance the effects of aging, increasing NGF levels in old rats (Rebimbas-Cohen 2005). Aging is known to be one of the main risk factors for the onset of cognitive impairments, dementia, AD, with a concomitant decrease in neurotrophic factor levels, such as NGF, BDNF and GDNF (reviewed in Budni et al., 2015). Exercise has been suggested to promote recovery of central and peripheral nerve injuries and delays the progression of neurodegenerative diseases, possibly directed by neurotrophic factor signaling (reviewed by Cobianchi et al., 2017). Six weeks of exercise endurance training increased levels of NGF and BDNF mRNAs, NGF and BDNF proteins in the cerebellum of healthy and diabetic rats (Taheri et al., 2020). Work from Wehrwein et al. (2002) found that a 4-week walking exercise plan resulted in increased GDNF content in soleus, gastrocnemius, and pectoralis major muscles of rats. Furthermore, 2 weeks of hindlimb unloading resulted in a decrease in GDNF content in the hindlimb muscles. However, hindlimb unloading also led to an increase in GDNF content in the pectoralis major. These results suggest that GDNF production by skeletal muscles in rats is

regulated by physical activity, suggesting that exercise may help promote remodeling and recovery of the NMJ after injury and disease via a mechanism involving increased neurotrophic factor expression (Wehrwein et al. 2002). Ultimately, little is known about the impacts of sedentary lifestyle, exercise, and aging on neurotrophic factor protein expression and on the innervation of the heart. In this study, the impact of aging, sedentarism, and exercise on NF levels and structural plasticity of all branches of the nervous system in the heart throughout the entire lifetime of rats will be examined. The goals of our study was to examine age-related changes in the innervation pattern of the heart; and in GDNF and NGF protein content in the heart, and to examine the effects of exercise in the innervation pattern of the heart; and in GDNF and NGF protein content in the heart. We hypothesize that NF content will decrease with age, leading to changes in the structure and function of cardiac innervation. These changes are hypothesized to result in increased blood pressure and heart rate that is typically observed with increased age.

Methods

Animal Model, Exercise Regimen, and Distance Measurements

All animal experiments were performed in accordance with the "Guide for the Care and Usage of Laboratory Animals" (National Research Council) and protocols were approved by the Institutional Animal Care and Usage Committee at Western Michigan University. Male Sprague Dawley rats (Charles River Laboratories) were used for all studies. For the first part of this study, 18 rats were used. Six animals were sacrificed at 4 weeks of age (4wk-sed), 6 were maintained for 10 weeks without access to running wheels (14wk-sed) and 6 were maintained with access to running wheels (14wk-ex). For the second part of this study, eighteen 6-month-old rats were used. Six animals were sacrificed at 6 months of age (6mo-sed), 6 were maintained for 6 months

without access to running wheels (12mo-sed) and 6 were maintained for 6 months with access to running wheels (12mo-ex). For the third part of this study, twelve 12-month-old rats were used. Six animals were maintained for 6 months without access to running wheels (18mo-sed) and the other 6 animals were maintained for 6 months with access to running wheels (18mo-ex). For the fourth part of this study, twelve 18-month-old rats were used. Six animals were maintained for 6 months without access to running wheels (24mo-sed) and the other 6 animals were maintained for 6 months with access to running wheels (24mo-ex). Control animals were housed individually in cages with access to food and water. Exercised animals had access to a running wheel monitored with Activity Wheel Monitoring System software (Lafayette Instrument Company, Inc., North Lafayette, IN, USA) for total distance measurements. Animals were cared for according to standard operating procedures outlined by WMU's IACUC committee.

Weight

Weights from all animals were taken on the first and last day of each study segment.

Blood Pressure and Heart Rate

Animals were placed in animal holder tubes and a dark blanket was put on top of the tubes for acclimation. A warm electrical blanket was placed underneath the tubes and set to 37ºC. A tail-cuff was placed on the animals' tails and a 15-minute interval was allowed for additional acclimation. Resting, noninvasive, blood pressure and heart rate were measured weekly using a CODA® Monitor Module (Kent Scientific Corp., Torrington, CT, USA). The first and last blood pressure and heart rate measurements of the studies were considered for statistical analysis.

Tissue Harvesting

Animals were sacrificed via CO2 asphyxiation followed by thoracotamy. Hearts were removed and cleaned of any fat and connective tissue. Heart chambers were separated as follows: right atria (RA), left atria (LA), and top and bottom ventricles. Heart chambers were flash frozen by contact with dry ice and stored at -80ºC for later determination of NGF and GDNF content and immunohistochemical processing.

Tissue Processing for Enzyme-Linked Immunosorbant Assay (ELISA)

Frozen heart chambers were dipped into liquid nitrogen and crushed on a metal block chilled on dry ice. The pulverized heart chamber was suspended in sample buffer consisting of 0.1M phosphate buffered saline (PBS: 0.225 M NaCl, 0.02 M NaH2PO4, 0.08 M NaHPO4), containing 0.1% Tween-20, 0.05% bovine serum albumin (BSA), aprotinin [6.6 trypsin inhibitor unit/mL, Sigma, St. Louis, MO], 0.2 mM Benzamidine, 0.01 mM Benzethonium Chloride, and 0.2 mM ethylenediaminetetra-acetic acid (EDTA). The suspension was chilled on wet ice and homogenized for 30 seconds using a variable speed Tissue Tearor (Biospec Products, Inc., Bartlesville, OK, USA). The homogenate was centrifuged at 13,000xg in a cold room and the supernatant was pipetted to another assay tube and stored at -20ºC for later quantification of NGF and GDNF.

ELISA for NGF and GDNF

Nunc-Immuno 96 well plates (ThermoFisher, Waltham, MA, USA) were coated with 100 µl/well of 1 µg/ml of anti-GDNF or 0.4 µg /ml of anti-NGF monoclonal antibody (R&D Systems, Minneapolis, MN, USA) in PBS (pH 7.4) and incubated overnight at room temperature (RT) in a humidified chamber. Plates were washed with wash buffer containing 0.4 M NaCl and 0.05% Tween-20 in 0.1 M PBS (pH 7.4) and blocked with 200 µl/well of 1.0% BSA, 5% sucrose

in PBS for 1 hour at RT in a humidified chamber. GDNF or NGF standard and samples were added and incubated for 2 hours at RT in a humidified chamber. After 2 hours, plates received 100 µl of biotinylated anti-GDNF secondary antibody (100 ng/ml) or biotinylated anti-NGF secondary antibody (100 ng/ml) (R&D Systems, Minneapolis, MN), diluted in Tris buffered saline (TBS, pH 7.3) containing 1% BSA and 0.05% Tween-20. Plates were placed in a humidified chamber and incubated at RT for 2 hours. Plates received 50 µl of Streptavidin-HRP (PierceTM High Sensitivity, ThermoFisher, Waltham, MA, USA) diluted in PBS containing 1% BSA and were placed in a humidified chamber and incubated at RT for 30 minutes. Then, 100 µl of 1-step Turbo TMB-ELISA (ThermoFisher, Waltham, MA, USA) was added in each well for 20 minutes. The HRP reaction was stopped by adding 100 µl of HCl into each well and absorbance was measured at 488nm, using a microplate spectrophotometer.

Tissue Processing for Immunohistochemistry

Heart chambers were removed from -20º C and immediately exposed to 4% paraformaldehyde at RT for 15 minutes. Samples were washed 3 times for 15 minutes and tissues were blocked for 1 hour at 4º C with PBS + 1% BSA + 0.4% Triton. After blocking, tissues were incubated for five days (4º C) with guinea pig anti-αCGRP (Synaptic Systems) at a dilution of 1:250, rabbit anti-tyrosine hydroxylase (TH) (Abcam) at a dilution of 1:250, and goat anti-choline acetyltransferase (ChAT) (Abcam) at a dilution of 1:100. Next the tissues were washed and incubated for 24 hours at 4º C with goat anti-guinea pig IgG conjugated to Alexa Fluor 568 (Abcam), goat anti-rabbit IgG conjugated to Alexa Fluor 405 (Abcam) and donkey anti-goat IgG conjugated to Alexa Fluor 488. After washing, the tissues were placed in welled slides in a 1:1 solution of phosphate buffer saline (PBS) and glycerol. Controls were incubated without primary antibodies.

Confocal Microscopy and Nerve Count

For determination of nerve fiber density, images containing staining for sympathetic, parasympathetic, and sensory nerve fibers were obtained from the epicardium of each heart chamber, from each animal. Images were captured using a Nikon C2+ laser scanning confocal microscope. A 60X water objective was used to capture 40 μm-deep z-stack images from the epicardium. NIS-Element AR was used to analyze nerve densities. For each z-stack image, all layers were combined to form one image. Two large 10,000 μm² grids were placed on each image, and nerves counts were determined by counting each time a stained fiber would cross any horizontal or vertical line in the smaller grids inside the 10,000 μm². An average of the two large fields (10,000 μm²) from each image were obtained for statistical analysis.

Statistical Analysis

Kruskal-Wallis and Wilcoxon Rank Sum tests were performed to determine significance between groups. Significance was set to $P<0.05$. Each group in this study had 6 animals (N=6).

Results

Gross Observations

The total weight gain throughout the study of 12mo-sed group (50.5 ± 20.9 g) was significantly greater than that for the 12mo-ex group (17.6 ± 16.2 g). For other sedentary groups, there was a trend towards greater weight gain throughout the study when compared to age-matched exercised groups, but none of the differences were significant. Old adults (24mo-sed group) had significantly greater weight than the young adults (6mo-sed group). Following tissue removal, it was noted that the hearts from young animals (4wk-sed, 14wk-sed, and 14wk-ex), from young adult animals (6mo-sed) and from exercised adult animals (12mo-ex, 18mo-ex, 24mo-ex) had a healthy and lean appearance, in contrast hearts from adult sedentary animals

(12mo-sed, 18mo-sed, 24mo-sed) had increased deposits of fat and connective tissue surrounding the heart.

Resting Blood Pressure

Our results show that the resting mean arterial blood pressure significantly increased with aging (Figure 1). BP was significantly increased in 18mo-sed (127.8 ± 3.8 mmHg) and 24mo-sed (133 ± 7.9 mmHg) groups when compared to that in the 6mo-sed group (102.6 ± 7.64 mmHg). Our results also show that exercise lowered BP (Figure 2). Blood pressure in the 12mo-ex (88.5 ± 2.4 mmHg) and 18mo-ex (120.8 ± 3.9 mmHg) groups had significantly lower BP than the age-matched sedentary groups. The effect of exercise to lower resting BP diminishes with aging. Our results show that exercise did not lower the BP in the 24mo-ex group.

Resting Heart Rate

Similar to BP, our results show that the resting heart rate significantly increases with aging (Figure 3). HR significantly increased in 18mo-sed (440.8 ± 20.4 bpm) and 24mo-sed (421.6 ± 21.9 bpm) groups when compared to that in the 6mo-sed group (327.9 ± 12 bpm). Our results also show that exercise lowers HR. The 12mo-ex (269.3 ± 11.2 bpm) and 18mo-ex groups (394.8 ± 5.4 bpm) had significantly lower HR than the age-matching sedentary groups. The effect of exercise on lowering resting HR diminishes with aging. Our results show that exercised did not lower the HR in the 24mo-ex group (Figure 4).

Distance Ran

Our results demonstrate that the distance ran by the exercised animals significantly decreased as the animals aged (14wk-ex: 116.2 km ± 62 km; 12mo-ex: 74.6 km ± 20.8 km; 18mo-ex: 37.1 km ± 16 km; 24mo-ex: 14.8 km ± 7.4 km).

Effects of Aging and Exercise on GDNF Protein Levels

GDNF protein content significantly increased at early ages in right atria, left atria, and ventricles. The 14wk-sed group had higher GDNF protein content in all heart chambers (RA: 1.2 ± 0.16; LA: 1.3 ± 0.13; Vent: 1.27 ± 0.1 pg of GDNF/mg of tissue) when compared to 4wk-sed group (RA: 0.95 ± 0.14; LA: 0.91 ± 0.28; Vent: 0.53 ± 0.35 pg of GDNF/mg of tissue). At 6 months of age, GDNF protein levels (RA: 0.17 ± 0.02; LA: 0.13 ± 0.07; Vent: 0.03 ± 0.02 pg of GDNF/mg of tissue) significantly decrease in all heart chambers and continued to decline as the animals aged (Figure 5). Exercise significantly increased GDNF protein levels in right atria and ventricles at all ages, and in left atria of 14wk-ex and 12mo-ex groups. There was a trend towards an increase in GDNF levels in left atria at older ages (18mo-ex and 24mo-ex groups) when compared to the sedentary age-matching groups, although the difference was not significant (Figure 6).

Effects of Aging and Exercise on NGF Protein Levels

In general, NGF levels decline with age in heart tissues. At 4 weeks of age, NGF was at its highest levels in any heart chambers (RA: 1.7 ± 0.24; LA: 2.19 ± 0.48; Vent: 1.39 ± 0.36 pg of NGF/mg of tissue). At 14 weeks of age, NGF protein levels significantly decreased in all heart chambers (RA: 0.36 ± 0.17; LA: 0.66 ± 0.49; Vent: 0.67 ± 0.37 pg of NGF/mg of tissue). At 6 months of age (6mo-sed), NGF levels significantly increased in RA (1.0 ± 0.38 pg of NGF/mg of tissue). At 12 months of age (12mo-sed), NGF levels were not different from that in 6-month-old animals. However NGF levels at 12 months of age (RA: 0.92 ± 0.1; LA: 0.78 ± 0.14; Vent: 0.57 ± 0.15 pg of NGF/mg of tissue) were significantly lower than that in 4-week-old animals in all heart chambers. NGF protein content significantly decreased at 18 months of age in all heart chambers (RA: 0.0026 ± 0.0021; LA: 0.0019 ± 0.0015; Vent: 0.0037 ± 0.002 pg of NGF/mg of

tissue) when compared to the 12-month-old animals. At 24 months of age, NGF levels were significantly lower in all heart chambers (RA: 0.0011 ± 0.0005; LA: 0.0006 ± 0.0003; Vent: 0.0001 ± 5.35^{-5} pg of NGF/mg of tissue) than the 4-week-old and 12-month-old animals. In addition, NGF levels were significantly lower in the ventricles of 24-month-old animals compared to that in 18-month-old animals (Figure 7).

Exercise significantly impacted NGF levels in the heart of rats. At 14 weeks of age, voluntary exercise significantly decreased NGF levels in all heart chambers (RA: 0.028 ± 0.021; LA: 0.11 ± 0.10; Vent: 0.04 ± 0.03 pg of NGF/mg of tissue) when compared to the sedentary group. At 12 months of age, voluntary exercise how no effect on NGF protein levels when compared to the 12mo-sed group. At 18 months of age, voluntary exercise significantly increased NGF levels in all heart chambers (RA: 0.08 ± 0.05; LA: 0.04 ± 0.02; Vent: 0.08 ± 0.05 pg of NGF/mg of tissue) when compared to the 18mo-sed group. At 24 months of age, voluntary exercise significantly increased NGF levels in all heart chambers (RA: 0.0062 ± 0.0015; LA: 0.0031 ± 0.0011; Vent: 0.00018 ± 6.18^{-5} pg of NGF/mg of tissue) when compared to 24-month-old sedentary group (Figure 2.8).

Effects of Aging and Exercise on the Innervation Pattern of Right Atria

The innervation pattern of the heart changes as the animals age. In the RA, sympathetic nerve density significantly increased from the 4wk-sed group (41 ± 2.3 grid count - 1000μm²) to the 14wk-sed group (57 ± 13.8 grid count - 1000μm²). Sympathetic innervation was significantly increased in the 18mo-sed group (54.8 ± 6.6 grid count - 1000μm²) when compared to the 4wk-sed group, and there was a trend towards an increase in the 24mo-sed group (59.4 ± 20.7 grid count - 1000μm²) (Figure 2.9) though the change was not significant.

Parasympathetic nerve density significantly decreased in RA in the 6mo-sed group (RA: 14.8 ± 7 grid count - 1000µm²) when compared to the 4wk-sed group (RA: 28 ± 4.18 grid count - 1000µm²). In the 12mo-sed group, parasympathetic nerve density also decreased (RA: 8.1 ± 6.6 grid count - 1000µm²) when compared to 4wk-sed group. In the 18mo-sed group, parasympathetic nerve density significantly increased in RA (19 ± 2.2 grid count - 1000µm²) when compared to the 12mo-sed group but was still significantly decreased when compared to the 4wk-sed group. In the 24mo-sed group (16.8 ± 8.1 grid count - 1000µm²), parasympathetic nerve density was increased when compared to the 12mo-sed group, and decreased when compared to the 4wk-sed (Figure 2.9).

Sensory nerve density significantly decreased from the 4wk-sed group (21.2 ± 1.9 grid count - 1000µm²) to the 14wk-sed (4.25 ± 2 grid count - 1000µm²). In the 6mo-sed group (11.6 ± 6.2 grid count - 1000µm²), sensory nerve density increased when compared to the 14wk-sed group and decreased when compared to the 4wk-sed group. In the 12mo-sed group (4 ± 3.9 grid count - 1000µm²) and older ages (18mo-sed [4.2 ± 2 grid count - 1000µm²]; 24mo-sed [3.4 ± 2.9 grid count - 1000µm²]), sensory nerve density was significantly decreased when compared to the 6mo-sed and 4wk-sed groups (Figure 2.9).

Exercise did not impact sympathetic and sensory nerve density in RA. However, exercised had significant effects on parasympathetic nerve density in RA. Increase in parasympathetic innervation was observed in the 12mo-ex (17.5 ± 3.6 grid count - 1000µm²) and 18mo-ex groups (47.4 ± 18.9 grid count - 1000µm²); and trended to increase in 24mo-ex (29.8 ± 14.6 grid count - 1000µm²) when compared to the age-matching sedentary groups (Figure 2.10). There were significant differences in staining robustness between sedentary and exercised

animals. Exercised animals had denser innervation in the epicardium of all heart chambers when compared to sedentary animals (Figure 2.14).

Effects of Aging and Exercise on the Innervation Pattern of Left Atria

In left atria, aging had no significant effect in sympathetic nerve density from the 4wk-sed to the 18mo-sed groups (4wk-sed [37.6 ± 2.3]; 14wk-sed [31.2 ± 13.5]; 6mo-sed [41.6 ± 18.6]; 12mo-sed [36 ± 7.7]; 18mo-sed [54.2 ± 16.9] grid count - 1000μm²) but significantly increased in the 24mo-sed group (87.2 ± 26.4 grid count - 1000μm²) (Figure 2.11).

In the 4wk-sed group (24.2 ± 3.83 grid count - 1000μm²), parasympathetic nerve density was significantly higher when compared to the 6mo-sed group (11.3 ± 4.6 grid count - 1000μm²), 12mo-sed (8.5 ± 6.4 grid count - 1000μm²), and 18mo-sed groups (10 ± 5.4 grid count - 1000μm²). In the 24mo-sed group (36.8 ± 23.3 grid count - 1000μm²), parasympathetic nerve density was significantly increased when compared to 6mo-sed, 12mo-sed, and 18mo-sed (Figure 2.11).

Sensory nerve density was at its highest denseness in the 4wk-sed group (21.2 ± 3.8 grid count - 1000μm²) and significantly decreased in the 14wk-sed group (4.25 ± 2.2 grid count - 1000μm²). After that, sensory nerve density remained significantly low as the animals aged (6mo-sed [7 ± 4.9]; 12mo-sed [2.5 ± 2.7]; 18mo-sed [4.2 ± 2.2]; 24mo-sed [4.6 ± 4.2] grid count - 1000μm²), compared to that in the 4wk-sed group (Figure 2.11).

Similar to RA, exercise did not impact sympathetic and sensory nerve density in LA. However, exercise significantly increased parasympathetic nerve density in the 18mo-ex group (30.6 ± 13.1 grid count - 1000μm²); and trended to increase in the 12mo-ex (8.5 ± 6.4 grid count - 1000μm²) and 24mo-ex (56 ± 27.2 grid count - 1000μm²) groups (Figure 2.12) though the difference was not statistically significant.

Effects of Aging and Exercise on the Innervation Pattern of Ventricles

Aging increased sympathetic nerve density in the ventricles. The 24mo-sed group (48.8 ± 15.1 grid count - 1000μm²) had significantly denser sympathetic innervation than the 4wk-sed (19.2 ± 1.3 grid count - 1000μm²), 6mo-sed (13.8 ± 15.6 grid count - 1000μm²) and 12mo-sed (20.1 ± 19.2 grid count - 1000μm²) groups.

Parasympathetic nerve density significantly increased from the 4wk-sed group (9.2 ± 1 grid count - 1000μm²) to the 14wk-sed group (16.5 ± 3.6 grid count - 1000μm²). In the 12mo-sed group (4.8 ± 5.6 grid count - 1000μm²), parasympathetic nerve density decreased when compared to that in the 14wk-sed group. Parasympathetic nerve density significantly increased from 12 months to 24 months of age.

Sensory nerve density significantly decreased from 4 weeks of age (11.8 ± 1.3 grid count - 1000μm²) to 14 weeks of age (4.2 ± 1.7 grid count - 1000μm²), 6 months of age (2.8 ± 3.6 grid count - 1000μm²), and 12 months of age (3.16 ± 2.8 grid count - 1000μm²), and there was a trend towards a decrease in the 24mo-sed group (6.4 ± 4 grid count - 1000μm²) when compared to the 4wk-sed group (Figure 2.13). Exercise had no significant effect on innervation pattern of the ventricles.

Discussion

In this study, novel findings concerning the impacts of aging/sedentarism and exercise on HR and BP; GDNF and NGF protein levels; and innervation pattern in the hearts of rats were reported. As sedentary control rats age, resting HR and BP significantly increases compared to that in young animals, with BP increasing to levels that would meet the clinical definition of hypertension. Results of other studies have shown that increased resting HR has been linked with increased BP (Shen et al. 2020; Reule & Drawz 2012) and increased resting HR and BP have

been associated with increased risk for stroke (Hu et al., 2019; Reule & Drawz, 2012) and heart failure (Zhao et al., 2020).

The onset of many cardiac diseases such as hypertension, heart failure, myocardial infarction, and arrythmias have been related to deficiencies in the innervation of the heart (Barron and Lesh, 1996; Nolan et al., 1998; La Rovere et al., 1998; Floras, 2003; Shen and Zipes, 2014; reviewed by Hanna et al., 2017). Increased sympathetic tone and activity in the heart is observed in early onset of hypertension (Julius & Nesbitt, 1996). Our results demonstrate that sympathetic nerve density significantly increases with age in the left atria and ventricles of sedentary animals. Sympathetic nerve density is at its highest in the 24mo-sed group, coinciding with the highest levels of resting BP and HR. In RA, parasympathetic and sensory innervation densities of 18mo-sed and 24mo-sed groups are significantly decreased when compared to the 4wk-sed group. In LA, sensory innervation density is decreased in aged groups when compared to the 4wk-sed group. Our data demonstrates that, throughout the animal's lifespan, aging combined with sedentary behavior may lead to an increase in sympathetic nerve density and a decrease in parasympathetic and sensory nerve densities in the heart, which may contribute to the development of hypertension. Increased activity of the sympathetic system is suggested to promote left ventricular hypertrophy, which may lead to hypertension. In addition, increased sympathetic activity may increase the production of renin, primarily by the kidneys, which will promote the formation of angiotensin II, leading to a chronic increase in BP and HR (Palatini 2001; Campos 2015). Many other studies have shown that increased sympathetic activity may lead to hypertension (Lambert et al., 2007; Esler, 2011; Menuet et al., 2017; Dissanayake et al., 2018). Ultimately, as animals age sedentary, the innervation pattern of the heart shifts from a

balanced sympathetic/parasympathetic/sensory innervation to a predominantly sympathetic innervation, leading to hypertension and increased resting HR.

Our results demonstrate that exercise significantly increases parasympathetic nerve density in the RA of the 12mo-ex and 18mo-ex groups; and shows trend towards an increase in the 24mo-ex groups when compared to the age-matching sedentary groups. In addition, our results show that exercise significantly increases parasympathetic nerve density in LA in the 18mo-ex group; and shows a trend towards an increase in the 12mo-ex and 24mo-ex groups. Concomitantly with the significant increases in parasympathetic nerve density in RA and LA, our results demonstrate that exercise significantly decreases BP and HR in the 12mo-ex and 18mo-ex groups when compared to the age-matching sedentary groups. Exercise has been shown to prevent and treat hypertension (Wallace, 2003; Goodman et al., 2011; Waki et al., 2019). In SHR, exercise prevented hypertension and disrupted the correlation between age-related elevation of BP and dynamic changes of vascular sympathetic activity (Li et al., 2019). Our results suggest that exercise may significantly increase parasympathetic nerve density in the heart and reduce resting BP and HR, which could prevent or treat high blood pressure. Our immunohistochemical imaging shows that exercised animals have more robust and denser nerve staining than sedentary animals. This suggests that exercise may promote changes in the structure of the nervous system in the heart. The development, growth, maintenance, and regeneration of nerve fibers are directed by neurotrophic factors such as GDNF and NGF. Our results demonstrate that aging/sedentarism alters neurotrophic factor levels in the heart. From four- to fourteen-week-old animals, GDNF protein levels increase in all heart chambers. This increase in GDNF levels coincides with the steady growth and development of the animals. For six-month-old and older groups, GDNF protein levels progressively decrease in all heart

chambers. Studies have shown that, in the heart, the parasympathetic nervous system is developed, maintained, and regenerated by GDNF (Hiltunen et al., 2000; Martinelli et al., 2002; Martinelli et al., 2006; Alves et al., 2019). Our results show that as GDNF levels decrease with aging/sedentarism, parasympathetic nerve density in the heart also decreases. Voluntary exercise increases GDNF protein levels in all heart chambers, with a concomitant increase in parasympathetic nerve density and a decrease in HR and BP. Exercise has been shown to increase GDNF protein levels and the levels of other GFL in a variety of tissues, while improving the overall functionality of these tissues (Saarma & Sariola, 1999; Rebimbas-Cohen, 2005; Wehrwein et al. 2002; McCullough et al. 2013; Gyorkos et al., 2014; Cobianchi et al., 2017; Alves et al., 2019; Correia et al., 2021).

Our results demonstrate that aging impacts NGF protein levels in the heart as well. NGF protein levels decrease in all heart chambers as the animals age. In 18- and 24-month-old animals, NGF levels is significantly lower when compared to all younger ages. However, exercise significantly increases NGF levels in all heart chambers when compared to the age-matched sedentary groups. Results of studies by Rebimbas-Cohen (2005) suggest that NGF decreases with aging in the mesenteric vessels of sedentary rats, and that the pattern of innervation shifts from a balanced sympathetic/sensory innervation in young rats to predominantly sympathetic innervation in older animals. Immunohistochemical analysis revealed that NGF colocalized with Calcitonin Gene-Related Peptide (CGRP), a marker for sensory innervation and potent vasodilator, in mesenteric vessels of young rats. In old rats, NGF colocalized with sympathetic nerve fibers. Moreover, blood pressure (BP) increased in old rats. However, voluntary exercise regimen was able to lower blood pressure, restore NGF protein content, and reverse the imbalance of the sympathetic/sensory innervation in the mesenteric

vessels of old animals. These results suggest that exercise increases NGF protein content in mesenteric vessels and may participate in restoring the balance of sympathetic/sensory innervation (Rebimbas-Cohen, 2005).

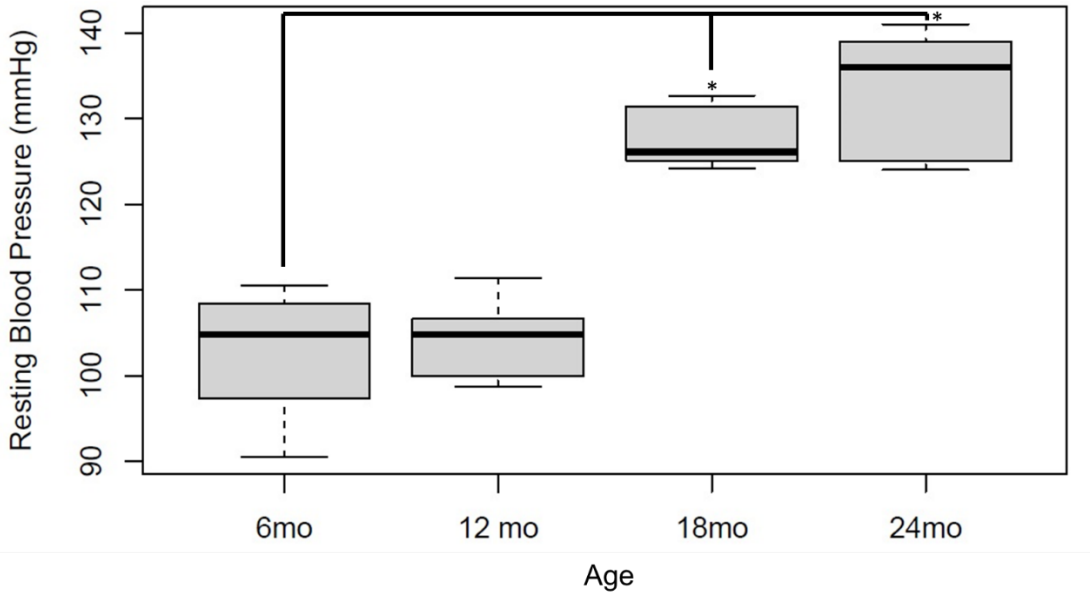
Research suggests that NGF may also contribute to the growth and survival of parasympathetic fibers (Ekman et al., 2017). We propose that the increase in parasympathetic innervation observed in our studies may also be due, in part, to an increase in NGF following exercise. Furthermore, interactions between sympathetic and parasympathetic nervous system can be found throughout the entire heart, including in the pacemaker region. The proper interactions between the two branches of the ANS is essential for a balanced cardiac function. For example, during the development of HF, disarrangement of autonomic nerves can decrease parasympathetic influence over the sympathetic system, leading to sympathetic over activity. It has been suggested that NGF may play a role in maintaining appropriate coupling of parasympathetic and sympathetic axons in the heart (Wetzel and Brown, 1985, Loiacono and Story, 1986; Fan and Smith, 1993; Warn et al., 1997; Smith et al. 2002; Dunlap et al. 2003; Randall et al. 2003; Nihei et al. 2005; Hasan & Smith 2009). Additional research found that decreased NGF expression in congestive HF leads to alterations in sympathetic neuronal function and neuroanatomy of the heart (Kaye et al. 2000).

In conclusion, our results suggest that neurotrophic factor content in the heart peaks in young animals and declines with aging. GDNF content declines with aging earlier and more drastically than NGF content. These results support the hypothesis that NGF primarily supports sympathetic nervous system, which does not seem to change much with age, while GDNF supports the parasympathetic system, which does decline with age. NGF supports the sensory nervous system. Therefore, the changes in NGF content that were observed in these studies may

be linked to the changes in sensory nerve density. Density of parasympathetic and sensory innervation decline with aging, while sympathetic innervation does not decline as much with aging, which may be the cause for an increase in BP and HR. Therefore, both BP and HR increase with age, as balance between sympathetic and parasympathetic innervation is impaired. With exercise, GDNF content increases, parasympathetic innervation increases, and BP and HR decrease. The effects of exercise on neurotrophic factor expression may be a possible mechanism by which exercise exerts positive effects on cardiac innervation, promoting the prevention and treatment of cardiovascular diseases.

Figure 2.1

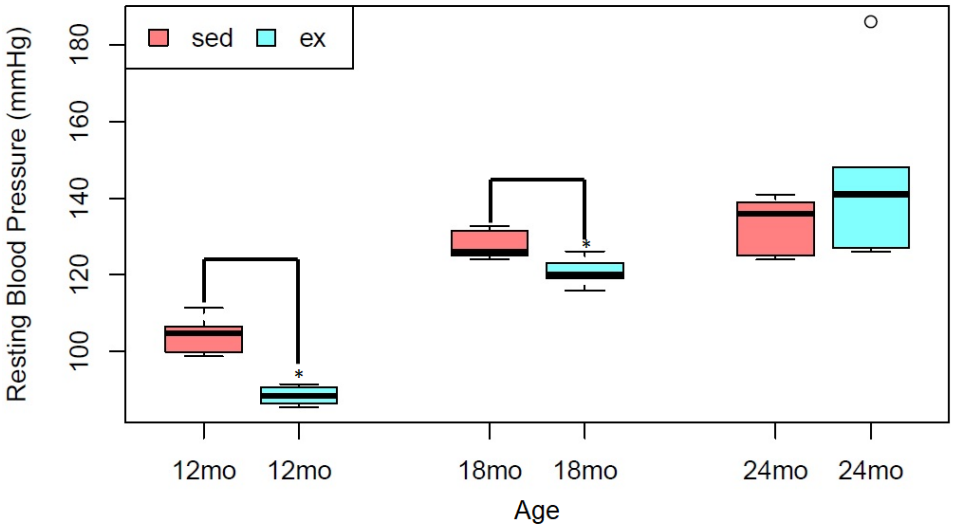
Blood Pressure Increases With Age



Note. Mean arterial blood pressures were obtained using tail-cuff plethysmography in rats maintained in a dark chamber, at 37ºC. The data show that BP was significantly elevated at 18 and 24 months of age compared to that at 6 months of age. Wilcoxon signed-rank test – 95% of confidence interval. The asterisk (*) indicates a significant difference in BP from that measured in 6-month-old animals.

Figure 2.2

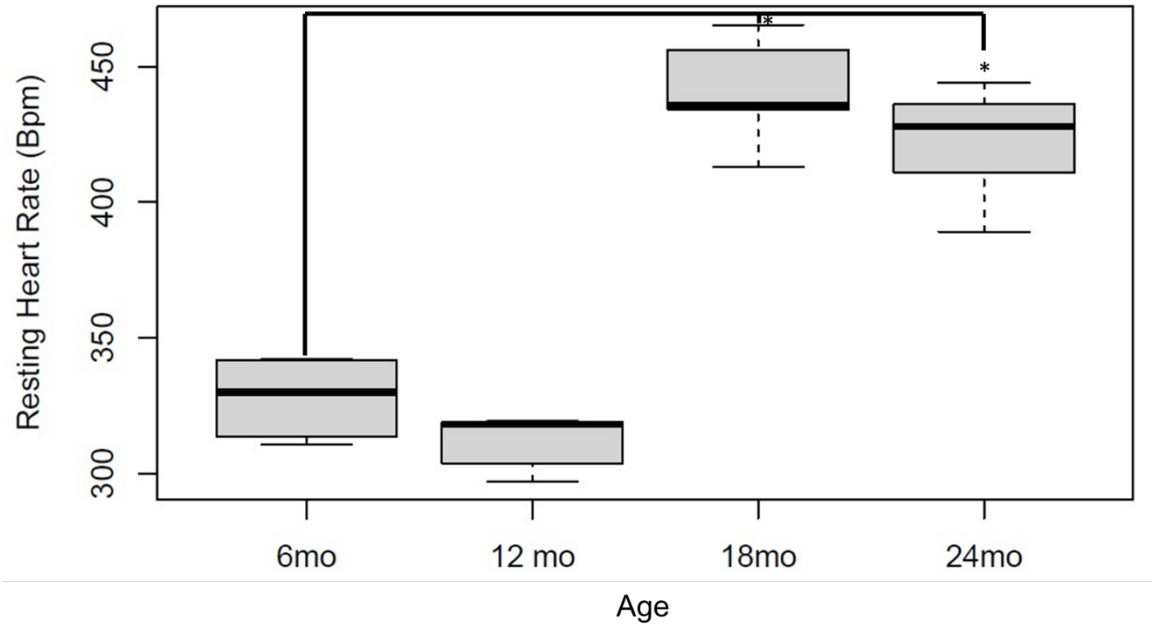
Exercise Decreases Blood Pressure



Note. Mean arterial blood pressures were obtained using tail-cuff plethysmography in rats maintained in a dark chamber, at 37ºC. Exercise significantly ($p<0.05$) reduced BP at 12 and 18 months of age and there is a trend towards a decrease in 24-month-old animals when compared to the age-matching sedentary groups. Wilcoxon signed-rank test – 95% of confidence interval. The asterisk (*) indicates a significant difference in BP in exercised animals compared to that in age-matched sedentary controls.

Figure 2.3

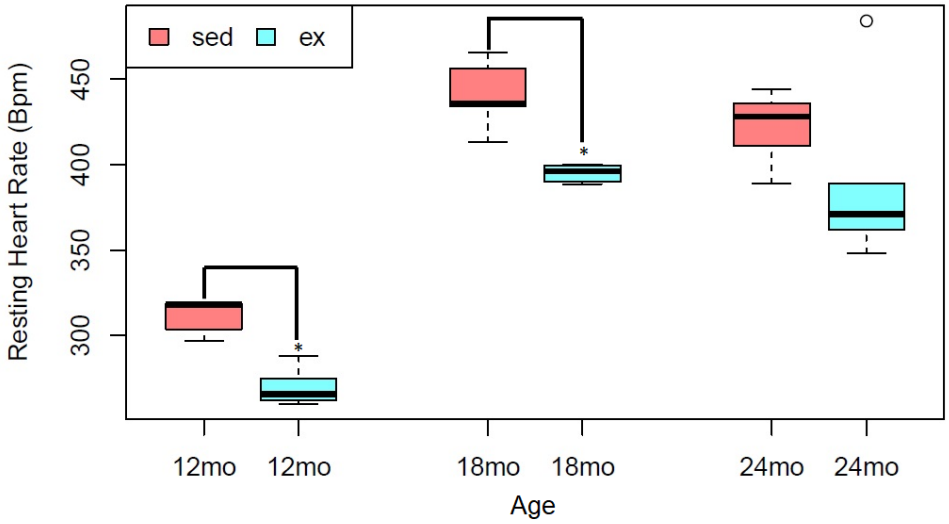
Resting Heart Rate Increases With Aging



Note. Resting heart rates were obtained using tail-cuff plethysmography in rats maintained in a dark chamber, at 37ºC. Our data shows that HR significantly increased at 18 and 24 months of age compared to 6 months of age. Wilcoxon signed-rank test – 95% of confidence interval. The asterisk (*) indicates a significant difference in HR from that measured in 6-month-old animals.

Figure 2.4

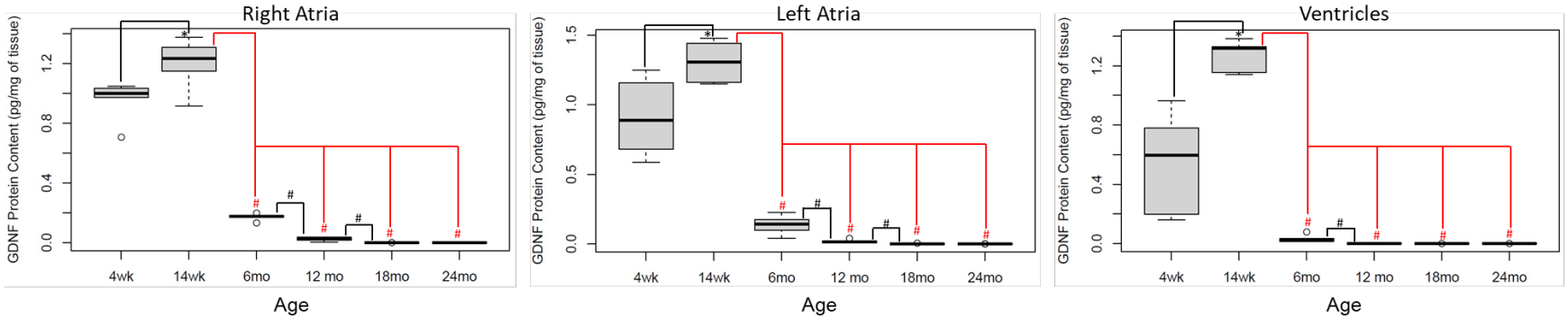
Exercise Decreases Resting Heart Rate



Note. Resting heart rates were obtained using tail-cuff plethysmography in rats maintained in a dark chamber, at 37ºC. Voluntary exercise significantly reduced HR at 12 and 18 months of age, and there was a trend towards a decrease in the 24-month-old animals when compared to the age-matching sedentary groups. Wilcoxon signed-rank test – 95% of confidence interval. The asterisk (*) indicates a significant difference in HR in exercised animals compared to that in age-matched sedentary controls.

Figure 2.5

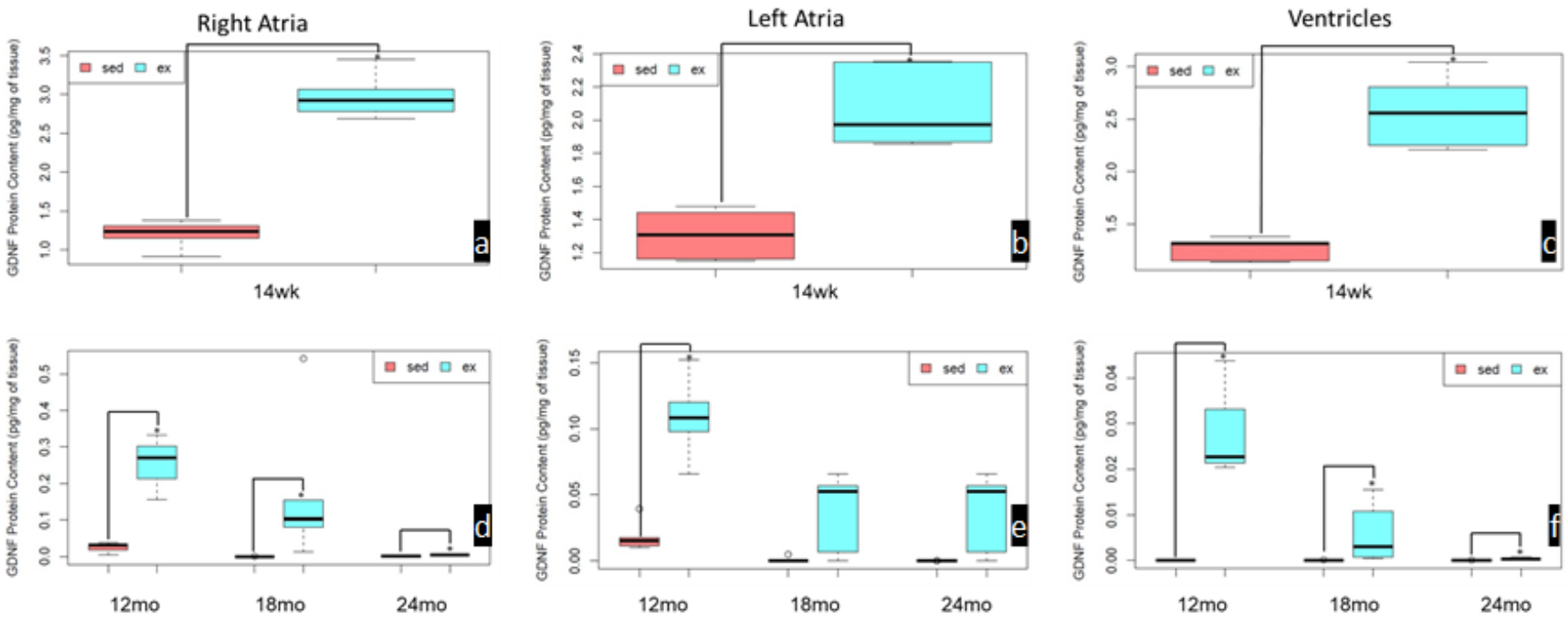
Impacts on Aging on GDNF Levels in all Heart Chambers



Note. GDNF protein content was measured by analyzing supernatant from heart chamber homogenate by ELISA. From the 4wk-sed group to the 14wk-sed group, GDNF protein levels significantly increased (*) in all heart chambers. At 6 months of age, GDNF levels significantly decreased (#) in all heart chambers and kept decreasing as animals aged. Wilcoxon signed-rank test – 95% of confidence interval.

Figure 2.6

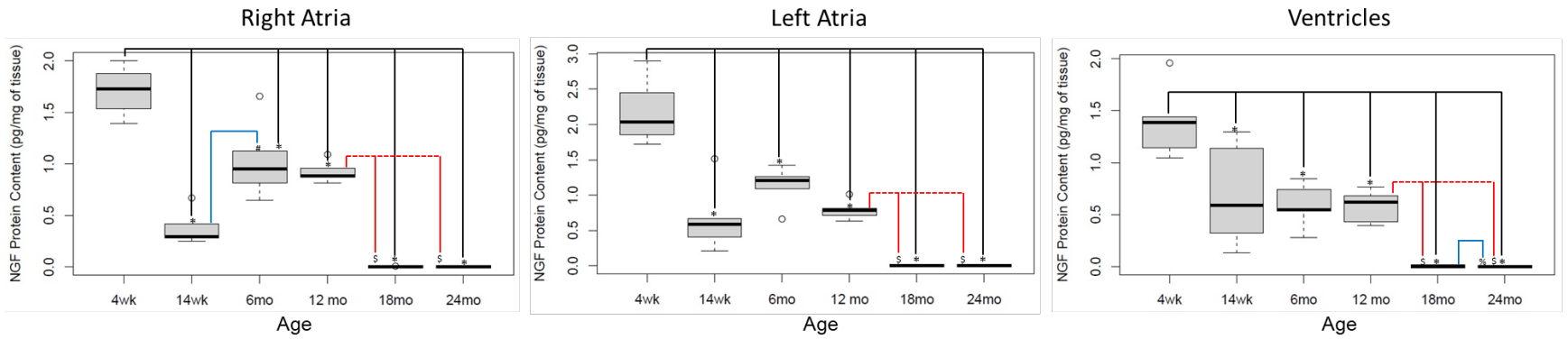
Impacts of Voluntary Exercise on GDNF Levels in all Heart Chambers



Note. GDNF protein content was measured by analyzing supernatant from heart chamber homogenate by ELISA. GDNF protein content was significantly (*) higher in all voluntary exercised groups in right atria and ventricles (**a, c, d, f**). Voluntary exercise significantly (*) increased GDNF content in left atria in the 14wk-ex and 12mo-ex groups; and had a trend towards an increase in the 18mo-ex and 24mo-ex groups (**b, e**). Wilcoxon signed-rank test – 95% of confidence interval.

Figure 2.7

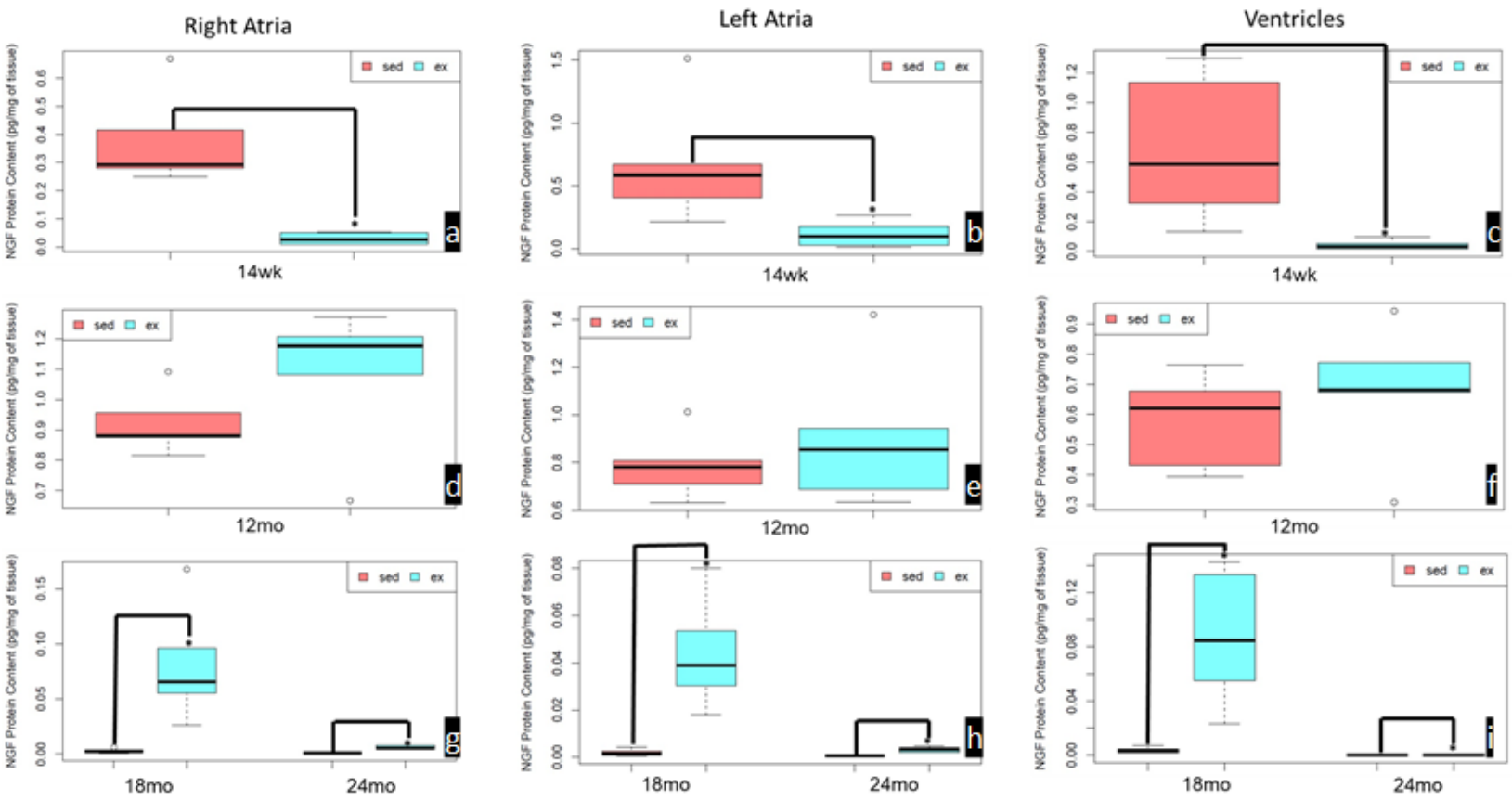
Impacts on Aging on NGF Levels in all Heart Chambers



Note. NGF protein content was measured by analyzing supernatant from heart chamber homogenate by ELISA. NGF protein levels were significantly (*) higher in all heart chambers from the 4wk-sed group when compared to all older ages. In right atria, NGF protein levels significantly increased from 14 weeks of age to 6 months of age (#). In all heart chambers, NGF protein levels significantly ($) decreased in 18-month-old and 24-month-old animals when compared to 12-month-old animals. In ventricles, NGF protein levels significantly (%) decreased in 24-month-old animals when compared to 18-month-old animals. Wilcoxon signed-rank test – 95% of confidence interval.

Figure 2.8

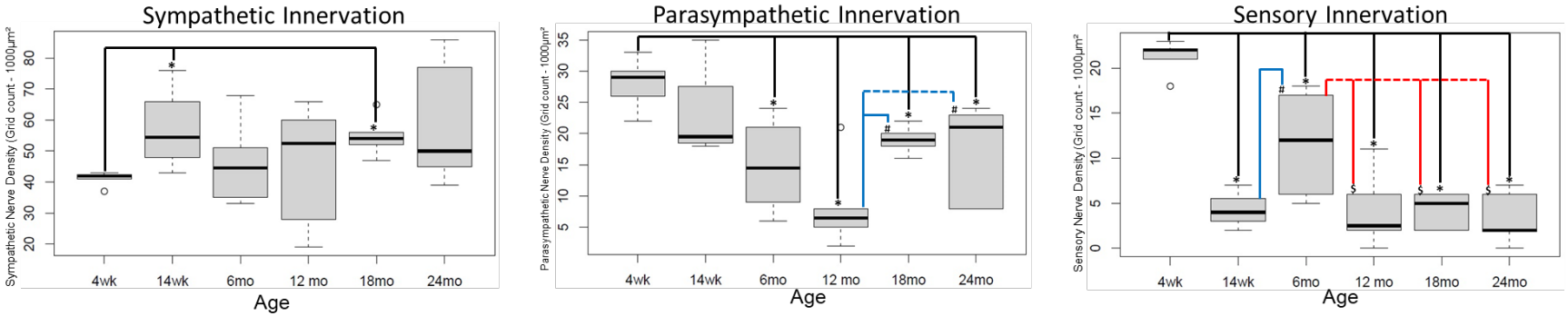
Impacts of Voluntary Exercise on NGF Levels in all Heart Chambers



Note. NGF protein content was measured by analyzing supernatant from heart chamber homogenate by ELISA. Voluntary exercise significantly (*) decreased NGF protein levels in all heart chambers at 14-week-old animals (**a, b, c**). Exercise did not impact NGF protein content in none of the heart chambers in the 12-month-old group (**d, e, f**). At 18 and 24 months of age, voluntary exercise significantly (*) increased NGF protein content in all heart chambers (**g, h, i**). Wilcoxon signed-rank test – 95% of confidence interval.

Figure 2.9

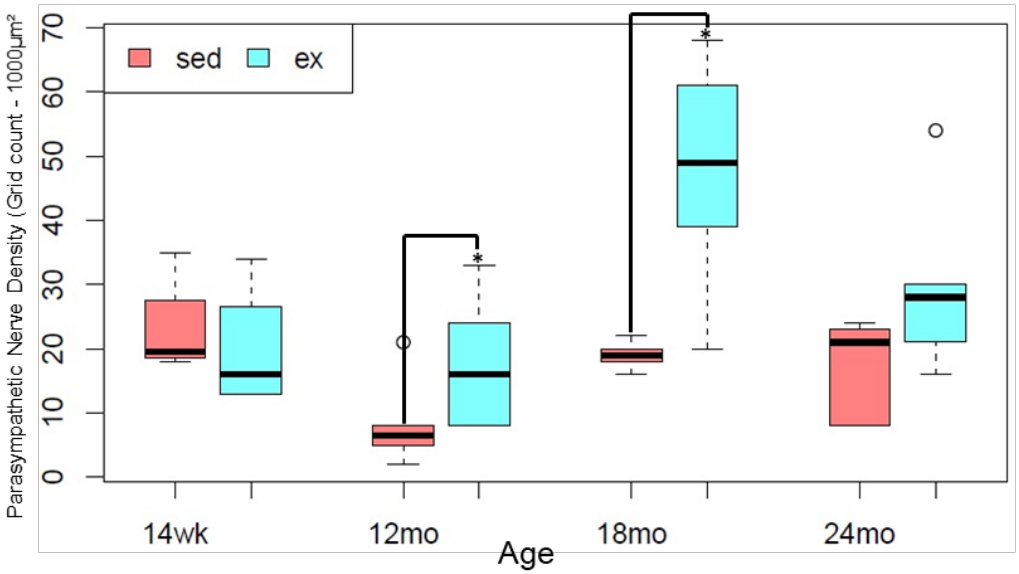
Impacts of Aging on the Innervation Pattern in Right Atria



Note. Innervation pattern of sympathetic, parasympathetic, and sensory nervous systems were measured on the epicardium of each heart chamber from each animal by using a Nikon C2+ laser scanning confocal microscope. 60X water objective was used to capture 40 µm-deep z-stack images from the epicardium. NIS-Element AR was used to analyze nerve densities. Innervation pattern of right atria significantly changes with aging. Sympathetic nerve density is significantly (*) increased in the 14wk-sed and 18mo-sed groups when compared to the young 4wk-sed group, and it trend towards an increased in the 24mo-sed group. Parasympathetic nerve density significantly (*) decreased in the 6mo-sed, 12mo-sed, 18mo-sed, and 24mo-sed groups when compared to the 4wk-sed group. Parasympathetic nerve density significantly (#) increased in the 18mo-sed and 24mo-sed groups when compared to the 12mo-sed group. Sensory nerve density was at its highest density (*) in the 4wk-sed when compared all other groups. Sensory nerve density significantly (#) increased from the 14wk-sed group to the 6mo-sed group, but significantly decreased ($) from the 6mo-sed group to all older ages. Wilcoxon signed-rank test – 95% of confidence interval.

Figure 2.10

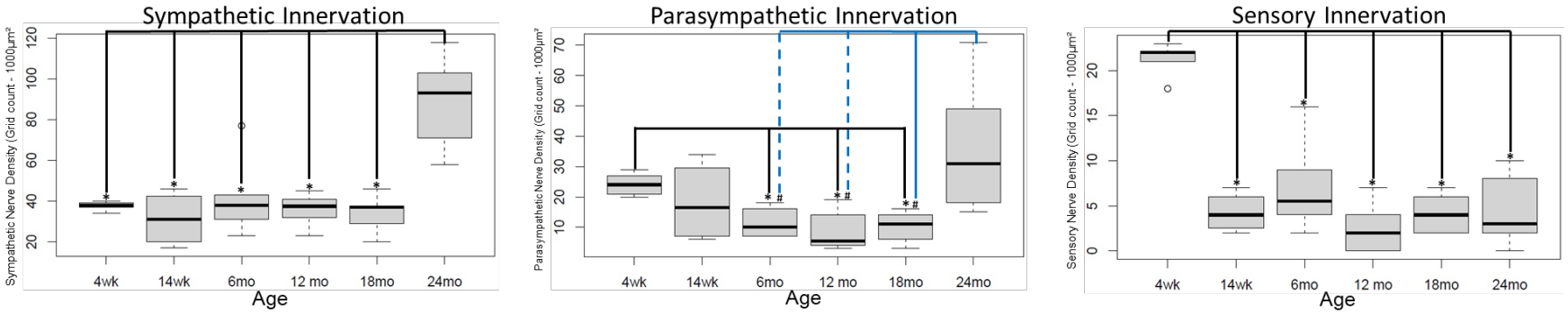
Impacts of Exercise on Parasympathetic Innervation in Right Atria



Note. Innervation pattern of sympathetic, parasympathetic, and sensory nervous systems were measured on the epicardium of each heart chamber from each animal by using a Nikon C2+ laser scanning confocal microscope. 60X water objective was used to capture 40 µm-deep z-stack images from the epicardium. NIS-Element AR was used to analyze nerve densities. Voluntary exercise significantly (*) increased parasympathetic nerve density in right atria in the 12mo-sed and 18mo-sed groups, and trend towards an increase in the 24mo-sed group. Wilcoxon signed-rank test – 95% of confidence interval.

Figure 2.11

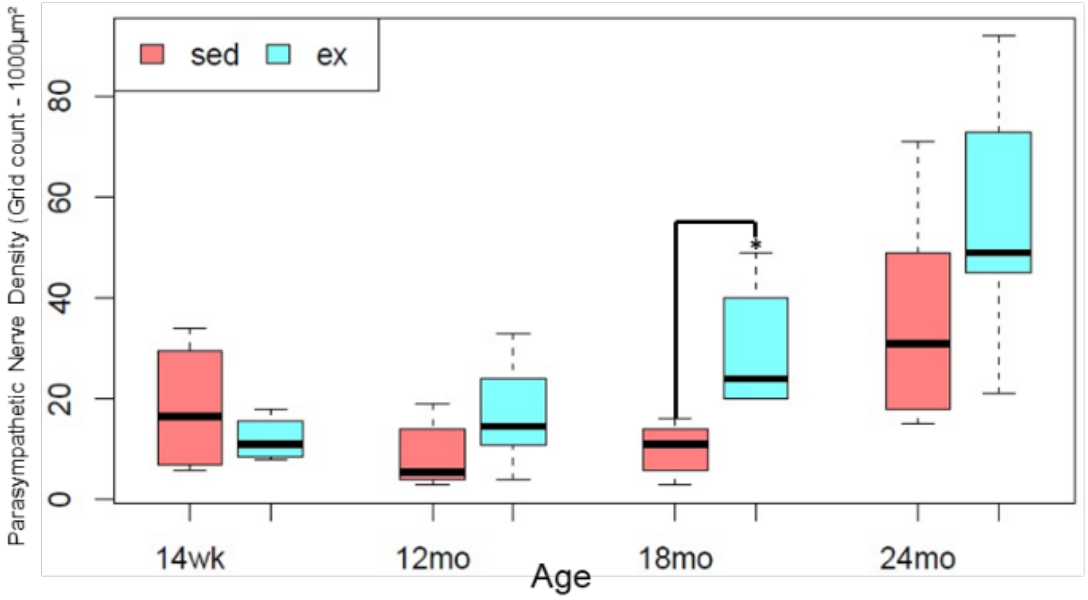
Impacts of Aging on the Innervation Pattern in Left Atria



Note. Innervation pattern of sympathetic, parasympathetic, and sensory nervous systems were measured on the epicardium of each heart chamber from each animal by using a Nikon C2+ laser scanning confocal microscope. 60X water objective was used to capture 40 μm-deep z-stack images from the epicardium. NIS-Element AR was used to analyze nerve densities. Innervation pattern of left atria significantly changed with aging. Sympathetic nerve density significantly (*) increased in the 24mo-sed group when compared to all other groups. Parasympathetic nerve density was significantly decreased in the 6mo-sed, 12mo-sed, and 18mo-sed groups when compared to the 4wk-sed (*) and 24mo-sed (#) groups. Sensory nerve density was significantly increased in the 4wk-sed group when compared to all other groups. Wilcoxon signed-rank test – 95% of confidence interval.

Figure 2.12

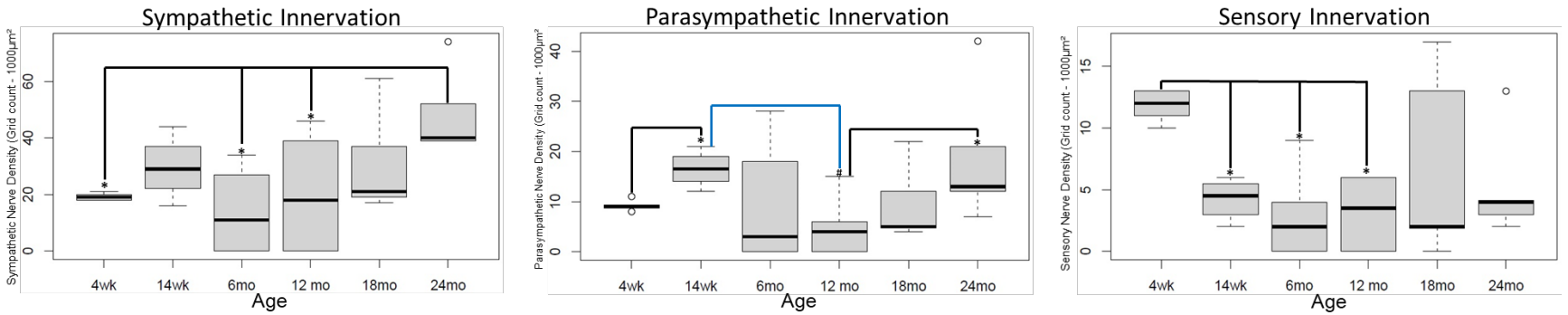
Impacts of Exercise on Parasympathetic Innervation in Left Atria



Note. Innervation pattern of sympathetic, parasympathetic, and sensory nervous systems were measured on the epicardium of each heart chamber from each animal by using a Nikon C2+ laser scanning confocal microscope. 60X water objective was used to capture 40 µm-deep z-stack images from the epicardium. NIS-Element AR was used to analyze nerve densities. Voluntary exercise significantly (*) increased parasympathetic nerve density in left atria in the 18mo-sed group, and trend towards an increase in the 12mo-sed and 24mo-sed groups. Wilcoxon signed-rank test – 95% of confidence interval.

Figure 2.13

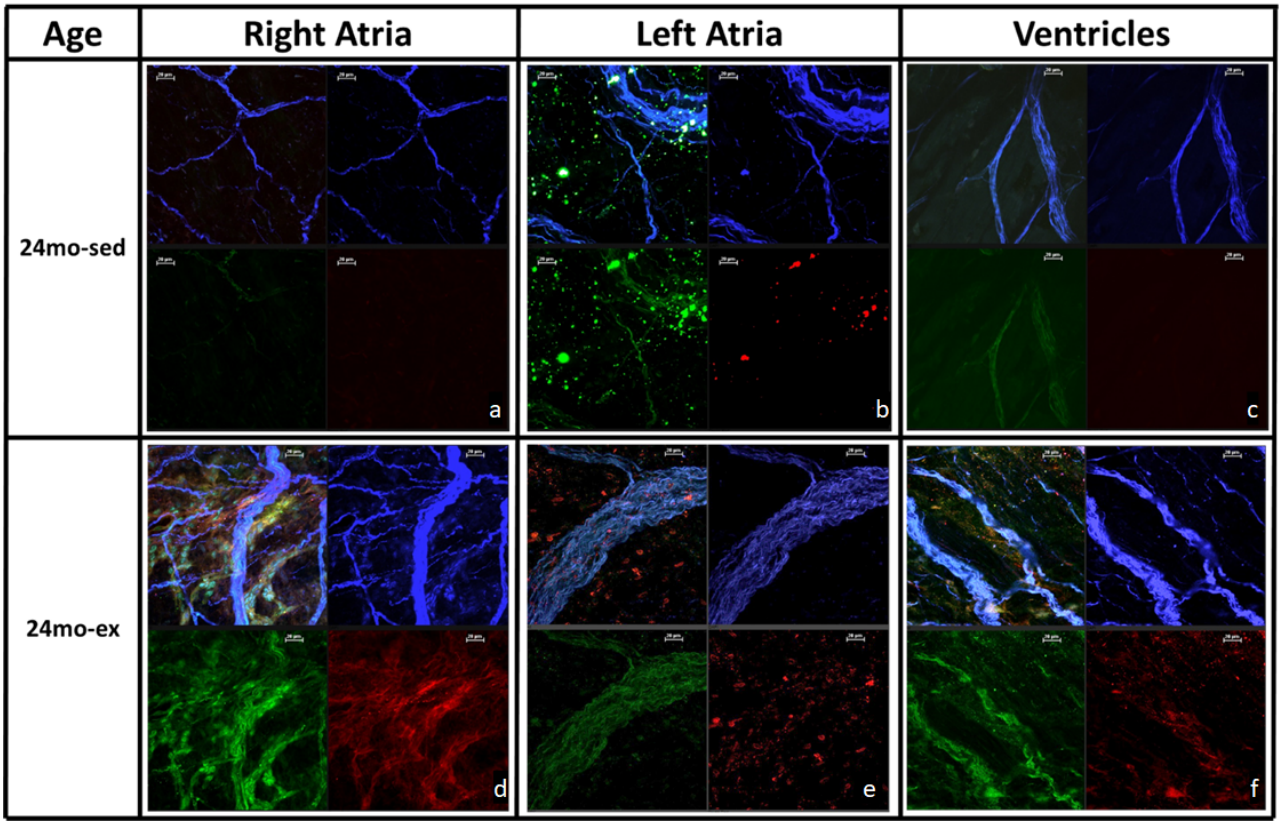
Impacts of Aging on the Innervation Pattern in Ventricles



Note. Innervation pattern of sympathetic, parasympathetic, and sensory nervous systems were measured on the epicardium of each heart chamber from each animal by using a Nikon C2+ laser scanning confocal microscope. 60X water objective was used to capture 40 µm-deep z-stack images from the epicardium. NIS-Element AR was used to analyze nerve densities. Innervation pattern of ventricles significantly changes with aging. Sympathetic nerve density was significantly (*) increased at the 24mo-sed group when compared to the 4wk-sed, 6mo-sed and 12mo-sed groups, and there was a trend towards an increase when compared to 14wk-sed and 18mo-sed groups. Parasympathetic nerve density was significantly (*) increased in the 14wk-sed group when compared to the 4wk-sed group. At 12 months of age, parasympathetic nerve density is significantly (#) decreased when compared to the 14wk-sed group. Sensory nerve density was significantly (*) higher in the 4wk-sed group when compared to 14wk-sed, 6mo-sed, and 12mo-sed groups, and trends to be higher than the 24mo-sed group. Wilcoxon signed-rank test – 95% of confidence interval.

Figure 2.14

Nerve Fiber Staining in Heart Chambers From 24mo-sed and 24mo-ex groups



Note. Heart chambers from rat – whole mount preparation – Confocal imaging – 60X objective – anti-CGRP conjugated to AlexaFluor 568 (red), anti-TH conjugated to AlexaFluor 405 (blue), and anti-ChAT conjugated to AlexaFluor 488 (green). Comparison between exercised (**d, e, f**) and sedentary (**a, b, c**) lifestyles at 24 months of age. There were significant differences in staining robustness between sedentary and exercised animals. Exercised animals had denser innervation in the epicardium when compared to sedentary animals. In sedentary animals, sympathetic innervation

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CHAPTER III

EFFECTS OF TREATMENT WITH α-CALCITONIN GENE-RELATED PEPTITDE ON HEART FUNCTION

Abstract

Calcitonin gene-related peptide (CGRP) plays an important role as a potent vasodilator, which may help prevent cardiac and pulmonary hypertension, ischemia, migraine, and ultimately, improve blood flow distribution, and wound healing. It has been suggested that CGRP may play a role in cardiovascular regulation. However, the effects of exogenous CGRP on cardiac physiology have not been adequately investigated. The primary goal of this research is to investigate the effects of exogenous αCGRP on heart function. Adult bullfrogs (*Lithobates catesbeianus*) were divided into one control group and three treatment groups receiving 40 nM, 100 nM, and 400 nM of CGRP. To investigate the role that sympathetic and parasympathetic neurotransmitters may play in the effects of CGRP. For some treatments, antagonists for one or both branches of the autonomic nervous system were used with CGRP. Our results demonstrate that all groups treated with CGRP alone exhibited a significantly lower force of contraction (FOC) than the control group five minutes following treatment. Treatment with CGRP in the presence of the muscarinic receptor antagonist, atropine, significantly decreased FOC and HR. Immunohistochemical analysis revealed CGRP positive fibers on the epicardium. Our results suggest that CGRP positive fibers with varicosities are present in the heart and CGRP may be released from these fibers causing the effects observed in this study. Exogenous CGRP treatment reduced FOC and HR in the heart of frogs, even when the parasympathetic nervous system was blocked, suggesting that CGRP may act directly on the cardiac muscle.

Introduction

Calcitonin gene-related peptide (CGRP) is a 37 amino-acid peptide that serves as a neurotransmitter in enteric, somatic motor, and sensory neurons. Studies have shown that CGRP is processed into two distinct isoforms, that result from different genes (reviewed on Russell et al., 2014). βCGRP is found in the central and enteric nervous system, as well as in the thyroid and pituitary glands (Petermann et al., 1987). βCGRP is produced by splicing of the CALCII gene on the short arm of chromosome 11 (11p 12-14.2), playing a similar role to αCGRP (Alevizaki et al., 1986; Brain & Grant, 2004; Muddhrry et al., 1988; Steenbergh et al., 1986). αCGRP is produced by alternative splicing of the CALCI gene and stored in vesicles in sensory and somatic motor nerve terminals. αCGRP can be found in peripheral and central neural tissues throughout the body and may regulate cell and tissue function (Alevizaki et al., 1986; Steenbergh et al., 1986; Russell et al., 2014).

CGRP acts via the CGRP receptor. Previous studies suggest that the CGRP receptor is a combination of two receptors, the calcitonin-like receptor (CLR) and receptor activity modifying protein 1, 2, or 3 (RAMP1, RAMP2, RAMP3) (Fluhmann et al., 1995; Hay et al., 2008; Russell et al., 2014). These receptors are translated into the endoplasmic reticulum (ER) and combined to form a binding site for CGRP. CLR:RAMP complexes are transported from the ER into the plasma membrane where it binds CGRP. The individual CGRP receptor components are unable to recognize CGRP by themselves. Instead, they must be combined to recognize the CGRP molecule (McLatchie et al., 1998; Spielman & Parameswaran, 2012). CGRP also has an affinity with the CLR:RAMP3 complex, also known as adrenomedullin 2 (AM2) receptor (Choksi et al., 2002; Muff et al., 1998; Russell et al., 2014). Finally, a third CGRP receptor component named receptor component protein (RCP) is an intracellular peripheral membrane protein that binds to

the second intracellular loop of the CLR. Inhibiting RCP caused a shutdown of the CGRP receptor-intracellular signaling (Evans et al., 2000; Luebke et al., 1996).

A well-known pathway triggered by CGRP binding to its receptor is the activation of adenylate cyclase, which increases intracellular cyclic AMP (cAMP), leading to activation of protein kinase A (PKA). PKA influences many pathways downstream, including activation of extracellular signal-related kinases and transcription factors (Drake et al., 1999, 2000). Phosphorylated PKA may also open potassium channels and promote smooth muscle relaxation in arteries (Nelson et al. 1990). In addition, CGRP may also cause the release of calcium by the endoplasmic reticulum, through the production of inositol trisphosphate (reviewed on Russell et al., 2014). Binding of CGRP to its receptor can also increase intracellular levels of diacylglycerol, which may activate protein kinase C (Pin & Bahr, 2008).

CGRP plays an essential role as a potent vasodilator, which may help to prevent cardiac and pulmonary hypertension, ischemia, migraine, and acts to improve blood flow distribution, and wound healing (Hasbak et al., 2001, 2003; Jonhagen, 2006; Russell et al., 2014; Schlier et al., 2009; Toda et al., 2008; Vause & Durham, 2010). CGRP is also found to promote vasodilation of in-vitro parenchymal microvessels from hippocampal slices of rat brains (Fergus et al., 1995). CGRP immunoreactive fibers are found in coronary arteries in the heart of rats, and the action of CGRP regulates blood flow in these vessels (Goto et al., 1991).

Exercise may increase CGRP production and secretion by the sensory nerve fibers present in skeletal and cardiac muscle (Jonhagen, 2006; Onuoha et al., 1998; Russell et al., 2014; Sun & Pan, 2014). Exercise also may increase CGRP-positive sensory nerve fibers density in arteries and veins of exercised animals (Rebimbas-Cohen, 2005). Research suggests that during exercise, CGRP may target epididymal fat, promoting lipolysis (Aveseh et al., 2018). Finally,

exercise is suggested to increase CGRP production, which may reduce myocardial ischemia via collateral circulation development promoted by CGRP (Wang et al., 2016).

The two branches from the autonomic nervous system (ANS), the sympathetic (SNS) and parasympathetic (PNS) nerve systems work antagonistically to modulate the heart's function (Bush et al., 2016; Hiltunen et al., 2000). It has been suggested that CGRP may play a role in cardiovascular regulation by inhibiting sympathetic nervous system activity in mice (Kurihara et al., 2003). Intravenous injection of CGRP in humans caused an increase in heart rate, decreased blood pressure, and produced skin redness, which indicates vasodilation (Lundberg et al., 1989). A study using anesthetized rats and conscious dogs and treating them with intravenous human CGRP, found that CGRP caused dose-dependent peripheral vasodilation. Pre-treatment with CGRP 8-37, a CGRP receptor antagonist, prevented the vasodilatory actions of CGRP. Other findings from this study suggested that CGRP has a greater effect on increasing blood flow in rat hearts than vasodilatory effects in the brain. However, by systemically blocking β-adrenergic receptors, the increase of regional blood flow caused by CGRP in these two organs was identical. Their findings suggest that CGRP increases blood flows in coronary, renal, and carotid arteries of dogs (Shen et al., 2001). Studies in αCGRP knockout mice (αCGRPKO) suggest that treatment with angiotensin II increases mRNA levels of CLR and RAMP1 in wild-type animals, but not in the αCGRPKO animals. Findings from these studies suggest that αCGRP mRNA was also elevated in the aorta, mesenteric vessels, and dorsal root ganglia of WT animals treated with angiotensin II compared to the WT control animals (Smillie et al., 2014).

At present, it is unclear concerning the effects exogenous treatment with CGRP on heart function. The aim of the current study was to investigate the effects of CGRP treatment on heart

function to test the hypothesis that CGRP decreases the force of contraction (FOC) and heart rate (HR) in frogs.

Methods

Heart Preparations

All animal experiments were performed in accordance with the "Guide for the Care and Use of Laboratory Animals" (National Research Council) and protocols were approved by the Institutional Animal Care and Use Committee at Western Michigan University. Adult bullfrogs (*Lithobates catesbeianus*) were euthanized, pinned on their backs and the body cavity was opened to expose the heart and assemble an i*n-situ* preparation. Each heart was connected to a PowerLab data acquisition system for the collection of FOC and HR data using a force transducer. Data was collected and analyzed using LabChart software (ADInstruments). Before treating the hearts with CGRP and/or autonomic antagonists, a 60-second stable baseline was recorded from each animal and those levels of HR and FOC were recorded as the initial control level (0 on the y-axis of graphs). Following treatment, FOC and HR were recorded for 5 minutes.

Treatment With CGRP

Twelve adult frogs were divided into one control group and three treatment groups. Treatment groups received CGRP (rat αCGRP, Tocris Bioscience [catalog# 83651-90-5] loaded into frog ringer's solution, at concentrations of 40, 100, and 400 nM. The control group received frog ringer's solution only.

Treatment With Autonomic Antagonists

Additional frogs were used to investigate the effects of CGRP (40 nM) when one or both branches of the autonomic nervous system were blocked. To examine the role that the sympathetic neurotransmitter norepinephrine may play in the observed effects of CGRP, we

blocked beta-adrenergic receptors using propranolol and alpha-adrenergic receptors using phentolamine and CGRP. To examine the role that the parasympathetic neurotransmitter acetylcholine may play in the observed effects of CGRP, we blocked muscarinic acetylcholine receptors on the heart using atropine and CGRP. Phentolamine, propranolol, and atropine were used at a concentration of 10mM and applied directly to the heart. The treatment groups were divided as follows: Atropine alone; Atropine and CGRP (AC); Phentolamine alone; Phentolamine and CGRP (PhC); Propranolol alone; Propranolol and CGRP (PrC); Propranolol + Phentolamine and CGRP (PPC); Propranolol + Phentolamine + Atropine and CGRP (PPAC). Hearts were pre-treated with autonomic antagonist to block all available receptors, then the mixture containing the autonomic antagonist and CGRP were added.

Immunohistochemistry

Frog hearts were removed, and the heart chambers were separated in PBS solution. Heart chambers were fixed with 4% paraformaldehyde for 15 minutes. After washing, the tissues were blocked for 1 hour at 4ºC with PBS + 1% BSA + 0.4% Triton. After blocking, tissues were incubated for five days (4º C) with guinea pig anti-αCGRP (Synaptic Systems) at a dilution of 1:500, rabbit anti-tyrosine hydroxylase (TH) (Abcam) at a dilution of 1:500, and chicken anti-neurofilament (Abcam) at a dilution of 1:250. Next the tissues were washed and incubated (24 hr at 4º C) with goat anti-guinea pig IgG conjugated to Alexa Fluor 568 (Abcam), goat anti-rabbit IgG conjugated to Alexa Fluor 488 (Abcam) and/or goat anti-chicken IgG conjugated to Alexa Fluor 488 (Abcam). All secondary antibodies were used at dilutions of 1:500. After washing, the tissues were placed in welled slides in a 1:1 solution of phosphate buffer saline (PBS) and glycerol. Control tissue was incubated without primary antibodies. Without the primary

antibody, no fluorescence was observed. Images were captured using a Nikon C2+ laser scanning confocal microscope.

Statistical Analysis

Kruskal-Wallis and Wilcoxon Rank Sum tests were performed to determine significance following treatment with CGRP with and without autonomic antagonists. Significance was set to $p<0.05$.

Results

Staining of Nerve Fibers in the Wall of the Heart

Our immunocytochemical studies suggest that CGRP nerve fibers are found in the walls of the frog heart. Confirmation that CGRP was present in neural tissues was done by co-staining with antibodies against neurofilament; a general neuronal marker (Figure 3.1). Bundles of TH and CGRP positive fibers, with numerous varicosities, were found in the epicardium of the right atria (Figure 3.2). Bundles of CGRP positive fibers with smaller bundles branching off at intervals are seen in Figure 3.3. Figure 3.4 shows a 3-dimensional (3D) view image of the fiber bundles displayed in Figure 3.3. With this topographic 3D representation, it was possible to visualize bundles of axons on the top of the epicardium (red) and a smaller bundle branching out and projecting into the muscle of the epicardium (purple/blue).

Effects of Treatment With CGRP on Heart Force of Contraction (FOC) and Heart Rate (HR)

Following treatment with CGRP, changes in cardiac physiology were observed five minutes following exposure. All treatment groups (40, 100 and 400nm) exhibited significantly lower FOC than the control group (Figure 3.5). The 40 nM and 100 nM, treatment groups exhibited a significantly lower FOC than the 400 nM treatment group. All control groups were treated with frog ringer's solution alone, thus the FOC for controls was different than zero (FOC

at the point of initiation of treatment). Despite the trend to decrease HR, there were no significant differences in HR in any treatment with CGRP alone.

Effects of Treatment With Autonomic Antagonists Alone and in Combination With CGRP

To better understand the mechanisms by which CGRP impacts heart function, we conducted additional experiments in which CGRP was added in combination with receptor antagonists for autonomic neurotransmitters. Significant results revealing decreased FOC (Figure 3.6) and HR (Figure 3.7) were observed in the AC group when the muscarinic acetylcholine receptors were blocked with atropine. Wilcoxon signed-rank test comparisons were significant at the 10% level for the remainder of the other treatment groups**;** PhC, PPC, PrC, and PPAC, suggesting a trend towards a decreased FOC and HR for these groups.

When we compared the effects of the autonomic antagonists alone and the autonomic antagonists in combination with CGRP in the heart physiology, we observed significant changes in the AC group. When we treated the hearts with atropine and CGRP combined (AC), it significantly decreased HR (Figure 3.8) and FOC (Figure 3.9) when compared to atropine alone.

Discussion

The Sensory Innervation of the Heart

Previous research from Woods (1970) demonstrated the presence of postganglionic sympathetic and parasympathetic innervation in the heart of frogs (Woods, 1970). Additional research from Woods (1970) found sensory-vagal ganglion cells in the heart of frogs. Histochemical methods also suggest that all cells in the heart's vagosympathetic branches are parasympathetic cells (Woods, 1970). Vagal innervation of the heart was further investigated by Cheng et al. (1997). To eliminate sensory efferent fibers, Cheng and colleagues (1997) surgically removed them by supranodose vagotomy. Their confocal investigations found sensory afferent

nerves in the epicardium of rat hearts and some in contact with cardiac ganglia, where principal neurons and small intensely fluorescent (SIF) cells are located. Principal neurons may directly control SA and AV nodes and muscle physiology. SIF cells, alongside sensory afferent fibers, may indirectly affect cardiac physiology through principal neurons. Cheng et al. (1997) suggests that these afferent sensory fibers present in the heart do not have a vagal origin (Cheng et al., 1997). Rysevaite et al.'s (2011) research found CGRP positive fibers in close proximity with ChAT (choline acetyltransferase) and TH fibers and in colocalization with TH fibers in whole-mount of the mouse heart (Rysevaite et al., 2011). Our immunocytochemical studies suggest that CGRP is available in nerve fibers in the wall of the frog heart. CGRP could be released by these nerve fibers, possibly released from their varicosities, and could cause the effects we observed in these studies.

Supporting this theory, studies from Chanez et al. (1998) have found sensory fibers with many prominent nerve endings, enlarged varicosities, and nerve bundles in the bronchial mucosa of a pig. The same study found CGRP positive sensory nerves with varicosities present beneath and within the epithelium, around blood vessels and sub-mucosal glands, and within the bronchial smooth-muscle layer (Chanez et al., 1998). Additional immunohistological studies from Mense (2019) found CGRP positive sensory fibers in thoracolumbar fascia, and some of these fibers exhibit varicosities. In our studies, the varicosities can be seen in the wall of the heart in detail on Figure 3.3. Studies have reported that αCGRP is produced and stored in vesicles in sensory and somatic motor nerve terminals and may regulate cell and tissue function (Alevizaki et al., 1986; Russell et al., 2014; Steenbergh et al., 1986).

Exogenous CGRP Treatment Causes Changes in Cardiac Physiology

The demonstration from our studies that all exogenous CGRP treatment groups have lower FOC compared to the control strengthens the suggestion that CGRP and the sensory nervous system may have potent parasympathetic-like roles. These results agree with other research findings in the literature, which have shown the potent relaxative effects of CGRP in many types of target tissues. CGRP is known to regulate the coronary arterial tone in the heart of rats (Goto et al., 1991) and to promote *in-vitro* vasodilation of parenchymal microvessels from the hippocampus of rats (Fergus et al., 1995). Other findings from Nelson et al. (1990) suggest that CGRP causes smooth muscle relaxation in arteries by hyperpolarizing the smooth muscle. In addition, CGRP effects are inhibited when potassium channels are blocked, which would contribute to hyperpolarization of the smooth muscles. Intracoronary injections of CGRP caused dose-depended vasodilation of epicardial coronary arteries in humans (McEwan et al., 1986). These studies suggesting negative inotropic roles of CGRP, is strengthened by a study from Takami et al. (1985) in which CGRP was found in colocalization with acetylcholine in single cells of the hypoglossal and facial nerves and nucleus ambiguus of rats. Research from Machado and Brody (1985) demonstrated that the nucleus ambiguous, besides being an area in the medulla filled with motor and sensory cell bodies, also contains preganglionic parasympathetic neurons in which will further connect with postganglionic parasympathetic neurons. Additional experiments from Takami et al. (1985) found CGRP in neuromuscular junctions in the tongue muscles. Therefore, the results from these experiments, combined with others in the literature, strongly suggest that CGRP and the sensory nervous system may play a role in regulating muscle physiology and may be co-expressed with ACh in somatic motor neurons and in parasympathetic

neurons. The sensory nervous system may cause alterations in cardiac and smooth-muscle physiology in a parasympathetic-like manner.

Information on the effects of CGRP on heart rate is contradictory in the literature, although exogenous CGRP has been found to play a role in important cardiac events. A study from Ono and Giles (1991) using single-cell voltage-clamp techniques demonstrated that low concentrations of CGRP are responsible for causing relevant chronotropic effects in single myocytes from bullfrog and Guinea pig atria. Lundberg et al. (1989) reported that intravenous injection of αCGRP and βCGRP in humans caused increased heart rate and decreased blood pressure due to systemic vasodilation (Lundberg et al., 1989). By exposing the hearts to αCGRP, our results show that all treatment groups exhibited a trend towards a lower HR at the fifth minute following treatment. Research from Rigel (1988) treating dogs with CGRP showed no significant physiological changes in heart rate. Work from Zeller et al. (2008) suggests that treatment with anti-CGRP antibody prevented the vasodilatory actions of CGRP but had no effect on heart rate in rats. Treating rats with an αCGRP receptor antagonist (BIBN4096BS) did not alter heart rate suggesting that endogenous CGRP may not participate in cardiac function regulation (Arulmani et al., 2004). Supporting these findings, research studying the safety of a humanized monoclonal antibody that antagonizes CGRP binding to its receptor named LBR-101 found that this antibody did not alter blood pressure, heart rate, or temperature in human subjects (Bigal et al., 2013). Treatments using another CGRP-receptor antagonist (Telcagepant – MK-0974) also did not alter HR or blood pressure in patients with migraines (Depré et al., 2013). A possible explanation on why CGRP may reduce heart rate comes from Nelson et al. (1990), where it is suggested that CGRP hyperpolarizes arterial mesenteric smooth muscle by activating potassium channels. This efflux of potassium may cause muscle relaxation and contributes to a

reduction of HR by hyperpolarization-activated current, which may influence the action potential and contraction frequency of the cardiac cells (Momin et al., 2008; Nelson et al., 1990).

Our results corroborate previous literature examining the effects of autonomic antagonists on HR and FOC. Atropine is a non-selective muscarinic antagonist and blocks acetylcholine's (ACh) parasympathetic effects. ACh is used in emergency cardiovascular care and resuscitation; and treats bradycardia, atrioventricular block, acute sinus node dysfunction, organophosphate and beta-blocker poisoning (Dick, 2000; Montano et al., 1998; Olshansky et al., 2008; Schweitzer & Mark, 1980). Atropine also causes tachyarrhythmias and can increase HR if given before treadmill exercise (Jost et al., 2000; Schweitzer & Mark, 1980). Furthermore, atropine is found to increase FOC and HR in human atrial myocardium and the hearts of both wild-type and receptor (M2 or M1/3-) knockout mice by inhibiting cAMP-specific phosphodiesterase type 4 (PDE4), a novel pathway that atropine may act independently of muscarinic receptors (Perera et al., 2017). However, our treatments with atropine and CGRP (AC) significantly promoted negative inotropic and chronotropic effects in the heart. Even if CGRP could cause the release of ACh, the parasympathetic nervous system is blocked by atropine, which binds to muscarinic receptors, therefore, the effects observed with CGRP treatment is suggested by our results to be a direct action of CGRP on cardiac muscle. These results suggest and strengthen the proposition that CGRP and the sensory nervous system may play parasympathetic-like roles and act as a compensatory mechanism in a lack of parasympathetic stimulation.

Propranolol blocks beta-1 and beta-2 adrenergic receptors present in the heart causing a direct decrease in FOC and HR and an indirect decrease in norepinephrine release (reviewed by Al-Majed et al., 2017). In our experiments, propranolol promoted a greater decrease in HR than in FOC. There were no significant differences between the treatments with propranolol alone and

PrC in HR and FOC. Research suggests that injection of CGRP in lateral cerebroventricles significantly increased HR in normotensive rats. This positive chronotropic effect was mitigated by pretreatment with propranolol (Lappe et al., 1988). Additional studies from Marshal et at. (1986) also suggest that propranolol antagonized the systemic effects of intravenous CGRP in rats (Marshal et al., 1986). Taken together, these results suggest that propranolol may conceal the effects of CGRP and that the systemic effects of exogenous CGRP treatments are different than treating the heart directly with CGRP.

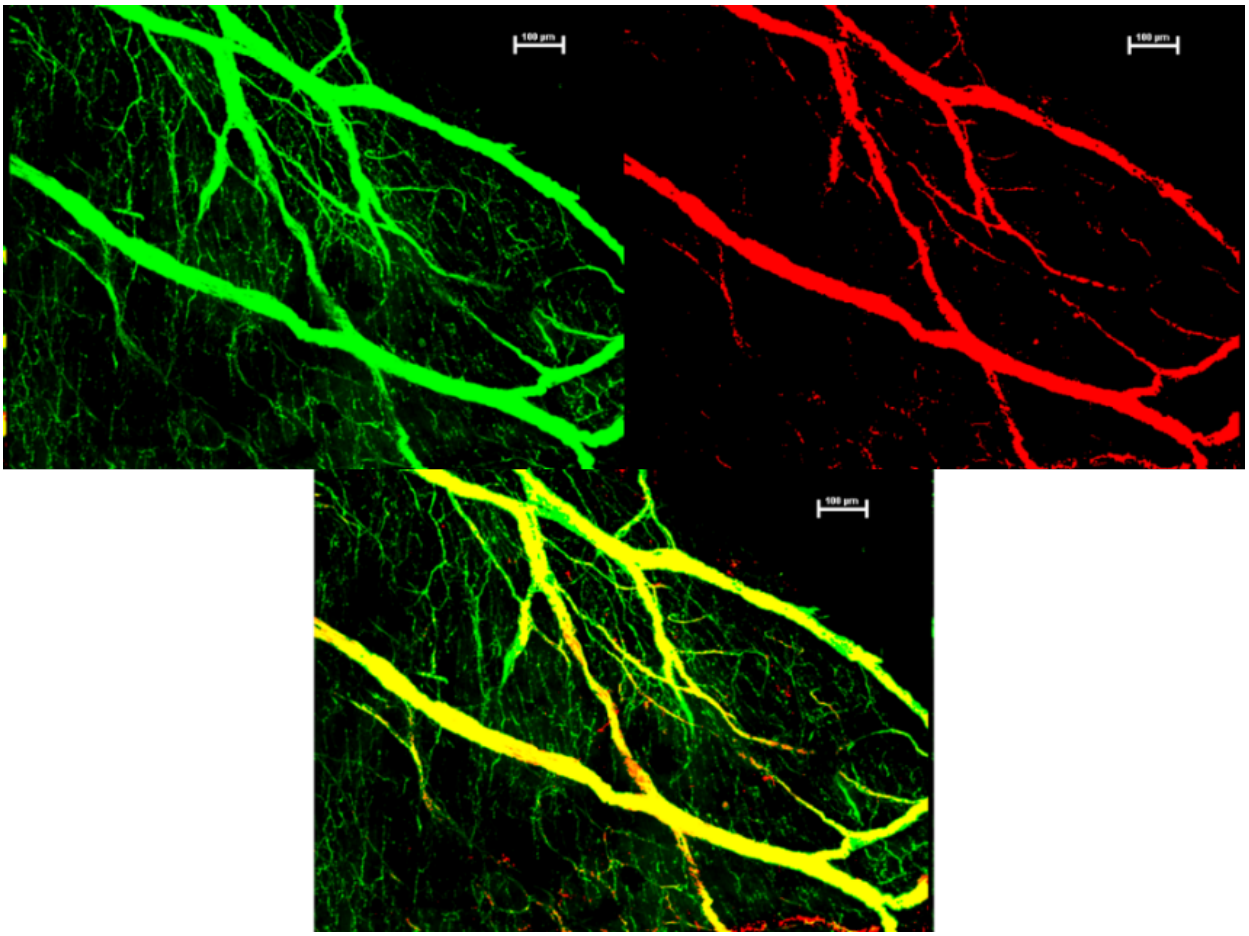
Alpha-1 adrenergic receptors compose the minority of the adrenergic receptors in the heart. It signals via G-protein coupled receptors and when activated, increases force of contraction and heart rate, and promotes hypertrophic adaptations and induction of ischemic preconditioning (reviewed by O'Connell et al., 2013). Phentolamine blocks alpha-1 adrenergic receptors and may cause a decrease in HR and FOC (Aronson, 2016). There were no significant differences between the treatments with phentolamine alone and PhC in HR at the 5% level. Phentolamine may have counterbalanced the effects of CGRP on HR via increase in norepinephrine levels and beta-activation. Although no significance in the treatment with PhC on FOC was observed, there was a substantial trend on lowering FOC by an additive effect of CGRP.

In conclusion, our experiments suggest that CGRP has direct effects in the heart and may contribute to the regulation of cardiac function. Exogenous CGRP treatment significantly reduces FOC and and there is a trend towards decreasing HR, even when the parasympathetic nervous system is blocked. We were also able to confirm the presence of immunoreactive CGRP fibers with varicosities in the heart of frogs. Previous literature and the results from this study support the hypothesis that CGRP and the sensory nervous system may actively play additional

and important roles in the regulation of function in heart and other organs and systems (reviewed on Maggi, 1995; and on Russell et al., 2014). Additional research on the roles of CGRP and the afferent and efferent sensory innervation roles in the heart should be conducted. By acquiring further knowledge on the role of these in the heart, we may find potential therapies for cardiovascular diseases (Tullio et al., 2017) such as cardiac anaphylaxis (Dai et al., 200), cardiac dysfunction in diabetes (Sun & Pan, 2014), hypertension (Smillie & Brain, 2011), and be a target of neural remodeling in the heart health/disease processes.

Figure 3.1

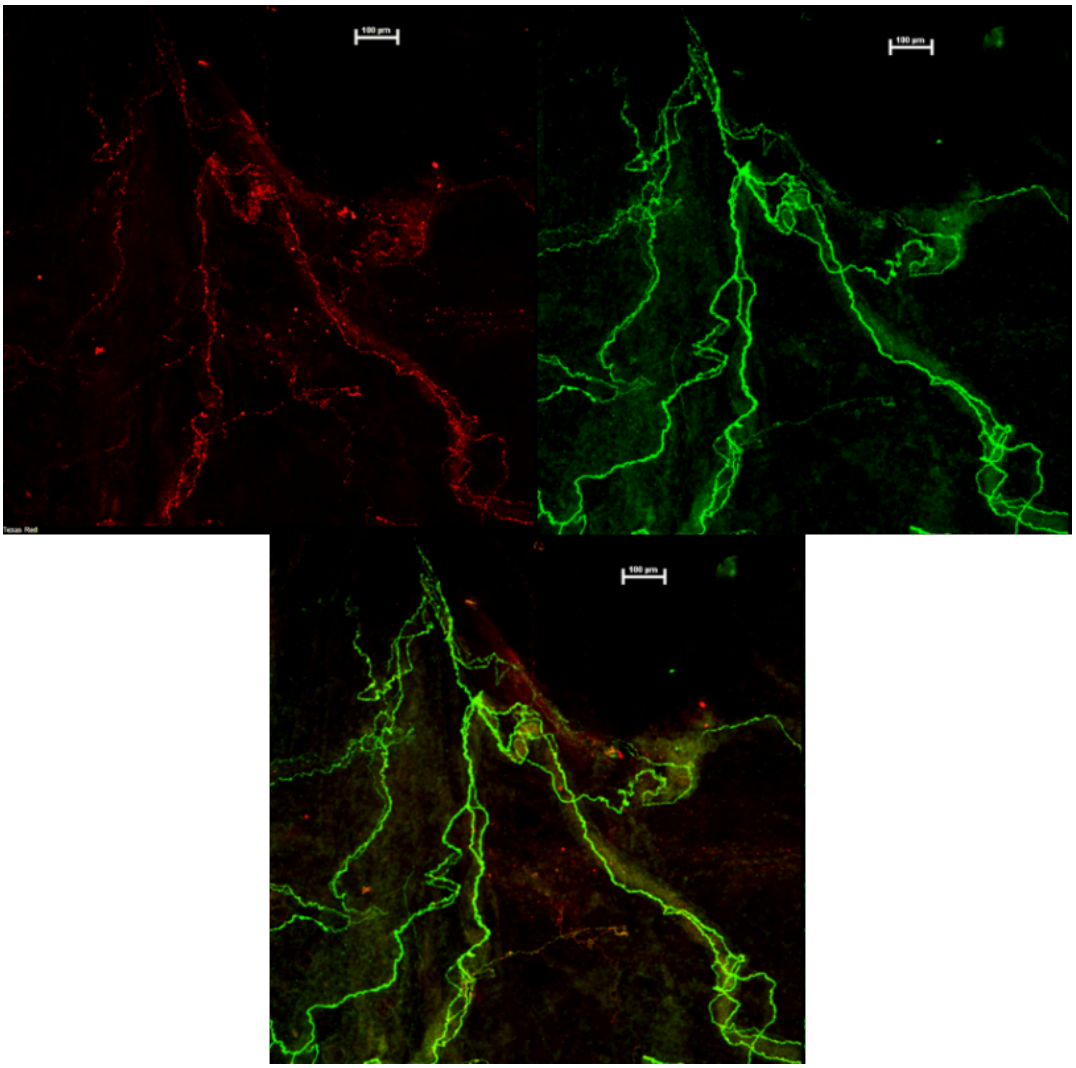
Co-localization of Anti-CGRP and Anti-neurofilament in Fibers in the Heart



Note. Immunohistochemical staining using antibodies against CGRP (red) and neurofilament (green). Whole-mount preparations of frog right atria were fixed and stained using antibodies against CGRP (red) and neurofilament (green) and images were captured using a Nikon C2+ laser scanning confocal microscope. The image shows positive staining for CGRP and neurofilament co-localizing within the same fibers, suggesting these are neural tissues containing the neurotransmitter CGRP.

Figure 3.2

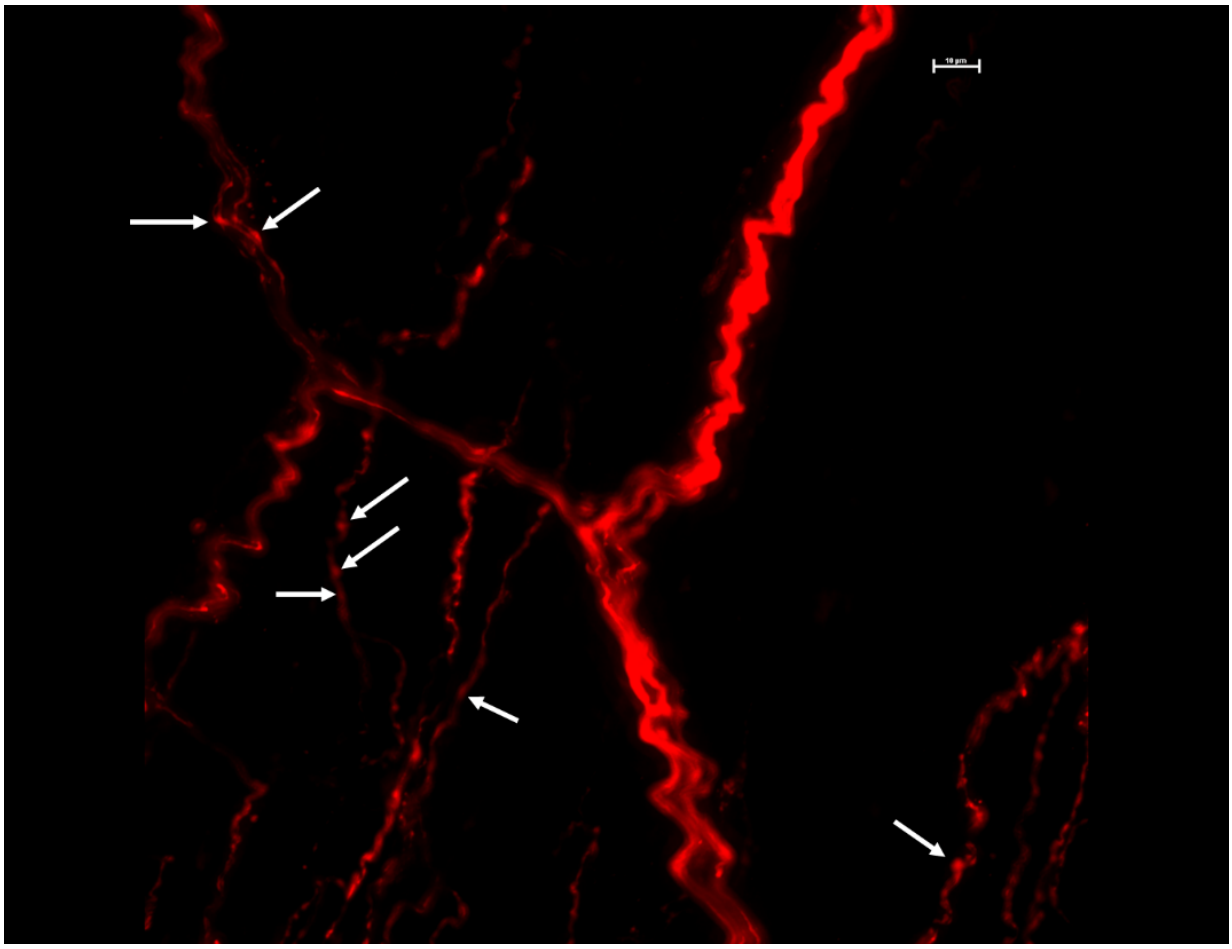
TH and CGRP Positive Fibers Running Side-by-side in the Wall of the Heart



Note. Immunohistochemical staining using antibodies against CGRP (red) and TH (green). Whole-mount preparations of frog right atria were fixed and stained using antibodies against CGRP (red) and neurofilament (green) and images were captured using a Nikon C2+ laser scanning confocal microscope. The image shows positive bundles of TH (sympathetic) and CGRP positive fibers running together on the epicardium.

Figure 3.3

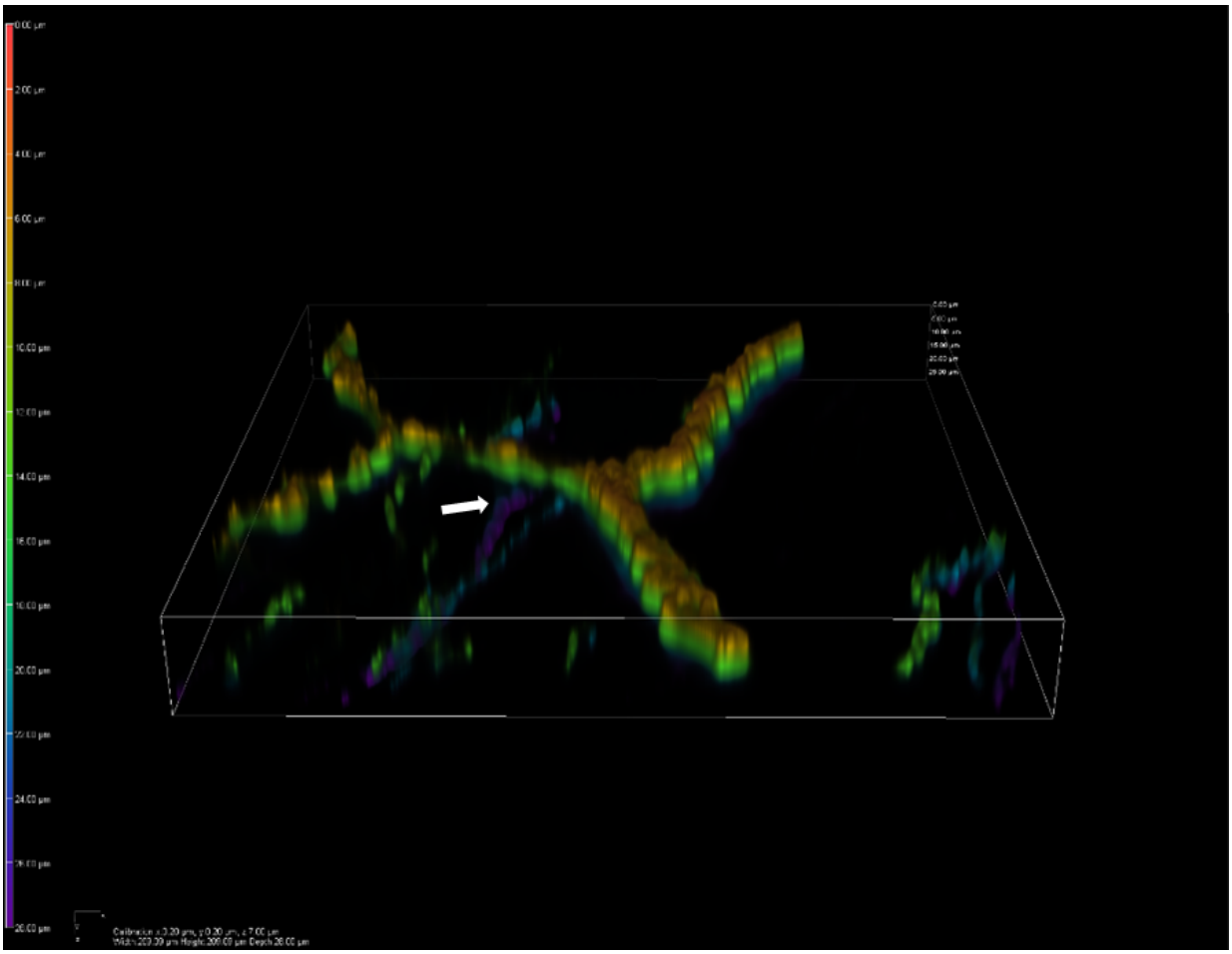
Large and Small Bundles of CGRP Positive Fibers



Note. Immunohistochemical staining using antibodies against CGRP (red). Whole-mount preparations of frog right atria were fixed and stained using antibodies against CGRP (red) and neurofilament (green) and images were captured using a Nikon C2+ laser scanning confocal microscope. Arrows indicate CGRP positive post-ganglionic varicosities.

Figure 3.4

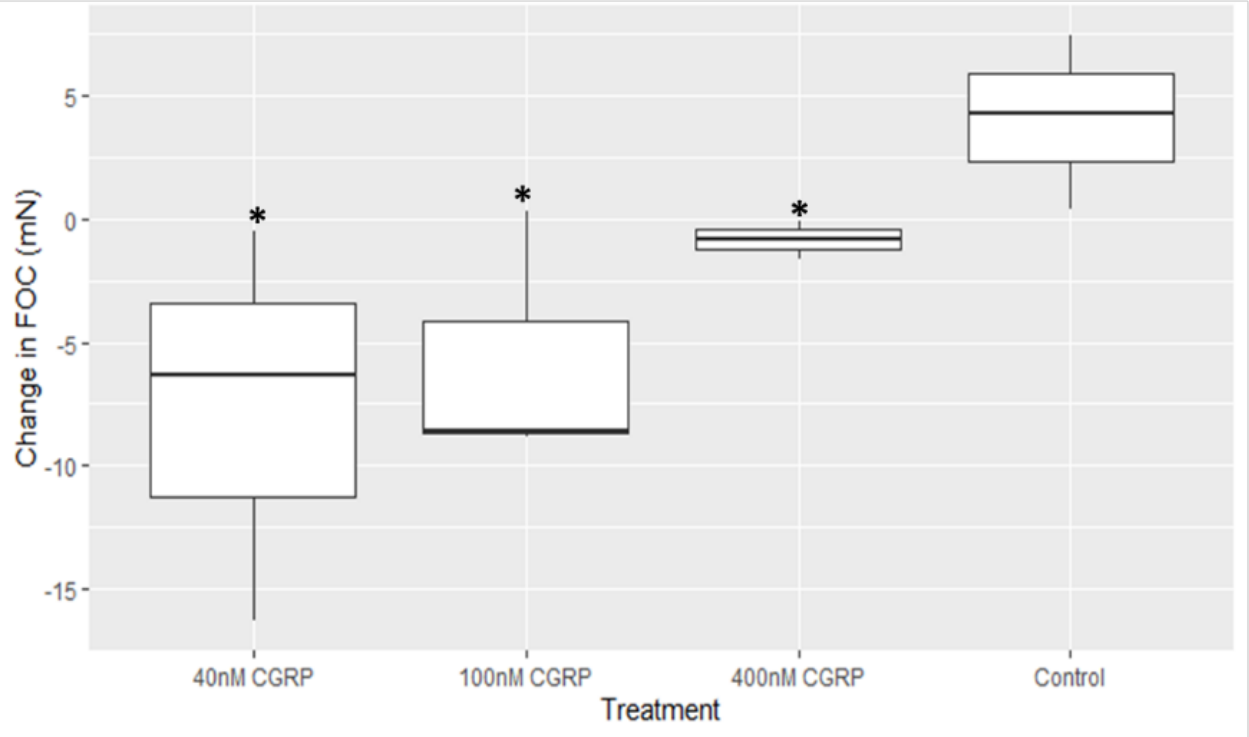
3D Representation



Note. Immunohistochemical staining using antibodies against CGRP. Whole-mount preparations of frog right atria were fixed and stained using antibodies against CGRP (red) and neurofilament (green) and images were captured using a Nikon C2+ laser scanning confocal microscope. This 3D representation of Figure 3.3 shows a bundle of CGRP positive fibers entering and running on deeper layers of the epicardium. Bundles of axons on the top of the epicardium are red colored and deeper bundles are blue and purple colored. Arrow indicates a smaller bundle branching away from a major superficial bundle and entering deeper layers of the epicardium.

Figure 3.5

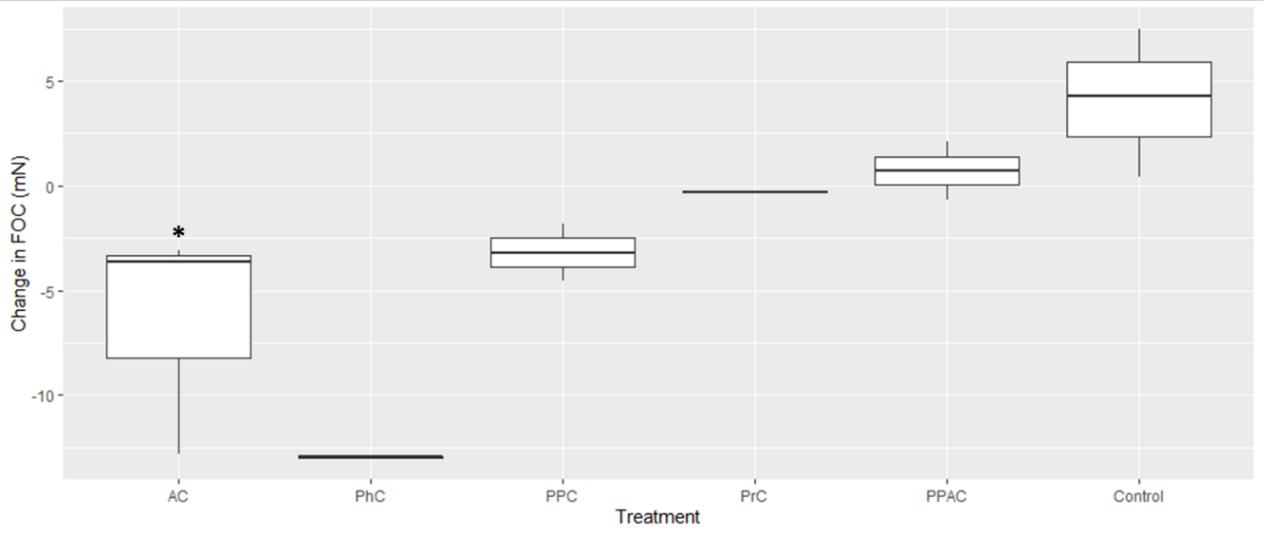
Change in FOC at the 5th Minute Across Treatments With CGRP



Note. In-situ preparations of hearts were performed by connecting each heart to a PowerLab data acquisition system for the collection of FOC data using force transducers and LabChart software. A 60-second stable baseline was recorded from each animal before treatment, and the FOC was recorded as the initial control level (0 on the y-axis of graphs). Following treatment, FOC was recorded for 5 minutes. Exogenous CGRP treatments (40 nM, 100 nM and 400 nM) significantly (*) lowered FOC in frogs. Wilcoxon signed-rank test – 95% of confidence interval.

Figure 3.6

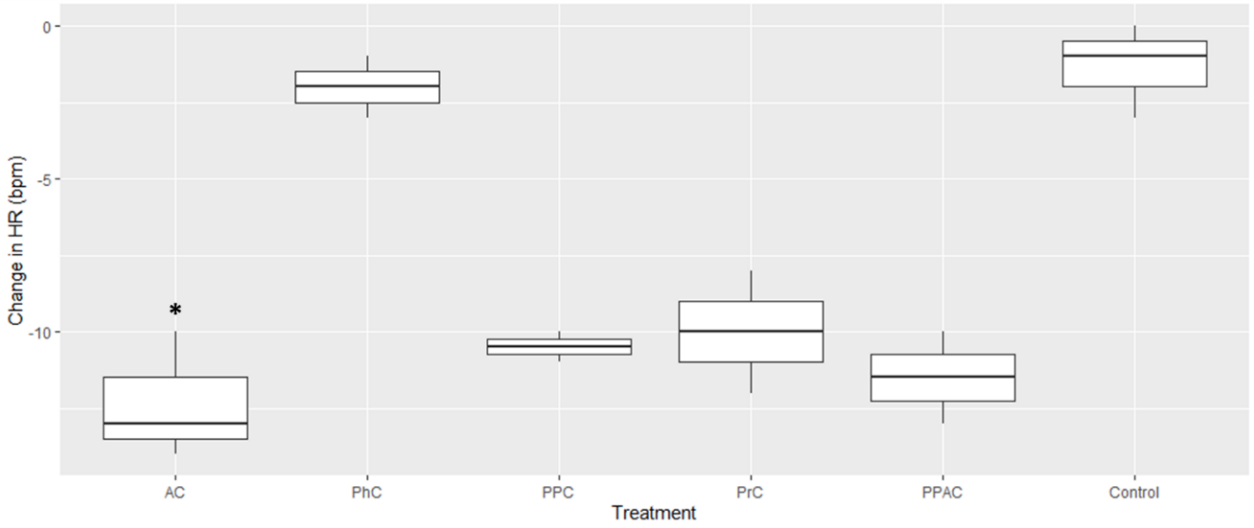
Change in FOC at the 5th Minute Across Treatments With CGRP and Autonomic Blockers



Note. In-situ preparations of hearts were performed by connecting each heart to a PowerLab data acquisition system for the collection of FOC data using force transducers and LabChart software. A 60-second stable baseline was recorded from each animal before treatment, and the FOC was recorded as the initial control level (0 on the y-axis of graphs). Following treatment, FOC was recorded for 5 minutes. Exogenous CGRP (40 nM) + 1mM of atropine treatment (AC) significantly (*) lowered FOC in frogs. Wilcoxon signed-rank test – 95% of confidence interval. Note: Atropine + CGRP (AC); Phentolamine + CGRP (PhC); Propranolol + Phentolamine + CGRP (PPC); Propranolol + CGRP (PrC); Propranolol + Phentolamine + Atropine + CGRP (PPAC).

Figure 3.7

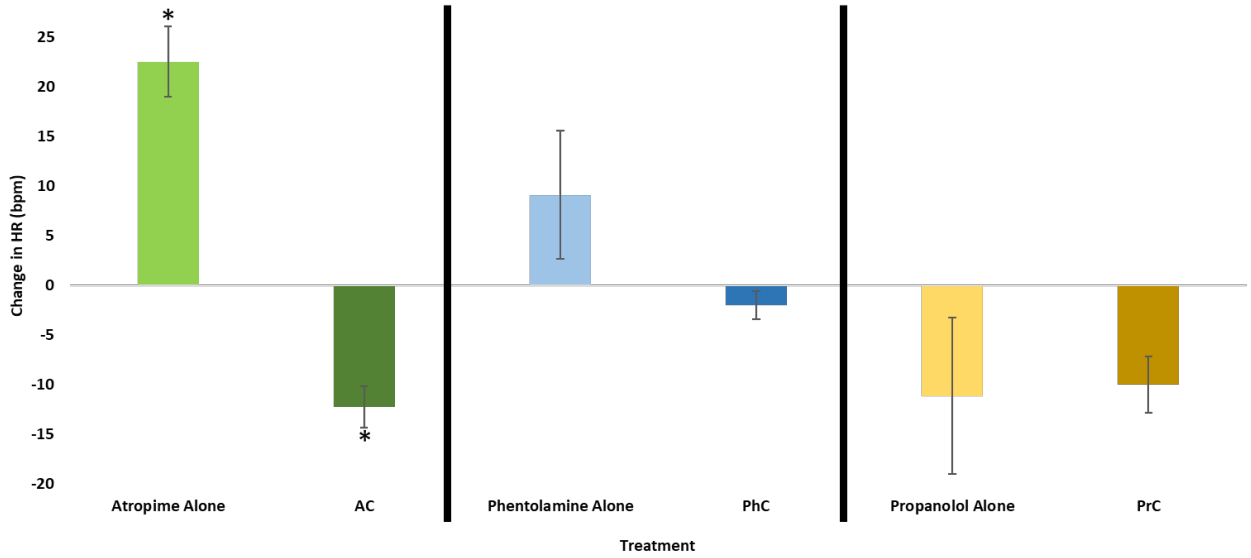
Change in HR at the 5th Minute Across Treatment With CGRP and Autonomic Blockers



Note. In-situ preparations of hearts were performed by connecting each heart to a PowerLab data acquisition system for the collection of HR data using force transducers and LabChart software. A 60-second stable baseline was recorded from each animal before treatment, and the HR was recorded as the initial control level (0 on the y-axis of graphs). Following treatment, HR was recorded for 5 minutes. Exogenous CGRP (40 nM) + 1mM of atropine treatment (AC) significantly (*) lowered HR in frogs. Wilcoxon signed-rank test – 95% of confidence interval. Note: Atropine + CGRP (AC); Phentolamine + CGRP (PhC); Propranolol + Phentolamine + CGRP (PPC); Propranolol + CGRP (PrC); Propranolol + Phentolamine + Atropine + CGRP (PPAC).

Figure 3.8

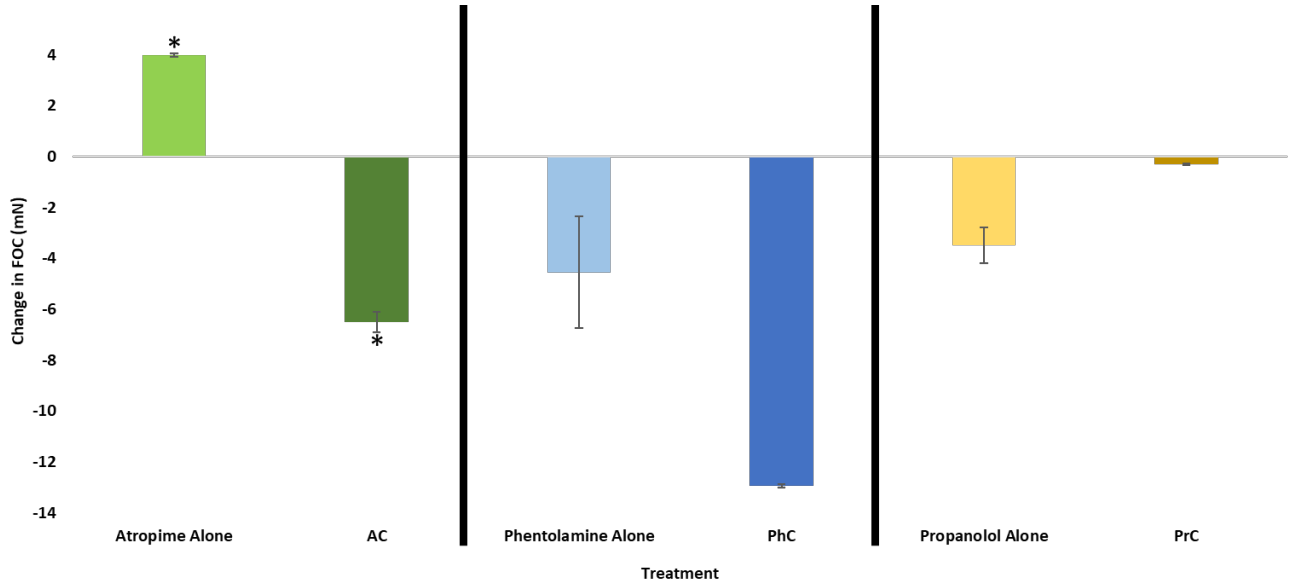
Impacts in HR of Autonomic-receptor Antagonist Alone vs. Antagonist and CGRP Treatments



Note. In-situ preparations of hearts were performed by connecting each heart to a PowerLab data acquisition system for the collection of HR data using force transducers and LabChart software. A 60-second stable baseline was recorded from each animal before treatment, and the HR was recorded as the initial control level (0 on the y-axis of graphs). Following treatment, HR was recorded for 5 minutes. Comparison between autonomic antagonist alone and autonomic antagonist combined with CGRP. Exogenous CGRP (40 nM) + 1 mM of atropine treatment (AC) significantly (*) lowered HR in frogs when comparing with atropine alone. Wilcoxon signed-rank test – 95% of confidence interval. Note: Atropine + CGRP (AC); Phentolamine + CGRP (PhC); Propranolol + CGRP (PrC).

Figure 3.9

Impacts in FOC of Autonomic-receptor Alone vs. Antagonist and CGRP Treatments



Note. In-situ preparations of hearts were performed by connecting each heart to a PowerLab data acquisition system for the collection of FOC data using force transducers and LabChart software. A 60-second stable baseline was recorded from each animal before treatment, and the FOC was recorded as the initial control level (0 on the y-axis of graphs). Following treatment, FOC was recorded for 5 minutes. Comparison between autonomic antagonist alone and autonomic antagonist combined with CGRP. Exogenous CGRP (40 nM) + 1mM of atropine treatment (AC) significantly (*) lowered FOC in frogs when comparing with atropine alone. Wilcoxon signed-rank test – 95% of confidence interval. Note: Atropine + CGRP (AC); Phentolamine + CGRP (PhC); Propranolol + CGRP (PrC).

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CHAPTER IV

DISCUSSION

The goal of this study was to investigate if levels of expression of neurotrophic factors in heart muscle, and patterns of innervation of heart muscle, change with age, exercise, and sedentary lifestyle; and to investigate additional roles that the sensory nervous system may play in the heart. The results demonstrated that resting heart rate (HR) increased with aging in sedentary animals, but age-matched exercised animals had lower resting HR when compared to their sedentary counterparts. In sedentary animals older than 12 months of age, resting mean arterial blood pressure (BP) increased above 100mmHg, which is considered to be hypertensive. At 12 months of age, voluntary exercise lowered BP below 100mmHg, reversing the hypertensive state. At 18 months of age, voluntary exercise decreased BP, however BP measurements were still above 100mmHg in the exercised group. Studies from Rebimbas-Cohen (2005) suggests that exercise may reverse or prevent hypertension possibly due to increased neurotrophic factor support, which in turn could provide neuroprotection and neural remodeling favoring a more balanced innervation pattern in mesenteric vessels (Rebimbas-Cohen 2005). Exercise contributing to sympathetic attenuation have also been reported to control BP in SHR (Krieger et al., 1999). Other types of exercise regimen have shown to have positive impacts in BP. Research has shown that high-resistance strength-training normalizes BP in old men and women (Martel et al., 1999). Exercise has been shown to prevent and treat hypertension (Goodman et al., 2011; Waki et al., 2019; Wallace, 2003).

Research suggests that deficiencies in the innervation of the heart may be the cause of many cardiovascular diseases (Barron & Lesh, 1996; Floras, 2003; La Rovere et al., 1998; Nolan et al., 1998; Shen & Zipes, 2014; reviewed by Hanna et al., 2017). Sympathetic hyperinnervation

and overactivity have been suggested to play a role in a variety of cardiovascular diseases including hypertension and left ventricular hypertrophy (Campos, 2015; Dissanayake et al., 2018; Esler, 2011; Julius & Nesbitt, 1996; Lambert et al., 2007; Menuet et al., 2017; Palatini, 2001; Rebimbas-Cohen, 2005; Xing et al., 2014).

In the vasculature, sympathetic hyperinnervation is suggested to be caused by overexpression of NGF. Spitsbergen et al. (1995) investigated the levels of NGF in vascular smooth muscle cells (VSMCs) from normotensive Wistar-Kyoto (WKY) rats and spontaneously hypertensive rats (SHR). Their results suggest that NGF protein secretion was higher in VSMCs maintained in serum free medium from SHR than in WKY (Spitsbergen et al., 1995). Research from Clemow et al. (1998) suggests that NGF mRNA is elevated in a hypertensive strain of WKY rats (WKHT) compared to a hyperactive strain of WKY rats (WKHA).

Nerve fibers are developed, maintained, and regenerated by neurotrophic factors such as GDNF and NGF. Our studies demonstrate that aging and sedentarism combined alters GDNF and NGF protein levels and consequently, the innervation pattern of the heart. GDNF and NGF protein levels decrease in all heart chambers as the animals age while sedentary. While the levels of these NF are decreasing with aging/sedentarism, we observed that parasympathetic and sensory nerve densities are also decreased in right atria, sensory nerve density is decreased in left atria and ventricles, and sympathetic nerve density is increased in all heart chambers of old groups of animals when compared to younger animals. These events are concomitant with the increase in resting HR and BP. These results support the hypothesis that the decrease in NF protein contents in the heart due to aging and sedentarism combined may be the underlining cause of decreased sensory and parasympathetic nerve densities, and increased sympathetic

nerve density, which creates an innervation imbalance in the heart, leading to increased resting HR and hypertension.

The results from this study demonstrates that exercise increases GDNF levels in all heart chambers in all ages and increases NGF in all heart chambers in adult animals. Cycling has been shown to increase BDNF and NGF levels in patients with multiple sclerosis. Patients that underwent this exercise regimen had increased neural plasticity and improved cognitive function (Petajan & White, 1999; Gold et al., 2003). Exercise has been found to promote neurogenesis in hippocampus and recover septohippocampal cholinergic arrangement and function with concomitant increase in NGF levels in rats (Chae et al., 2013; Hall et al., 2018). Wehrwein et al. (2002) found that a 4-week walking exercise plan resulted in increased GDNF production by soleus, gastrocnemius, and pectoralis major muscles of rats. Furthermore, 2 weeks of hindlimb unloading provoked a decrease in GDNF production in the hindlimb muscles although it led to an increase in GDNF production by the pectoralis major. These results suggest that GDNF production by skeletal muscles in rats is activity-dependent, indicating that exercise may promote remodeling and recovery of NMJs in injury and disease (Wehrwein et al., 2002). Therefore, we proposed that exercise-induced changes in neurotrophic factor expression may be a possible mechanism by which exercise exerts positive effects on cardiac innervation.

Along with the increases in NF protein levels, our results demonstrate that exercise significantly increased or trended to increase parasympathetic nerve density in RA and LA with concomitant decrease in BP and HR in the 12mo-ex and 18mo-ex when compared to the age-matching sedentary groups. Research suggests that GDNF is the main NF which supports the growth, development, and maintenance of parasympathetic nerve fibers. In addition, cardiomyocytes may promote the growth, development, and maintenance of all branches of the

nervous system by secreting GDNF and other members of the GFL. GDNF mRNA was found in atrial and ventricular myocytes in rats and GDNF protein is synthesized by cardiomyocytes in the heart of normal and sympathectomized animals (Alves et al., 2019; Hiltunen et al., 2000; Martinelli et al., 2002, 2006; Rebimbas-Cohen 2005). Research also suggests that NGF may also contribute to the growth and survival of parasympathetic fibers (Ekman et al., 2017). We propose that the increase in parasympathetic innervation observed in our studies may also be due to an increase in NGF following exercise. We propose that the increase in parasympathetic innervation observed in our studies may be due to an increase in GDNF and NGF protein levels following exercise. Interactions between sympathetic and parasympathetic nervous system can be found throughout the entire heart, including in the pacemaker region. The proper interactions between the two branches of the autonomic nervous system are essential for a balanced cardiac function. Sympathetic overactivity may be observed during heart failure due to a disarrangement of autonomic nerves, which decreases parasympathetic influence over the sympathetic system. It is suggested that NGF may play a role in maintaining appropriate coupling of parasympathetic and sympathetic axons in the heart (Dunlap et al., 2003; Fan & Smith, 1993; Hasan & Smith, 2009; Loiacono & Story, 1986; Nihei et al., 2005; Randall et al., 2003; Smith et al., 2002; Warn et al., 1997; Wetzel & Brown, 1985).

Our results suggest that aging combined with a sedentary lifestyle may alter NF protein content and lead to an innervation imbalance in the heart, which increases resting HR and BP. Exercise may cause an increase in neurotrophic factor expression, which may be a possible mechanism to promote positive effects on cardiac innervation that could prevent or treat cardiovascular diseases.

In our final studies, we chose to investigate the effects of CGRP on the regulation of heart function. CGRP is produced and stored in vesicles in sensory varicosities and somatic motor nerve terminals and can regulate cell and tissue function (Alevizaki et al., 1986; Russell et al., 2014; Steenbergh et al., 1986). The presence of sensory nerve fibers, with varicosities, has been reported in a variety of tissues, including thoracolumbar fascia (Mense 2019), bronchial mucosa (Chanez et al., 1998) and in the epicardium of rat hearts (Cheng et al., 1997). Sensory-vagal ganglion cells have also been found in the heart of frogs (Woods, 1970). Cheng et al. (1997) reported that some sensory nerve fibers were in contact with cardiac ganglia, where they could indirectly affect cardiac physiology (Cheng et al., 1997). Our immunohistochemical investigations showed the presence of sensory nerve fibers, with varicosities, running next to sympathetic nerve fibers in the epicardium in the heart of frogs.

CGRP is mainly known for its potent vasodilatatory effects, which may help to prevent cardiac and pulmonary hypertension, ischemia, migraine, and improve blood flow distribution and wound healing (Hasbak et al., 2001, 2003; Jonhagen, 2006; Russell et al., 2014; Schlier et al., 2009; Toda et al., 2008; Vause & Durham, 2010). It has been suggested that CGRP may play a role in regulating cardiovascular function by inhibiting sympathetic nervous system activity in mice (Kurihara et al., 2003).

The functional CGRP receptor is a combination of two receptors, the calcitonin-like receptor (CLR) and receptor activity modifying protein 1, 2, or 3 (RAMP1, RAMP2, RAMP3) (Fluhmann et al., 1995; Hay et al., 2008; Russell et al., 2014). An important pathway triggered by CGRP binding to its receptor is the activation of adenylate cyclase, which increases intracellular cyclic AMP (cAMP), leading to activation of protein kinase A (PKA). PKA influences many pathways downstream, including activation of potassium channels and

promotes smooth muscle relaxation in arteries. Potassium efflux from the smooth muscle cells causes hyperpolarization of these cells, leading to relaxation. CGRP effects are inhibited when potassium channels are blocked (Nelson et al., 1990).

Our results also demonstrated that all groups treated with CGRP alone exhibited a lower force of contraction (FOC) and heart rate (HR) compared to the control group. To examine whether CGRP was acting on sympathetic or parasympathetic neural tissues, we compared the effects of CGRP treatment alone and in combination with autonomic antagonists. Our results showed significant changes in the group in which CGRP was combined with atropine (AC). When we treated the hearts with AC, it significantly decreased HR and FOC when compared to atropine alone. Our results suggest that atropine does not block the effects of CGRP, suggesting that CGRP is not exerting its effects by causing release of ACh.

Results from our CGRP studies have several implications. Traditionally, it is suggested that the two branches of the autonomic nervous system, the sympathetic and parasympathetic branches, work antagonistically to regulate heart function (Bush et al., 2016; Hiltunen et al., 2000). However, our results and those of others, suggest that sensory nerve fibers present in the heart may participate in regulating cardiac physiology in an afferent manner (Alevizaki et al., 1986; Cheng et al., 1997; Russell et al., 2014; Steenbergh et al., 1986; Woods, 1970). Results from our studies, combined with those of others, strongly suggest that the sensory nervous system and CGRP may play a role in regulating cardiac muscle physiology in a manner similar to that of the parasympathetic nervous system. Additional studies on the role that sensory nervous system plays in regulating heart function are needed and it is likely that the sensory nervous system could serve as a target for future therapies (Ajijola & Shivkumar, 2012; Huang et al. 2017; Ieda & Fukuda, 2009). Some researchers have already described how the sensory

nervous system and CGRP could be prospective therapies for cardiovascular diseases (Tullio et al., 2017) such as cardiac anaphylaxis (Dai et al., 2000), cardiac dysfunction in diabetes (Sun et al., 2014), and hypertension (Smillie & Brain, 2011).

Finally, declines in neurotrophic factor expression with sedentary aging may impact multiple aspects of neural function and could be a possible cause of the decline in density of both parasympathetic and sensory innervation and increases in BP. Exercise can increase NT expression and may protect/restore neural elements, but more studies are needed to determine which NTs may support parasympathetic and sensory innervation in the heart. Exercise may prevent the majority of cardiovascular diseases and has neuroprotective effects. Exercise should be prescribed as a treatment of several diseases, including neurodegenerative diseases such as ALS and MS. Additional research on exercise, NF, and diet interventions should be conducted. Diet restriction has been a notable intervention as research has shown that restricting the number of calories that animals eat can have a substantial effect on their vitality and longevity, as much as by 40-50% (Weindruch & Sohal, 1997). Perhaps, it is not the number of calories, but the type of food they are eating. It would be significant in looking at diets that are known to have neuroprotective effects, such as ketogenic diet. In addition, long-term fasting and intermittent fasting have been shown to have positive effects in neuroprotection, metabolism, chronic diseases such a Type II Diabetes, blood pressure, among several other conditions.

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Appendix A

Institutional Animal Care and Use Committee Approval

WESTERN MICHIGAN UNIVERSITY

Institutional Animal Care and Use Committee

ANNUAL REVIEW OF VERTEBRATE ANIMAL USE

RECEIVED
JUN 08 2018
I.A.C.U.C.
Western Michigan University

PROJECT OR COURSE TITLE: The Effect Of Exercise, Aging, And Hypertension On The Pattern Of Innervation And Neurotrophic Factor Expression In Heart

IACUC Protocol Number: 16-06-02

Date of Review Request: 06/20/18 Date of Last Approval: 06/22/2017

Purpose of project (select one): ☐ Teaching ☒ Research ☐ Other (specify):

PRINCIPAL INVESTIGATOR OR ADVISOR

Name: John Spitsbergen Title: Professor

Department: Biological Sciences Electronic Mail Address: john.spitsbergen@wmich.edu

CO-PRINCIPAL OR STUDENT INVESTIGATOR

Name: Gabriel Alves Title: Graduate Student

Department: Biological Sciences Electronic Mail Address: gabriel.almeidaalves@wmich.edu

1. The research, as approved by the IACUC, is completed:
   ☐ Yes (Continue with items 4-5 below.) ☒ No (Continue with items 2-5 below.)

If the answer to any of the following questions (items 2-4) is "Yes," please provide a detailed explanation on an attached sheet of paper. Include details of any modifications made to the protocol based on new findings or publications, adverse events or mortalities.

2. Have there been any changes in Principal or Co-Principal Investigators? ☐ Yes ☒ No
3. Have there been any new findings or publications relative to this research that require you to alter your study? ☐ Yes ☒ No

   Describe the sources used to determine the availability of new findings or publications:

   ☐ No search conducted (Please provide a justification on an attached sheet.)
   ☐ Animal Welfare Information Center (AWIC)
   ☐ Search of literature databases (select all applicable)
   ☐ AGRICOLA ☐ Current Research Information Service (CRIS)
   ☐ Biological Abstracts ☐ Medline
   ☐ Other (please specify): google.scholar.com
   Date of search: 05/30/18 Years covered by the search: 2016-18
   Key words: exercise, GDNF, cardiac, neuroplasticity
   ☐ Additional search strategy narrative:

4. Are there any adverse events, in terms of animal well-being, or mortalities to report as a result of this research? ☐ Yes ☒ No

   Cumulative number of mortalities:

5. Animal usage: Number of animals used during this quarter (3 months): 18 Cumulative number of animals used to date: 42

John 5/30/18

Principal Investigator/Faculty Advisor Signature Date

Gabriel A. Alves 6/04/18

Co-Principal or Student Investigator Signature Date

IACUC REVIEW AND APPROVAL

Upon review of the relevant information regarding this protocol, the IACUC approval for this project has been extended for one year from the date of this signature.

Revised 01/2012 WMU IACUC All other copies obsolete.